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Short Communications

Respiration-Driven Proton Translocation in *Escherichia coli*

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Measurements were made of the stoicheiometry of respiration-driven proton translocation coupled to the oxidation of NAD(P)-linked or flavin-linked substrates in intact cells of *Escherichia coli*. Observed stoicheiometries ($\rightarrow H^+/O$ quotient; Mitchell, 1966) were approx. 4 with L-malate as substrate and approx. 2 for succinate, D-lactate and glycerol oxidation. It is concluded that the potential number of equivalent energy-conservation sites associated with the respiratory chain is 2 in aerobically grown cells of *E. coli* harvested during the exponential phase of growth.

Mitchell's (1966) chemiosmotic hypothesis proposes that the respiratory carriers and enzymes of the energy-transducing membrane of both mitochondria and bacteria are so arranged that electron flow through the respiratory chain during respiration is obligatorily coupled to the translocation of protons (written $\rightarrow H^+$) across the membrane. The number of protons translocated per pair of reducing equivalents transferred along a span of the respiratory chain (i.e. $\rightarrow H^+/2e^-$ quotient) equivalent to a single energy-conservation site (Lehninger, 1966) is 2.0. Evidence supporting this conclusion comes primarily from experiments in which the length of the respiratory chain under study has been altered by using (1) different oxidants (Mitchell & Moyle, 1967*b*; Lawford & Garland, 1972), (2) different reductants (Mitchell & Moyle, 1967*b*; Lawford & Garland, 1973) or (3) mitochondria from phenotypic variants of *Candida utilis* possessing modified respiratory chains (Downie & Garland, 1973).

Transient acidification of the suspending medium, associated with short bursts of respiratory activity when anaerobic bacterial suspensions were 'pulsed' with O_2, has been observed for *Micrococcus denitrificans* (Scholes & Mitchell, 1970) and *Staphylococcus aureus* (Jeacocke *et al.*, 1972). However, in analogous experiments with *Escherichia coli* proton translocation was either not observed (Wimpenny, 1970) or was characterized by a significantly lower $\rightarrow H^+/O$ ratio than has been observed in other bacterial systems (West & Mitchell, 1972). In none of these studies was any attempt made to define the substrate being oxidized. An additional problem associated with the use of *E. coli* is the ability of this bacterium to form a variety of membrane-bound electron-transport carriers depending on the conditions chosen for growth (Kamen & Horio, 1970; Horio & Kamen, 1970; White & Sinclair, 1971).

In the present work we have measured respiration-driven proton translocation associated with the oxidation of different added substrates in *E. coli*, enabling us to distinguish two energy-conservation sites associated with the aerobic respiratory chain, which in this organism is composed of ubiquinone, cytochrome b_{556} and cytochrome *o* as the terminal electron acceptor (Haddock & Schairer, 1973).

Materials and methods

Bacterial strain, growth conditions and starvation procedure. E. coli strain EMG-2 (K12 Ymel, prototroph) was provided by Dr. M. Peacey (Department of Molecular Biology, University of Edinburgh, Edinburgh, U.K.). The cells were grown aerobically at 37°C in a mineral salts medium (Cohen & Rickenburg, 1956) containing vitamin-free casamino acids (0.1%, w/v) and either L-malate (0.5%, w/v) or glycerol (0.5%, w/v). The cells were harvested during early exponential phase of growth ($E_{420} \leqslant 0.35$; 10mm light-path), washed twice with mineral salts medium lacking any carbon source and resuspended to a final protein concentration of approx. 0.1 mg/ml in this medium. The cells were incubated in 2-litre flasks at 37°C for 2h with vigorous shaking to starve them of endogenous substrates. The cells were reharvested by centrifugation, washed twice with 150mM-KCl and resuspended to a final protein concentration of 25–35 mg/ml.

Measurement of proton translocation. pH measurements were made, with apparatus as described by Lawford & Garland (1972), in 1.0ml of N_2-saturated

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100mM-KCl-50mM-KSCN-1.5mM-glycylglycine buffer, pH7.0, at 25°C with approx. 2–4mg of bacterial protein; further details are given in the legend to Fig. 1. Calibration of the pH scale was accomplished with additions of 5mM-HCl in 150mM-KCl. The peak value for the H^+ concentration was measured directly from the recorder tracing, and correction for decay of this peak during its formation was not made. The O_2 concentration of air-saturated 150mM-KCl was taken to be 0.47μg-atom of O/ml (Chappell, 1964).

Reagents. Carbonyl cyanide *m*-chlorophenylhydrazone (Heytler, 1963), valinomycin and D-lactic acid (lithium salt) were from Calbiochem Ltd., London W1H 1AS, U.K., and vitamin-free casamino acids were from Difco Laboratories, Detroit, Mich., U.S.A. All other reagents were from British Drug Houses Ltd., Poole, Dorset, U.K., and were of the highest available purity.

Other assays. Polarographic measurements of O_2 uptake were made with a Clark oxygen electrode (type YSI 4004; Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.) calibrated as described by Chappell (1964). Protein was assayed by the method of Lowry *et al.* (1951), with dry bovine serum albumin as standard.

Results and discussion

We observed that the rate and extent of proton ejection and the rate of proton back-flow were enhanced if the cells were treated with valinomycin and suspended in a KSCN-containing medium. In addition, we noted that starving the cells appeared to preclude the necessity of a Tris–EDTA preconditioning procedure (Pavlasová & Harold, 1969), as employed by West & Mitchell (1972) to render the cells susceptible to valinomycin and hence increase their K^+ permeability.

Fig. 1 shows a typical proton 'pulse' achieved with intact starved cells of *E. coli*. The acidification of the suspending medium is transient and decays exponentially with a half-time of 80s. Addition of a proton-conducting agent such as carbonyl cyanide *m*-chlorophenylhydrazone (Mitchell & Moyle, 1967*a*) to the cell suspension shortly after an O_2 'pulse' results in a rapid collapse of ΔpH, and subsequent injections of O_2 fail to elicit a proton 'pulse' (Fig. 1).

Poisoning of the respiratory chain with KCN resulted in a dramatic lowering of the $\rightarrow H^+/O$ ratio. That proton translocation was not completely abolished may reflect the relative inefficiency of cyanide as a respiratory inhibitor in intact cells.

In the absence of suitable respiratory inhibitors

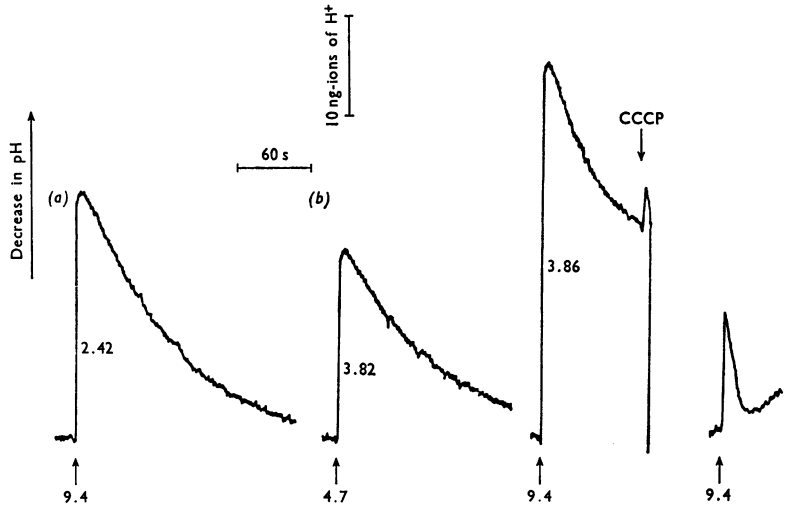

Fig. 1. *pH recordings of proton translocation in response to O_2 'pulses' by anaerobic suspensions of E. coli*

The pH-measuring apparatus was as described by Lawford & Garland (1972). Cells were grown on L-malate as sole carbon source, harvested during early exponential phase of growth and starved as described in the text under 'Materials and methods'. Cells (3.2mg of bacterial protein) were added to 1.0ml of N_2-saturated 100mM-KCl–50mM-KSCN–1.5mM-glycylglycine buffer, pH7.0, at 25°C containing valinomycin (5μg/ml) and in (*a*) 1mM-succinate and in (*b*) 1mM-L-malate as their potassium salts. Additions of air-saturated 150mM-KCl were made (indicated by arrows), after 15min incubation, by injection with a micro-syringe, and the amount of O_2 (ng-atoms of O) added in each 'pulse' is indicated. In (*b*) the addition of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; 4.0nmol) is indicated by an arrow. The $\rightarrow H^+/O$ ratio is shown for each O_2 'pulse'.

Table 1. *Observed $\rightarrow H^+/O$ ratios for E. coli cells respiring different added substrates*

Apparatuses used to measure O_2 uptake and pH of cell suspensions are described in the text under 'Materials and methods'. The values for $\rightarrow H^+/O$ shown are presented as the means ±S.E.M., with the numbers of observations in parentheses; N.T., not tested.

Carbon source for growth	Substrate added	O_2 uptake (ng-atoms of O/min per mg of protein)	Observed $\rightarrow H^+/O$ ratio
L-Malate	—	86	3.36±0.32 (6)
	L-Malate	600	3.86±0.12 (10)
	Succinate	1000	2.42±0.06 (4)
	D-Lactate	530	2.26±0.08 (4)
Glycerol	—	58	3.53±0.22 (6)
	Glycerol	800	2.36±0.12 (6)
	L-Malate	315	3.78±0.08 (8)
	D-Lactate	420	N.T.

to ensure that we were observing proton translocation associated with the oxidation of a specific added substrate, the cells were starved for a period of 1–2 h before pH measurements were made. That such starved cells were indeed depleted of substrate is demonstrated by their low rate of endogenous respiration (Table 1). Rates of O_2 uptake were measured to test the ability of the starved cells to oxidize substrates different from the carbon source used to support growth (Table 1). An interesting finding was that the $\rightarrow H^+/O$ quotient altered depending on the substrate being utilized by the cells. Table 1 demonstrates that the oxidation of the flavin-linked substrates such as succinate (Sedar & Burde, 1965), D-lactate (Kline & Mahler, 1965) and glycerol 3-phosphate (Kung & Henning, 1972) yielded an $\rightarrow H^+/O$ ratio of approx. 2 whereas the oxidation of an NAD(P)-linked substrate such as L-malate (Courtright & Henning, 1970) produced a value for $\rightarrow H^+/O$ ratio close to 4. More variable results were obtained with cells respiring endogenous substrates.

These observations clearly establish that *E. coli* possesses an outwardly directed proton-translocating respiratory chain. Assuming a value for the $\rightarrow H^+/2e^-$ ratio of 2 per energy-conservation site (Mitchell & Moyle, 1967*b*), our observations with *E. coli* suggest that only two energy-conservation sites are associated with the respiratory chain along the span from NAD(P) to O_2. Further, one of these sites must be located in the region between the junction of the flavin-linked dehydrogenases (e.g. succinate dehydrogenase, EC 1.3.99.1, or D-lactate dehydrogenase, EC 1.1.99.–) with the respiratory chain and the terminal oxidase, cytochrome *o*.

Alternative methods exist for assessing the number of energy-conservation sites in *E. coli*. From molar growth yield experiments Harrison & Loveless (1971) concluded that two energy-conservation sites are present under aerobic growth conditions, although direct measurements of phosphorylation coupled to NAD(P)H oxidation in *E. coli* suggest three phosphorylation sites under these conditions (Hempfling, 1970). More recently both these approaches have been criticized (Stouthamer & Bettenhaussen, 1973; van der Beek & Stouthamer, 1973). Indeed, the concept of assessing the number of potential energy-conservation sites in terms of phosphate esterification must be reconsidered in view of the demonstration that mutants of *E. coli* defective in the membrane-bound Mg^{2+}-dependent adenosine triphosphatase (EC 3.6.1.3) are still able to accumulate thiomethyl galactoside (Schairer & Haddock, 1972) or proline (Klein & Boyer, 1972; Simoni & Shallenberger, 1972; Berger, 1973) by using a proton gradient established by the respiratory chain directly. We believe that the technique used in the present work is a more direct method for measuring the number of energy-conservation sites in bacteria, although we realize that our conclusions rest on the assumption that 2 protons are translocated per pair of reducing equivalents transferred along a span of the respiratory chain, as has been demonstrated in mitochondria.

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