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Oxidative Phosphorylation in *Escherichia coli* K12

MUTATIONS AFFECTING MAGNESIUM ION- OR CALCIUM ION-STIMULATED ADENOSINE TRIPHOSPHATASE

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1. Two mutants of *Escherichia coli* K12 were isolated which, although able to grow on glucose, are unable to grow with succinate or D-lactate as the sole source of carbon. 2. Genetic mapping of these mutants showed that they both contain a mutation in a gene (designated *uncA*) mapping at about minute 73.5 on the *E. coli* chromosome. 3. The *uncA*$^-$ alleles were transferred by bacteriophage-mediated transduction into another strain of *E. coli* and the transductants compared with the parent strain to determine the nature of the biochemical lesion in the mutants. 4. The mutants gave low aerobic growth yields when grown on limiting concentrations of glucose, but oxidase activities in membranes from both the mutants and the normal strain were similar. 5. Measurement of P/O ratios with D-lactate as substrate indicated that a mutation in the *uncA* gene causes uncoupling of phosphorylation associated with electron transport. 6. Determination of the Mg^{2+},Ca^{2+}-stimulated adenosine triphosphatase activities in the mutant and normal strains indicated that the *uncA* gene is probably the structural gene for Mg^{2+},Ca^{2+}-stimulated adenosine triphosphatase. 7. Mg^{2+},Ca^{2+}-stimulated adenosine triphosphatase therefore appears to be essential for oxidative phosphorylation in *E. coli*.

Oxidative phosphorylation coupled to electron transport, in both fractionated mitochondrial (Linnane & Titchener, 1960) and bacterial (Pinchot, 1953) systems, has been shown to require, in addition to a respiring membrane fraction, a number of soluble coupling factors. These factors have been classified as phosphoryltransferases, energy-transfer factors or oligomycin-sensitizing factors (see Lardy & Ferguson, 1969). Purification of the various coupling factors has been attempted but, with the exception of the Mg^{2+}-stimulated adenosine triphosphatase activity from mitochondria (Penefsky & Warner, 1965), most preparations seem to consist of mixtures of coupling factors or mixtures of coupling factors and other membrane components (Lam, Warshaw & Sanadi, 1968; Racker, 1964).

It is a difficult practical problem to damage membrane preparations specifically and reproducibly in such a way as to obtain a system lacking only the one factor under consideration (see Lehninger, 1964). The methods used generally involve sonication followed by treatment with trypsin, trypsin–urea, or phosphatides (Racker, 1963). In theory, the study of microbial mutants that lack specific enzymic activities concerned in the coupling reactions could be used to overcome the problem of lack of specificity inherent in other approaches. A mutant of *Saccharomyces cerevisiae* that had the phenotype expected for an 'uncoupled' mutant, i.e. lack of growth on oxidizable non-fermentable carbon sources, normal amounts of cytochromes and apparently normal respiration, has been described (Kovac, Lachowicz & Slonimsky, 1967). However, a detailed biochemical investigation (Somlo, 1970) seems to indicate that strains carrying this mutation cannot be considered as uncoupled. The use of bacteria with their simpler cellular organization than eucaryotic cells, and of *Escherichia coli* in particular, with its amenability to genetic manipulations, seems a promising experimental system for a combined genetic and biochemical approach to the problem of coupling of phosphorylation to electron transport. The present paper describes an investigation of two mutant strains of *E. coli* which have the phenotypic characteristics expected for mutants in which phosphorylation is uncoupled from electron transport.

MATERIALS AND METHODS

Chemicals and enzymes. Piericidin A was kindly provided by Professor S. Tamura, Department of Agricultural Chemistry, University of Tokyo, Japan. Chemicals generally were of the highest purity available commercially and were not further purified. Glucose 6-phosphate dehydrogenase and hexokinase were obtained from Boehringer G.m.b.H., Mannheim, Germany.

Organisms. All the bacterial strains used in this work were derived from *E. coli* K12 and are shown in Table 1.

Media. The mineral-salts minimal medium 56 described by Monod, Cohen-Bazire & Cohn (1951) was used. Sterile solutions of various carbon sources to give a final concentration of 30mM were added to the sterilized mineral-salts base. Additional amino acid supplements (0.2mM) and thiamin (0.2μM) were added as required. The cells used for genetic experiments were grown on a tryptone–yeast extract broth (Luria & Burrous, 1957). The nutrient broth was solidified with 2% (w/v) Difco agar as required.

Streptomycin-resistant mutants. Spontaneous streptomycin-resistant mutants were obtained by the method of Cox, Gibson & Pittard (1968).

Transduction techniques. The generalized transducing bacteriophage Plkc was used for transduction experiments as described by Pittard (1965).

Conditions for conjugation experiments. The conditions under which mating was carried out were similar to those described by Taylor & Thoman (1964), with the modifications used by Cox *et al.* (1968).

Growth conditions. Growth yields of various strains were obtained as described by Cox, Newton, Gibson, Snoswell & Hamilton (1970), by using a medium containing limiting glucose. The turbidities were measured at intervals until growth was complete, i.e. when two successive readings taken at 60min intervals on a Klett–Summerson colorimeter were similar.

For growth under anaerobic conditions, the cultures were incubated at 37°C in tubes with loosely fitting tops in an atmosphere of H_2, in a Gallenkamp anaerobic culture jar fitted with a platinum catalyst.

For the preparation of cell extracts (from strains AN120 and AN180) the growth from a nutrient agar slope was suspended in medium 56 and added to 1 litre of medium 56 supplemented with glucose, arginine and thiamin. This culture was shaken overnight at 37°C, and then used to inoculate 9 litres of medium in a 14 litre New Brunswick fermenter. The fermenter was kept at 37°C, aerated at 12 litres/min, stirred at 600rev./min and the culture harvested at about 0.6mg dry wt. of cells/ml.

Preparation and fractionation of cell extracts. The cells were washed, a cell extract was prepared with a Sorvall Ribi Cell Fractionator at 20000lb/in^2 and the membranes were separated by $(NH_4)_2SO_4$ precipitation as described by Cox *et al.* (1970). The procedure was modified slightly for the preparation of membranes for adenosine triphosphatase determinations. In this case, the cells were washed in 0.05M-imidazole buffer (pH 7.2), resuspended in fresh imidazole buffer (4ml of buffer/1.0g wet wt. of cells) and then fractionated as described above. The precipitate obtained after $(NH_4)_2SO_4$ fractionation was resuspended in 4ml of imidazole buffer per original 1.0g wet wt. of cells. All operations on the harvested cells were conducted at 0–4°C. Proteins were determined with Folin's phenol reagent (Lowry, Rosebrough, Farr & Randall, 1951) with bovine serum albumin (fraction V; Sigma Chemical Co., St Louis, Mo., U.S.A.) as standard.

Determination of fermentation products. The supernatants obtained after centrifuging cultures grown in the

Table 1. *Strains of* E. coli *K12 used*

Genes coding for enzymes in various biosynthetic pathways are denoted as follows: *thi*, thiamin; *ilv*, isoleucine–valine; *arg*, arginine; *met*, methionine; *leu*, leucine; *pro*, proline; *pur*, purine; *trp*, tryptophan; *thr*, threonine; *ubi*, ubiquinone. Streptomycin resistance is denoted by str^R. The two mutant alleles *uncA401* and *uncA402* are described in this paper. Abbreviation: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

Strain	Sex	Relevant genetic loci	Other information
AB259	Hfr	*thi*$^-$	Hfr Hayes. Obtained from J. Pittard
AN118	♀	*thi*$^-$, *uncA401*	Derived from strain AB259 after MNNG treatment
AN119	♀	*thi*$^-$, *uncA401*, str^R	Derived from strain AN118 by spontaneous mutation
JP58	♀	*ilvC7*, *argE3*, *thi-1*, str^R	Obtained from J. Pittard
AN120	♀	*argE3*, *thi-1*, str^R, *uncA401*	Isolated after transduction with strain AN119 as donor and strain JP58 as recipient
AN180	♀	*argE3*, *thi-1*, str^R	Isolated after transduction with strain AN119 as donor and strain JP58 as recipient
AB3311	Hfr	*metB*$^-$, *thi*$^-$	Hfr Reeves, Hfr1 (Echols *et al.* 1961). Obtained from J. Pittard
AN181	Hfr	*metB*$^-$, *thi*$^-$, *uncA402*	Derived from strain AB3311 after MNNG treatment
AB1515	♀	*leu*$^-$, *proC*$^-$, *purE*$^-$, *trp*$^-$, str^R	Obtained from J. Pittard
AN182	♀	*proC*$^-$, *purE*$^-$, *trp*$^-$, *thi*$^-$, *uncA402*	Recombinant obtained after conjugation between strains AN181 and AB1515
AN183	♀	*argE3*, *thi-1*, str^R, *uncA402*	Isolated after transduction with strains AN182 as donor and strain JP58 as recipient
AN59	Hfr	*thr-1*, *leu-6*, *ubiB*$^-$	See Cox, Young, McCann & Gibson (1969)
AB2154	Hfr	*metE*$^-$, *thr-1*, *leu-6*	Obtained from J. Pittard
AB2826	♀	*aroB351*	Obtained from J. Pittard

New Brunswick fermenters were examined by g.l.c. for volatile and non-volatile fermentation products (Doelle, 1969). Volatile fermentation products were determined by g.l.c. on a Porapak Q column at 180°C with N_2 as carrier gas. Trimethylsilyl derivatives of the non-volatile fermentation products were determined on a 10% SE-30 Chromosorb W(60–80) column with N_2 as carrier gas, the temperature being raised from 100°C to 250°C at 4°C/min.

Measurement of oxygen uptake. Measurements of oxidase activity were made by using an oxygen electrode as described by Cox *et al.* (1970). The reaction mixture contained (final concentrations): 1.3mg of protein, 15mM-sodium–potassium phosphate buffer (pH7.4), 1.9mM-$MgCl_2$, in a final volume of 2.5ml. The substrates, succinate (8mM), lactate (4mM) or NADH (1.2mM), were added as indicated. Buffer solutions were calibrated for oxygen content by the method of Chappell (1964).

Difference spectra. Difference spectra for the determination of cytochromes b_1, a_1, a_2 and o and the flavoproteins in the membrane preparations were recorded as described by Cox *et al.* (1970) except that an Aminco Chance spectrophotometer was used.

Determination of quinones. The ubiquinone and menaquinone contents of 5g samples of whole cells were determined as described by Cox *et al.* (1968).

Assay of adenosine triphosphatase. Membrane fractions prepared in imidazole buffer were assayed for Mg,Ca-ATPase* activity as described by Evans (1969). The reaction mixture contained, in a final volume of 1ml, 0.1M-tris–HCl buffer (pH9), 5mM-ATP and other additions as described in the text. The reaction was initiated by the addition of 50μl of membrane preparation containing about 250μg of protein. The tubes were shaken at 37°C in a water bath for 30min and the reaction was terminated by the addition of 1.0ml of 5% (v/v) $HClO_4$. Phosphate was determined by the method of King (1932) on 0.5ml samples of the reaction mixture, with correction for acid hydrolysis of ATP.

P/O *ratios.* Oxygen uptake was measured in constant-volume Warburg manometers at 30°C (Umbreit, Burris & Stauffer, 1951). CO_2 was removed by 0.2ml of 10% (w/v) KOH in the centre well of the manometer flasks. The flasks were incubated for 10min before being sealed, after which membrane preparations (150μl) containing 3.9mg of protein were tipped from the side arm into the main section of the flasks, which contained, in a total volume of 3.0ml, 20mM-glucose, 13mM-KF, 5mM-sodium–potassium phosphate buffer (pH7), 5mM-$MgCl_2$, 16.7mM-AMP, 4.2mM-ADP, hexokinase and other additions as indicated in the text. Lactate (20mM, pH7) was used as substrate. The reactions were terminated after 30min by the addition of 0.3ml of 35% (v/v) $HClO_4$, the precipitated protein was removed by centrifugation and the supernatants were neutralized with 10% KOH. The precipitated $KClO_4$ was removed by centrifugation and the glucose 6-phosphate contents of samples of the supernatants were determined fluorimetrically (Greengard, 1963). The reaction mixture contained 0.8mM-$NADP^+$, 17mM-$MgCl_2$, 3mM-EDTA (pH7.4) and 17mM-triethanolamine buffer (pH7) in a total volume of 1.5ml. The formation of NADPH after the addition of glucose 6-phosphate dehydrogenase (0.5 Kornberg unit) was determined fluorimetrically with an Aminco–Bowman spectrophotofluorimeter (excitation wavelength, 360nm; emission wavelength, 460nm, uncorrected). The estimated amount of glucose 6-phosphate formed was corrected for the amount formed in the absence of lactate, which amounted to about 25% of the total glucose 6-phosphate formed by membranes from the normal strain.

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

RESULTS

During the examination of a number of mutants able to grow with glucose but not succinate as the sole source of carbon (Suc^- mutants), two strains were found which gave low aerobic growth yields in a glucose medium, but appeared to possess normal activities of the NADH oxidase and D-lactate oxidase systems. The mutant strains (AN118 and AN181) were therefore examined in detail.

Genetic mapping. Strain AN118, although derived from an Hfr male, was found to be a female and therefore the mutation causing the Suc^- phenotype could be mapped by using interrupted-mating experiments. A spontaneous streptomycin-resistant derivative of strain AN118 was obtained and designated AN119. The latter strain was then used in an interrupted mating experiment with the Hfr strain AB3311, and Suc^+ recombinants selected on a medium containing succinate as sole carbon source together with streptomycin to kill the ex-conjugant

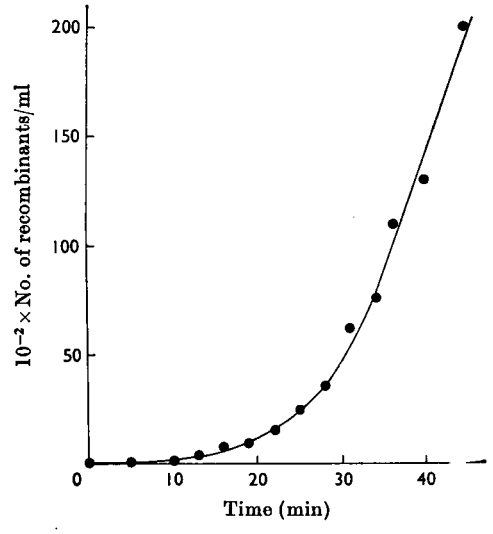

Fig. 1. Kinetics of zygote formation when the female strain AN119 was mated with the Hfr strain AB3311. The ordinate scale shows the interval between the time when the parental cultures were mixed together (zero time) and the time at which the sample was vortexed to interrupt mating.

males. A time of entry of about 10 min for the wild-type allele was obtained (Fig. 1) indicating that the mutation causing the Suc$^-$ phenotype was in a gene transferred early by the Hfr strain AB3311. The marker (*phoS*) nearest to the origin of transfer of this Hfr strain maps at about minute 73.5 on the *E. coli* genome (Echols, Garen, Garen & Torriani, 1961; Taylor, 1970). The mutation causing the Suc$^-$ phenotype was located more precisely by tests for co-transduction with two genes, namely *metE* at minute 74.5 and *ilvC* at minute 74. The generalized transducing bacteriophage Plkc was grown on strain AN119 and used for co-transduction experiments with strains AB2154 (*metE*$^-$) and JP58 (*ilv*$^-$). All the 200 *metE*$^+$ transductants examined were able to grow with succinate as sole carbon source, whereas only 50 out of 84 *ilv*$^+$ transductants were able to grow on succinate. The mutation affecting growth on succinate and the *ilvC* gene were therefore co-transducible at a frequency of about 40%. The co-transduction results are consistent with the mutation present in strain AN119 being located at about minute 73.5 on the *E. coli* genome, and the gene carrying this mutation was designated *uncA401*. An *ilv*$^+$, *unc*$^-$ transductant (strain AN120) and an *ilv*$^+$, *unc*$^+$ transductant (strain AN180) were retained for further study.

The mutation in the second strain (AN181), in which oxidative phosphorylation was possibly uncoupled, was also mapped. Strain AN181 was an Hfr male, and a mating was carried out with the female AB1515 (*leu*$^-$, *proC*$^-$, *purE*$^-$, *trp*$^-$) selecting for either Leu$^+$, Pro$^+$, Pur$^+$ or Trp$^+$ recombinants. Ex-conjugant males were killed by the inclusion of streptomycin in the selective media. The recombinants were then examined for the Suc$^-$ phenotype. Aerobic growth yields of the Suc$^-$ recombinants showed that a Leu$^+$, Suc$^-$ strain (AN182) had the same low growth yield as strain AN120 (*unc*$^-$). Strain AN182 was then used as a recipient in an interrupted mating experiment with the Hfr male AB3311. A time of entry which was similar to that described above for the mating with strain AN119 was obtained for the wild-type allele. Co-transduction tests were carried out with strain AN182 as donor and strain JP58 as recipient. Transductants that were Ilv$^+$ were then screened for their ability to grow on succinate as sole source of carbon, and 45% of the Ilv$^+$ recombinants (27 out of 60 tested) were found to be of the Suc$^-$ phenotype. One of the Suc$^-$ transductants (strain AN183) was retained for further investigation.

The mutation in strain AN183 affecting growth on succinate was apparently in the same region of the chromosome as the *unc* mutation in strain AN120. A bacteriophage lysate prepared on strain AN120 (*uncA401*) was used as donor and strain AN182 as recipient, selecting for Suc$^+$ transductants. The numbers of Suc$^+$ transductants obtained were normalized with respect to the numbers of Pro$^+$ transductants obtained by using the same bacteriophage lysates. The Suc$^+$ transductants were detected at frequencies of about 2% of the frequencies obtained by using a bacteriophage lysate prepared on a wild-type strain (AB2826) (Table 2). The mutations in strains AN182 and AN120 affecting growth on succinate were probably in the same gene and the mutation in strains AN182 and AN183 was therefore designated *uncA402*.

A study of the biochemical effects of mutations in the *uncA* gene was made by a comparison of three of the strains referred to above. These were strain AN120 carrying the *uncA401* allele, strain AN183 carrying the *uncA402* allele and, as a control, the Ilv$^+$, Unc$^+$ transductant (strain AN180). The latter strain will be referred to as the normal strain. The results obtained with both mutant strains were identical within experimental error, and therefore only the results with one of them (strain AN120) are described in detail.

Growth characteristics and the metabolic products of normal and mutant strains. Apart from the inability of the mutant strains to grow on a medium with succinate or D-lactate as sole source of carbon, the mean generation time in glucose–mineral salts medium was longer for the mutants, being 1.5 h compared with 1 h for the normal strain. Examination by g.l.c. of the products of glucose metabolism by the mutant and the normal strains grown under aerobic conditions showed that the concentrations of succinate, lactate and ethanol in the culture supernatants were the same for the normal and mutant strains. However, the concentrations of acetate in the culture supernatants of the normal

Table 2. *Transduction crosses involving strains AN*120 (uncA401) *and AN*182 (uncA402)

The transducing bacteriophage Plkc was grown on either strain AB2826 (Pro$^+$, Suc$^+$) or on strain AN120 (Pro$^+$, Suc$^-$) and used as donor with strain AN182 (Pro$^-$, Suc$^-$) as recipient. Either Pro$^+$ or Suc$^+$ transductants were selected.

Donor	Recipient	Number of Pro$^+$ transductants	Number of Suc$^+$ transductants
AB2826	AN182 (*uncA402*)	125	290
AN120 (*uncA401*)	AN182 (*uncA402*)	370	10

and mutant strains differed, and were 9mM and 14mM respectively.

Determination of the growth yields, measured as turbidities, of the strains growing on media containing limiting concentrations of glucose showed (Fig. 2) that the aerobic growth yield of strain AN120 (*unc*$^-$) was lower than that of the normal strain. However, the aerobic growth yield of strain AN120 (*unc*$^-$) was higher than the anaerobic growth yield of the normal strain.

*Concentrations of membrane components in strains AN*180 *and AN*120 (unc$^-$). Concentrations of total flavin and cytochromes in the membrane fractions from strain AN120 (*unc*$^-$) and strain AN180 were determined from the reduced-minus-oxidized difference spectra. It was found (Table 3) that the concentrations in the normal and mutant strains did not differ significantly. Similarly the concentrations of ubiquinone and vitamin K in the normal and mutant strains, determined after extraction and partial purification, were not significantly different (Table 3). Examination of the membranes by electron-spin-resonance spectroscopy showed that the signal attributed to ubisemiquinone (Hamilton, Cox, Looney & Gibson, 1970) and the signal attributed to 'non-haem iron' could be demonstrated in both the normal and the mutant strains (J. A. Hamilton, personal communication).

*Oxidase systems in strains AN*180 *and AN*120 (unc$^-$). The NADH oxidase and lactate oxidase systems have been shown to be quantitatively the most significant in membranes from *E. coli* K12 grown with glucose as carbon source (Cox *et al.* 1970). Comparison of these two oxidase systems in strains AN180 and AN120 showed that there was no significant difference in NADH oxidase activity between the normal and mutant strains (about 1000ng-atoms of O/min per mg of protein), whereas the lactate oxidase activity in membranes from the mutant (340ng-atoms of O/min per mg of protein) was 35% higher than that in membranes from the normal strain. It was found that there was considerable variation in the succinoxidase activities of different batches of membranes, although a similar range of activities was found in both the normal and mutant strains.

*Oxidative phosphorylation by membranes from strains AN*180 *and AN*120 (unc$^-$). Oxidative phosphorylation was examined by using lactate rather than NADH as the oxidizable substrate, since the presence of the reduced nicotinamide nucleotide interfered with the assay system used. A mixture of AMP and ADP was used for the determination of oxidative phosphorylation to inhibit adenylate kinase activity (Slater, 1953) present in the membrane preparations. The P/O ratio of 0.12 found for the normal strain (Table 4) is low but consistent with the values found for most bacterial systems (see Gel'man, Lukoyanova & Ostrovskii, 1967).

2,4-Dinitrophenol (0.3 mM) uncoupled oxidative phosphorylation by 75% whereas piericidin A (0.13mM) completly prevented phosphorylation. This concentration inhibited lactate oxidase activity by only 41% (Table 4).

There was no detectable phosphorylation by strain AN120 (*unc*$^-$) and it therefore appears that

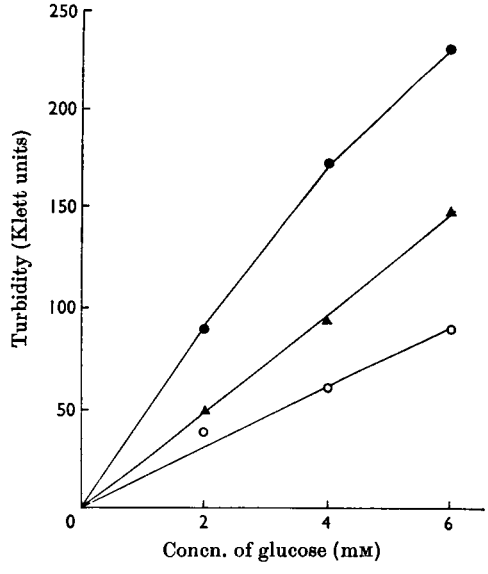

Fig. 2. Growth yields (turbidity) for strains AN180 and AN120 (*unc*$^-$) grown on limiting concentrations of glucose. Cultures were aerated by shaking at 37°C and anaerobic cultures were incubated in an anaerobic jar. ●, Strain AN180, aerobic; ○, strain AN180, anaerobic; ▲, strain AN120 (*unc*$^-$), aerobic.

Table 3. *Concentrations of some membrane components in strains AN*180 *and AN*120 (unc$^-$)

Experimental methods are described in the Materials and Methods section.

	Concn. of component (nmol/mg of protein)	
Membrane component	Strain AN180	Strain AN120 (*unc*$^-$)
Total flavin	1.3	1.0
Cytochrome b_1	0.54	0.65
Cytochrome a_2	0.02	0.02
Cytochrome *o*	0.24	0.28
Cytochrome a_1*	+	+
Ubiquinone	6.1	6.7
Menaquinone	2.0	2.3

* Cytochrome a_1 was present but the quantities were too low for determination.

Table 4. *Oxidative phosphorylation by membranes from strains AN180 and AN120* (unc$^-$)

Experimental details are given in the Materials and Methods section.

Membrane from strain	Alteration to basal systems in Warburg flask	Oxygen uptake* (ng-atoms/min per mg of protein)	Glucose 6-phosphate formed (nmol/min per mg of protein)	P/O ratio
AN180	—	103	12.4	0.12
AN180	Flask gassed with oxygen-free nitrogen	0	<0.1	0
AN180	2,4-Dinitrophenol (300 μM) included	84	2.3	0.03
AN180	Piericidin A (130 μM) included	58	<0.1	<0.001
AN120 (*unc*$^-$)	—	121	<0.1	<0.001

* The presence of AMP and ADP in the reaction mixture used for the determination of P/O ratios inhibits the lactate oxidase system.

Table 5. Mg,Ca-*ATPase activity of membranes from strains AN180 and AN120* (unc$^-$)

Adenosine triphosphatase activity was determined by the method of Evans (1969) except that P_i was determined by the method of King (1932). Membranes were incubated with ATP (5 mM) for 30 min at 37°C in 0.1 M-tris–HCl buffer, pH 9, with other additions as indicated, in a final volume of 1 ml. $MgCl_2$ was added at a final concentration of 2 mM or $CaCl_2$ at 1 mM.

Additions and final concn.	P_i released (μmol/mg of protein in 30 min)		Stimulation of adenosine triphosphatase activity by metal ions (μmol of P_i released/mg of protein in 30 min)	
Membranes from strain ...	AN180	AN120 (*unc*$^-$)	AN180	AN120 (*unc*$^-$)
None	12	6	—	—
Mg^{2+}	45	7.5	33	1.5
Ca^{2+}	30	6	18	0
EDTA (7.5 μM)	1.6	0.5	—	—
EDTA (7.5 μM)+Mg^{2+}	38	1.3	36	0.8
EDTA (7.5 μM)+Ca^{2+}	18	1.3	16	0.8
Piericidin A (400 μM)	12	—	—	—
Piericidin A (400 μM)+Mg^{2+}	40	—	28	—
Piericidin A (400 μM)+Ca^{2+}	30	—	18	—

a consequence of a mutation in the *uncA* gene is uncoupling of phosphorylation from electron transport.

Mg,Ca-*ATPase activity of membranes from strains AN180 and AN120* (unc$^-$). Evans (1969) studied adenosine triphosphatase activity in membranes from *E. coli* and suggested that there may be a single adenosine triphosphatase present activated by either Mg^{2+} or Ca^{2+}. Preliminary experiments with the membrane preparations used in the present study indicated that they possessed about the same amount of Mg^{2+}- or Ca^{2+}-stimulated adenosine triphosphatase activity as found by Evans (1969) and that Na^+ or K^+ inhibited the activity in the presence of Ca^{2+}. The stimulation by Ca^{2+} was less than that reported by Evans (1969) and the reason for this is not known. The addition of EDTA to the assay system in the absence of added Mg^{2+} or Ca^{2+} decreased the endogenous adenosine triphosphatase activity (Table 5), but the stimulation by the bivalent metal ions remained about the same. Piericidin A had no effect on the adenosine triphosphatase activity (Table 5) and, as found by Evans (1969), 2,4-dinitrophenol did not stimulate, and azide and *p*-chloromercuribenzoate inhibited adenosine triphosphatase.

Comparison of the Mg,Ca-ATPase activity from strains AN180 and AN120 (*unc*$^-$) shows that the mutation in the *uncA* gene resulted in a virtually complete (>95%) loss of activity.

DISCUSSION

The *uncA* gene mapping near minute 73.5 on the *E. coli* genome is probably the structural gene for the membrane-bound Mg,Ca-ATPase. Mutations in this gene resulted in loss of Mg^{2+},Ca^{2+}-activated adenosine triphosphatase activity, prevented growth on succinate or D-lactate as carbon source

and decreased the growth rate with glucose as carbon source. Aerobic growth yields of the mutant strain grown on limiting glucose were less than those from the normal strain but not as low as the anaerobic growth yield of the normal strain. Unlike membrane preparations from the normal strain, those from the mutant strain lacked the ability to couple phosphorylation to electron transport with D-lactate as substrate. The oxidase activities in the membranes and the concentrations of a number of membrane components were not altered by lack of Mg,Ca-ATPase.

The P/O ratio of 0.12 for the normal strain *in vitro* with D-lactate as substrate makes it difficult to decide whether phosphorylation at more than one site is being measured. However, the mutants, although possessing derepressed values for D-lactate oxidase activity, are unable to grow with D-lactate as sole source of carbon, indicating that the Mg,Ca-ATPase is required for phosphorylation at both the sites coupled to lactate oxidation.

The aerobic growth yield of the Mg,Ca-ATPase-deficient strain was higher than the anaerobic growth yield of the normal strain. If it is assumed that Mg,Ca-ATPase is required for ATP production at each of the three postulated sites for NADH oxidation in the electron-transport system it would appear that some oxidative energy may be conserved via the 'energy-linked' reactions. These reactions, such as the NAD(P) transhydrogenase, utilize high-energy intermediates rather than ATP (Snoswell, 1962).

Recent work on the function of ubiquinone in *E. coli* K12, using mutants unable to form ubiquinone, has led to the proposal of a scheme for electron transport in this organism (Cox *et al.* 1970). It was suggested (Cox *et al.* 1970) that piericidin A separated ubiquinone from the remainder of the electron-transport chain. In the present experiments it has been shown that piericidin A completely uncouples oxidative phosphorylation when used at a concentration that inhibited lactate oxidation by only 41%. Piericidin A may have two points of action, the more sensitive reaction lying between the proposed iron–ubisemiquinone complex and the Mg,Ca-ATPase. Vallin & Löw (1968) have found, using mitochondria, that piericidin A has an uncoupler-like action when used at concentrations that do not affect NADH oxidase activity.

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