ASCORBATE-PHENAZINE METHOSULFATE-DEPENDENT MEMBRANE ENERGIZATION IN RESPIRATORY CHAIN MUTANTS OF ESCHERICHIA COLI

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SUMMARY

Ascorbate with phenazine methosulfate was able to energize the membrane of inside-out membrane vesicles from cytochrome-containing but not cytochrome-deficient cells of the E. coli hem A⁻ mutant SASX76 as measured by the quenching of the fluorescence of acridine dyes. This substrate could also energize vesicle membranes from the ubiquinone-deficient mutant E. coli AN59 in the absence of exogenous ubiquinone. These results suggest that there is site of membrane energization coupled to substrate oxidation in the respiratory chain of E. coli in the cytochrome region between ubiquinone and oxygen.

INTRODUCTION

Ascorbate in the presence of phenazine methosulfate is more effective in energizing active transport of certain solutes in membrane vesicles of E. coli than are the physiological electron donors NADH, D-lactate, succinate and glycerol-3-phosphate (1). The reason for this is unknown. Moreover, the site at which electrons are introduced into the respiratory chain by this artificial system has not been defined. The redox potential of ascorbate (80 mV) suggests that electrons might enter the respiratory chain at the level of ubiquinone or cytochrome b_1 (2). We have examined the energization by ascorbate-PMS of inside-out vesicles from ubiquinone- and cytochrome deficient mutants of E. coli by measuring the energy-dependent quenching of the fluorescence of quinacrine and 9-aminoacridine. The

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Abbreviation: PMS, phenazine methosulfate

fluorescence of these acridine dyes alters with changes in the energized state of phosphorylating membranes of E. coli and other sources (3), and appears to respond to the magnitude of the pH difference across the membrane (3). We have found that ascorbate-PMS can energize the membrane in ubiquinone-deficient but not in cytochrome-deficient vesicles. This indicates that there is a site of membrane energization coupled to substrate oxidation in the respiratory chain of E. coli in the cytochrome region between ubiquinone and oxygen.

METHODS

E. coli SASX76 (formerly SHSP 18) (F^-, hem A^-, met^-, trp^-, lac^-, str^-) (4) and E. coli AN59 (Hfr, thr^-, leu^-, ubi B^-) (5) were generous gifts of Dr. S. Sasarman (University of Montreal, Canada) and Professor F. Gibson (Australian National University, Canberra, Australia), respectively. Both strains were grown aerobically at 37°C in trypticase-soy broth and harvested at the end of the exponential phase of growth. Cytochrome synthesis was induced in E. coli SASX76 by growing in the above medium in the presence of 25 µg/ml of 5-aminolevulinic acid. It was noted that strain AN59 exhibited a high reversion rate to ubi B^+. Therefore, all cultures were checked for ubi B^+ revertants after growth. Only those cultures having 1% revertants, or less, were used to prepare inside-out membrane vesicles.

The preparation of inside-out membrane vesicles (membrane particles) and the measurement of the quenching of the fluorescence of quinacrine and 9-aminoacridine were carried out as previously described (6,7). Protein was determined by the Folin method (8). The reduction of cytochrome b_1 was followed at a wavelength pair of 558-540 nm using a Perkin-Elmer/Hitachi model 356 spectrophotometer operating in the dual wavelength mode.

Ubiquinone-1, a generous gift of Dr. A.F. Wagner (Merck, Sharp and Dohme, Rahway, N.J.), was used as a solution in ethanol.

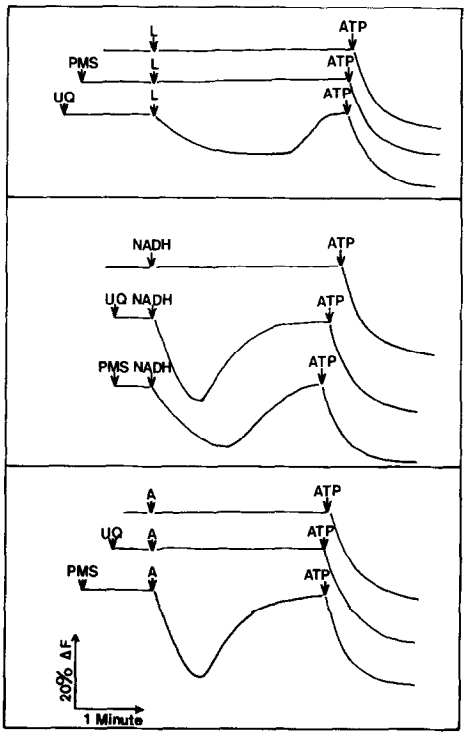

FIGURE 1. Substrate-dependent quenching of the fluorescence of 9-aminoacridine in inside-out membrane vesicles of the ubiquinone-deficient strain E. coli AN59. The reaction mixture in a total volume of 2 ml contained 10 mM HEPES-KOH buffer, pH 7.5, 300 mM KCl, 5 mM $MgCl_2$, 5 μM 9-aminoacridine, and 2.4 mg membrane protein. The concentrations of the other components were: D-lactate (L), 10 mM; NADH, 0.5 mM; ascorbate (A), 10 mM; PMS, 10 μM; ubiquinone-1 (UQ), 25 μM; ATP, 0.5 mM. Fluorescence was excited at 420 nm and the emmission measured at 500 nm.

RESULTS

The fluorescence of 9-aminoacridine was measured in the presence of inside-out vesicles prepared from the ubiquinone-deficient strain of E. coli, AN59. Under our growth conditions about 1% of the vesicles were derived from revertants. Although energy-dependent quenching of fluorescence by ATP could be observed, D-lactate and NADH had no effect on fluorescence unless exogenous ubiquinone-1 was added (Fig. 1). This resulted in the formation of an intact respiratory chain as shown by the restoration of NADH and D-lactate oxidase

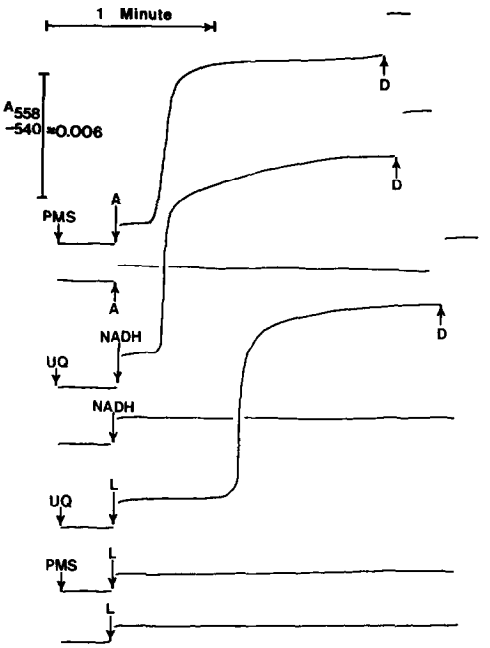

FIGURE 2. Reduction of cytochrome b_1 by substrates in inside-out membrane vesicles of the ubiquinone-deficient strain *E. coli* AN59. The reaction mixture contained 300 mM KCl, 5 mM $MgCl_2$, 4 mg membrane protein/ml, in 10 mM HEPES-KOH buffer, pH 7.5. The concentrations of the other components were: PMS, 10 μM; ubiquinone-1 (UQ), 25 μM; ascorbate (A), 10 mM; NADH, 0.5 mM; D-lactate (L), 10 mM. Cytochrome reduction was measured at a wavelength pair of 558-540 nm.

activities. Then, addition of these substrates caused fluorescence quenching which was reversed when the system became anaerobic. Ubiquinone acted at a site in the respiratory chain prior to cytochrome b_1 as shown by the restoration by exogenous ubiquinone of NADH- and D-lactate-cytochrome b_1 reductase activities (Fig. 2). Ascorbate with PMS caused both quenching of 9-aminoacridine fluorescence and reduction of cytochrome b_1 in the ubiquinone-deficient vesicles in the absence of exogenous ubiquinone (Figs. 1 and 2). Thus, electrons from ascorbate must be introduced into the respiratory chain between ubiquinone and oxygen.

The requirement of a cytochrome chain for the functioning of

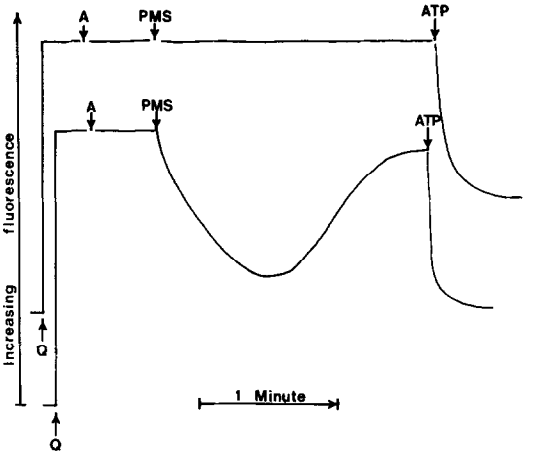

FIGURE 3. Substrate-dependent quenching of the fluorescence of quinacrine in inside-out membrane vesicles of cytochrome-deficient (upper curve) and cytochrome-containing (lower curve) cells of E. coli SASX76. The reaction mixture contained 300 mM KCl, 5 mM $MgCl_2$, 5 μM quinacrine (Q), 0.64 mg membrane protein/ml in 10 mM HEPES-KOH buffer, pH 7.5. The concentrations of the other components were: ascorbate (A), 10 mM; PMS, 10 μM; ATP, 0.5 mM. Fluorescence was excited at 430 nm and the emmission measured at 505 nm.

the ascorbate-PMS system was shown as follows. Cytochrome-deficient inside-out vesicles were prepared from E. coli SASX76 grown in the absence of 5-aminolevulinic acid (6). In a similar manner to D-lactate

TABLE 1. Effect of inhibitors on the initial rate of quenching of quinacrine fluorescence in inside-out vesicles of cytochrome-containing cells of E. coli SASX76 by ascorbate with PMS

Inhibitor	% control rate
KCN, 4 mM	29
Piericidin A, 18 μM	78
36 μM	25
2-Heptyl-4-hydroxy-quinoline N-oxide, 28 μM	37
55 μM	16
Dicumarol, 62 μM	12
Bathophenanthroline, 75 μM	25

and DL-glycerol-3-phosphate (6), ascorbate and PMS, added alone or together, were unable to quench the fluorescence of quinacrine (Fig. 2). However, in cytochrome-containing vesicles prepared from cells grown with 5-aminolevulinic acid ascorbate with PMS caused quenching which was reversed when the system became anaerobic. Quenching was inhibited in cytochrome-containing vesicles by the respiratory chain inhibitors KCN, piericidin A, 2-heptyl-4-hydroxy-quinoline N-oxide, dicumarol and bathophenanthroline (Table 1) which had previously been shown to inhibit the oxidation of ascorbate (2).

DISCUSSION

Electrons from ascorbate appear to enter the respiratory chain of *E. coli* at an electron carrier(s) between ubiquinone and oxygen. Since cytochrome *b*1 can be reduced by this artificial electron donor it is probable that the electrons are introduced at a level close to this cytochrome. Cox *et al.* (9), using the same ubiquinone-deficient strain of *E. coli*, have proposed that ubiquinone is located at two sites in the respiratory chain of this organism, one before cytochrome *b*1 in the sequence, and the other between cytochrome *b*1 and oxygen. Our results show that, in the absence of ubiquinone, cytochrome *b*1 can be reduced by ascorbate with PMS, and that this substrate can be oxidized with resulting energization of the membrane. Thus, either ubiquinone cannot be located in the respiratory chain between cytochrome *b*1 and oxygen, or ascorbate can feed electrons into the chain both at the level of cytochrome *b*1 *and* after the second ubiquinone site. If the last alternative is correct, then there must be a membrane energization site in the region of the respiratory chain between the second ubiquinone site and oxygen. At present, the only components which have been found in this region are cytochromes *o* and *d* (9). It is of interest that Lawford and Haddock (10) have found a site of coupled proton extrusion in the

cytochrome region of the respiratory chain of E. coli. It seems likely that it is this site of energization which is responsible for ascorbate-PMS energized active transport.

ACKNOWLEDGMENT

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