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Proton Translocation Coupled to Electron Flow from Endogenous Substrates to Fumarate in Anaerobically Grown *Escherichia coli* K12

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Observed $\rightarrow H^+/2e^-$ values for proton translocation during the reduction of fumarate by endogenous substrates in anaerobic cells of *Escherichia coli* K12 varied with fumarate concentration. This variation was probably due mainly to incomplete fumarate utilization. Under optimum conditions a minimum value for $\rightarrow H^+/2e^-$ of 1.04 ± 0.20 was obtained.

Anaerobic electron flow to fumarate in *Escherichia coli* energizes ATP synthesis (Miki & Lin, 1975) and thus can form the 'high-energy' membrane state (Harold, 1974). As predicted by the chemiosmotic hypothesis (Mitchell, 1967), such electron flow causes outward proton translocation; approximately one proton is translocated per electron pair transferred (Brice *et al.*, 1974). In contrast, approximately two protons are extruded by *E. coli* per site of energy conservation per electron pair transferred to nitrate or to oxygen (Garland *et al.*, 1975; Lawford & Haddock, 1973).

The low value for $\rightarrow H^+/2e^-$* with fumarate could result from the uptake of protons with fumarate (Gutowski & Rosenberg, 1975) or from slow and incomplete metabolism of added fumarate. The present study was undertaken to examine these possibilities.

Materials and Methods

Materials

E. coli strain AN283 (*argH*, *entA*, *uncB401*) defective in coupled membrane Mg-ATPase (EC 3.6.1.3) activity was donated by Dr. G. B. Cox (of this department). [2,3-^{14}C]-Fumaric acid was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Carbonyl cyanide *m*-chlorophenylhydrazone (Calbiochem, Sydney, N.S.W., Australia) was used as the potassium salt in 50% (v/v) ethanol. Other chemicals were Reagent Grade. N_2 was passed before use through 5% (w/v) pyrogallol in 14M-KOH, then through water.

* Abbreviations: $\rightarrow H^+/2e^-$, number of protons translocated per electron pair transferred from the donor to the acceptor; Mg-ATPase, Mg^{2+}-stimulated adenosine triphosphatase.

Growth of cells

Cells were grown anaerobically at 37°C as described previously (Gutowski & Rosenberg, 1976*a*) in growth medium supplemented with 10μM-2,3-dihydroxybenzoate, 3.3μM-thiamin, 10mM-DL-α-glycerophosphate, 40mM-potassium fumarate, 2mM-L-arginine and 0.1% Bacto casamino acids (Difco, Detroit, MI, U.S.A.).

Measurement of proton movements and of the intracellular radioactive 'pool'

Cells were prepared and stored in lightly buffered medium as described previously (Gutowski & Rosenberg, 1976*b*). Portions (20ml) of this cell suspension were concentrated approx. sevenfold by centrifugation (8000*g*, 1 min), and 2.5ml of the concentrated suspension was incubated anaerobically at 37°C in the presence of KSCN as described previously (Gutowski & Rosenberg, 1976*b*). All solutions added after the start of incubation were N_2-purged and pH-matched.

When the cells reached pH equilibrium between pH6.9 and 7.3 (after 15–20min of incubation), one of the following assays was performed: (*a*) unlabelled fumarate was added to 24–200μM and the resultant proton movements were recorded, or (*b*) [^{14}C]fumarate was added to similar concentrations and its uptake measured as described previously (Rosenberg *et al.*, 1975).

Cell densities were measured and proton movements calibrated as described previously (Gutowski & Rosenberg, 1976*b*). Cells were preincubated with inhibitors for 5min before the addition of fumarate.

Examination of dicarboxylic acids in the medium

Cells were equilibrated as described above and [^{14}C]fumarate was added. Samples (0.2ml) of the sus-

pension were withdrawn at maximum acidification (see below) and were filtered and washed. The filtrate and washings were acidified with HCl and continuously extracted with diethyl ether for 2h. The ether layer was dried, the residue extracted with 200μl of 2mM-succinate/2mM-fumarate in water, and portions of this solution were spotted on to cellulose plates. Succinate and fumarate were separated and located as described by Myers & Huang (1966). Spots were eluted and counted for radioactivity as described previously (Gutowski & Rosenberg, 1975).

Results

Addition of fumarate to anaerobic cells caused a reproducible decrease in medium pH after a short lag. Slower alkalinization followed, reflecting re-equilibration of protons (x, Fig. 1), but the pH did not relax back to its original value. The reason for this is unclear, since the production of net acid equivalents during the oxidation of endogenous substrates (in the presence of uncoupler) was small (result not shown).

The slow rate of medium acidification and the lag in this rate precluded accurate determination of the $\rightarrow H^+/2e^-$ value. However, the method of Mitchell & Moyle (1967) was adapted and used to extrapolate the phase of proton re-entry to the point of maximum medium acidification, to obtain minimum estimates for this value. Such estimates varied with the concentration of fumarate added (Fig. 2). Under optimum conditions a value for $\rightarrow H^+/2e^-$ of 1.04 ± 0.20 was found (mean$\pm$S.D., five determinations). Similar variation in observed $\rightarrow H^+/2e^-$ values was obtained when the alkalinization phase was extrapolated to the halfway point of medium acidification (Mitchell & Moyle, 1967), but in this case the optimum value of $\rightarrow H^+/2e^-$ was 1.25 ± 0.28.

The rate of medium acidification was inhibited 98% by 1mM-potassium succinate and 87% by 2mM-potassium tartrate.

Labelling of the intracellular 'pool' by added [^{14}C]fumarate was complete within 1min or less and remained constant for at least 5min after that. The proportion of added radioactivity found in the cells decreased from 18% to 6.5% when the concentration of added fumarate increased from 12 to 150μM.

After fumarate addition, succinate appeared in the medium (Table 1). At the peak of medium acidification, succinate and fumarate accounted for over 85% of the radioactivity in the filtrate and, depending on the concentration of fumarate added, 19–48% of the added fumarate was unchanged.

Discussion

The lag in medium acidification after the addition of fumarate to anaerobic cells (Fig. 1), and its inhibition by tartrate, suggest that fumarate uptake by the

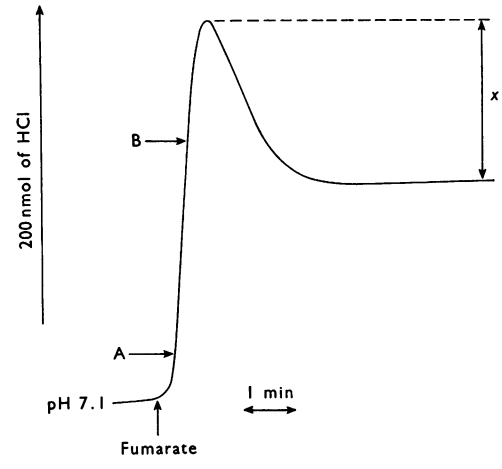

Fig. 1. *pH changes on the addition of fumarate to anaerobic cells*

Cells were grown, prepared and incubated at an A_{660} of 20 as described in the Materials and Methods section. Potassium fumarate (80μM) and the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (40μM) were added as shown. Rates of proton extrusion were calculated from the linear part of the acidification phase between points A and B.

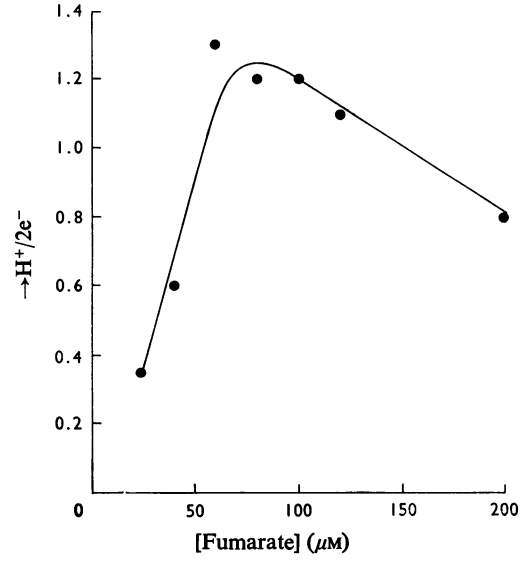

Fig. 2. *Variation in $\rightarrow H^+/2e^-$ estimates with fumarate concentration*

Cells were incubated at an A_{660} of 20, and potassium fumarate was added to various concentrations. For further details see the Materials and Methods section.

Table 1. *T.l.c. of dicarboxylic acids*

Cells were prepared and incubated at an A_{660} of 14. Potassium [^{14}C]fumarate was added to equilibrated cells to the concentrations shown. Samples (0.2ml) of the cell suspension were withdrawn at the peak of medium acidification, filtered and processed. Radioactively labelled succinate and fumarate in the filtrate were separated and determined. For further details, see the Materials and Methods section.

Expt.	[Fumarate] (μM)	$10^{-3}\times$[^{14}C]Succinate radioactivity (c.p.m./ml of filtrate)	$10^{-3}\times$[^{14}C]Fumarate radioactivity (c.p.m./ml of filtrate)	$\frac{\text{Succinate}}{\text{fumarate}}$	$\rightarrow H^+/2e^-$
1	24	10.3	8.3	1.2	0.3
	120	74.6	19.5	3.8	0.8
	200	134.0	124.9	1.1	0.35
2	40	25.2	5.1	4.3	0.8
	80	38.5	11.4	3.4	1.0
	120	94.0	31.0	3.1	1.2
	200	110.0	44.5	2.5	0.9

dicarboxylate transport system (Kay & Kornberg, 1971) preceded proton extrusion in fumarate reduction. However, the addition of fumarate did not cause any initial alkalinization of the medium. As one possible explanation of these observations, we postulate that fumarate, transported together with two protons (Gutowski & Rosenberg, 1975), was immediately reduced to succinate with the concomitant extrusion of two protons. Thus initially proton extrusion balanced proton uptake with fumarate. As fumarate reduction continued, succinate efflux (with two protons per succinate molecule) commenced (Table 1) and increased until it matched fumarate influx, and a steady rate of medium acidification resulted from proton extrusion and the production of some net acid equivalents.

Estimates of $\rightarrow H^+/2e^-$ obtained here varied with fumarate concentration (Fig. 2). Under optimum conditions, a minimum value of 1.04 was obtained. Brice *et al.* (1974) suggested that the low $\rightarrow H^+/2e^-$ values obtained in their study during the fumarate-dependent oxidation of endogenous substrates may have been due to the uptake of protons with fumarate. However, we found that only 6–18% of added radioactivity was sequestered by the cells (see the Results section). Thus, although net uptake of protons in the dicarboxylate flux occurred, it was relatively unimportant.

From 20 to 50% of added fumarate was still unchanged when medium acidification was maximal (Table 1). In the calculations of $\rightarrow H^+/2e^-$ values made here, the corrected number of protons translocated (obtained from a semi-logarithmic plot of the alkalinization phase) was divided by the number of fumarate molecules added. Incomplete metabolism of added fumarate will thus have caused overestimation of the denominator and underestimation of the numerator in these calculations, leading to underestimation of $\rightarrow H^+/2e^-$.

This underestimation may well be the major reason for the low observed $\rightarrow H^+/2e^-$ values and for their variation. The point of maximum medium acidification is the point at which the rates of proton extrusion and re-entry are equal. Both these rates will depend on fumarate concentration but in different ways. Thus the point of maximum medium acidification, the proportion of added fumarate metabolized at that point and the degree of underestimation of $\rightarrow H^+/2e^-$ due to incomplete metabolism will depend on fumarate concentration. The form of this dependency will, however, be complex, and Table 1 shows that considerable day-to-day variation in this dependency occurs.

The results highlight the difficulties in determining $\rightarrow H^+/2e^-$ values where substrate uptake is accompanied by proton uptake and where substrate metabolism is slow.

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