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DIFFERENT EFFECTS OF INHIBITORS ON TWO MUTANTS OF *ESCHERICHIA COLI* K12 AFFECTED IN THE F_0 PORTION OF THE ADENOSINE TRIPHOSPHATASE COMPLEX

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SUMMARY

The effects of the inhibitors dicyclohexyl-carbodiimide (DCCD), bathophenanthroline and tertiary octylcatechol, on some enzyme activities in membranes from strains of *Escherichia coli* carrying mutations in the *uncB* or *uncC* genes have been studied. Membranes prepared from *uncC* mutants retain a normal DCCD-sensitive Mg^{2+}-stimulated adenosine triphosphatase (Mg-ATPase) activity whereas in *uncB* mutants this enzyme activity is insensitive to DCCD. The membrane-bound Mg-ATPase activity from the *uncC* mutant strain, as compared with that from the normal strain, is only partially sensitive to the inhibitors bathophenanthroline or tertiary-octylcatechol. Both of these inhibitors stimulate the membrane-bound Mg-ATPase from *uncB* mutant strains. A DCCD-insensitive Mg-ATPase activity is found in the cytoplasmic fraction following cell disruption of either the *uncB* or the *uncC* mutants. The lipophilic chelators bathophenanthroline and tertiary-octylcatechol stimulate the activity of the 'soluble' Mg-ATPase in the *uncB* mutant but partially inhibit the activity in the *uncC* mutant. The NADH oxidase activities in membranes from both mutant and normal strains are strongly inhibited by tertiary-octylcatechol and bathophenanthroline but not by DCCD.

INTRODUCTION

Dicyclohexylcarbodiimide (DCCD) inhibits the membrane-bound Mg^{2+}-stimulated adenosine triphosphatase (Mg-ATPase) complex of *Escherichia coli* as it does the analogous Mg-ATPases form other bacteria, and in mitochondria and chloroplasts [1, 2]. The Mg-ATPase activity in *E. coli* is insensitive to DCCD when the enzyme has been solubilised by washing membranes in low ionic strength solutions [3]. Mutants of the *uncB* type also have a DCCD-insensitive Mg-ATPase activity [4] as do specific DCCD-resistant mutant strains [5]. Electron transport in *E. coli* is

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unaffected by DCCD at the concentrations used to inhibit the membrane-bound Mg-ATPase [6]. In contrast Crane and co-workers have shown that the lipophilic chelator bathophenanthroline inhibits both electron transport and the membrane-bound Mg-ATPase [7, 8]. This compound inhibits both activities at about the same inhibitor concentration and, as for DCCD, the solubilised Mg-ATPase is uninhibited by bathophenanthroline. In addition bathophenanthroline does not inhibit the membrane-bound Mg-ATPase of *uncB*-type mutants [8].

A new mutant allele (*uncC424*) has recently been described [9] and mutant strains carrying mutations in the *uncC* gene have a similar phenotype to *uncB* mutants. In the present paper, the effects of the inhibitors DCCD, bathophenanthroline and tertiary-octylcatechol on the Mg-ATPases, electron transport and some energy-linked functions in membranes from *uncB* or *uncC* mutants are compared. The Mg-ATPase activity, found in the cytoplasmic fraction following cell disruption of the *uncC* mutant, retains some of the inhibition characteristics of the membrane-bound form.

MATERIALS AND METHODS

Chemicals. Chemicals were of the highest purity available commercially and were not further purified.

Organisms. All the bacterial strains used are derived from *E. coli* K12 and are described in Table I.

Media and growth of organisms. The minimal medium used was that described by Gibson et al. [9] .Growth supplements were added where required as sterile solutions at the following final concentrations: glucose, 30 mM; thiamine HCl, 0.2 μM; 2,3-dihydroxybenzoate, 40 μM; L-arginine-HCl, 0.8 mM; L-isoleucine, 0.3 mM; L-valine, 0.3 mM; L-leucine, 0.6 mM; 5-aminolevulinic acid, 20 μM.

Cells for the preparation of membranes were grown in 14-l New Brunswick fermenters as described previously [10].

Preparation of cell membranes. Membranes were prepared as described previously [11]. Briefly, washed cells were disintegrated by using a Sorvall Ribi Cell

TABLE I

STRAINS OF *ESCHERICHIA COLI* K12 USED

Bacterial strain	Relevant genetic loci	Other information
AN812	*uncC424*	Gibson et al. [9]
AN248	*ilvC*$^-$, *argH*$^-$, *entA*$^-$	Butlin et al. [16]
AN259	*argH*$^-$, *entA*$^-$	Butlin et al. [16]
AN283	*uncB402*, *argH*$^-$, *entA*$^-$	Butlin et al. [16]
AN771	*uncC424*, *argH*$^-$, *entA*$^-$	Isolated following transduction with AN812 as donor and AN248 as recipient
AN704	*ilvC*$^-$, *argH*$^-$, *entA*$^-$, *hemA*$^-$, *leu*$^-$	
AN716	*uncB402*, *argH*$^-$, *entA*$^-$, *hemA*$^-$, *leu*$^-$	Isolated following transduction with AN283 as donor and AN704 as recipient
AN770	*uncC424*, *argH*$^-$, *entA*$^-$, *hemA*$^-$, *leu*$^-$	Isolated following transduction with AN771 as donor and AN704 as recipient

fractionator and the membranes were separated by ultra-centrifugation and resuspended in a 0.1 M Tris (hydroxymethyl) methyl-2-aminoethanesulphonic acid (Tes) buffer system (pH 7.0) containing magnesium acetate, sucrose and ethanedioxybis (ethylamine) tetracetate (EGTA). The membrane suspension was recentrifuged and the pellet resuspended in the same buffer system to give the 'membrane preparation'. The supernatant fraction from the first ultracentrifugation was retained as the "cytoplasmic" fraction.

Protein concentrations were determined by using Folin's phenol reagent [12] with bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, Mo., U.S.A.) as standard.

Assay of Mg-ATPase. Assays for Mg-ATPase activity were carried out as described by Gibson, et al. [9].

Measurement of the ATP-dependent transhydrogenase activity. The reduction of $NADP^+$ was assayed by coupling the reaction to the NADPH-dependent glutathione reductase and measuring the decrease in E_{340} [13]. Details of the technique used are described by Cox et al. [14].

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. [14].

Measurement of atebrin fluorescence. Atebrin fluorescence was measured at 30 °C as described by Haddock and Downie [15] using an Aminco-Bowman fluorimeter with the excitation wave length set at 450 nm and emission wave length set at 510 nm.

Oxidase activities. Oxygen uptake by membrane preparations was measured using an oxygen electrode as described previously [10].

Measurement of inhibition. The inhibitors DCCD, bathophenanthroline and tertiary-octylcatechol were added as ethanolic solutions to the reaction mixture either after or at the same time as the addition of substrate. The kinetics of the reaction in the presence or absence of the inhibitor were measured and the inhibited or stimulated rates determined after linearity was achieved. Linearity normally occurred after 1–2 min.

RESULTS

The mutant alleles *uncB402* and *uncC424* have been transduced into different parental strains giving two sets of isogenic strains in which the effects of the three inhibitors DCCD, bathophenanthroline and tertiary-octylcatechol have been compared. Strains AN259(*unc*$^+$), AN283(*uncB*$^-$) and AN771(*uncC*$^-$) comprise one isogenic set and strains AN704(*unc*$^+$), AN716(*uncB*$^-$) and AN770(*uncC*$^-$) the other. Where the results obtained with both sets of strains were similar, only results from a typical experiment with one isogenic set of strains have been given.

Energy-linked reactions. As expected from previously published data [9], the presence of the *uncB402* or *uncC424* alleles causes a loss of the ATP-dependent transhydrogenase activity in membrane preparations as well as a loss of ATP-dependent atebrin fluorescence quenching (Table II). Neither the non-energy-linked transhydrogenase activity nor the NADH-dependent quenching of atebrin fluorescence is

TABLE II

ENERGY-LINKED ACTIVITIES IN MEMBRANES FROM MUTANT AND NORMAL CELLS AND SENSITIVITY TO INHIBITORS

Details are given in Materials and Methods.

Membranes from strain	Additions (final concn.)	ATP-dependent transhydrogenase nmol NADPH formed/ min per mg protein)	Non-energy-linked transhydrogenase (nmol NADH formed/ min per mg protein)	% Atebrin fluorescence quenching	
				NADH	ATP
AN770 (*uncC424*)	–	4	62	78	13
AN716 (*uncB402*)	–	5	49	82	10
AN704 (*unc*$^+$)	–	44	76	64	80
AN704	BP* (60 μM)	3	59	31	20
AN704	BP (120 μM)	0	35	0	0
AN704	TOC** (260 μM)	0	54	0	0
AN704	DCCD (80 μM)	8	86	68	0

* BP, bathophenanthroline

** TOC, tertiary-octylcatechol

TABLE III

INHIBITION OF NADH OXIDASE ACTIVITIES IN MEMBRANES FROM NORMAL AND MUTANT STRAINS

Details are given in Materials and Methods.

Membranes from strain	NADH oxidase (ng atoms O_2/ min per mg protein	Percent inhibition of NADH oxidase by						
		DCCD		bathophenanthroline			tertiary-octylcatechol	
		40 μM	80 μM	60 μM	120 μM	240 μM	260 μM	520 μM
AN771 (*uncC424*)	1134	28	45	72	91	99	83	100
AN283 (*uncB402*)	1089	26	44	74	90	97	81	100
AN259 (*unc*$^+$)	297	16	28	54	75	92	62	95

significantly affected by the presence of these two mutant alleles (Table II).

The lipophilic chelators, bathophenanthroline and tertiary-octylcatechol, give essentially complete inhibition of the ATP-dependent transhydrogenase activity in membranes from the normal strain while only partially inhibiting the non-energy-linked transhydrogenase activity (Table II). The effect of DCCD was more specific, in that the ATP-dependent transhydrogenase activity was inhibited by about 80 % while the non-energy-linked activity was slightly stimulated. Atebrin fluorescence quenching was totally inhibited by both bathophenanthroline and tertiary-octylcatechol with either ATP or NADH as energy source. In contrast DCCD specifically inhibited the ATP-induced atebrin fluorescence quenching.

NADH oxidase activities. The NADH oxidase activities in membranes from the mutant strains were 3–4 times higher than that found in the normal strain. The activity in the normal strain was lower than previously reported [16], presumably due to the different growth medium used. The NADH oxidase activities in membrane preparations from both mutant and normal strains were almost completely inhibited by the lipophilic chelators at the concentrations used (Table III). In contrast, DCCD only partially inhibited the NADH oxidase activities in membrane preparations from both the normal and mutant strains (Table III).

Mg-ATPase activities. The Mg-ATPase activities in membrane preparations from the "normal" strains, AN704(*unc*$^+$) and AN259(*unc*$^+$) are both inhibited approximately 50 % by 50 μM DCCD (Table IV). However, the Mg-ATPase activity in membrane preparations from strain AN259(*unc*$^+$) is totally inhibited by the chelator tertiary-octylcatechol whereas the Mg-ATPase activity from strain AN704 (*unc*$^+$) is only about 20 % inhibited (Table IV). The same trend is observed with the inhibitor bathophenanthroline (Table IV).

Following cell disruption of *uncB* or *uncC* mutant types, Mg-ATPase activity is found in both the membrane preparation and the cytoplasmic fraction. The Mg-ATP-

TABLE IV

INHIBITION[a] OF MEMBRANE-BOUND AND CYTOPLASMIC Mg-ATPase ACTIVITIES IN NORMAL AND MUTANT STRAINS

Details are given in Materials and Methods

Strain	Fraction	Mg-ATPase (μmol P_i released/min per mg protein)	Percent inhibition by		
			DCCD (50 μM)	tertiary-octylcatechol (640 μM)	bathophenanthroline (300 μM)
AN704 (*unc*$^+$)	membrane	0.42	57	17	32
AN716 (*uncB402*)	membrane	0.43	− 7	− 21	−10
AN770 (*uncC424*)	membrane	0.48	46	22	34
AN259 (*unc*$^+$)	membrane	0.39	51	100	82
AN283 (*uncB402*)	membrane	0.37	− 9	− 20	−20
AN771 (*uncC424*)	membrane	0.28	48	39	15
AN283 (*uncB402*)	cytoplasm	0.17	0	−122[b]	−48
AN771 (*uncC424*)	cytoplasm	0.73	0	28[b]	29

[a] Negative values for % inhibition indicate % stimulation

[b] The concentration of tertiary-octylcatechol used was 1.28 mM.

ase activity in membranes from the *uncC* mutant is inhibited by DCCD to the same extent as the enzyme in normal membranes whereas in the *uncB* mutant the enzyme is slightly stimulated by DCCD (Table IV). Both bathophenanthroline and tertiary-octylcatechol also stimulate the Mg-ATPase activity in membranes from the *uncB* mutant but these inhibitors only partially inhibit the enzyme in the *uncC* mutant (Table IV).

The Mg-ATPase activities in the cytoplasmic fractions from either the *uncB* or *uncC* mutants are not inhibited by DCCD (Table IV). However, the lipophilic chelators tertiary-octylcatechol and bathophenanthroline have markedly different effects on the cytoplasmic Mg-ATPase activity in the two mutant strains depending on whether the mutation occurs in the *uncB* or *uncC* genes. Thus, bathophenanthroline or tertiary-octylcatechol stimulate by 50–120 % the cytoplasmic Mg-ATPase activity from the *uncB* mutant but inhibit by approximately 30 % the enzyme from the *uncC* mutant (Table IV). This degree of inhibition is about the same as that found for the membrane-bound Mg-ATPase activity in the *uncC* mutant and the parental type AN704 (Table IV).

DISCUSSION

The membrane-bound Mg-ATPase activity in an uncoupled mutant strain carrying a mutation in the *uncC* gene is similar to the Mg-ATPase in the normal strain in that the enzyme is inhibited to the same degree by DCCD. In contrast a mutation in the *uncB* gene causes the membrane-bound Mg-ATPase activity to be insensitive to DCCD. The DCCD-binding protein has been identified as an "intrinsic" membrane protein with an approximate molecular weight of 9000 and the inhibition of the Mg-ATPase activity by DCCD is a transmitted rather than a direct effect [5]. A mutant strain carrying the *uncB402* allele has a normal DCCD binding protein [5] and a mutation in the *uncB* gene therefore prevents transmission of the inhibitory effect; a mutation in the *uncC* gene does not.

Crane and co-workers [7, 8] have suggested that the chelation of non-heme iron by bathophenanthroline results in the inhibition of both electron transport and the membrane-bound Mg-ATPase. The inhibition of the Mg-ATPase activity would then presumably be a transmitted, rather than a direct, effect. Mutations in either the *uncB* gene or the *uncC* gene cause a reduction in the degree of inhibition by either bathophenanthroline or tertiary-octylcatechol of the membrane-bound Mg-ATPase activity without affecting the inhibition of the NADH oxidase activity. These results are not inconsistent with the suggestion of a common site for bathophenanthroline and tertiary-octylcatechol inhibition of both electron transport and the Mg-ATPase, but they are not substantive evidence.

The membrane-bound Mg-ATPases of the parental strain AN704 and the "isogenic" *uncC* mutant AN770 both have reduced sensitivity to bathophenanthroline and tertiary-octylcatechol, although inhibition of electron transport is the same as that in the other parental strain, AN259. It would appear that AN704 carries a mutation affecting the inhibition of the Mg-ATPase by bathophenanthroline or tertiary-octylcatechol without causing uncoupling of oxidative phosphorylation. Strain AN704 has a normal growth yield and membrane preparations from this strain have normal energy-linked activities (Cox, G. B., unpublished observations).

This observation emphasizes the importance of studying the effects of particular mutations in different genetic backgrounds and comparing those effects with normal strains that are as closely isogenic as possible.

The 'residual' chelator sensitivity of the membrane-bound Mg-ATPase in strains AN704(*unc*$^+$), AN770(*uncC*$^-$) and AN771(*uncC*$^-$) would suggest a second less sensitive site of action of bathophenanthroline and tertiary-octylcatechol. The effect of the inhibitor at the less sensitive site is still likely to be a transmitted effect as the membrane-bound Mg-ATPase in the *uncB* mutant is not inhibited by bathophenanthroline or tertiary-octylcatechol. The 'soluble' Mg-ATPase accumulated in the cytoplasmic fraction of the *uncC* mutant appears to retain this second less sensitive chelator site although DCCD sensitivity has been lost. In contrast the Mg-ATPase activity found in the cytoplasmic fraction from the *uncB* mutant is stimulated by bathophenanthroline and tertiary-octylcatechol as is the Mg-ATPase solubilised by low-ionic strength washing of normal membranes [17].

The further characterisation of the two chelator inhibitor sites discussed above combined with a detailed study of the polypeptide compositions of the soluble Mg-ATPases from both *uncB* and *uncC* mutants should assist in understanding structure/function relationships in the Mg-ATPase complex.

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