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Proton Translocation in Cytochrome-Deficient Mutants of *Escherichia coli*

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Cytochrome-deficient cells of a strain of *Escherichia coli* lacking 5-aminolevulinate synthetase have been used to study proton translocation associated with the reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase region of the electron transport chain. Menadione was used as electron acceptor, and mannitol was used as the substrate for the generation of intracellular NADH. The effects of iron deficiency on NADH- and D-lactate-menadione reductase activities were studied in iron-deficient cells of a mutant strain unable to synthesize the iron chelator enterochelin; both activities were reduced. The NADH-menadione reductase activity in cytochrome-deficient cells was associated with proton translocation and could be coupled to the uptake of proline. However proton translocation associated with the NADH-menadione reductase activity was prevented by a mutation in an *unc* gene. It was concluded that there is no proton translocation associated with the NADH-dehydrogenase region of the electron transport chain in *E. coli* and that the proton translocation obtained with mannitol as substrate is due to the activity of membrane-bound adenosine triphosphatase.

A scheme depicting the functional organization of the redox carriers responsible for aerobic electron transport in *Escherichia coli* membranes has recently been proposed by Haddock and Jones (10). The redox carriers are arranged in two proton-translocating loops, the first of which is equivalent to the site 1 region of the mitochondrial system and involves the flavin and nonheme iron species associated with the NADH dehydrogenase. Proton translocation associated with the NADH dehydrogenase part of the electron transport chain in *E. coli* has not yet been unequivocally demonstrated. Apart from the difficulty of finding a suitable electron acceptor, NADH does not readily enter whole cells of *E. coli*, and a permeable substrate must be used which is oxidized to yield intracellular NADH. It has been shown recently that mutants lacking NADH dehydrogenase activity are unable to grow aerobically on mannitol (19). NADH is generated by the oxidation of mannitol-1-phosphate to fructose-6-phosphate, and the inability to oxidize NADH via the electron transport chain apparently prevents growth. On this basis, normal cells grown aerobically might be expected to accumulate appreciable quantities of NADH when incubated anaerobically in the presence of mannitol.

In the present paper, proton translocation and active transport of proline is examined in cytochrome-deficient cells of *E. coli* K-12, using mannitol as substrate and menadione as electron acceptor.

MATERIALS AND METHODS

Chemicals. Chemicals were of the highest purity available commercially and were not further purified. L-[*U*-^{14}C]proline (290 mCi/nmol) was purchased from the Radiochemical Centre, Amersham, Bucks, U.K.

Organisms. The bacterial strains used are derived from *E. coli* K-12 and are described in Table 1.

Media. The minimal medium used was that described by Monod et al. (14) as medium 56. Growth supplements were added as sterile solutions at the following final concentrations, unless otherwise stated in the text: 30 mM mannitol; 3 μM thiamine; 0.8 mM L-leucine; 0.4 mM L-isoleucine; 0.4 mM L-valine; 1 mM L-arginine; 30 μM 2,3-dihydroxybenzoate; 0.1 mM 5-aminolevulinic acid. Solid media were prepared by the addition of 2% (wt/vol) agar to the minimal medium described above.

Growth of cells. For the preparation of heme-deficient cells, a 100-ml inoculum of cells was grown overnight in the presence of 0.4 μM 5-aminolevulinic acid. This culture was added to 900 ml of medium without added 5-aminolevulinic acid, and the cells were incubated for 6 to 8 h until growth was limited at about 2×10^8 cells per ml. Cytochromes were reconstituted in the heme-deficient cells by incubation of the cells for 2 h in minimal medium containing mannitol and 0.1 mM 5-aminolevulinic acid. These cells are referred to as heme reconstituted.

TABLE 1. *E. coli K-12 strains used*

Bacterial strain	Relevant genetic loci	Reference/other information
AN704	*ilvC argH entA hemA leu*	2
AN249	*uncA401 argH entA*	5
AN283	*uncB402 argH entA*	1
AN285	*uncD405 argH entA*	6
AN721	*uncA401 argH entA hemA leu*	Isolated after transduction with AN249 as donor and AN704 as recipient
AN706	*uncB402 argH entA hemA leu*	Isolated after transduction with AN283 as donor and AN704 as recipient
AN723	*uncD405 argH entA hemA leu*	Isolated after transduction with AN285 as donor and AN704 as recipient

Starvation procedure. The cells were harvested, washed once in medium 56, and resuspended in 1 liter of growth medium but without mannitol. After incubation with shaking for 2 h, the cells were harvested, washed twice with 100 mM KCl–2 mM glycyglycine buffer, pH 7.0 (17), and resuspended in the same buffer to a density of 7 to 8 mg (dry weight)/ml.

Measurement of proton translocation. The measurement of proton extrusion by starved cells was carried out by using the apparatus and general method described by Gutowski and Rosenberg (9). Equilibration of the cells to a steady pH and calibration of the pH scale were made by additions of N_2-saturated 20 mM HCl (13). N_2-saturated menadione solution (400 nmol) and air-saturated 100 mM KCl–2 mM glycylglycine buffer, pH 7.0 (42 ng-atoms of O), were added as oxidant pulses. Peak values for calculation of the H^+ concentration were measured directly from the recorder tracing, and corrections for decay of the peaks during formation were made.

Measurement of oxygen uptake. Oxygen uptakes at 37°C were measured as described by Cox et al. (7).

Fluorometric measurement of menadione reduction. The reaction mixture, containing 100 mM KCl–2mM glycylglycine buffer (pH 7.0) and bacterial cells (2 to 4 mg, dry weight) in a final volume of 2.0 ml, was bubbled with N_2 gas for 5 min in a cuvette incubated at 37°C. N_2-saturated additions of substrate were made, the reaction mixture was incubated for an additional 5 min, and the N_2 inlet tube was raised above the level of the cell suspension. The reaction was started by the addition of menadione (400 nmol), and the formation of reduced menadione was measured as the increase in fluorescence at 440 nm with the activation wavelength at 340 nm (15).

Measurement of proline uptake. The cells were washed twice, starved, and then resuspended in medium 56 to a density of 0.15 to 0.2 mg (dry weight)/ml and stored on ice until used. Uptake experiments were performed under nitrogen of high purity, which was further treated to remove traces of oxygen by passage through a double NILOX scrubber supplied by Jemcons, Hemel Hempstead, U.K.

Mannitol, 20 mM, or 10 mM D-lactate was added to 3-ml cell suspensions, which were incubated at 37°C for 7 min. Controls without added substrates were included. The uptake experiment was started by the addition of 10 μM [^{14}C]proline. Samples (0.5 ml) were then withdrawn at 2 and 4 min. After a further 10 s, 1 μmol of menadione was added, and samples were withdrawn at intervals over a period of 3 min. The samples were filtered, washed, and counted for radioactivity as described by Rosenberg et al. (16).

RESULTS

Reduction of menadione by suspensions of cytochrome-deficient cells. A number of artificial electron acceptors were screened for their ability to induce a transient acidification of the medium when added to anaerobic cell suspensions of a normal strain of *E. coli*. Menadione was found to promote such activity (see below), and some characteristics of the menadione reductase activity were investigated.

Strain AN704 carries a mutation in the *hemA* gene, the structural gene concerned with the biosynthesis of 5-aminolevulinic acid. Cytochrome-deficient cells and cytochrome-reconstituted cells were prepared from strain AN704 as described in Materials and Methods. Such cells were then starved in the absence of the carbon source, mannitol, and the rate of reduction of menadione by cell suspensions was measured as the increase in fluorescence due to formation of menadiol, with either mannitol or D-lactate as substrate (Fig. 1, Table 2). The results indicate that the menadione reduction rate, in contrast to the oxidase rate (Table 2), is not affected by the presence or absence of cytochromes. The menadione reductase rate is dependent on the substrate added and is equal to or faster than the oxidase rates. The menadiol formed remained in the suspending medium after removal of the cells by centrifugation, and greater than 90% of the added menadione could be accounted for as menadiol.

Menadione reduction by iron-deficient cells. Strain AN704 has a mutation affecting the *entA* gene in addition to the mutation affecting the *hemA* gene. The *entA* mutation prevents the formation of enterochelin, an iron chelator required for the uptake of iron in *E. coli* (18). The requirement for enterochelin in an *entA* mutant can be satisfied by the addition of the biosynthetic intermediate 2,3-dihydroxybenzoate. The particular mutant *entA* allele in strain AN704, in contrast to that previously described (4), causes an absolute requirement for 2,3-dihydroxybenzoate, and deficient cells were produced by starvation after growth on a limiting concentration of 2,3-dihydroxybenzoate.

Menadione reductase activities with either mannitol or D-lactate as substrate were low in the iron-starved cells compared with normal

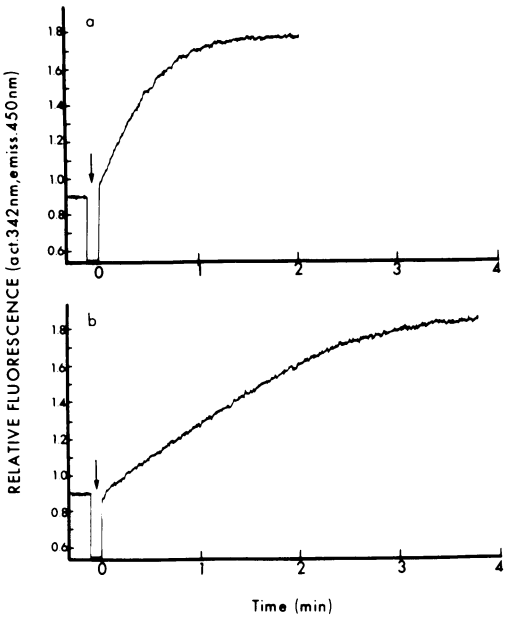

FIG. 1. *Menadione reduction in heme-deficient cells of strain AN704. Cells were incubated with mannitol (a) or* D*-lactate (b), and menadione was added as indicated by the arrows. Fluorescence was measured with the activation wavelength set at 342 nm and the emission wavelength set at 450 nm.*

TABLE 2. *Oxidase and menadione reductase activities in cell suspensions of strain AN704*

Substrate	Menadione reduction (nmol/min per mg [dry wt])		Oxygen uptake (ng-atoms of O/min per mg [dry wt])	
	Cytochrome deficient	Cytochrome reconstituted	Cytochrome deficient	Cytochrome reconstituted
Mannitol	336	354	22	181
D-Lactate	147	157	37	136
Endogenous	14	16	19	34

cells (Table 3). The oxidase activities, as expected, were also lower in the iron-starved cells (Table 3). These results, taken in conjunction with those obtained with the cytochrome-deficient cells, indicate that nonheme iron is required for both the mannitol- and D-lactate-menadione reductase activities in whole cells.

Measurement of proton translocation with menadione as oxidant pulse. Cytochrome-deficient cells of strain AN704 were prepared and tested for menadione-induced proton translocation. Figure 2 illustrates the extent of acidification of the medium on the addition of menadione as the added electron acceptor in the presence of mannitol or D-lactate as substrate. The proton pulse obtained with mannitol as substrate (Fig. 2a) was rapidly collapsed by the

TABLE 3. *Oxidase and menadione reductase activities in iron-starved*[a] *cell suspensions of strain AN704*

Substrate	Menadione reduction (nmol/min per mg [dry wt])		Oxygen uptake (ng-atoms of O/min per mg [dry wt])	
	Iron sufficient	Iron deficient	Iron sufficient	Iron deficient
Mannitol	300	15	211	26
D-Lactate	123	37	238	44
Endogenous	25	19	26	22

[a] Cells were grown in the presence of 5-aminolevulinate.

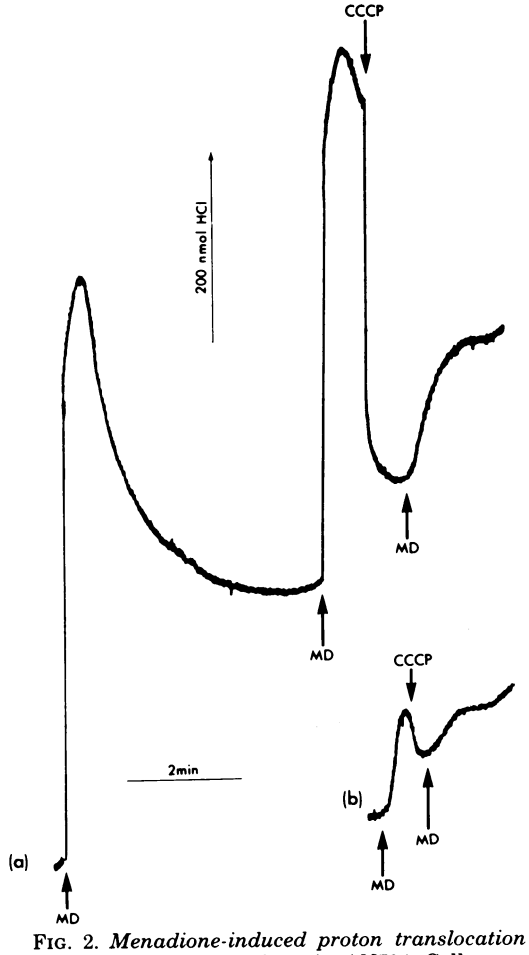

FIG. 2. *Menadione-induced proton translocation by heme-deficient cells of strain AN704. Cells were incubated with mannitol (a) or* D*-lactate (b), and 400 nmol of menadione (MD) was added as indicated. CCCP (100 nmol) was added as indicated.*

addition of carbonylcyanide-*m*-chlorophenylhydrazone (CCCP), but the pH did not completely return to the value prior to the addition of menadione. Further addition of menadione caused acidification only to an extent equivalent to the CCCP-insensitive acidification obtained in the previous pulse. Only a small CCCP-collapsible proton pulse was obtained with D-lactate as substrate (Fig. 2b). The $H^+/2e$ ratio calculated for the CCCP-collapsible portion of the proton pulse with mannitol as substrate gave a value of 1.7 compared with 0.2 for D-lactate.

Measurement of proton translocation with oxygen as oxidant pulse. Cytochrome-reconstituted cells of strain AN704 were prepared as described in Materials and Methods. The reconstituted cells preincubated in the presence of either mannitol or D-lactate were then tested for proton translocation on the addition of air-saturated buffer. An H^+/O ratio of 3.5 was calculated for the CCCP-collapsible proton pulse with mannitol as substrate, and a value of 1.7 was obtained with D-lactate as substrate. These values were obtained in freshly prepared cells, but if the cells were held for 12 h at 4°C, the H^+/O ratio obtained with mannitol as substrate decreased from 3.5 to 2.4. The ratio obtained with D-lactate as substrate remained unchanged and, similarly, the oxidase activities with both substrates were unchanged.

Proline uptake coupled to menadione reduction in cytochrome-deficient cells. Cytochrome-deficient cells were assayed for proline uptake under anaerobic conditions with either mannitol or D-lactate as the oxidizable substrate. Under these conditions no proline uptake was observed (Fig. 3). However, the addition of menadione caused the uptake of proline with mannitol as substrate but not with D-lactate as substrate (Fig. 3). These results are consistent with those obtained in the proton translocation experiments.

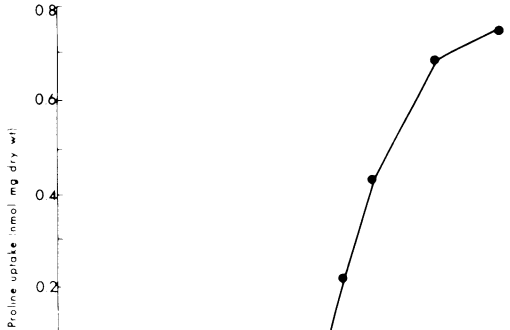

FIG. 3. *Proline uptake under anaerobic conditions by heme-deficient cells of strain AN704. Menadione (1 μmol) was added as indicated. The substrate present was mannitol (●), D-lactate (□), or endogenous (○).*

Proton translocation in cytochrome-deficient cells carrying mutations in various *unc* genes. Strain AN704 carries a mutation in the *ilv* genes which made it possible to prepare the transductant strains AN721 (*uncA401*) AN716 (*uncB402*), and AN723 (*uncD405*). Each of these *unc* mutant alleles causes the loss of oxidative phosphorylation (3). The results obtained with each of the strains was essentially the same, and only those obtained with strain AN721 (*uncA401*) will be presented.

Menadione reductase activities in whole cells of cytochrome-deficient and cytochrome-reconstituted AN721 (*uncA401*) are shown in Table 4. As for strain AN704, the presence or absence of cytochromes had no effect on the menadione reductase activities with either mannitol or D-lactate as substrates. The oxidase activities were cytochrome dependent, and although the D-lactate oxidase rate was similar in reconstituted cells from both strains AN704 and AN721 (*uncA401*), the oxidase activity in the latter strain with mannitol as substrate was about 50% of that in strain AN704 (Tables 2 and 4).

Typical proton pulses obtained in cytochrome-deficient cells of strain AN721 (*uncA401*) are shown in Fig. 4. Acidification of the external medium on the addition of menadione with mannitol as substrate was not affected by the addition of CCCP, in contrast to that observed in cells from strain AN704 (see Fig. 2). Thus, no proton gradient coupled to menadione reduction was generated in cytochrome-deficient cells of uncoupled mutants. This conclusion was supported by the observation that cytochrome-reconstituted cells of strain AN721 (*uncA401*) gave H^+/O ratios of only 1.9 with mannitol as substrate and 1.4 with D-lactate as substrate. In addition, no anaerobic menadione-dependent proline uptake was ob-

TABLE 4. *Oxidase and menadione reductase activities in cell suspensions of strain AN721 (uncA)*

Substrate	Menadione reduction (nmol/min per mg [dry wt])		Oxygen uptake (ng-atoms of O/min per mg of protein)	
	Cytochrome deficient	Cytochrome reconstituted	Cytochrome deficient	Cytochrome reconstituted
Mannitol	244	207	15	90
D-Lactate	187	177	30	136
Endogenous	12	15	14	27

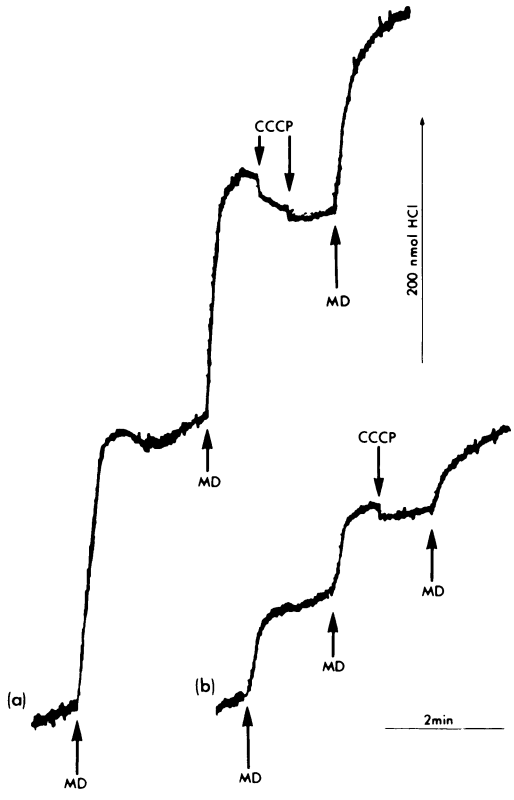

FIG. 4. *Absence of menadione-induced proton translocation by heme-deficient cells of the uncoupled mutant strain AN721 (uncA). As in Fig. 2, the cells were incubated with mannitol (a) or* D*-lactate (b), and 400 nmol of menadione (MD) was added as indicated. CCCP (100 nmol) was added as indicated.*

served with cytochrome-deficient cells of strain AN721 (*uncA401*) (data not shown).

DISCUSSION

Proton translocation by anaerobic cell suspensions of cytochrome-deficient cells can be induced by the addition of menadione in the presence of mannitol but not in the presence of D-lactate. However, it would appear from the data obtained with the *unc* mutants that the proton translocation obtained with mannitol as substrate and menadione as electron acceptor is due to the hydrolysis of ATP by the membrane-bound adenosine triphosphatase. The ATP is apparently produced by substrate-level phosphorylation after the oxidation of NADH (and subsequently mannitol-1-phosphate) that occurs on addition of a pulse of menadione (see above).

A similar situation occurs with the cytochrome-reconstituted cells in that about half the proton translocation obtained with mannitol as substrate on addition of a pulse of oxygen appears to be due to the hydrolysis of ATP by the membrane-bound adenosine triphosphatase. It would seem preferable, therefore, to determine H^+/O ratios in mutant strains lacking adenosine triphosphatase. Caution should be exercised in interpreting data using normal strains, particularly with endogenous substrates or added substrates likely to give substrate-level phosphorylation.

The NADH-menadione reductase activity does not involve cytochromes but does involve nonheme iron as judged by the loss of activity in cells starved of enterochelin. A ubiquinone-deficient strain has been prepared from strain AN704 (8) but is unable to grow on mannitol. Preliminary experiments with ubiquinone-deficient cells grown on glucose would indicate that the NADH-menadione reductase activity does not involve ubiquinone (J. Brookman, unpublished observations). The requirement for nonheme iron would suggest that the menadione is accepting electrons after the interaction of the flavoprotein and nonheme iron associated with the NADH dehydrogenase and, according to the scheme of Haddock and Jones (10), should result in proton translocation. The results reported in the present paper support an alternative scheme recently proposed for *E. coli* in which the NADH dehydrogenase, equivalent to the site 1 region in the mitochondrial system, is not proton translocating (8). The H^+/O ratios obtained in the cytochrome-reconstituted *unc* mutant cells are low for both NADH and D-lactate when compared with values obtained by others for normal strains of *E. coli* (12). However, Haddock and Schairer (11) have shown that cells reconstituted for cytochromes under conditions similar to those used in this paper do not form a normal pattern of aerobic cytochromes but form predominantly cytochromes b_{558} and *d*. As discussed above, there may also be a contribution from the membrane-bound adenosine triphosphatase in the studies using normal strains.

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