The Reconstitution of Functional Respiratory Chains in Membranes from Electron-Transport-Deficient Mutants of *Escherichia coli* as Demonstrated by Quenching of Atebrin Fluorescence

By BRUCE A. HADDOCK* and J. ALLAN DOWNIE†

Department of Biochemistry, Medical Sciences Institute, University of Dundee,
Dundee DD1 4HN, U.K.

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Measurements were made of electron-transport-dependent quenching of atebrin fluorescence in particles prepared from mutants of *Escherichia coli* unable to synthesize either ubiquinone or haem. Such quenching was either absent (in haem-deficient particles) or decreased (in ubiquinone-deficient particles), but was restored under conditions used to reconstitute oxidase activities. It is concluded that the reconstitution of oxidase activity, in both cases, is associated with the formation of a functional, proton-translocating, respiratory chain.

Mutants of *Escherichia coli* unable to synthesize either ubiquinone (Cox *et al.*, 1970; Newton *et al.*, 1972) or haem (Haddock & Schairer, 1973; Haddock, 1973) have been described in which the oxidation of a variety of substrates, using O₂ as terminal electron acceptor, is impaired. The subsequent reconstitution of oxidase activities in membrane preparations from such mutants has been achieved either by the addition of ubiquinone-1, for ubiquinone-deficient mutants, or by incubating the membranes with haematin and ATP, for haem-deficient mutants. Most of these reconstitution studies have been concerned simply with demonstrating an increase in oxidase activity, and little attention has been directed towards assessing if such reconstituted activity is functionally similar to that shown by particles from the parental strain, although the ubiquinone-1-deficient reconstitution of energy-linked NAD⁺ reduction, in membranes from a ubiquinone-deficient mutant, has been reported (Poole & Haddock, 1974).

The quenching of acridine dye fluorescence has been shown to alter with changes in the energized state of phosphorylating membrane preparations from many sources (Azzone & Massari, 1973), including particles from *Azotobacter vinelandii* (Eilermann, 1970) and *E. coli* (Nieuwenhuis *et al.*, 1973). Although there is still some disagreement as to whether this phenomenon is related to the distribution of the dye across the membrane in response to the formation of a transmembrane pH gradient or to the appearance of dye-binding sites on energization of the membrane, it is clear that the quenching of acridine dye fluorescence is a property of topologically closed vesicles, that possess an inside-out orientation

with respect to the original cell, capable of oxidative phosphorylation (Azzone & Massari, 1973; Nieuwenhuis *et al.*, 1973). We have therefore used electron-transport-dependent quenching of atebrin fluorescence as an indicator for the reconstitution of a functional, proton-translocating, respiratory chain in particles prepared from two electron-transport-deficient mutants of *E. coli*.

Materials and methods

Bacterial strains, growth conditions and preparation of cells and particles. *E. coli* strain EMG-2 (K12Ymel, prototroph) was grown in the glycerol medium described by Lawford & Haddock (1973). The haem-deficient strain A1004a (*hem* A⁻; Haddock, 1973) and the ubiquinone-deficient strain AN59 (*ubi* B⁻; Cox *et al.*, 1970) were grown as described by Poole & Haddock (1974).

Particles were prepared from harvested cells by the method of Haddock (1973) except that all procedures were done in a medium containing 300 mM-KCl, 15 mM-MgCl₂ and 10 mM-2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid-KOH (pH 7.5), rather than the medium described previously. For the measurement of proton translocation in strain AN59, whole cells were prepared by the procedure of Lawford & Haddock (1973).

Assay techniques. Atebrin fluorescence was measured with an Eppendorf fluorimeter modified as described by Light & Garland (1971) (filters: excitation 449 nm, emission 500–3000 nm) 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) with H₂O₂ (about 1 mM), catalase (EC 1.11.1.6, 50000 units/ml), atebrin (3.3 μM) and particles (0.3–0.7 mg of protein/ml). The temperature of the reaction was 30°C and

* To whom correspondence should be addressed.

† Present address: Department of Radiation Biology and Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, N.Y. 14642, U.S.A.

Fig. 1. Atebrin fluorescence in particles from various *E. coli* strains

Particles used in traces (a), (b) and (c) were from strains EMG-2, AN59 and A1004a respectively, and were prepared as described under 'Materials and methods'. The reconstituted particles from strain A1004a used in trace (d) were made by incubating particles prepared for trace (c) at 1.3 mg of protein/ml with haematin ($6\mu\text{M}$) and ATP (20 mM) for 30 min at 37°C as described by Haddock (1973); after this time the particles were diluted 20-fold in the buffer used for particle preparation and re-centrifuged at $225\,000g$ for 60 min. Fluorescence measurements were performed as described under 'Materials and methods' with the following concentrations of particles (mg of protein/ml): trace (a), 0.4, (b) 0.7, (c) 0.4 and (d) 0.3. Additions were made as indicated by the arrows: atebrin ($3.3\mu\text{M}$), NADH (0.7mM), KCN (3mM), ATP (2 mM), dio 9 ($6\mu\text{g/ml}$), ubiquinone-1 (UQ_1 , $2\mu\text{M}$) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, $1\mu\text{M}$). At point A in trace (b) the decrease in atebrin quenching was caused by the exhaustion of NADH. The NADH oxidase activities, assayed in parallel polarographic experiments at 30°C , in ng-atoms of O/min per mg of protein were: trace (a) 410; (b) before addition of ubiquinone-1 30, after addition of ubiquinone-1 450; (c) 42; and (d) 450.

other additions were made as indicated in the legend to Fig. 1.

Measurements of proton translocation and O_2 uptake and protein determinations were performed as described by Lawford & Haddock (1973), except that all experiments with particles were performed in the buffer used for their preparation as indicated above, and the apparatus of Lawford & Garland (1973) was used for the proton-translocation studies.

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. Valinomycin and D-lactate (lithium salt) were from Calbiochem Ltd., London W1H 1AS, U.K., and dio 9 was obtained from Gist Brocades NV, Delft, The Netherlands. Ubiquinone-1 was a generous gift from Hoffmann-La Roche, Basel, Switzerland, and nigericin was a generous gift from Eli Lilly and

Co., Indianapolis, Ind., U.S.A. All other reagents were from BDH Chemicals Ltd., Poole, Dorset, U.K., and were of the highest available purity.

Results and discussion

Electron-transport-induced quenching of atebrin fluorescence in particles prepared from the prototrophic strain EMG-2 resembles in its characteristics the phenomenon previously described in other systems (Fig. 1a, and results not shown). The steady-state amount of quenching is dependent on the amount of membrane added, but reaches a maximum around 75–85%. Also the rate and extent of electron-transport-induced atebrin quenching alter depending on the substrate added, e.g. NADH, D-lactate or succinate, in a manner that reflects the relative oxidase activity of the particular substrate; thus NADH is oxidized much faster than other substrates

and the rate and extent of quenching are larger. Electron-transport-induced atebtrin quenching is sensitive to detergents (Triton X-100, 0.04%, v/v), uncouplers (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, 1 μ M; Heytler, 1963), electron-transport inhibitors (KCN, 3 mM), nigericin (10 μ g/ml) in the presence of K⁺ ions (300 mM), and NH₄⁺ ions (5 mM). Valinomycin (10 μ g/ml) plus K⁺ ions (300 mM) stimulates the rate and, less markedly, the steady-state amount of quenching. The quenching of atebtrin fluorescence is also induced by ATP and is affected by all the conditions described above except that in this case the quenching is insensitive to electron-transport inhibitors and blocked or reversed by inhibitors (Roisin & Kepes, 1973) of the proton-translocating adenosine triphosphatase (EC 3.6.1.3) activity of particles, e.g. dio 9 (Fig. 1*a*) and dicyclohexylcarbodi-imide (100 μ M, results not shown). These observations confirm that the quenching of atebtrin fluorescence is dependent on an energized state of the membrane that is induced either by electron transport or by ATP hydrolysis. More specifically, the characteristics of the response in the presence of ionophorous antibiotics or NH₄⁺ ions suggest that atebtrin distributes between the inner and outer compartments of the particles, at least qualitatively, in response to a pH gradient rather than a membrane potential (Schuldiner *et al.*, 1972; Melandri *et al.*, 1972).

Energy-dependent atebtrin quenching in particles prepared from the ubiquinone-deficient strain AN59 is shown in Fig. 1(*b*). Although such particles exhibit an ATP-dependent quenching similar to that observed with particles from the wild-type strain EMG-2 (Fig. 1*a*), it is apparent that both the rate and extent of electron-transport-dependent quenching are much decreased and may be stimulated extensively by the subsequent addition of ubiquinone-1. There is a linear relationship between the extent of the ubiquinone-1-dependent atebtrin quenching and the ubiquinone-1-dependent reconstitution of NADH oxidase activity by particles, both values being maximal at about 4 nmol of added ubiquinone-1/mg of protein (results not shown).

The electron-transport-dependent atebtrin quenching in the absence of added ubiquinone-1 is the particularly interesting feature of Fig. 1(*b*), since it has been concluded previously that electron transport in strain AN59 cannot be coupled either to phosphorylation with oxygen as terminal electron acceptor, as judged by comparison of molar growth yields (Cox *et al.*, 1970), or to the accumulation of galactose (Parnes & Boos, 1973), serine and P_i (Cox & Gibson, 1974). As an independent assay for functional electron transport in whole cells of strain AN59, the technique of respiration-driven proton translocation was employed (Lawford & Haddock, 1973). If an anaerobic whole-cell suspension was

'pulsed' with O₂ an uncoupler-sensitive transient acidification of the suspending medium was observed. The apparent $\rightarrow$ H⁺/O quotient was 2.3 ± 0.28 (s.d., seven values) with endogenous substrates, and was unaffected by the addition of L-malate, which had no effect on the oxidase activity, or D-lactate, which stimulated the oxidase rate from 15 ng-atoms of O/min per mg of protein to 20 ng-atoms of O/min per mg of protein. The addition of ubiquinone-1 (10 nmol/mg of protein) stimulated the oxidase activities due to endogenous substrates or D-lactate by 50%, and the apparent rate of proton translocation with D-lactate as substrate from 304 ng-ions of H⁺/min per mg of protein to at least 890 ng-ions of H⁺/min per mg of protein (limited by the mixing time of the pH-measuring system; Lawford & Garland, 1973), but had no effect of the $\rightarrow$ H⁺/O quotient.

Thus two independent experimental approaches indicate that the respiratory chain of strain AN59 is still functionally organized and capable of translocating protons; however, this activity does not contribute significantly to the cell's metabolism either in generating ATP by oxidative phosphorylation (Cox *et al.*, 1970) or in the accumulation of nutrients (Parnes & Boos, 1973; Cox & Gibson, 1974). It has been shown previously that the ubiquinone content of strain AN59 is less than 1% of that of the parental strain, but that the menaquinone content is increased fourfold (Cox *et al.*, 1970). Thus it is not clear if the proton-translocating ability of the respiratory chain of strain AN59 is due to residual ubiquinone or to a partial replacement of ubiquinone by menaquinone in electron transport.

Energy-dependent atebtrin quenching in particles prepared from the haem-deficient strain A1004a is shown in Figs. 1(*c*) and 1(*d*). Although an ATP-dependent quenching is observed, electron-transport-dependent quenching with either NADH (Fig. 1*c*) or D-lactate (results not shown) as donor is absent. We have been unable to demonstrate O₂-dependent proton translocation in whole cells from strain A1004a (Haddock *et al.*, 1974), so it may be concluded that, in the absence of cytochromes, the respiratory chain of aerobically grown *E. coli* is completely non-functional. In agreement with this, Singh & Bragg (1974) have reported the absence of electron-transport-dependent phenylalanine accumulation in another haem-deficient mutant.

If particles from strain A1004a are incubated under conditions resulting in the reconstitution of oxidase activity and substrate-reducible cytochromes (Haddock, 1973), then these reconstituted particles demonstrate KCN-sensitive, electron-transport-dependent, atebtrin quenching with either NADH (Fig. 1*d*) or D-lactate (results not shown) as donor. Thus the reconstitution of oxidase activity and cytochromes results in the formation of a functional respiratory chain capable of proton translocation.

It may be concluded from the data presented here, and by Nieuwenhuis *et al.* (1973) using 9-amino-6-chloro-2-methoxyacridine, that energy-linked acridine-dye-fluorescence quenching may be used as a sensitive, and rapid, qualitative indicator for the ability to reconstitute either a functional respiratory chain or adenosine triphosphatase activity in membranes from a variety of oxidative-phosphorylation-deficient mutants of *E. coli*.

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