Reconstitution of the Energy-Linked Transhydrogenase Activity in Membranes from a Mutant Strain of *Escherichia coli* K12 Lacking Magnesium Ion- or Calcium Ion-Stimulated Adenosine Triphosphatase

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1. We have isolated a mutant of *Escherichia coli* K12 (strain AN295) that forms de-repressed amounts of Mg^{2+} , Ca^{2+} -stimulated adenosine triphosphatase. 2. The Mg^{2+} , Ca^{2+} -stimulated triphosphatase activity was separated from membrane preparations from strain AN295 by extraction with 5 mM-Tris-HCl buffer containing EDTA and dithiothreitol, resulting in a loss of the ATP-dependent transhydrogenase activity. The non-energy-linked transhydrogenase activity remained in the membrane residue. 3. The solubilized Mg^{2+} , Ca^{2+} -stimulated adenosine triphosphatase activity from strain AN295 was partially purified by repeated gel filtration. The addition of the purified Mg^{2+} , Ca^{2+} -stimulated adenosine triphosphatase to the membrane residue from strain AN295 reactivated the ATP-dependent transhydrogenase activity. 4. Strain AN296, lacking Mg^{2+} , Ca^{2+} -stimulated adenosine triphosphatase activity, was derived by transducing the mutant allele, *uncA401*, into strain AN295. The ATP-dependent transhydrogenase activity was lost but the non-energy linked transhydrogenase was retained. 5. The ATP-dependent transhydrogenase activity in membrane preparations from strain AN296 (*uncA*⁻) could not be re-activated by the purified Mg^{2+} , Ca^{2+} -stimulated adenosine triphosphatase from strain AN295. However, after extraction by 5 mM-Tris-HCl buffer containing EDTA and dithiothreitol, the ATP-dependent transhydrogenase activity could be re-activated by the addition of the purified Mg^{2+} , Ca^{2+} -stimulated adenosine triphosphatase from strain AN295 to the membrane residue from strain AN296 (*uncA*⁻).

Strains of *Escherichia coli* K12 carrying mutations in the *uncA* gene (see Table 1) have been used to study the function of Mg , Ca -ATPase† by comparing the properties of the mutant strain with those of a normal strain. However the mutation in the *uncA* gene has not yet been used as a means of specifically depleting the cell membrane of Mg , Ca -ATPase activity with a view to studying its reconstitution. The reconstitution of the membrane could be detected by the reactivation of an energy-linked reaction dependent on Mg , Ca -ATPase. Such a reaction would be the ATP-dependent transhydrogenase, the activity of which is lost in strains carrying mutations in the *uncA* gene (Cox *et al.*, 1971).

The present paper describes a study in which partially purified Mg , Ca -ATPase from *E. coli* was used to reactivate the ATP-dependent transhydrogenase in membrane preparations from a strain carrying a mutation in the *uncA* gene.

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

Experimental

Materials

Chemicals. Chemicals generally were of the highest purity available commercially, and were not further purified. Lactate dehydrogenase was supplied by Sigma Chemical Co., St. Louis, Mo., U.S.A. and glutathione reductase was from Calbiochem (Australia) Pty. Ltd., Carlingford, N.S.W., Australia.

Organism. All the bacterial strains used were derived from *E. coli* K12 and are described in Table 1. The method for isolating mutants unable to grow with succinate as sole source of carbon has been described previously (Cox *et al.*, 1968).

Methods

Media and growth of organisms. The medium used was that described by Monod *et al.* (1951) as medium 56. To the sterilized mineral-salts base were added the appropriate L-amino acids to give a final concentration of 0.2 mM, thiamin was added to a final concentration of 0.2 μ M and 2,3-dihydroxybenzoate to

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

Genes coding for enzymes in various biosynthetic pathways are denoted as follows: *met*, methionine; *ilv*, isoleucine-valine; *arg*, arginine; *ent*, enterochelin. The gene coding for Mg,Ca-ATPase activity is denoted by *uncA*.

Strain	Relevant genetic loci	Other information
AB3311	<i>metB</i> ⁻	Hfr Reeves
AN233	<i>metB</i> ⁻	Unable to grow with succinate as sole source of carbon; de-repressed Mg ²⁺ , Ca ²⁺ -ATPase activity present. Responds to a mixture of adenine and L-serine on succinate medium. Derived from strain AB3311 after treatment with <i>N</i> -methyl- <i>N'</i> -nitro- <i>N</i> -nitrosoguanidine
AN248	<i>ilvC</i> ⁻ , <i>argH</i> ⁻ , <i>entA</i> ⁻	Isolated after transduction with strain AN233 as donor and strain AN248 as recipient. Phenotype as for strain AN233
AN295	<i>ilvC</i> ⁻ , <i>entA</i> ⁻	
AN120	<i>argE</i> ⁻ , <i>uncA401</i>	Butlin <i>et al.</i> (1971)
AN296	<i>entA</i> ⁻ , <i>uncA401</i>	Isolated after transduction with AN120 as donor and strain AN295 as recipient

a final concentration of 40 μ M. The carbon source was added as a sterile solution at a final concentration of 30 mM.

Cells were grown in 7-litre New Brunswick fermentors as described by Cox *et al.* (1970).

Preparation and fractionation of cell membranes. The cells were washed in a buffer system, pH 7.0, containing 0.1 M-*N*-tris(hydroxymethylmethyl-2-aminoethanesulphonic acid (Tes), 0.02 M-magnesium acetate, 0.25 M-sucrose and 0.25 mM-ethanedioxybis-(ethylamine)tetra-acetate (EGTA). The cells were then resuspended in fresh buffer (1 ml/0.5 g wet wt. of cells) and disrupted by passage through a Sorvall Ribi cell fractionator at 137 MPa (20000 lb/in²). The cell extract was then centrifuged at 25000g for 20 min and the supernatant fraction separated. The supernatant fraction was then centrifuged at 100000g for 4 h and the pellet retained. The pellet consisted of 2 layers, a pale-yellow translucent heavy layer and a red-brown light layer.

The red-brown layer was separated and resuspended in 1 ml of the Tes buffer system for each original 1 g wet wt. of cells, after which it was centrifuged at 160000g for 1 h. The supernatant was then discarded and the pellet resuspended in an equal volume of 50 mM-Tris-HCl buffer (pH 7.4) to give the membrane fraction (A, Table 2). The suspended membranes were centrifuged at 160000g for 1 h and the supernatant decanted (50 mM-Tris wash; B, Table 2). The pellet was then resuspended in the same volume of 5 mM-Tris-HCl buffer (pH 7.4) containing 0.25 mM-EDTA and 0.5 mM-dithiothreitol and the suspension centrifuged at 160000g for 2 h. The supernatant was retained and the pellet was again resuspended and centrifuged. The two supernatants were pooled and the protein was concentrated by using a Diaflo XM-50 ultra-filter (Scientific and Research Equipment Co.

Pennant Hills, N.S.W., Australia) until the final volume was equal to about 20% of the original volume of membrane fraction. This fraction is designated the 'concentrated 5 mM-Tris wash' (C, Table 2). The pellet remaining after the low-ionic-strength washes was resuspended in the low-ionic-strength buffer system to a volume equal to two-thirds of the original volume of membrane fraction. This suspension was designated the 'membrane residue' (E, Table 2).

All operations on the whole cells and membranes were carried out at 4°C. Solubilized Mg,Ca-ATPase preparations were either stored at room temperature or at 4°C in the presence of 20% (v/v) glycerol. Membrane residues were stored at 4°C in the presence of 20% (v/v) glycerol.

Protein concentrations were determined by using Folin's phenol reagent (Lowry *et al.*, 1951) with bovine serum albumin (Fraction V; Sigma Chemical Co., St. Louis, Mo., U.S.A.) as standard.

Transduction technique. The technique for transduction experiments, in which the generalized transducing bacteriophage PIKC was used, were carried out as described by Pittard (1965).

Measurement of the ATP-dependent transhydrogenase activity. The reduction of NADP⁺ by NADH was assayed by coupling the reaction to the NADPH-dependent glutathione reductase and measuring the decrease in *E*₃₄₀ (Ernster & Lee, 1967). Details of the technique used are described by Cox *et al.* (1971).

Measurement of the non-energy-linked transhydrogenase activity. Reduction of NAD⁺ by NADPH was assayed by coupling the reaction to the NADH-dependent reduction of pyruvate by lactate dehydrogenase and measuring the decrease in *E*₃₄₀. Details of the technique used are described by Cox *et al.* (1971).

Assay of Mg,Ca-ATPase. During the present work only the ATPase activity in the presence of Mg²⁺ was measured as a routine. The reaction mixture contained 0.1M-Tris-HCl buffer (pH9), 20mM-ATP, 10mM-MgCl₂, 0.75mM-EDTA and 1mM-dithiothreitol in a final volume of 1ml. The reaction was started by the addition of 5-50μl of enzyme preparation. After incubation for 10min at 30°C in a water bath, the tubes were transferred to an ice bath and 0.5ml samples of the reaction mixture added to 9.5ml of King's reagent (King, 1932). After 15min the E₆₆₀ was measured. The assay was checked to ensure that the activity was proportional to the amount of protein added. Controls without substrate or without enzyme preparation were also included.

Column chromatography. Column chromatography was carried out by using Agarose (Biogel A-5m, 200-400 mesh; Bio Rad, Richmond, Calif., U.S.A.) in a 2.5cm × 90cm column that was eluted with 5mM-Tris-HCl buffer pH7.4, containing 0.5mM-dithiothreitol and 0.25mM-EDTA. The elution rate was 25ml/h and 3.5ml fractions were collected. Active column-fractions were pooled and then concentrated by using a Diaflo XM-50 ultrafilter.

Results

Preparation and preliminary examination of bacterial strains

During the examination of a number of mutants able to grow with glucose, but not succinate, as the sole source of carbon, a strain (AN233) was found that had two properties not previously noted in mutants with this phenotype. First, the strain formed higher amounts of Mg,Ca-ATPase than normal and secondly, it could grow if the succinate medium was supplemented with adenine and serine. These two characteristics are probably the result of a single mutation, the mutation being co-transduced with the *argH* gene at a frequency of about 12%. The *argH* gene maps at minute 77 on the *E. coli* chromosome (Taylor, 1970).

A set of strains suitable for the present investigation was then prepared by inserting the new mutation into strain AN248 by co-transduction with the *argH* gene to give strain AN295. The allele *uncA401*, which affects Mg,Ca-ATPase activity (Butlin *et al.*, 1971) was then transduced into strain AN295 thereby deriving strain AN296 (*uncA*⁻).

The Mg,Ca-ATPase activities (μmol of P_i released/min per mg of protein) of membrane preparations from the three strains examined were as follows: AN248, 0.25; AN295, 1.08; AN296, <0.005. Strain AN295 usually showed a three- to five-fold depression of Mg,Ca-ATPase when compared with the normal strain (AN248) whereas strain AN296

Table 2. Enzymic activities and protein content of fractions obtained during solubilization of Mg,Ca-ATPase from *E. coli* AN295

Assays and the procedure for fractionating the membranes are described in the Experimental section. One unit of Mg,Ca-ATPase activity is defined as the amount of enzyme that releases 1 μmol of inorganic phosphate from ATP/min at 30°C.

Fraction	Enzymic activity					Protein		
	Units of Mg,Ca-ATPase	Mg,Ca-ATPase (μmol of P _i released/mg protein per min)	Energy-linked transhydrogenase (nmol of NADPH formed/min per mg of protein)	Non-energy-linked transhydrogenase (nmol of NADH formed/min per mg of protein)	NADH oxidase (ng-atoms of O/min per mg of protein)	Volume (ml)	Concentration (mg/ml)	Recovery (%)
A Membranes	273	0.790	60	55	1260	30	11.5	100
B 50mM-Tris wash	<6	<0.1	—	—	—	30	2	17
C Concentrated 5 mM-Tris wash	162	7.3	0	0	<100	6	3.7	6
D Filtrate obtained during concentration of 5 mM-Tris wash	—	<0.20	—	—	—	60	0.2	3
E Membrane residue	32	0.150	3	94	2630	20	10.5	61

(*uncA*⁻) showed no detectable Mg,Ca-ATPase activity.

Solubilization of the Mg,Ca-ATPase activity

Treatment of the membrane preparations with 5 mM-Tris-HCl buffer containing EDTA and dithiothreitol (see the Experimental section) resulted in the separation of a crude soluble Mg,Ca-ATPase preparation (concentrated 5 mM-Tris wash) and a membrane residue. The membrane residue retained NADH oxidase and non-energy-linked transhydrogenase activities (Table 2). During this fractionation the ATP-dependent transhydrogenase activity was lost. There was an overall recovery of about 70% of the Mg,Ca-ATPase activity present in the original membranes with 59% being recovered in the concentrate of the low-ionic-strength wash.

Reactivation of the ATP-dependent transhydrogenase activity

The ATP-dependent transhydrogenase activity lost during the fractionation of the membranes could be restored by the addition of the concentrated 5 mM-Tris wash to the membrane residue (Fig. 1). Fig. 1 shows that the restoration of energy-linked transhydrogenase activity is not a linear function of the

amount of concentrated 5 mM-Tris wash added, but that there is a linear relationship between the extent of reactivation and the square of the amount of concentrated 5 mM-Tris wash added.

Further purification of Mg,Ca-ATPase

Further purification of the Mg,Ca-ATPase in the concentrated 5 mM-Tris wash was achieved by repeated gel filtration by using agarose beads (Biogel A-5 m). The results can be seen in Fig. 2. The specific activity of the peak fraction obtained from column B was 18 μmol of P_i /min per mg of protein which represents a 23-fold purification of Mg,Ca-ATPase activity in the membrane fraction from strain AN295 and an approximately 80-fold purification relative to membranes from normal cells.

The purified Mg,Ca-ATPase was then tested for its ability to reactivate the ATP-dependent transhydrogenase when combined with the membrane residue from strain AN295. Fig. 3. shows that the purified Mg,Ca-ATPase, when added to the membrane residue, caused reactivation of the ATP-dependent transhydrogenase. A comparison of the ability of the crude and purified Mg,Ca-ATPase preparations to reactivate the ATP-dependent transhydrogenase, calculated on the basis of Mg,Ca-ATPase activity, indicated that the purified Mg,Ca-ATPase was

Fig. 1. Reactivation of the ATP-dependent transhydrogenase activity

The ATP-dependent transhydrogenase activity was measured in the membrane residue (1.1 mg of protein) from strain AN295 by using the assay system described in the Experimental section. The basal level of activity was found to be 33 nmol of NADPH formed/min and was subtracted to give the values shown. The increase in ATP-dependent transhydrogenase activity was determined on the addition of increasing amounts of concentrated 5 mM-Tris wash from membranes of strain AN295.

Fig. 2. Gel filtration of solubilized Mg,Ca-ATPase from concentrated 5 mM-Tris wash from strain AN295 on Biogel A-5m

Details of the chromatography are given in the Experimental section. The fractions indicated from column (a) were pooled, concentrated and applied to column (b) and active fractions again chromatographed on column (c). ●, Mg,Ca-ATPase activity; ○, protein concentration.

about 70% as active as the concentrated 5 mM-Tris wash.

Reactivation of the ATP-dependent transhydrogenase in strain AN296 (*uncA*⁻)

As expected, membranes from strain AN296 (*uncA*⁻) also lacked ATP-dependent transhydrogenase activity (Cox *et al.*, 1971). The addition of either the concentrated 5 mM-Tris wash (Fig. 4, curve A) or the purified Mg,Ca-ATPase did not result in any appreciable increase in the ATP-dependent transhydrogenase activity. However, if the membranes

from strain AN296 (*uncA*⁻) were first exposed to a low-ionic-strength wash, identical with that described above for the removal of the Mg,Ca-ATPase activity from the membranes of strain AN295, then the resulting membrane residue could be reactivated by the addition of either the concentrated 5 mM-Tris wash from strain AN295 (Fig. 4, curve B) or the purified Mg,Ca-ATPase from strain AN295.

Discussion

The solubilization of the Mg,Ca-ATPase from *E. coli* has been the subject of studies by Davies &

Fig. 3. Activation of the ATP-dependent transhydrogenase activity in membrane residue from strain AN295 by purified Mg,Ca-ATPase

The ATP-dependent transhydrogenase activity was measured as described in the Experimental section. The reaction mixture included membrane residue (0.7 mg of protein) and a Mg,Ca-ATPase preparation (0.07 mg of protein) consisting of concentrated column fractions 52–55 from the third chromatography on Agarose (see Fig. 2c).

Bragg (1972) and Kobayashi & Anraku (1972). The technique used in these studies for solubilizing the Mg,Ca-ATPase activity has been to treat membrane preparations with low-ionic-strength buffer as described for the solubilization of the ATPase from *Streptococcus faecalis* by Abrams (1965). The method of solubilization of the Mg,Ca-ATPase described in the present paper is similar to that of Abrams (1965) although the lithium chloride treatment was omitted and EDTA and dithiothreitol were included in the buffer used. The specific activity of the Mg,Ca-ATPase after repeated gel filtration was $18 \mu\text{mol}$ of P_i /min per mg of protein, which is somewhat higher than that reported by Davies & Bragg (1972) but not as high as that obtained by Kobayashi & Anraku (1972),

Fig. 4. Comparison of reactivation of ATP-dependent transhydrogenase activity

The fractions tested were, curve A, membrane preparation from strain AN296 (*uncA*⁻) and curve B, membrane residue from strain AN296 (*uncA*⁻). The ATP-dependent transhydrogenase activity was measured as described in the Experimental section. To the reaction mixtures were added membrane preparation (1.1 mg of protein) or membrane residue (1.1 mg of protein) and concentrated 5 mM-Tris wash (0.4 mg of protein) from strain AN295.

although the different temperatures used for the assay of Mg,Ca-ATPase activity make the results difficult to compare.

The reactivation of the ATP-dependent transhydrogenase activity in membranes from strain AN296 (*uncA*⁻) by the purified Mg,Ca-ATPase could not be achieved unless the membranes had been submitted to the low-ionic-strength washing procedure. This may indicate that the mutant allele *uncA401* codes for an altered Mg,Ca-ATPase protein that has lost activity but is still bound to the membrane at the sites normally occupied by the active Mg,Ca-ATPase. Alternatively, the Mg,Ca-ATPase protein may be a component of an aggregate analogous to the 'knobs' purified from mitochondria (see Razin, 1972). Strain AN296 (*uncA*⁻) may still form an aggregate, albeit of altered composition and lacking Mg,Ca-ATPase activity, but sufficiently similar to occupy the membrane sites of normal 'knobs'.

The reconstitution experiments indicate that the time taken for the reassociation of the purified Mg,Ca-ATPase activity and the membrane residue is about 2 min under the conditions used. The non-linear relationship between the amount of Mg,Ca-ATPase added and the extent of the reactivation

indicates that co-operative effects may be involved, reminiscent of those described by Kagawa (1972) for the restoration of ^{32}P i-ATP exchange reactions by adding preparations of coupling factors to vesicles derived from mitochondria. It would also appear, from the reactivation with the purified Mg,Ca-ATPase, that gel filtration does not separate a protein from the Mg,Ca-ATPase of *E. coli* which is analogous to the 'nectin' protein in the *Strep. faecalis* system (Baron & Abrams, 1971).

The strain of *E. coli* (AN295) that has depressed amounts of Mg,Ca-ATPase is of considerable interest. No attempt has been made to assign a name to the gene mapping at about minute 77 which directly or indirectly affects the Mg,Ca-ATPase activity. Whether the gene concerned is one of the known genes in this region indirectly affecting the amount of Mg,Ca-ATPase activity or whether a new gene is concerned is a subject for further investigation. The complete loss of Mg,Ca-ATPase activity from strain AN295 after the insertion of the *uncA401* allele indicates that there is a real de-repression of Mg,Ca-ATPase activity and not an additional ATPase being expressed.

It is assumed that both the ATP-dependent and non-energy-linked transhydrogenase activities involve different forms of the same enzyme. It has previously been shown that both activities are inhibited by tri-iodothyronine and thyroxine (Ball & Cooper, 1959; Stein *et al.*, 1959; Hommes & Estabrook, 1963) and antibodies prepared against the purified non-energy-linked transhydrogenase inhibited both transhydrogenase activities (Kawasaki *et al.*, 1964). Further, a strain of *E. coli* has recently been isolated in which a mutation, in a gene mapped by co-transduction, affected both ATP-dependent and non-energy-linked transhydrogenase activities (J. D. Butlin, unpublished work). The process by which the high-molecular-weight ATPase complex reassociates with the transhydrogenase protein in the membrane

residue to reactivate the ATP-dependent transhydrogenase activity presents an intriguing problem for future work.

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References

- Abrams, A. (1965) *J. Biol. Chem.* **240**, 3675-3681
Ball, E. G. & Cooper, O. (1959) *Proc. Nat. Acad. Sci. U.S.A.* **43**, 357-364
Baron, C. & Abrams, A. (1971) *J. Biol. Chem.* **246**, 1542-1544
Butlin, J. D., Cox, G. B. & Gibson, F. (1971) *Biochem. J.* **124**, 75-81
Cox, G. B., Gibson, F. & Pittard, J. (1968) *J. Bacteriol.* **95**, 1591-1598
Cox, G. B., Newton, N. A., Gibson, F., Snoswell, A. M. & Hamilton, J. A. (1970) *Biochem. J.* **117**, 551-562
Cox, G. B., Newton, N. A., Butlin, J. D. & Gibson, F. (1971) *Biochem. J.* **125**, 489-493
Davies, P. L. & Bragg, P. D. (1972) *Biochim. Biophys. Acta* **266**, 273-284
Ernster, L. & Lee, C. (1967) *Methods Enzymol.* **10**, 738-744
Hommes, F. & Estabrook, R. W. (1963) *Biochem. Biophys. Res. Commun.* **11**, 1-6
Kagawa, Y. (1972) *Biochim. Biophys. Acta* **265**, 297-338
Kawasaki, T., Satoh, K. & Kaplan, N. O. (1964) *Biochem. Biophys. Res. Commun.* **17**, 648-654
King, E. J. (1932) *Biochem. J.* **26**, 292-297
Kobayashi, H. & Anraku, Y. (1972) *J. Biochem. (Tokyo)* **71**, 387-399
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
Monod, J., Cohen-Bazire, G. & Cohn, M. (1951) *Biochim. Biophys. Acta* **7**, 585-599
Pittard, J. (1965) *J. Bacteriol.* **89**, 680-686
Razin, S. (1972) *Biochim. Biophys. Acta* **265**, 241-296
Stein, A. M., Kaplan, N. O. & Ciotti, M. M. (1959) *J. Biol. Chem.* **234**, 979-986
Taylor, A. L. (1970) *Bacteriol. Rev.* **34**, 155-175