The F_1F_0 -ATPase of *Escherichia coli*

Substitution of proline by leucine at position 64 in the *c*-subunit causes loss of oxidative phosphorylation

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(Received 10 March 1983/Accepted 27 April 1983)

The *uncE410* allele differs from the normal *uncE* gene in that C→T base changes occur at nucleotides 190 and 191, resulting in proline at position 64 in the *c*-subunit of the F_1F_0 -ATPase being replaced by leucine. Two partial-revertant strains were isolated in which alanine-20 of the *c*-subunit was replaced by proline, owing to a G→C base change at nucleotide 58. These *c*-subunits, coded for by the *uncE501* and *uncE502* alleles, therefore contained two amino acid changes, namely proline-64→leucine, and alanine-20→proline. Membranes prepared from the partial-revertant strains lacked ATP-dependent atebirin-fluorescence-quenching activity but were able to carry out oxidative phosphorylation. The ATPase activity of the F_1 -ATPase was inhibited when bound to membranes from strains carrying the *uncE410*, *uncE501* and *uncE502* alleles. It is concluded that a bend in the helix axis in one of the arms of the *c*-subunit hairpin structure is required for integration of the *c*-subunit into a functional F_1F_0 -ATPase.

The genes forming the *unc* operon in *Escherichia coli* code for the components of the energy-coupling ATPase complex and map at about 82.5 min on the *E. coli* chromosome (Downie *et al.*, 1979a). The *uncE* gene, the third gene from the promoter end of the *unc* operon (Gay & Walker, 1981), codes for the DCCD-binding protein or *c*-subunit of the F_1F_0 -ATPase (Downie *et al.*, 1981). Six mutations in the *uncE* gene have been described for which the resulting amino acid substitutions in the mutant *c*-subunits have been established. Mutations which resulted in isoleucine-28 being replaced by either valine or threonine have been shown to cause a loss of reactivity of aspartic acid-61 with the inhibitor DCCD (Hoppe *et al.*, 1980a). It was concluded that the *c*-subunit formed a hairpin structure such that isoleucine-28 was near to aspartic acid-61. Mutations resulting in aspartic acid-61 being re-

placed by either glycine or asparagine have confirmed that this amino acid plays a key role in proton translocation and oxidative phosphorylation (Hoppe *et al.*, 1980b, 1982; Wachter *et al.*, 1980). The substitutions of glycine-23 by aspartic acid and of leucine-31 by phenylalanine prevent the incorporation of the mutant *c*-subunits into the membrane, although in the latter case assembly does occur if the gene dosage is increased (Jans *et al.*, 1983).

The amino acid substitutions in the *c*-subunits coded for by the *uncE410*, *uncE501* and *uncE502* alleles and their effects on assembly and function of the F_1F_0 -ATPase are the subject of the present paper.

Materials and methods

Enzymes and chemicals

Restriction endonucleases and virus-T4 DNA-ligase were obtained from Boehringer Mannheim Australia Pty. Ltd. All chemicals used were of the highest quality available.

Bacterial strains and plasmids

All of the bacterial strains used were derived from *E. coli* K12 and are described, together with the plasmids used, in Table 1.

Abbreviations used: ATPase, Mg^{2+} -stimulated adenosine triphosphatase; DCCD, dicyclohexylcarbodiimide.

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Genetic techniques

The techniques used for genetic experiments were as outlined previously (Downie *et al.*, 1980; Gibson *et al.*, 1977b; Gibson *et al.*, 1978).

Preparation of plasmids

Plasmid DNA was prepared as described by Selker *et al.* (1977). Chromosomal DNA was prepared by the phenol-extraction method of Saito & Miura (1963).

DNA sequencing

Nucleotide sequences were determined by the method of Maxam & Gilbert (1980). End-labelling of DNA was carried out by using DNA polymerase and [α - 32 P]deoxyadenosine triphosphate (Maxam & Gilbert, 1980).

Purification of the c-subunit of the F_1F_0 -ATPase

The c-subunit of the F_1F_0 -ATPase was purified from chloroform/methanol extracts (Fillingame, 1976) of membranes (about 6g of membrane protein) by chromatography on CM-cellulose (Graf & Sebald, 1978; Hoppe *et al.*, 1980a).

Amino acid analysis

Peptides were hydrolysed in 6M-HCl at 100°C for 22 h. Amino acid analysis was carried out by using a Beckman System 6300 high-performance analyser.

CNBr treatment and peptide purification

The purified c-subunit was cleaved by CNBr and the peptides purified as described by Jans *et al.* (1983).

Media and growth of organisms

The mineral-salts minimal medium used and additions were as described previously (Gibson *et al.*, 1977b). Cells for the preparation of membranes were grown in 14-litre fermenters essentially as described previously (Cox *et al.*, 1970). The media in the fermenter vessels was supplemented with 5% (v/v) Luria broth (Luria & Burrous, 1957). Growth yields were measured as described previously (Cox *et al.*, 1970). Turbidities of cultures were measured with a Klett-Summerson colorimeter.

Preparation of cell membranes

The preparation and treatment of membranes were as described previously (Cox *et al.*, 1983).

ATPase activity

ATPase activity was measured by using the method described by Gibson *et al.* (1977a).

Atebrin-fluorescence quenching

Atebrin-fluorescence quenching was measured as described by Gibson *et al.* (1977a).

Table 1. *Bacterial strains and plasmids used*

The chromosomal gene nomenclature is that given by Bachmann *et al.* (1976); plasmid nomenclature is that given by Novick *et al.* (1976).

Strain no.	Relevant genotype	Notes and references
(a) Bacterial strains		
AN1006	pAN14, <i>uncE410/argH ilvC pyrE recA</i>	Partial diploid strain carrying <i>uncE410</i> allele on F' plasmid (prepared as described previously; Gibson <i>et al.</i> , 1977b)
AN1521	<i>uncF476 argH pyrE entA recA</i>	Downie <i>et al.</i> (1981)
AN1440	<i>uncF469 argH pyrE entA recA</i>	Downie <i>et al.</i> (1981)
AN955	<i>uncE410 argH pyrE entA</i>	Downie <i>et al.</i> (1979b)
AN1782	<i>argH pyrE entA</i>	U.v.-induced revertant of AN955 (The present paper)
AN1781	<i>uncE501 argH pyrE entA</i>	U.v.-induced partial revertant of AN955 (The present paper)
AN1892	<i>uncE502 argH pyrE entA</i>	U.v. partial revertant of AN955 (The present paper)
AN1707	<i>uncF476 thr leu rk⁻ mk⁺ recA</i>	Obtained by transducing <i>uncF476</i> allele into C600 background
AN1959	pAN175, <i>uncE501/uncF476 thr leu rk⁻ mk⁺ recA</i>	The present paper
AN1971	pAN177, <i>uncE502/uncF476 thr leu rk⁻ mk⁺ recA</i>	The present paper
(b) Plasmids		
pAN14	<i>PyrE⁺ uncE410 ilvC⁺ argH⁺</i>	F' plasmid carrying <i>uncE410</i> allele (prepared as described previously; Gibson <i>et al.</i> , 1977b)
pHC79	<i>Ap^r Tc^r</i>	pBR322 derivative obtained from Dr. B. Hohn, (Friedrich Miescher Institute, Basel, Switzerland)
pAN175	<i>Ap^r Tc^s uncB⁺E501F⁺H⁺A⁺</i>	pHC79 derivative carrying <i>uncE501</i> allele (The present paper)
pAN177	<i>Ap^r Tc^s uncB⁺E502F⁺H⁺A⁺</i>	pHC79 derivative carrying <i>uncE502</i> allele (The present paper)

Oxidative phosphorylation

Oxidative phosphorylation was measured as described by Cox *et al.* (1973).

Protein determination

Protein concentrations were determined by using Folin's phenol reagent (Lowry *et al.*, 1951), with bovine serum albumin (fraction V, Sigma) as standard.

Results

Further investigation of the *unc-410* allele

The *unc-410* allele had, on the basis of genetic complementation data and on the failure to isolate revertants, been previously thought to affect a gene or genes in addition to the *uncE* gene (Downie *et al.*, 1979b). The mutant strain carrying the *unc-410* allele had been isolated after u.v. mutagenesis, and it was suggested that a deletion involving the *uncE* gene and an unknown adjacent gene had occurred. The *uncF* gene is the gene immediately after the *uncE* gene (Gay & Walker, 1981) and the *uncF469* and *uncF476* alleles (Downie *et al.*, 1981) had not been described at the time the previous work with the *unc-410* allele was published. The partial-diploid strain AN1006 carries the *unc-410* allele on an F-plasmid (pAN14) and was used as a donor strain in complementation tests with the female strains AN1521 (*uncF476*) and AN1440 (*uncF469*). Complementation was obtained since colonies were formed on the succinate medium in the region where the donor and recipient strains mixed in the cross-streaking test described previously (Gibson *et al.*, 1977a). The reverse cross, in which either *uncF* mutant allele was on the plasmid and the *unc-410* allele on the chromosome, also gave complementation. The *unc-410* mutation does not, therefore, affect the *uncF* gene.

The medium used previously (Downie *et al.*, 1979b) in the attempted isolation of revertants was a succinate/minimal medium. In a further attempt, cells of strain AN955 (*unc-410*) were spread on solid succinate/minimal medium supplemented with 0.05% casamino acids and irradiated with u.v. light for 40 s. Under these conditions revertant colonies were obtained. However, of about 50 revertant colonies examined only one, strain AN1782, was found to be a full revertant (see below). Of the remaining partial revertants, two were selected (AN1781 and AN1892) for further examination. Strains AN1781 and AN1892 represented the group of partial-revertant strains giving growth yields closest to that obtained for a normal strain (see below).

Amino acid substitutions in the *c*-subunits of the F_1F_0 -ATPases from strain AN955 (*unc-410*) and the revertant strains AN1782, AN1781 and AN1892

The *c*-subunits of the F_1F_0 -ATPase were extracted from membranes of strains AN955 (*unc-410*), AN1782, AN1781 and AN1892 and purified by chromatography on CM-cellulose. The purified proteins were solubilized in formic acid and cleaved with CNBr. Cleavage of the *c*-subunit with CNBr produces nine fragments (Fig. 1). The peptide fragments soluble in 0.1% phosphoric acid were separated from insoluble material by centrifugation and examined by high-pressure liquid chromatography. The elution profiles obtained by measurement of A_{210} indicated that fragment B7 of the *c*-subunit from strain AN1782 was different from the B7 fragments from strains AN955 (*unc-410*), AN1781 and AN1892 (Fig. 2). The eluate corresponding to each of the peaks of absorption was collected, freeze-dried and the amino acid compositions determined. Fragment B7 derived from strains AN955 (*unc-410*), AN1781 and AN1892 contained two leucine residues and no proline residues, whereas B7 derived from strain AN1782 had the expected one leucine residue and one proline residue (Table 2). The amino acid compositions of the remaining soluble fragments derived from all four strains were the same (L. B. James, unpublished work).

The material insoluble in 0.1% phosphoric acid was dissolved in chloroform/methanol (1/1, v/v) and the B6 fragments purified by cellulose t.l.c. Amino acid analysis indicated that the B6 fragments from strains AN1781 and AN1892 contained one less alanine residue and one more proline residue than the B6 fragments from strains AN955 (*unc-410*) and AN1782 (Table 2).

It was concluded that the *unc-410* mutation occurs in the *uncE* gene and that the partial-revertant strains AN1781 and AN1892 each carry a second mutation in the *uncE* gene. The mutant *uncE* genes present in strains AN1781 and AN1892 result in the formation of *c*-subunits with two amino acid changes and have been given the allele numbers *uncE501* and *uncE502* respectively. The *c*-subunit from strain AN1782 appeared to be normal.

Cloning and sequencing of the *uncE501* and *uncE502* alleles

In order to locate which alanine residue has been replaced by proline in the B6 fragments derived from strains AN1781 (*uncE501*) and AN1892 (*uncE502*), the DNA coding for the mutant *c*-subunits was sequenced and compared with the DNA sequence for a normal *uncE* gene.

Chromosomal DNA was prepared from strains AN1781 (*uncE501*) and AN1892 (*uncE502*) and digested with the restriction endonuclease *Hind*III.

Fig. 1. Nucleotide sequence of the DNA coding for the *c*-subunit of the F_1F_0 -ATPase of *E. coli* together with the corresponding amino acid sequence

The fragments of the *c*-subunit generated by CNBr are indicated, and the designation of the fragments (B1 to B9) follows that used by Wachter *et al.* (1980). The base changes and the corresponding amino acid change due to the *uncE410* allele (proline-64→leucine) are indicated as are the additional changes due to the *uncE501* and *uncE502* alleles (alanine-20→proline).

Each of the DNA preparations were mixed with *Hind*III-digested pHC79-plasmid DNA and treated with virus-T4 DNA-ligase. Strain AN1707 (*uncF476*, *recA*) was then transformed with each of the ligated-DNA mixtures and selection made for growth on succinate/ampicillin minimal medium. One colony was selected from each of the transformation experiments, purified, and each strain shown to carry a plasmid with an insert corresponding in size to that of pAN51 (Downie *et al.*, 1980). Strain AN1959 carried the plasmid pAN175 with an insert derived from the chromosomal DNA of strain AN1781 (*uncE501*). Strain AN1971 carried the plasmid pAN177 with an insert derived from the chromosomal DNA of strain AN1892 (*uncE502*).

The *Hinf*I- and *Bam*HI-restriction-endo-nuclease-generated DNA fragments of plasmids pAN175 and pAN177 were used to derive the nucleotide sequences of the *uncE501* and *uncE502* alleles, respectively, as described previously (Jans *et al.*, 1983). The *uncE501* and *uncE502* mutant DNA sequences were identical and differed from the normal sequence in that C→T base changes occurred at nucleotides 190 and 191 and a G→C base

change occurred at nucleotide 58 (see Fig. 1). The changes at nucleotides 190 and 191 result in proline being replaced by leucine at position 64 and the change at nucleotide 58 results in alanine being replaced by proline at position 20 (see Fig. 1).

Effect of the uncE501 and uncE502 mutant c-subunits on the function of the F₁F₀-ATPase

The growth yields of strains AN1781 (*uncE501*) and AN1892 (*uncE502*) on limiting concentrations of glucose were determined and compared with those obtained for the original mutant strain AN955 (*uncE410*) and the full-revertant strain AN1782 (*unc*⁺) (Table 3). Strain AN1782 (*unc*⁺) gave a value expected for a normal strain (Cox & Gibson, 1974), whereas strains AN1781 (*uncE501*) and AN1892 (*uncE502*) gave values intermediate between those of the mutant strain AN955 (*uncE410*) and strain AN1782 (*unc*⁺).

Membranes were prepared from strains AN955 (*uncE410*), AN1782 (*unc*⁺), AN1781 (*uncE501*) and AN1892 (*uncE502*) and ATPase activities, fluorescence-quenching activities and oxidative-phosphorylation activities examined. The ATPase

Fig. 2. High-pressure liquid chromatography of soluble fragments of the CNBr-treated c-subunits from strain AN955 (*uncE410*) (a), AN1781 (*uncE501*) (b) and AN1782 (*unc+*) (c)

The elution profile of the soluble CNBr fragments derived from the c-subunit from strain AN1892 (*uncE502*) was the same as that obtained for strain AN1781 (*uncE501*). The CNBr fragments indicated were identified after amino acid analysis. Experimental details are given in the Materials and methods section.

activities of membranes from the partial-revertant strains AN1781 (*uncE501*) and AN1892 (*uncE502*) were about twice the value obtained for membranes from strain AN955 (*uncE410*) (Table 3). The ATPase activities of the membranes from the partial-revertant strains could be about doubled by growing the cells on media containing succinate as energy source (Table 3). The phosphorylation rates and P/O ratios obtained with membranes from the partial-revertant strains AN1781 (*uncE501*) and AN1892 (*uncE502*) were about 25% of the values

Table 2. Amino acid compositions of CNBr fragments B6 and B7 of the c-subunits from four strains of *Escherichia coli*

Amino acid compositions of the purified CNBr fragments were determined by using a Beckman System 6300 high-performance analyser after hydrolysis in 6M-HCl for 22h. Leucine always analysed as seven residues in the B6 fragment of the c-subunit from normal strains. The numbers in parentheses are the numbers of residues expected for each fragment as calculated from the known amino acid sequence. The molar ratios were determined by dividing the concentrations of the amino acids derived from a particular fragment by the concentrations of an amino acid known to be in that fragment as a single residue.

		Molar ratio			
Frag- ment	Amino acid	AN1782 (<i>unc+</i>)	AN955 (<i>uncE410</i>)	AN1781 (<i>uncE501</i>)	AN1892 (<i>uncE502</i>)
B7	Gly	1.0 (1)	1.0	1.0	1.0
	Asp	1.2 (1)	1.0	1.1	1.3
	Pro	1.2 (1)	0	0	0
	Ala	1.0 (1)	1.0	1.1	1.3
	Val	0.9 (1)	0.9	1.1	1.0
	Ile	1.0 (1)	0.9	0.8	0.9
	Leu	1.2 (1)	2.0	2.0	2.3
	Lys	1.0 (1)	1.0	1.0	1.0
B6	Arg	1.7 (2)	1.5	1.6	1.6
	Asp	1.4 (1)	1.5	1.5	1.4
	Thr	1.1 (1)	1.0	1.1	0.9
	Glu	3.0 (3)	2.9	2.9	2.7
	Pro	2.1 (2)	2.0	2.9	2.7
	Gly	6.6 (7)	6.8	7.2	7.1
	Ala	6.0 (6)	6.2	5.4	5.3
	Val	1.4 (1)	1.5	1.5	1.6
	Ile	5.7 (6)	6.0	5.9	5.7
	Leu	7.0 (6)	7.2	7.4	7.4
	Phe	2.7 (3)	3.0	2.9	2.7

obtained for normal membranes (Table 3). Membranes from strains AN1781 (*uncE501*) and AN1892 (*uncE502*) lacked ATP-dependent atebrin-fluorescence-quenching activity (Fig. 3). NADH-dependent atebrin-fluorescence-quenching activity was present in membranes from strains AN1781 (*uncE501*) and AN1892 (*uncE502*), and this activity was retained in membranes from which the F₁-ATPase was removed (Fig. 3).

Inhibition of ATPase activity on binding of the F₁-ATPase to membranes from strain AN955 (uncE410)

The ATPase activity of membranes from strain AN955 (*uncE410*) was about 15% of that obtained for normal membranes (see Table 3). However, on examination of these membranes by two-dimensional gel electrophoresis, it was clear that the five subunits of the F₁-ATPase were present in amounts at least equal to those found in normal membranes

Table 3. *Growth yields and membrane properties of various strains of Escherichia coli*

Growth yields were measured as turbidities (Klett units) after growth had ceased on media containing 5 mM-glucose. ATPase activities were determined on membrane preparations as described previously (Gibson *et al.*, 1977a). 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.

Strain	Growth yields (Klett units)	Activity		ATP formation (μ mol/min per mg of protein)	P/O
		ATPase (μ mol/min per mg of protein)	NADH oxidase (ng-atoms of O/min per mg of protein)		
AN1782 (<i>unc</i> ⁺)	198	1.5	101	16.7	0.17
AN1781 (<i>uncE501</i>)	160	0.42	149	5.4	0.04
AN1781 (<i>uncE501</i>)*		0.80	89	3.4	0.04
AN1892 (<i>uncE502</i>)	158	0.44	152	4.9	0.03
AN1892 (<i>uncE502</i>)*		1.0	64	2.6	0.04
AN955 (<i>uncE410</i>)	120	0.19	184	<0.1	<0.001

* Membranes were prepared from cells grown on media containing succinate as energy source.

Fig. 3. *Atebrin-fluorescence quenching in membranes prepared from strains AN1892 (uncE502) and AN1782 (unc⁺)* Atebrin-fluorescence quenching was measured as described previously (Gibson *et al.*, 1977a). Atebrin was added to give a final concentration of 4 μ M, NADH to 2 mM, NaCN to 2.5 mM, ATP to 1 mM and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to 20 μ M. (a) Membranes from strain AN1892 (*uncE502*); (b) stripped membranes from strain AN1892 (*uncE502*); (c) membranes from strain AN1782 (*unc*⁺); (d) stripped membranes from strain AN1782 (*unc*⁺).

Fig. 4. Proposed structures of the membrane forms of the c -subunits of the F_1F_0 -ATPase in mutant strains carrying the *uncE410*, *uncE501* or *uncE502* alleles

Structures are shown of the c -subunit in (a) normal strains, (b) strains carrying the *uncE410* allele and (c) partial revertant strains (derived from the *uncE410* mutant strain) carrying either the *uncE501* or *uncE502* alleles. The changed amino acids are indicated. The broken line ($\leftarrow\cdots\rightarrow$) indicates the distance between the C-terminal alanine residue and the aspartic acid residue at position 7.

(G. B. Cox, unpublished work). A membrane preparation of strain AN955 (*uncE410*) was assayed for ATPase activity before and after dialysis against low-ionic-strength buffer in the absence of *p*-aminobenzamidine. Such treatment results in the dissociation of the F_1 -ATPase from the membrane (Senior *et al.*, 1979) and the ATPase activity of the membranes from strain AN955 (*uncE410*) increased from $0.19\ \mu\text{mol}/\text{min}$ per mg of protein before dialysis to $0.94\ \mu\text{mol}/\text{min}$ per mg of protein after dialysis. The ATPase activity of a normal membrane preparation was unchanged by this treatment. The activities are expressed per mg of membrane protein for purposes of comparison, as the ATPase activity is present in the supernatant after the membranes have been removed by centrifugation. A similar effect was found with membranes from strains AN1781 (*uncE501*) and AN1892 (*uncE502*), although the effect was not as marked, with the ATPase activities increasing from 0.40 – $0.45\ \mu\text{mol}/\text{min}$ per mg of protein before dialysis to 0.85 – $0.92\ \mu\text{mol}/\text{min}$ per mg of protein after dialysis.

Discussion

The mutant allele *uncE410* codes for a c -subunit of the F_1F_0 -ATPase in which the proline at position 64 is replaced by leucine. The G $\rightarrow$ T base changes at both nucleotide 190 and nucleotide 191 provide an explanation for the difficulty experienced in isolating revertants (Downie *et al.*, 1979b). CNBr fragment B7 of the c -subunit from the mutant strain AN955

(*uncE410*) contains two leucine residues and no proline residues, which is consistent with the DNA-sequencing data. Fragment B7 from the full-revertant strain AN1782 contains the expected one leucine and one proline residue. It is therefore clear that the uncoupled phenotype of strain AN955 (*uncE410*) is due to the substitution in the c -subunit of proline-64 by leucine and may be attributed either to direct mechanistic effects or to indirect structural effects. The nature and position of the amino acid changes in the partial-revertant strains AN1781 (*uncE501*) and AN1892 (*uncE502*) would suggest the second of these alternatives.

We propose that, in the normal c -subunit of the F_1F_0 -ATPase, a bend in the helix axis at proline-64 (see Fig. 4) is required for the successful integration of this subunit with the other F_0 -subunits during the assembly in the membrane of the F_1F_0 -ATPase. The distance between the *N*-terminal and C-terminal ends of the hairpin structure of the c -subunit would be different in the *uncE410* mutant c -subunit (proline-64 $\rightarrow$ leucine) as compared with a normal c -subunit (Fig. 4), and it is proposed that this would prevent the normal integration with the other F_0 -subunits. This incorrect assembly of the c -subunit into the F_0 structure could also provide an explanation for the observed inhibition of ATPase activity. It is the restoration, in the partial-revertant strains, of the distance between charged groups at the C-terminal and *N*-terminal ends of the c -subunit (see Fig. 4) that suggests that the phenotype of strain AN955 (*uncE410*) is due to the lack of normal integration of

the mutant *c*-subunit with the remaining F_0 subunits. The distance between a proline at position 64 and a proline at position 20 would also appear to preclude the direct involvement of proline in the mechanism of oxidative phosphorylation as an explanation for the phenotype of strain AN955 (*uncE410*).

The membranes of the partial-revertant strains do not have ATP-dependent atebirin-fluorescence-quenching activity but are capable of carrying out oxidative phosphorylation. However, membranes from these partial-revertant strains retain NADH-dependent atebirin-fluorescence-quenching activity after removal of the F_1 -ATPase. The properties of membranes from the partial-revertant strains are therefore similar to those of membranes from mutant strains carrying either the *uncE408* or *uncE463* alleles in that apparently proton-impermeable membranes are capable of carrying out oxidative phosphorylation (Cox *et al.*, 1983).

We thank Ms. D. Marsden, Mrs. B. Rowell and Mr. B. Webb for skilled technical assistance. Mr. D. Abigail is thanked for assistance with the growth of cells and preparation of membranes. A. E. S. is grateful for a grant from the National Science Foundation under the U.S./Australian Co-operative Science Agreement.

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