Functional Anaerobic Electron Transport Linked to the Reduction of Nitrate and Fumarate in Membranes from *Escherichia coli* as Demonstrated by Quenching of Atebrin Fluorescence

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Measurements were made of energy-dependent quenching of atebrin fluorescence in membrane particles prepared from *Escherichia coli* grown anaerobically with glycerol as carbon source in the presence of either nitrate or fumarate. It is concluded that this technique can be used to study the functional organization of the anaerobic proton-translocating electron-transport chains that use nitrate or fumarate as terminal electron acceptor.

Under anoxic conditions facultative anaerobes such as *Escherichia coli* derive energy for growth from either sugar fermentation or from oxidative phosphorylation by using fumarate or nitrate as terminal electron acceptor. The characterization of the redox carriers responsible for nitrate reduction has shown that electron transport involves dehydrogenases, a quinone (either ubiquinone or menaquinone; I. G. Young, personal communication), a *b*-type cytochrome (which is synthesized only in the presence of nitrate under anaerobic growth conditions; Ruiz-Herrera & De Moss, 1971), and nitrate reductase (EC 1.7.99.4, a molybdenum-containing iron-sulphur protein; Forget, 1974; MacGregor *et al.*, 1974) as minimal components (Enoch & Lester, 1974). Similar studies on the components responsible for fumarate reduction are not so advanced. Menaquinone (Newton *et al.*, 1971) and fumarate reductase (EC 1.3.99.1, Miki & Lin, 1973) are known to be required, but fumarate reduction is apparently cytochrome independent in *E. coli* (Singh & Bragg, 1975). Studies on the functional organization of the two electron-transport chains terminating in either nitrate reductase or fumarate reductase have shown that, in the absence of oxygen, both are responsible for respiration-dependent proton translocation in whole cells (Brice *et al.*, 1974; Downie, 1974) and can be used for the accumulation of various metabolites in whole cells and in membrane vesicles with an orientation similar to that of the original cell (Konings & Kaback, 1973; Rosenberg *et al.*, 1975).

The quenching of acridine dye fluorescence (Azzone & Massari, 1973) has been used to study the functional organization of the aerobic electron-transport chain (Haddock & Downie, 1974) and the proton-translocating adenosine triphosphatase (EC

3.6.1.3) activity (Nieuwenhuis *et al.*, 1973; Rosen & Adler, 1975) in membrane particles of *E. coli* that possess an inside-out orientation with respect to the original cell. Singh & Bragg (1975) demonstrated NADH-dependent atebrin quenching in the presence of fumarate under anaerobic conditions in membrane particles from a cytochrome-deficient mutant of *E. coli*. In the present paper we extend these studies and demonstrate that the quenching of atebrin fluorescence can be used to investigate the functional organization of the proton-translocating anaerobic electron-transport chains that use either nitrate or fumarate as terminal acceptor.

Materials and Methods

Bacterial strains, growth conditions and preparation of cells and particles

E. coli strain EMG-2 (K12 Ymel, prototroph) was grown in the mineral salts medium of Cohen & Rickenberg (1956) containing glycerol (0.5%, w/v) vitamin-free casamino acids (0.1%, w/v), potassium selenite (1 μ M), ammonium molybdate (1 μ M) and either KNO₃ (0.1 M) or potassium fumarate (50 mM). Growth was in 8-litre volumes of medium in a 10-litre fermentor vessel and anaerobic conditions were maintained by bubbling the cultures with O₂-free N₂ [(about 200 ml/min) 'White Spot' N₂, 99.9% pure; BOC, London S.W.19, U.K.] which had been bubbled through 1 litre of Fieser's solution (Vogel, 1956) and then 1 litre of degassed distilled water to remove traces of O₂. During growth, the temperature was maintained at 37°C and the medium was stirred at 25 rev./min by a Parvalux motor (type M1/112370/30, Parvalux Electric Motors, Bournemouth, Hants., U.K.), to prevent sedimentation of the cells. The pH of the growth medium was not controlled, and

cells were harvested in the late-exponential phase of growth. Particles were prepared from harvested cells as described previously (Haddock & Downie, 1974) in a medium containing 300 mM-KCl, 5 mM-MgCl₂ and 10 mM-2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid-KOH (pH 7.5); this medium was used in all the experiments described below.

Assay techniques

Atebrin fluorescence was measured with an Eppendorf fluorimeter modified as described previously (Haddock & Downie, 1974). The temperature was controlled at 30°C and other additions were made as indicated in the legend to Fig. 1. NADH-nitrate reductase and NADH-fumarate reductase were assayed spectrophotometrically at 340 nm under anaerobic conditions (Kemp *et al.*, 1975); the reaction was initiated by the addition of various concentrations of either nitrate or fumarate. Protein was determined by the method of Lowry *et al.* (1951), with dry bovine plasma albumin as standard.

Reagents

Catalase, NADH (disodium salt), ATP (disodium salt) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone were purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. D-Lactate was from Calbiochem, London W1H 1AS, U.K., and vitamin-free casamino acids were from Difco, Detroit, Mich., U.S.A. Nigericin was a generous gift from Eli Lilly and Co., Indianapolis, Ind., U.S.A. All other reagents were from BDH Chemicals, Poole, Dorset, U.K., and were of the highest available purity.

Results and Discussion

Atebrin fluorescence in particles prepared from cells grown in the presence of nitrate

Electron-transport-induced quenching of atebrin fluorescence in particles prepared from *E. coli* grown anaerobically in the presence of nitrate is shown in Fig. 1(a). After the addition of atebrin to the reaction cuvette, the first addition of formate caused a rapid but short-lived quenching of the fluorescence, which is probably due to the presence of low concentrations of O₂ in the incubation medium, since a second addition of formate elicited no such change. From Fig. 1(a) it can be concluded that energy-dependent atebrin quenching in these particles could be achieved in three ways: (a) by anaerobic electron-transport-dependent nitrate reduction, which was sensitive to low concentrations of azide (10–20 μM) sufficient to inhibit specifically nitrate reductase activity (Forget, 1974; MacGregor *et al.*, 1974); (b) by aerobic electron transport by using a KCN-sensitive cytochrome oxidase; and (c) by

ATP hydrolysis. In all three cases the energy-dependent atebrin quenching was sensitive to detergents (Triton X-100, 0.04%, v/v), uncouplers (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Heytler, 1963), nigericin (10 μg/ml) in the presence of K⁺ (300 mM) and NH₄⁺ (5 mM), suggesting that atebrin distributes between the inner and outer compartments of the particles in response to a pH gradient rather than a membrane potential (see Haddock & Downie, 1974).

Both the nitrate- and H₂O₂-dependent atebrin quenching in these particles could be induced by the oxidation of formate (1 mM), NADH (1.3 mM), D-lactate (5 mM) or DL-α-glycerophosphate (5 mM), although the rate and extent of quenching was always lower with nitrate as terminal electron acceptor. The rate and extent of nitrate-dependent atebrin quenching was also dependent on the amount of nitrate added over a given range and the concentration of nitrate required for a half-maximal change in the extent of NADH-induced atebrin quenching (60–120 μM in three separate particle preparations) was similar to the concentration of nitrate required for half-maximal rates of nitrate-dependent anaerobic NADH oxidation (25–65 μM) assayed spectrophotometrically.

These experiments demonstrate that the oxidation of NADH, formate, D-lactate and DL-α-glycerophosphate in these particles is associated with a proton-translocating electron-transport chain that functions anaerobically and uses nitrate as terminal electron acceptor. In addition these electron donors can donate reducing equivalents to an aerobic electron-transport chain via their respective dehydrogenases in these particles. Previous work has indicated that, of these electron donors, only formate-dependent nitrate reduction was capable of driving lactose uptake under anaerobic conditions in vesicles with an outside-out orientation with respect to the original cell (Konings & Kaback, 1973). The reason for this discrepancy is not known but may be due, at least in part, to the relative orientation of the two particle preparations and the permeability properties of the membrane, which limit access of certain electron donors to their respective dehydrogenases, as discussed by Hare *et al.* (1974).

Atebrin fluorescence in particles prepared from cells grown in the presence of fumarate

Energy-dependent atebrin quenching in particles prepared from cells grown anaerobically with added fumarate as terminal electron acceptor is shown in Fig. 1(b). Such energy-dependent quenching could be achieved, as before, in three ways: (a) by anaerobic electron-transport-dependent fumarate reduction, a process which was KCN-insensitive (results not shown); (b) by aerobic electron trans-

Fig. 1. Atebrin fluorescence in particles from *E. coli* grown anaerobically in the presence of either nitrate or fumarate

Particles used in traces (a) and (b) were from strain EMG-2 grown anaerobically in the presence of either nitrate or fumarate respectively and were prepared as described under 'Materials and methods'. Fluorescence measurements were performed as indicated under 'Materials and methods' in the presence of either 1.4 mg of particle protein/ml (trace a) or 1.7 mg of particle protein/ml (trace b): in both cases catalase (EC 1.11.1.6, 50000 units/ml) was also present at the start of the experiment. Additions were made as indicated by the arrows: atebrin (3.3 μ M), potassium formate (each addition 1 mM), KNO_3 (each addition 33 μ M), NaN_3 (7 μ M), H_2O_2 (about 1 mM), KCN (3 mM), ATP (2 mM), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, 1 μ M), NADH (each addition, 1.3 mM), potassium fumarate (first addition 1 mM, second addition 0.1 mM), and nigericin (3 μ g/ml).

port by using a KCN-sensitive cytochrome oxidase; and (c) by ATP hydrolysis. Interestingly, in three separate particle preparations only NADH and formate were able to act as electron donors for fumarate-dependent atebrin quenching in all preparations. In none of these preparations was D-lactate able to serve as a donor for fumarate-

dependent quenching though in all cases O_2 -dependent quenching was observed. DL- α -Glycerophosphate served as a donor for fumarate-dependent quenching with only one particle preparation, but in all cases O_2 -dependent quenching was observed. These results are in contrast with those reported by Konings & Kaback (1973) where oxidation of DL- α -

glycerophosphate and D-lactate, coupled to fumarate reduction under anaerobic conditions, could be used to drive lactose accumulation in vesicles with a similar orientation to the original cell: the use of NADH or formate as electron donor for such transport was not reported. The possible reason for this discrepancy is not known but obviously cannot be explained in terms of a difference in the orientation of the two types of particle preparation. Indeed, in the particles used here, there is no permeability barrier for either the electron donors (e.g. DL- α -glycerophosphate or D-lactate) or the electron acceptor (fumarate) since all the substrate-binding sites, associated with the various enzymes involved, should be on the outer surface of these inverted particles. One possible explanation for the observation that DL- α -glycerophosphate oxidation can drive O₂-dependent atebtrin quenching but not fumarate-dependent atebtrin quenching in all particle preparations lies in the nature and properties of the primary dehydrogenase involved. *E. coli* can synthesize at least three L- α -glycerophosphate dehydrogenases under appropriate growth conditions: (a) a membrane-bound flavin-linked dehydrogenase synthesized during aerobic growth conditions (EC 1.1.99.5; Koch *et al.*, 1964); (b) a soluble flavin-linked dehydrogenase synthesized during anaerobic growth conditions in the presence of fumarate (EC 1.1.99.-; Kistler *et al.*, 1969); and (c) NADP⁺-linked biosynthetic dehydrogenase which serves to produce L- α -glycerophosphate from dihydroxyacetone phosphate for phosphatidic acid biosynthesis (EC 1.1.1.8; Kito & Pizer, 1968). By using appropriate mutants it has been shown that dehydrogenase (b) is required to support anaerobic growth on glycerol with fumarate as terminal acceptor (Kistler & Lin, 1971) and can be purified as an enzyme complex with fumarate reductase (Miki & Lin, 1973); either enzyme (a) or enzyme (b) will allow anaerobic growth on glycerol with nitrate as terminal acceptor (Kistler & Lin, 1971). It seems likely, therefore, that the soluble dehydrogenase (b) is associated loosely with the membrane so that only the membrane-bound dehydrogenase (a), which can donate electrons to the O₂⁻ and nitrate-dependent but not the fumarate-dependent electron-transport chain, is retained as a routine under the conditions used for the preparation of particles with an inside-out orientation with respect to the original cell, but both are retained in vesicles with an orientation similar to that of the original cell.

The reason why D-lactate oxidation can drive O₂- but not fumarate-dependent atebtrin quenching is not known, and only one flavin-linked enzyme has been reported to catalyse the conversion of D-lactate into pyruvate in *E. coli* (Kline & Mahler, 1965), but it could possibly be due to a difference in the rates of D-lactate oxidase and D-lactate-fumarate oxidoreductase activities in these particles: a similar

argument could, of course, also serve to explain the observations with DL- α -glycerophosphate.

Although there is still some doubt whether the oxidation of DL- α -glycerophosphate and D-lactate can drive fumarate-dependent atebtrin quenching in these particles, oxidation of NADH and formate can be used for this purpose. The rate and extent of atebtrin quenching observed with both these electron donors was dependent on the amount of fumarate added over a given range, as shown in Fig. 1(b), and the concentration of fumarate required for a half-maximal change in the extent of NADH-induced atebtrin quenching (145–205 μ M in three separate particle preparations) was similar to the concentration of fumarate required for half-maximal rates of fumarate-dependent anaerobic NADH oxidation (110–150 μ M) assayed spectrophotometrically.

It has been concluded previously (Nieuwenhuis *et al.*, 1973; Haddock & Downie, 1974; Rosen & Adler, 1975) that the quenching of acridine dye fluorescence is a specific and sensitive assay for the sidedness and organization of various energy-linked functions in particles that possess an inside-out orientation with respect to the original cell. We have extended the preliminary studies of Singh & Bragg (1975) and demonstrated here that the technique can be applied to a physiological study of anaerobic electron transport linked to the reduction of either nitrate or fumarate, even though higher concentrations of lipophilic anions such as nitrate (>10 mM) have been shown to inhibit O₂-dependent atebtrin quenching in particles prepared from mammalian mitochondria (Dell'Antone & Azzone, 1974). In particular these results indicate that NADH-, formate-, D-lactate- and DL- α -glycerophosphate-dependent nitrate reduction and NADH- and formate-dependent fumarate reduction are associated with the generation of a transmembrane pH gradient which could be used to drive ATP synthesis by the reversal of the membrane-bound adenosine triphosphatase activity (West & Mitchell, 1974). In addition the results obtained for DL- α -glycerophosphate-dependent atebtrin quenching raise the possibility that the two flavin-linked dehydrogenases interact with distinct redox carriers in the membrane, implying a different functional organization and providing alternative routes for DL- α -glycerophosphate oxidation with different efficiencies of oxidative phosphorylation.

Note Added in Proof (Received 10 October 1975)

The initial studies on anaerobic transport in *E. coli* membrane vesicles (Konings & Kaback, 1973) have now been extended (Boonstra *et al.*, 1975) to include anaerobic electron-transport-dependent amino acid, as well as lactose, accumulation. The ability of various electron donors to stimulate active transport has also been re-examined and the results obtained

are now in general agreement with those reported in this paper. We thank Dr. Konings for making available a preprint of his manuscript.

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