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The F_1F_0-ATPase of *Escherichia coli*. The substitution of alanine by threonine at position 25 in the *c*-subunit affects function but not assembly

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A mutant strain of *Escherichia coli* carrying a mutation in the *uncE* gene which codes for the *c*-subunit of the F_1F_0-ATPase has been isolated and examined. The mutant allele, designated *uncE513*, results in alanine at position 25 of the *c*-subunit being replaced by threonine. The mutant F_1F_0-ATPase appears to be fully assembled and is partially functional with respect to oxidative phosphorylation. The ATPase activity of membranes from the mutant strain is resistant to the inhibitor dicyclohexylcarbodiimide, but this is due to the F_1-ATPase being lost from the membranes in the presence of the inhibitor. Mutant membranes from which the F_1-ATPase has been removed have a greatly reduced proton permeability compared with similarly treated normal membranes. The results are discussed in relation to a previously proposed mechanism of oxidative phosphorylation.

Introduction

The membrane-bound energy-transducing F_1F_0-ATPase has a remarkably similar structure in mitochondria, chloroplasts and bacteria. The membrane sector of the F_1F_0-ATPase derived from all sources contains a 'proteolipid' of 70–82 amino acid residues that is soluble in organic solvents. Many proteolipid subunits from various organisms have been sequenced and have been found to possess a number of features in common. There exists a conserved acidic residue in an otherwise hydrophobic segment of the protein which reacts with DCCD to form a stable adduct, resulting in inhihibition of ATPase activity. Also, each of the proteins consists of two hydrophobic segments separated by a central polar region containing a conserved sequence arginine-asparagine (or glutamine)-proline. Eighteen residues from this sequence, within the C-terminal hydrophobic segment, the DCCD-reactive conserved acidic residue resides, while within the N-terminal hydrophobic segment a conserved glycine-rich region occurs [1].

In *Escherichia coli*, the proteolipid (or *c*-subunit) is coded for by the *uncE* gene [2], the third gene from the promoter in the *unc* operon [3], and a number of mutations in this gene have been characterized. Mutations causing the isoleucine at position 28 to be replaced by either valine or threonine have been shown to affect the reactivity of the assembled protein with DCCD. It was inferred that the *c*-subunit is folded in a hairpin-like structure in the membrane such that the isoleucine at position 28 is close to the aspartic acid at position 61 [4]. Eight mutations in the *uncE* gene,

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Abbreviations: ATPase, Mg^{2+}-stimulated adenosine triphosphatase (EC 3.6.1.3); DCCD, dicyclohexylcarbodiimide; NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; P_i, inorganic phosphate.

resulting in loss of oxidative phosphorylation, have been characterised at the level of the amino-acid change in the corresponding *c*-subunit [5–10]. The study of the effects of these mutations has provided information about both assembly and function of the F_1F_0-ATPase.

In the present paper, we describe the amino acid substitution caused by the *uncE513* allele and the effects on F_1F_0-ATPase function.

Materials and Methods

Enzymes. Restriction endonucleases and virus-T4 DNA-ligase were obtained from Amersham Australia Pty Ltd.

Bacterial strains and plasmids. All the bacterial strains used were derived from *E. coli* K12 and are described, together with the plasmids used, in Table I.

Media and growth of organisms. The mineral-salts minimal medium used and additions were as described previously [11]. Cells for the preparation of membranes were grown in 14-l fermenters essentially as described previously [12]. The media in the fermenter vessels were supplemented with 5% (v/v) Luria broth [13]. Growth yields were measured as described previously [12]. Turbidities of cultures were measured with a Klett-Summerson colorimeter.

Purification and CNBr treatment of the c-subunit. The *c*-subunit was purified and treated with CNBr as described previously [7].

Separation of peptides and amino-acid analysis. The freeze-dried peptide mixture obtained after CNBr treatment was dissolved in 90% aqueous formic acid and a sample applied to a reverse phase C_{18} column (Varian MCH-10) on a Varian high-pressure liquid chromatograph, model 5000. The peptides were then eluted with a gradient of 0–40% acetonitrile in aqueous 0.1% phosphoric acid over a 40 min period, then 40–100% acetonitrile in aqueous 0.1% phosphoric acid over a 30 min period. The elution was monitored by measuring A_{210}. Peak fractions were collected, freeze-dried, dissolved in 6 M HCl, hydrolysed and analysed for amino acids.

In vivo mutagenesis of plasmid DNA. Strain AN1461 containing plasmid pAN45 (*unc*$^+$) was grown to logarithmic phase in 500 ml Luria broth containing chloramphenicol (100 μg/ml), centrifuged, washed and resuspended in 0.1 M sodium citrate buffer (pH 6.0). NTG was then added to a final concentration of 15 μg/ml and the cells incubated at 37°C for 20 min [14]. The cells were then washed twice with minimal medium, resuspended in Luria broth and grown for 3 h. The culture was centrifuged and cells were resuspended in 500 ml minimal medium and plasmid amplification carried out. Plasmid DNA was prepared as described by Selker et al. [15].

TABLE I

BACTERIAL STRAINS AND PLASMIDS USED

Strain No.	Relevant genotype	Reference
(a) Bacterial strains		
AN1453	*uncB402 thr leu recA rk*$^-$ *mk*$^-$	7
AN888	*unc416::MuB*$^+$*EFHAGDC argH pyrE entA recA*	16
AN943	*uncE429 argH pyrE entA recA*	7
AN1461	pAN45/*unc416::MuB*$^+$*EFHAGDC argH pyrE entA recA*	17
AN2188	pAN258/*unc416::MuB*$^+$*EFHAGDC argH pyrE entA recA*	This paper
AN2214	pAN268/*uncB402 thr leu recA*	This paper
(b) Plasmids		
pAN45	*Cm*^{r} *Tc*^{s} *uncB*$^+$*E*$^+$*F*$^+$*H*$^+$*A*$^+$*G*$^+$*D*$^+$*C*$^+$	17
pAN51	*Cm*^{r} *Tc*^{s} *uncB*$^+$*E*$^+$*F*$^+$*H*$^+$*A*$^+$	17
pAN258	*Cm*^{r} *Tc*^{s} *uncB*$^+$*E513F*$^+$*H*$^+$*A*$^+$*G*$^+$*D*$^+$*C*$^+$	This paper
pAN268	*Cm*^{r} *Tc*^{s} *uncB*$^+$*E513F*$^+$*H*$^+$*A*$^+$	This paper

Genetic techniques. The techniques used for genetic experiments were as outlined previously [11,16,17].

DNA sequencing. Nucleotide sequences were determined by the method of Maxam and Gilbert; end-labelling of DNA was carried out by using DNA polymerase and [α-^{32}P] deoxyadenosine triphosphate [18].

Preparation of cell membranes. The preparation and treatment of membranes were as described previously [19].

ATPase activity and DCCD inhibition. ATPase activity was measured by using the method described by Gibson et al. [20]. DCCD-sensitivity of the ATPase activity was determined using the method described by Cox et al. [19].

Atebrin-fluorescence quenching. Atebrin-fluorescence quenching was measured as described by Gibson et al. [20].

Oxidative phosphorylation. Oxidative phosphorylation was measured as described by Cox et al. [19].

Protein determination. Protein concentrations were determined by using Folin's phenol reagent [21].

Results

Plasmid mutagenesis and isolation of the uncE513 mutant allele

Strain AN1461, carrying plasmid pAN45 (*uncB*$^+$*E*$^+$*F*$^+$*H*$^+$*A*$^+$*G*$^+$*D*$^+$*C*$^+$) was mutagenised as described above. The mutated preparation of plasmid pAN45 was used to transform strain AN943 (*uncE429, recA*). Transformants were selected on nutrient medium containing chloramphenicol, and small colonies were screened for the inability to grow on succinate minimal medium supplemented with 0.05% (w/v) casamino acids. As strain AN943 (*uncE429*) is unable to grow utilising succinate as sole carbon source, transformant strains unable to grow on this medium are likely to carry a plasmid containing a mutation in the *uncE* gene.

One such transformant was purified, from which plasmid DNA was prepared and then used to transform a series of strains carrying a mutation in one of each of the eight *unc* genes (*uncA-uncH*). Transformants were selected on nutrient medium containing chloramphenicol, and screened for the ability to grow on succinate minimal medium. Thus plasmid, pAN258 (*uncB*$^+$*E513F*$^+$*H*$^+$*A*$^+$*G*$^+$*D*$^+$*C*$^+$), failed to complement the *uncE429* allele, whilst complementing all the other reference alleles (see Ref. 22). Plasmid pAN258 was transformed into strain AN888 (*unc416 : : MuB*$^+$ *EFHAGDC, recA*) and the resulting transformant (AN2188) used for further characterization of the *uncE513* allele. In this strain, the effects of the *uncE513*allele, located on the plasmid, could be studied without interference from the chromosomal *uncE* gene product.

Amino acid change in the mutant c-subunit

The *c*-subunit of the F_1F_0-ATPase was extracted from membranes of strain AN2188 and purified by chromatography on CM-cellulose. The purified protein was solubilised in formic acid and cleaved with CNBr. The freeze-dried peptide fragments were dissolved in 90% aqueous formic acid and examined by high pressure liquid chromatography. The elution profile obtained by measuring A_{210} is shown in Fig. 1. The eluate corresponding to each of the peaks of absorption was collected, freeze-

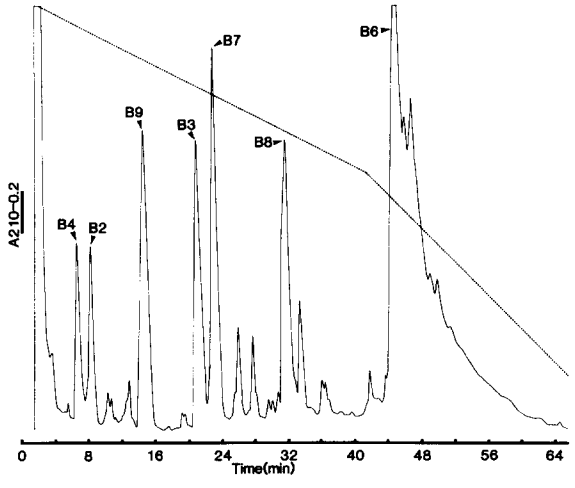

Fig. 1. High-pressure liquid chromatography elution profile of fragments of the CNBr-treated *c*-subunit from strain AN2188 (pAN258, *uncE513*). The CNBr fragments indicated were identified after amino acid analysis. Experimental details are described in the Materials and Methods. The broken line indicates a linear gradient ranging from 100% of 0.1% phosphoric acid to 100% acetonitrile.

TABLE II

AMINO ACID COMPOSITION OF CNBr FRAGMENT B6 OF THE *c*-SUBUNIT FROM STRAIN AN2188

The numbers in parenthesis are the numbers of residues expected from the known amino acid sequence for a normal *c*-subunit. The molar ratios were determined by dividing the concentrations of the amino acids by the concentration of lysine which is known to be present in fragment B6 of the *c*-subunit as a single residue.

Amino acids	Molar ratio
Asp	1.2(1)
Thr	1.9(1)
Glu	3.2(3)
Pro	2.2(2)
Gly	7.3(7)
Ala	5.4(6)
Val	1.2(1)
Ile	5.7(6)
Leu	6.4(6)
Phe	2.8(3)
Lys	1 (1)
Arg	2.0(2)

dried, and the amino acid compositions determined. The analyses for each of the cyanogen bromide fragments B2–B4 and B7–B9 [23] indicated that the amino acid composition was normal (results not shown). However, the B6 fragment appeared to contain one less alanine residue and one more threonine residue than a normal B6 fragment (Table II).

DNA sequence of the uncE513 allele

In order to facilitate DNA sequencing, plasmid pAN258 (*uncE513*) was reduced in size to that of pAN51 (*uncB*$^+$*E*$^+$*F*$^+$*H*$^+$*A*$^+$) by digestion with restriction endonuclease *Hin*dIII, followed by ligation with virus-T4 DNA-ligase. Strain AN1453 (*uncB402, recA*) was then transformed with the ligated DNA and selection made for groth on succinate-chloramphenicol minimal medium. A transformant, strain AN2214, was found to carry plasmid pAN268(*uncB*$^+$*E513F*$^+$*H*$^+$*A*$^+$) equivalent to plasmid pAN51 in size and restriction pattern. The DNA sequence of the *uncE513* allele was determined using the strategy described previously [7] and compared with the DNA sequence of the *uncE* gene present on pAN51 [7]. The sequence of the *uncE513* allele differed from normal only in that a G → A base change occurred at nucleotide 73 resulting in alanine being replaced by threonine at position 25 of the *c*-subunit.

Properties of strain AN2188(uncE513)

Strain AN2188 (pAN258, *uncE513*) grew slowly on succinate-minimal medium. Consistent with this observation, strain AN2188 was found to have a growth yield intermediate between the mutant strain AN888(*unc*416 : : *Mu*) and the normal strain AN1461(pAN45, *unc*$^+$) (Table III). Membrane preparations from strain AN2188 (pAN258, *uncE513*) were found to have an ATPase activity about 80% of that obtained for the control strain AN1461(pAN45, *unc*$^+$) (Table III). However, the ATPase activity of membranes from strain AN2188(pAN258, *uncE513*) was not coupled to proton translocation as judged by ATP-dependent

TABLE III

GROWTH YIELDS AND MEMBRANE PROPERTIES OF *ESCHERICHIA COLI* STRAINS

Growth yields were measured as turbidities (Klett units) after growth had ceased on media containing 5 mM glucose [12]. ATPase activities were determined on membrane preparations as described previously [20]. Oxygen uptake by the membrane preparations was measured by using an oxygen electrode. ATP formed from ADP in the presence of [^{32}P]P_i was trapped as glucose 6-phosphate. The reaction was stopped by the addition of trichloroacetic acid and P_i was extracted after the addition of molybdate [19].

Strain	Growth yield (Klett units)	ATPase activity (μmol/min per mg protein)	NADH oxidase activity (ngatoms of O/ min per mg protein)	ATP formation (μmol/min per mg protein)	P/O
AN888 (*unc416::Mu*)	140	< 0.1	178	< 0.1	0
AN1461 (pAN45, *unc*$^+$)	220	1.9	98	18.1	0.18
AN2188 (pAN258, *uncE513*)	175	1.5	132	6.4	0.05

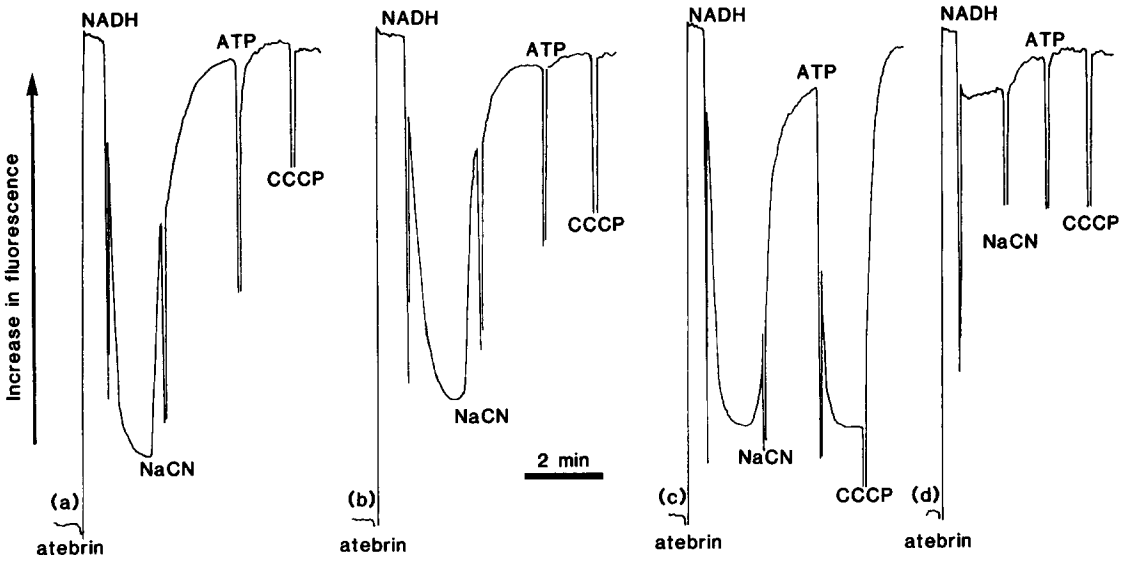

Fig. 2. Atebrin-fluorescence quenching in membranes prepared from strains of *E. coli*. Atebrin fluorescence quenching was measured as described previously [20]. Atebrin was added to give a final concentration of 4 μM, NADH to 2 mM, NaCN to 2.5 mM, ATP to 1 mM and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to 2 μM. (a) Membranes from strain AN2188(pAN258, *uncE513*); (b) F_1-stripped membranes from strain AN2188; (c) membranes from strain AN1461(pAN45, *unc*$^+$); (d) F_1-stripped membranes from strain AN1461.

atebrin fluorescence quenching (Fig. 2). The ATPase activity also differed from normal in membranes from strain AN2188 in that the activity was resistant to the inhibitor DCCD (Fig. 3). This observation was further investigated by incubating reaction mixtures identical to those containing 0 or 12 μg/ml of DCCD for 12 min at 30°C and separating the membranes by centrifugation. In the absence of DCCD, the membranes had a specific ATPase activity of 1.7 μmol P_i released/min per mg protein whereas in the presence of DCCD membrane-bound ATPase activity was only 0.16 μmol P_i released/min per mg protein.

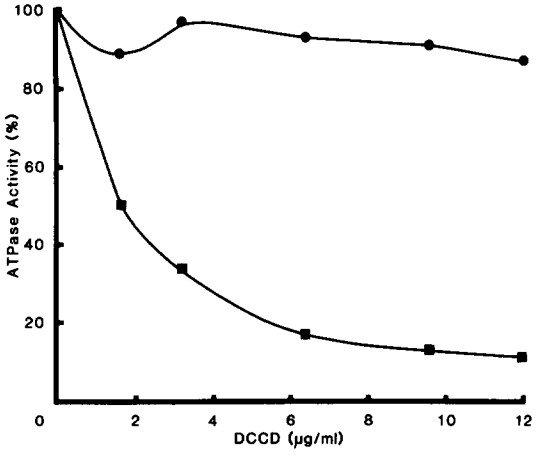

Fig. 3. Inhibition of ATPase activity by DCCD. Membranes (0.3 mg protein) were incubated in 5 ml of the ATPase assay mixture, together with the indicated amount of DCCD at 30°C. The mixture was sampled at intervals and the rate of ATP hydrolysis determined for each DCCD concentration and the percentage inhibition calculated. ●, Membranes from strain AN2188(pAN258, *uncE513*); ■, membranes from strain AN1461(pAN45, *unc*$^+$).

Membranes from strain AN2188(pAN258, *uncE513*) were capable of a low level of oxidative phosphorylation, phosphorylating at a rate of about 30% of the control strain AN1461(pAN45, *unc*$^+$) (Table III). NADH-dependent atebrin fluorescence quenching indicated that the membranes from strain AN2188, on removal of the F_1-ATPase, have greatly reduced permeability compared with normal membranes (Fig. 2).

Discussion

The mutant allele *uncE513* codes for a mutant *c*-subunit of the F_1F_0-ATPase in which alanine at position 25 is replaced by threonine. The mutant

F_1F_0-ATPase appears to be fully assembled, and is indeed partially functional. Mutant membranes from which the F_1-ATPase has been removed have greatly reduced proton permeability compared with similarly treated normal membranes. The ATPase activity of the F_1-ATPase is not inhibited on binding to F_1-depleted membranes and membrane-bound ATPase is apparently resistant to the inhibitor DCCD. However, this is due to the release of the F_1-ATPase from the membrane during the test.

Another mutant strain of *E. coli* has been described in which alanine-21 of the *c*-subunit has been replaced by valine [9]. This mutation therefore occurs one turn of an α-helix away from the mutated residue described in the present paper. If the α-helical hairpin structure of the *c*-subunit is correct, then the residues on the same helical arm and within the membrane bilayer would be alanine-14, glycine-18, alanine-21, alanine-25, glycine-29 and glycine-32. The two mutations referred to above both result in an increase in size of the amino acids at positions 21 and 25 on a helical arm of uniformly small amino acids. The threonine residue resulting from the *uncE513* allele may also allow intrahelical hydrogen bonding with the carbonyl oxygen atom in the preceding turn of the helix [24].

Of the nine mutations in the *uncE* gene [5–10] in which the corresponding amino acid change in the *c*-subunit has been determined, five occur in the N-terminal hydrophobic segment from residue 20 through to 31, and four occur in the C-terminal hydrophobic segment from residue 58 to 64 (Fig. 4). Proton conduction through the F_0 appears to be absent, or severely impaired, in membranes derived from all of the eight *c*-subunit mutants. Only glycine 23 → aspartate and glycine 58 → aspartate completely prevent incorporation of the *c*-subunit into the membrane, although inhibition of ATPase activity by proline 64 → leucine and aspartate 61 → asparagine would suggest that incorrect assembly of the corresponding mutant *c*-subunits into the F_0 structure had occurred. Mutations resulting in aspartate 61 → glycine, leucine 31 → phenylalanine, alanine 21 → valine and the mutation described in the present paper, alanine 25 → threonine all apparently allow incorporation of the mutant *c*-subunit into a correctly assembled

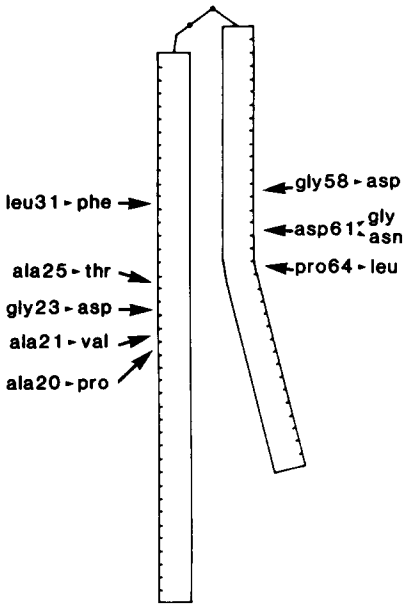

Fig. 4. Proposed structure of the membrane form of the *c*-subunit of the F_1F_0-ATPase [1,4,8], indicating mutations in the *uncE* gene affecting oxidative phosphorylation in which the corresponding amino acid change in the *c*-subunit has been determined – [Ala20 → Pro(8), Ala21 → Val(9), Gly23 → Asp(7), Ala25 → Thr(this paper), Leu31 → Phe(7), Gly58 → Asp(10), Asp61 → Gly(5), Asp61 → Asn(6), Pro64 → Leu(8)].

F_1F_0-ATPase complex. The loss of oxidative phosphorylation activity due to the aspartate 61 → glycine alteration is readily explained by the postulated key role this conserved acidic residue plays in proton conductance. The remaining three mutations which affect proton conduction through the F_0 all result in mutant *c*-subunits with larger amino acids compared to those in the normal *c*-subunit.

These observations provide support for a proposed mechanism for oxidative phosphorylation in which rotating *b*-subunits interact with a ring of *c*-subunits [25]. The increased size of particular amino acids may thus cause loss of activity due to steric hindrance.

Acknowledgements

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