CHARACTERIZATION OF THE MEMBRANE-BOUND NITRATE REDUCTASE ACTIVITY OF AEROBICALLY GROWN CHLORATE-SENSITIVE MUTANTS OF *ESCHERICHIA COLI* K12

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1. Introduction

Wild-type strains of *Escherichia coli* can grow in the presence of chlorate aerobically but under anaerobic conditions growth is inhibited. This observation has been explained on the assumption that under anaerobic conditions, chlorate, an analogue of nitrate, induces nitrate reductase (EC 1.7.99.4) and is converted by the enzyme to the toxic compound chlorite with the result that cell growth ceases: aerobic growth in the presence of chlorate is allowed since under these conditions nitrate reductase activity is repressed [1]. Recently mutants have been isolated which show a chlorate-sensitive phenotype when grown under aerobic conditions in the presence of a fermentable carbon source [2] and a detailed biochemical characterization of one of these mutants, strain 72, has indicated that the primary genetic lesion occurs in the biosynthetic pathway for ubiquinone$_8$ [3]. In addition, it was noticed that strain 72, when grown aerobically in the presence of nitrate, produced significantly higher activities of reduced benzylviologen-dependent nitrate reductase than an equivalent culture of the parent strain similarly grown, however this activity represented only 2–10% of that found in cultures of either strain 72 or the wild-type grown anaerobically in the presence of nitrate [2,3]. The nitrate reductase activity found in aerobically-grown strain 72 was destroyed by boiling cell extracts and shown to be sensitive to the inhibitor azide but it was not clear if this activity was catalyzed by the nitrate reductase enzyme that is normally synthesized only under anaerobic growth conditions or, alternatively, was the result of some side reaction of another unrelated enzyme that was induced (de-repressed) in strain 72 as a result of the mutation in the ubiquinone$_8$ biosynthetic pathway.

Using a variety of techniques, we demonstrate here that the protein responsible for nitrate reductase activity in aerobically and anaerobically grown strain 72 are identical, an observation that accounts for the chlorate-sensitive phenotype of the mutant when grown aerobically.

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2. Materials and methods

2.1. *Strains and growth media*

The wild-type strain 541 (F^-, *thr*$^-$, *leu*$^-$, *his*$^-$, *arg*$^-$, *thi*$^-$, *ade*$^-$, *gal*$^-$, *lacY*$^-$, *malE*$^-$, *xyl*$^-$, *ara*$^-$, *mtl*$^-$, *str*^{R}, T_1^R) and the derived chlorate-sensitive mutant strain 72 have been described [2]. The basic medium for growth contained (per litre) K_2HPO_4 (12 g), KH_2PO_4 (3 g), $(NH_4)_2SO_4$ (4 g), KNO_3 (10 g), $MgCl_2 \cdot 6\,H_2O$ (0.2 g), ammonium molybdate (1 μM), potassium selenite (1 μM), a sulphate-free mineral salts solution (10 ml) used in [4], glucose (2 g), vitamin-free casamino acids (1 g) and the amino acids required by these strains (all at 10 mg). For ^{35}S-labelling experiments, vitamin-free casamino acids and $(NH_4)_2SO_4$ were omitted and the latter replaced with NH_4Cl (4 g)

and $K_2{}^{35}SO_4$ (50 μM, spec. act. 20 μCi/μmol). Growth was at 37°C in 500 ml, either in 21 baffled conical flasks with vigorous shaking for aerobic conditions or in 500 ml tightly-stoppered bottles for anaerobic conditions. Cells were harvested, washed, broken by ultrasonic disruption and membrane particles prepared by differential centrifugation as in [4] except that all buffers were supplemented with the protease inhibitors benzamidine HCl (5 mM) and *N*-α-*p*-tosyl-L-lysine chloromethyl ketone HCl (0.1 mg/ml).

Triton X-100-soluble extracts from the membrane particles were prepared by suspending the particles (1–5 mg protein/ml) in Na phosphate buffer (50 mM, pH 7.4), benzamidine HCl (5 mM) and *N*-α-*p*-tosyl-L-lysine chloromethyl ketone HCl (0.1 mg/ml) in the presence of Triton X-100 (1%, w/v) at 0°C for 30 min, then centrifuging at 230 000 × *g* for 10 min at 4°C. The supernatants so obtained are termed Triton X-100 extracts.

2.2. *Enzyme assays*

Continuous, spectrophotometric assay of nitrate reductase using reduced benzyl viologen as reductant was carried out by the method in [5], and a unit of activity was defined at 1 μmol NO_3^- reduced/min at 30°C. Protein was assayed by the method in [6] with bovine serum albumin as standard.

Antiserum to *E. coli* nitrate reductase was raised in rabbits using enzyme purified essentially as in [7].

Double immunodiffusion analysis was performed in agar (1%, w/v) plates containing Na phosphate (50 mM, pH 7.4), Triton X-100 (0.5%, w/v). Immunoprecipitation was performed by the addition of 50 μl antiserum/mg protein to Triton X-100 extracts, incubating for 2 h at room temperature and then 14 h at 4°C. The immunoprecipitates were collected by centrifuging at 10 000 × *g* for 2 min and washing 3 times in Triton X-100 (1%, w/v), NaCl (0.3 M), Na phosphate (10 mM, pH 6.8).

2.3. *Polyacrylamide gels*

Electrophoresis under non-dissociating conditions was performed at pH 8.9 in 5% (w/v) polyacrylamide gels as in [8]. Activity staining of gels and agar plates for nitrate reductase activity was carried out as in [9].

Electrophoresis in the presence of dodecyl sulphate was performed either in 6.5% (w/v) polyacrylamide gels as in [10] or in 5% gels by the procedure in [11]. The distribution of radioactivity in polyacrylamide gels was determined by scintillation counting of 1 mm slices of the gel.

3. Results and discussion

3.1. *Initial characterization of the benzyl viologen-dependent nitrate reductase activity in the aerobically grown mutant strain 72*

3.1.1. Electrophoretic analysis

Triton X-100-solubilised extracts of the membranes isolated from the wild-type (strain 541) and mutant (strain 72) grown under aerobic and anaerobic conditions in the presence of nitrate, were obtained as in section 2. About 75% of the membrane-bound reduced benzyl viologen nitrate reductase activity was found in the detergent extracts for the wild-type growths and > 90% for the mutant preparations. The extracts were electrophoresed under conditions where the enzymic activity is retained, and activity staining of the gels produced the result summarized in fig.1. A

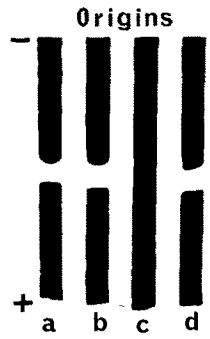

Fig.1. Electrophoresis at pH 8.9 of Triton X-100 extracts: activity stain. Triton X-100 extracts were prepared as in section 2. The clear zones on the plate represent the regions on the gels containing the reduced benzyl viologen-dependent NO_3^- reductase activity. The gels were photographed 10 min after the start of the activity stain. (a) Activity-stained gel of Triton X-100 extract from the wild-type organism (strain 514). Grown anaerobically; 0.2 unit enzyme activity applied to the gel. (b) As (a) but of mutant organism (strain 72). Grown anaerobically; 0.4 units enzyme activity applied to gel. (c) As (a) wild-type organism (strain 541). Grown aerobically; 0.02 unit enzyme activity applied to the gel. (d) As (a) but mutant organism (strain 72). Grown aerobically; 0.2 units enzyme activity applied to the gel.

band of activity appeared after ~5 min incubation in a corresponding position in each of the gels derived from the mutant grown aerobically and anaerobically and from the wild-type grown anaerobically. Insufficient activity was applied to the gel of the wild-type extract grown aerobically to produce a clear band of activity (fig.1(c)). After 30 min incubation 2 smaller faster migrating bands were also observed for the aerobic mutant. Faint bands migrating to this position are often observed for anaerobic cultures of *E. coli* (unpublished observation). This experiment therefore suggests that the enzyme responsible for the reduced benzyl viologen-dependent nitrate reductase activity in the mutant grown aerobically has similar gross molecular properties to the anaerobic nitrate reductase.

3.2.1. Immunological characterization

Identity between the enzyme catalysing the NO_3^--dependent benzyl viologen oxidation in the mutant and wild-type under the various growth conditions referred to above, is further supported by the results of double immunodiffusion analysis of the Triton X-100 extracts using antiserum specific for nitrate reductase. The antiserum produced a single precipitin line with Triton X-100 extracts from the anaerobically-grown mutant and wild-type. No precipitin line could be detected visually with the aerobic extracts but activity staining of the immunodiffusion plate produced a line of activity in a position that corresponded to that found for the anaerobically grown cultures (data not shown).

This demonstrates that the enzyme produced aerobically by the mutant shares common determinants with the nitrate reductase produced anaerobically.

3.2. *What is the subunit composition of the enzyme produced aerobically by the mutant?*

The subunit composition of the enzymes catalysing the reduced benzyl viologen-dependent NO_3^- reductase activities of the wild-type and mutant strains were investigated by immunoprecipitation using nitrate reductase specific antiserum. Uniformly ^{35}S-labelled cultures of the organisms grown under the appropriate conditions were obtained as in section 2.

Anaerobic nitrate reductase from *E. coli* consists of 2 subunits: (i) α subunit of mol. wt 150 000 and (ii) β subunit of mol. wt 65 000 [7]. Initially it was established that the subunits of the enzymes produced anaerobically by the mutant and wild-type, were the same. This can be seen to be so in fig.2(A,B). The polypeptide of mobility intermediate between that of the α and β subunits is often found in immunoprecipitates and is thought to be a proteolytic fragment of the α subunit. The apoprotein of the cytochrome $b_{556}^{NO_3^-}$ was not identified in the gels [12].

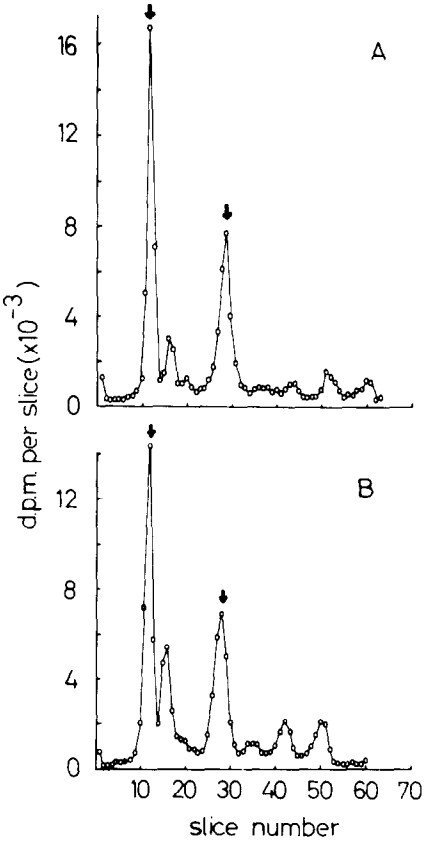

Fig.2. Dodecyl sulphate–polyacrylamide gel analysis of immunoprecipitates obtained from ^{35}S-labelled cultures of anaerobically-grown wild-type (strain 541) and mutant (strain 72). Immunoprecipitates were recovered using antiserum to nitrate reductase from Triton X-100 extracts obtained from equivalent cultures of the 2 organisms grown anaerobically. Electrophoresis was as in [10]. The arrows represent the α (nearer the gel origin) and β subunits. (A) Distribution of ^{35}S radioactivity in gel of immunoprecipitate from wild-type organism (strain 541) grown anaerobically. (B) As (A) but of mutant organism (strain 72).

In a separate experiment the subunit composition of the enzymes produced anaerobically and aerobically by the mutant were compared, fig.3(A,B). It can be seen that both the α and β subunits of nitrate reductase are produced by the mutant in aerobic culture. The additional material found in the lower molecular weight region of the gel (fig.2B) reflects the antiserum's limit of specificity for nitrate reductase. Control experiments showed that radioactivity was often associated with this region of the gel when similarly large amounts of radioactive Triton X-100 extracts are challenged by this antiserum.

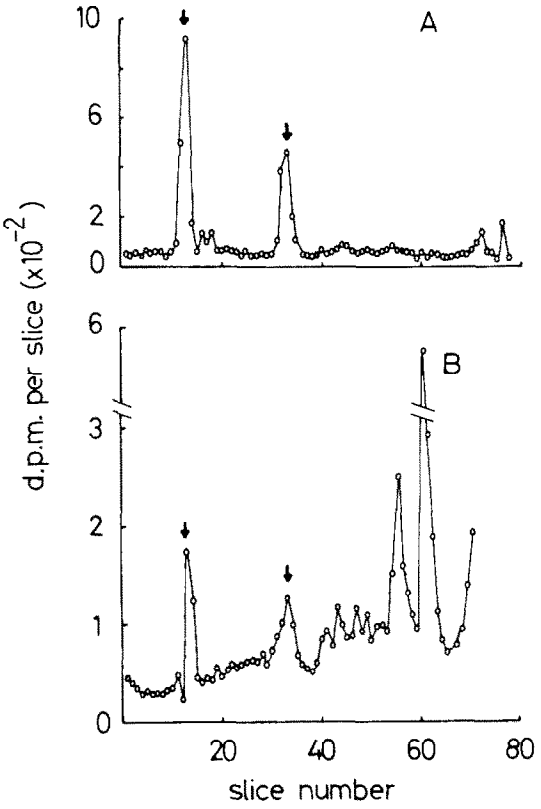

Fig.3. Comparison of immunoprecipitates obtained from anaerobic and aerobic ^{35}S-labelled cultures of mutant organism (strain 72). Immunoprecipitates were recovered using antiserum to nitrate reductase from Triton X-100 extracts from ^{35}S-labelled cultures of mutant organism (strain 72) grown under anaerobic and anaerobic conditions. The Triton X-100 extracts obtained were of equal specific radioactivity. About 3-times more Triton X-100 extract was used relative to the amount of antiserum for the immunoprecipitation from the aerobic culture than was used for the anaerobic culture. The ^{35}S-labelled aerobic Triton X-100 extract was supplemented with nonradioactive Triton X-100 extract from anaerobically-grown wild-type (strain 541) to give approximately the same concentration of enzyme activity in each immunoprecipitation mixture. The arrows locate the α and β subunits as in fig.2. Electrophoresis was by the procedure in [11]. (A) Distribution of ^{35}S radioactivity in gel of immunoprecipitate from anaerobically grown mutant (strain 72). (B) As (A) but aerobically grown.

4. Conclusion

The subunit composition of the enzyme responsible for the reduced benzyl viologen-dependent NO_3^- reductase activity present in aerobic cultures of mutant strain 72 is identical to that of nitrate reductase produced anaerobically by the wild-type organism. Since it has been thought that nitrate reductase is only functional during anaerobic growth conditions, a detailed analysis of the synthesis of this enzyme in aerobic cultures of strain 72 should provide greater insight into the factors regulating the expression of the genes coding for the various apoproteins of the nitrate reductase complex.

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