Effects of Dicyclohexylcarbodi-imide on Proton Translocation Coupled to Fumarate Reduction in Anaerobically Grown Cells of *Escherichia coli* K12

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The addition of dicyclohexylcarbodi-imide to anaerobic cells of *Escherichia coli* K12 decreases both the observed extent of proton translocation coupled to fumarate reduction by endogenous substrates and the $t_{\frac{1}{2}}$ of proton re-entry after such translocation, but does not affect fumarate uptake. Dicyclohexylcarbodi-imide also inhibits fumarate reductase activity in cell extracts.

Dicyclohexylcarbodi-imide inhibits the membrane-bound Mg^{2+} -ATPase* of *Escherichia coli* (Evans, 1970). On this basis dicyclohexylcarbodi-imide has been used, at concentrations of 0.1 mM and above, as a specific inhibitor of the Mg^{2+} -ATPase in several attempts to define the mechanism of energy coupling to active transport in bacteria (Klein & Boyer, 1972; Harold & Papineau, 1972; Maloney *et al.*, 1974; Singh & Bragg, 1976).

In spite of the isolation of a component of the Mg^{2+} -ATPase which binds dicyclohexylcarbodi-imide with high specificity (Fillingame, 1975), we considered that, owing to the high reactivity of the carbodi-imide bond, at concentrations of 0.1 mM and above, reaction of dicyclohexylcarbodi-imide with membrane components other than the Mg^{2+} -ATPase complex was likely. Singh & Bragg (1974) have, in fact, already shown that dicyclohexylcarbodi-imide at 0.1 mM increased the permeability of heptose-deficient mutants of *E. coli* to *o*-nitrophenyl β -galactoside and inhibited both the induction of β -galactosidase and the oxidation of some tri-carboxylic acid-cycle intermediates in these mutants, though it had little effect on wild-type cells.

The present report shows the results of our studies on the effects of dicyclohexylcarbodi-imide on anaerobic electron-transport systems in cells of *E. coli* K12, strain AN283 (*uncB401*).

Materials and Methods

Materials

Dicyclohexylcarbodi-imide was obtained from Fluka A.G., Buchs, Switzerland. A fresh solution was made up in ethanol for each experiment.

* Abbreviation: Mg^{2+} -ATPase, Mg^{2+} -stimulated adenosine triphosphatase.

DL- α -Glycerophosphate was obtained from Sigma Biochemicals, St. Louis, MO, U.S.A. Benzyl Viologen was obtained from BDH Laboratory Chemicals Division, Poole, Dorset, U.K. [2,3- ^{14}C]-Fumaric acid was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were of reagent grade.

N_2 was passed through two scrubbing towers containing 5% (w/v) pyrogallol in 14M-KOH, and then through water, before use.

E. coli strain AN283 (*argH*⁻, *entA*⁻, *uncB401*), defective in the coupling of the Mg^{2+} -ATPase to membrane energization, was kindly donated by Dr. G. B. Cox of this department and is described by Butlin *et al.* (1973).

Growth of cells

Cells were grown anaerobically as described previously (Gutowski & Rosenberg, 1976), but with 10mM- α -glycerophosphate as carbon source and 10mM-potassium fumarate as electron acceptor. Culture were checked daily for reversion to *suc*⁺, and all experiments reported were done with cultures containing no detectable revertants.

Measurement of proton extrusion and fumarate uptake

Cells were harvested by centrifugation, washed with 100mM-KCl/2mM-glycylglycine, pH 7.0 (West & Mitchell, 1973), resuspended in this medium to an E_{660} of 2.5–3.0 and stored on ice under N_2 . Portions of this suspension were concentrated approximately sevenfold by centrifugation, and the concentrated suspensions (2.5 ml) transferred to the glass-electrode cell described by Gutowski & Rosenberg (1975). KSCN was added to 50mM final concentration at the start of the incubation, and the cells were vigorously bubbled with N_2 for approx. 5 min. Incubation at

37°C was then continued under N₂ with magnetic stirring.

All solutions added after the start of incubation were N₂-purged. Whenever additions were made in ethanolic solution, control suspensions received appropriate amounts of ethanol.

Cells were allowed to reach pH equilibrium between pH 7.1 and 7.3, and dicyclohexylcarbodi-imide was then added to the required concentration. Incubation at 37°C was carried out for a further 10 min, and two types of assay were then carried out on alternate samples: (a) proton movements were recorded after the addition of 80 μ M- (final concentration) potassium fumarate or (b) potassium [¹⁴C]fumarate was added to 80 μ M final concentration, and its entry rates were determined by conventional membrane filtration techniques, as described by Rosenberg *et al.* (1975).

Values of counts for cellular radioactivity were corrected for background radiation and non-specific adsorption of radioactive material on the filters. Corrected counts were expressed as nmol of fumarate taken up/mg dry wt. of bacterial mass, by using suitable standards.

Cell densities were measured as described by Rosenberg *et al.* (1975). Proton movements were calibrated by the addition of known small amounts of HCl.

Assay of fumarate reductase activity

Spheroplasts of cells of *E. coli* strain AN283 were prepared by a method developed in this department by P. J. Wooley; cells were harvested, washed once in 30 mM-glycylglycine (pH 8.0)/30% (w/v) sucrose and resuspended in this medium to an *E*₆₆₀ of 1.6. Lysozyme and EDTA were added to final concentrations of 0.02 mg/ml and 0.4 mM respectively and the cell suspension stirred slowly for 15 min at 25°C. The resultant spheroplasts were then cooled on ice and sonicated for two 1 min intervals with a Branson Sonifier B12. No whole cells were detected in this sonicated suspension by phase-contrast microscopy. MgCl₂ was then added to the sonicated suspension to 10 mM final concentration, and the suspension was kept on ice under N₂ until required.

Fumarate reductase activity was measured spectrophotometrically by a modification of the method of Spencer & Guest (1973). Each cuvette received 1.5 ml of the sonicated suspension, 20 μ l of 1 M-D-lactate, 100 μ l of 4 mM-Benzyl Viologen, 1.25 ml of glycylglycine/sucrose medium and either dicyclohexylcarbodi-imide to the required concentration or the appropriate amount of ethanol. The cuvette was incubated at 30°C for 10 min and the Benzyl Viologen was reduced *in situ* by addition of approx. 100 μ l of N₂-purged 20 mM-sodium dithionite. Reoxidation of the reduced dye was initiated by adding 20 μ l of N₂-purged 1 M-potassium fumarate and was

followed at 550 nm by using a Cary 14 spectrophotometer.

Fumarate reductase activities, expressed as nmol of Benzyl Viologen reoxidized/min per mg of protein, were calculated by using a value of 7.73×10^{-3} litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹ for the molar extinction coefficient of the reduced dye (Spencer & Guest, 1973). Protein was assayed by the biuret reaction (Gornall *et al.*, 1949).

Results and Discussion

In confirmation of previous reports that fumarate reduction by endogenous substrates leads to proton translocation (Brice *et al.*, 1974), we found that the addition of fumarate to anaerobic cells of anaerobically grown *E. coli* strain AN283 (*uncB*) caused acidification of the medium, due to proton translocation and the production of some net acid equivalents during the oxidation of endogenous electron donors, followed by a phase of alkalization due to re-equilibration of translocated protons (result not shown).

Table 1 shows that both the observed rate and extent of medium acidification coupled to the reduction of fumarate by endogenous substrates are inhibited by dicyclohexylcarbodi-imide and that the extent of this inhibition depends on the concentration of this compound. The *t*_½ for proton re-entry into the cells after fumarate addition is greatly decreased by dicyclohexylcarbodi-imide (Table 1), which also decreases the *t*_½ for proton re-entry after proton extrusion coupled to either the reduction of O₂ by endogenous substrates in cells of *E. coli* strain AN283 (*uncB*) grown anaerobically on α -glycerophosphate plus fumarate or to the reduction of N₂ by formate in cells of *E. coli* strain AN480 (*uncB*, *frd*) grown anaerobically on glucose plus nitrate (result

Table 1. Effects of dicyclohexylcarbodi-imide on observed proton translocation

Cells of *E. coli* strain AN283 (*uncB*) were grown, prepared and incubated at an *E*₆₆₀ of 17 as described in the Materials and Methods section. Cells were preincubated with inhibitor for 10 min, then fumarate (80 μ M) was added and the proton movement recorded.

Final concn. of dicyclohexylcarbodi-imide (mM)	Maximum rate of medium acidification (nmol of H ⁺ /min per mg dry wt.)	Maximum extent of medium acidification (nmol of H ⁺)	<i>t</i> _½ proton re-entry (s)
0	10.7	145	93
0.1	8.2	125	50
0.25	3.6	68	<25
0.5	1.7	18.5	*

* No proton re-entry was observed.

not shown). Thus the effect of dicyclohexylcarbodi-imide on the $t_{\frac{1}{2}}$ of proton re-entry appears to be general and therefore 0.1–0.5 mM-dicyclohexylcarbodi-imide is behaving as a weak uncoupler.

At 0.5 mM-dicyclohexylcarbodi-imide no phase of alkalization is observed to follow the acidification phase (see Table 1). This suggests that this concentration completely inhibits the formation of a proton gradient during fumarate reduction by endogenous substrates, and the comparatively small acidification observed is probably due to the production of net acid equivalents in the oxidation of endogenous donors.

It should be noted that no preincubation of cells with EDTA was required to demonstrate these effects of dicyclohexylcarbodi-imide. Thus the strains used here, unlike strains used by previous workers (Singh & Bragg, 1974), do not seem to possess an effective outer-membrane permeability barrier to this compound. The reason for this discrepancy is unclear. However, our results highlight the fact that the presence of an outer-membrane permeability barrier to dicyclohexylcarbodi-imide cannot be assumed.

Table 2 shows that fumarate uptake by cells of *E. coli* strain AN283 is apparently unaffected by 0.5 mM-dicyclohexylcarbodi-imide. Thus inhibition of fumarate uptake is not responsible for the observed inhibition of proton extrusion by dicyclohexylcarbodi-imide. This finding contrasts with that of previous workers (Singh & Bragg, 1974), who from their results concluded that fumarate transport in *E. coli* was inhibited by 50 μ M-dicyclohexylcarbodi-imide. This discrepancy may be due to the fact that Singh & Bragg (1974) measured the aerobic uptake of fumarate at various times between 1 and 10 min after fumarate addition, whereas we measured the anaerobic uptake of fumarate over a

2 min period. Under aerobic conditions, over 90% of added succinate is metabolized to aspartate and other amino acids within 1 min of uptake (Gutowski & Rosenberg, 1975), and, since fumarate is an intermediate on this pathway, it is also likely to be almost completely metabolized to aspartate and other amino acids during uptake. Thus, under aerobic conditions, the amount of radioactive material accumulated by cells after [14 C]fumarate addition does not truly reflect the activity of the dicarboxylate-transport system (Kay & Kornberg, 1971), and an observed inhibition of fumarate uptake could well be due to an inhibition of fumarate metabolism.

Table 2 also shows that dicyclohexylcarbodi-imide (0.1–0.5 mM) does inhibit fumarate reductase activity in sonicated extracts of spheroplasts of *E. coli* strain AN283. The extent of the inhibition of fumarate reductase is considerably less than the corresponding inhibition of the observed rate and extent of proton translocation (Table 1). However, since the observed rate and extent of medium acidification are the net result of proton extrusion and the production of acid equivalents on the one hand and the re-entry of protons into the cell on the other, we believe that the inhibition of proton extrusion and acid formation due to the inhibition of fumarate reductase by dicyclohexylcarbodi-imide, coupled with the marked decrease in the $t_{\frac{1}{2}}$ of proton re-entry caused by dicyclohexylcarbodi-imide, is probably sufficient to explain the observed inhibition of medium acidification by this compound.

In the light of these results we contend that the observed inhibition of an energy-linked reaction, such as active transport, by 0.1–0.5 mM-dicyclohexylcarbodi-imide does not necessarily implicate the Mg^{2+} -ATPase in the energization of that reaction, and that dicyclohexylcarbodi-imide at these concentrations is therefore unsuitable as a specific inhibitor of the Mg^{2+} -ATPase, especially under conditions where fumarate reductase is induced. Specific mutations of the Mg^{2+} -ATPase complex do not suffer from this problem.

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Table 2. *Effects of dicyclohexylcarbodi-imide on fumarate uptake in whole cells and fumarate reductase activity in cell extracts*

Cells of *E. coli* strain AN283 were grown as described in the Materials and Methods section. Fumarate uptake was assayed in whole cells, at an E_{660} of 12, as described in the Materials and Methods section. Fumarate reductase activity was measured in sonicated spheroplast suspensions (5.4 mg of protein/ml), as described in the Materials and Methods section.

Final concn. of dicyclohexylcarbodi-imide (mM)	Fumarate uptake (nmol/mg dry wt.)		Fumarate reductase activity (nmol of Benzyl Viologen reduced/min per mg of protein)
	at 10 s	at 2 min	
0	0.5	1.2	60
0.1	—	—	52
0.5	0.5	1.2	34

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