Mutations in the *uncE* gene affecting assembly of the *c*-subunit of the adenosine triphosphatase of *Escherichia coli*

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The amino acid substitutions in the mutant *c*-subunits of *Escherichia coli* F_1F_0 -ATPase coded for by the *uncE429*, *uncE408* and *uncE463* alleles affect the incorporation of these proteins into the cell membrane. The DNA sequence of the *uncE429* allele differed from normal in that a G→A base change occurred at nucleotide 68 of the *uncE* gene, resulting in glycine being replaced by aspartic acid at position 23 in the *c*-subunit. The *uncE408* and *uncE463* mutant DNA sequences were identical and differed from normal in that a C→T base change occurred at nucleotide 91 of the *uncE* gene, resulting in leucine being replaced by phenylalanine at position 31 in the *c*-subunit. An increased gene dosage of the *uncE408* or *uncE463* alleles resulted in the incorporation into the membranes of the mutant *c*-subunits. The results are discussed in terms of the 'Helical Hairpin Hypothesis' of Engelman & Steitz [(1981) *Cell* 23, 411–422].

The membrane sector of the energy-coupling adenosine triphosphatase complex from mitochondria, chloroplasts or procaryotic-cell membranes contains a low-molecular-weight (about 8000) 'proteolipid' that reacts with DCCD. A conserved acidic residue in an otherwise hydrophobic segment of this protein reacts with DCCD to form a stable adduct, resulting in inhibition of ATPase activity (Sebald *et al.*, 1979, 1980). The DCCD-binding proteins from six different sources (*Neurospora crassa*, *Saccharomyces cerevisiae*, bovine heart, spinach chloroplasts, *Escherichia coli* and the thermophilic bacterium PS3) have been sequenced and have a number of features in common (Sebald *et al.*, 1979).

In *E. coli* the DCCD-binding protein (*c*-subunit) is coded for by the *uncE* gene (Downie *et al.*, 1981), the third gene from the promoter end of the *unc* operon (Gay & Walker, 1981). The aspartic acid residue at position 61 is the DCCD-reactive amino

acid and the substitution of this amino acid by glycine in an *uncE* mutant strain of *E. coli* has been shown to result in the loss of oxidative phosphorylation (Hoppe *et al.*, 1980a; Schairer *et al.*, 1976; Wachter *et al.*, 1980). However, whether this mutation prevents normal assembly of the F_1F_0 -ATPase, or whether it renders a normally assembled F_1F_0 -ATPase inactive, remains uncertain (see Fillingame, 1981). 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 the membrane in such a way that the isoleucine at position 28 is close to the aspartic acid residue at position 61 (Hoppe *et al.*, 1980b).

In the present paper we describe the amino acid substitutions caused by the three mutant *unc* alleles, *uncE429*, *uncE463* and *uncE408*, and their effects on the assembly of the F_1F_0 -ATPase complex.

Materials and methods

Enzymes and chemicals

Restriction endonucleases and T4-virus DNA ligase were obtained from New England Biolabs (Beverly, MA, U.S.A.). All chemicals used were of the highest quality available.

Abbreviations used: ATPase, Mg^{2+} -stimulated adenosine triphosphatase (EC 3.6.1.3); DCCD, dicyclohexylcarbodi-imide.

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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 or in the text.

Genetic techniques

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

Preparation of plasmids

Plasmid DNA was prepared as described by Downie *et al.* (1980). F-plasmid DNA was prepared by the method of Clewell & Helinski (1969) and included the 'alkali pulse' step of Hansen & Olsen (1978). Chromosomal DNA was prepared by the phenol-extraction method of Saito & Miura (1963).

Table 1. *Bacterial strains and plasmids used*

(a) Bacterial strains		Notes and references
Strain no.	Relevant genotype	
AN936	<i>uncE429 argH pyrE entA</i>	Downie <i>et al.</i> (1979)
AN1453	<i>uncB402 thr leu hsdR hsdM recA</i>	Prepared by transduction of <i>uncB402</i> allele into an <i>ilvC</i> derivative of C600 (Downie <i>et al.</i> , 1980)
AN1905	<i>pAN149/uncB402 thr leu hsdR hsdM recA</i>	Obtained by transformation of AN1453 with pAN149
AN1330	<i>pAN34/uncE463 argH pyrE entA recA</i>	Obtained by mating AN1308 with AN1307
AN1307	<i>uncE463 argH pyrE entA recA</i>	Obtained by P1 transduction of <i>uncE463</i> allele from original isolate into AN346 (Gibson <i>et al.</i> , 1977a)
AN1308	<i>pAN34/argH pyrE purE ilvC recA</i>	Strain carrying <i>uncE463</i> allele on an F-plasmid isolated as described previously (Gibson <i>et al.</i> , 1977b)
AN1332	<i>pAN10/uncE408 argH pyrE entA recA</i>	Obtained by mating AN859 with AN845
AN859	<i>pAN10/argH ilvC pyrE purE recA</i>	Strain carrying <i>uncE408</i> allele on an F-plasmid isolated as described previously (Gibson <i>et al.</i> , 1977b)
AN845	<i>uncE408 argH pyrE entA recA</i>	Obtained by P1 transduction of <i>uncE408</i> allele from original isolate into AN346 (Gibson <i>et al.</i> , 1977a)
AN1331	<i>pAN34/uncE408 argH pyrE entA recA</i>	Obtained by mating AN1308 with AN845
AN1333	<i>pAN10/uncE463 argH pyrE entA recA</i>	Obtained by mating AN859 with AN1307
AN1897	<i>argH pyrE entA</i>	Spontaneous revertant of AN795 (the present paper)
AN795	<i>uncE408 argH pyrE entA</i>	Downie <i>et al.</i> (1979)
AN1875	<i>pAN146/uncB402 argH pyrE entA recA</i>	Obtained by transformation of AN727 with pAN146
AN1915	<i>pAN159/unc416::MuB⁺EFHAGDC argH pyrE entA recA</i>	Obtained by transformation of AN888 with pAN159
AN888	<i>unc416::MuB⁺EFHAGDC argH pyrE entA recA</i>	Polar mutant isolated as described previously (Gibson <i>et al.</i> , 1978). Similar genetically to <i>unc418::Mu</i> in strain AN884
AN248	<i>ilvC argH entA</i>	Butlin <i>et al.</i> (1973)
AN1460	<i>pAN45/unc413::MuBEFHAGDC argH pyrE entA recA</i>	Downie <i>et al.</i> (1980)
AN724	<i>ilvC argH pyrE entA recA</i>	An isogenic set of strains carrying the reference 'unc' alleles (available from Coli Genetic Stock Centre) in AN346 background (Gibson <i>et al.</i> , 1977a; Downie <i>et al.</i> , 1979, 1980, 1981)
AN730	<i>uncA401 argH pyrE entA recA</i>	
AN727	<i>uncB402 argH pyrE entA recA</i>	
AN802	<i>uncC424 argH pyrE entA recA</i>	
AN818	<i>uncD409 argH pyrE entA recA</i>	
AN943	<i>uncE429 argH pyrE entA recA</i>	
AN1440	<i>uncF469 argH pyrE entA recA</i>	
AN1273	<i>uncG428 argH pyrE entA recA</i>	
(b) Plasmids		
pAN10	<i>pyrE⁺ uncE408 argH⁺ ilv⁺</i>	F-plasmid carrying <i>uncE408</i> allele (Gibson <i>et al.</i> , 1977b)
pAN34	<i>pyrE⁺ uncE463 argH⁺ ilv⁺</i>	F-plasmid carrying <i>uncE463</i> allele (Gibson <i>et al.</i> , 1977b)
pAN146	<i>Cm^r Tc^r uncB⁺ E408 F⁺H⁺A⁺</i>	The present paper
pAN51	<i>Cm^r Tc^r unc B⁺E⁺F⁺H⁺A⁺</i>	Downie <i>et al.</i> (1980)
pAN92	<i>Cm^r Tc^r</i>	The present paper
pAN45	<i>Cm^r Tc^r unc B⁺E⁺F⁺H⁺A⁺G⁺D⁺C⁺</i>	Downie <i>et al.</i> (1980)
pAN128	<i>Cm^r Tc^r unc B⁺ E408 F⁺H⁺A⁺G⁺D⁺C⁺</i>	The present paper
pAN149	<i>Ap^r Tc^r uncB⁺ E429 F⁺H⁺A⁺</i>	The present paper
pAN159	<i>Tc^r uncB⁺ E463 F⁺H⁺A⁺G⁺D⁺C⁺</i>	The present paper
pAN160	<i>Cm^r Tc^r uncB⁺ E463 F⁺H⁺A⁺</i>	The present paper
pACYC184	<i>Cm^r Tc^r</i>	Chang & Cohen (1978)
pHCY79	<i>Ap^r Tc^r</i>	pBR322 derivative. Obtained from Dr. B. Hohn

DNA sequencing

Nucleotide sequences were determined by the method of Maxam & Gilbert (1980). End-labelling of DNA was carried out with DNA polymerase and deoxyadenosine[α - 32 P]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 6 g of membrane protein) by chromatography on CM-cellulose (Graf & Sebald, 1978; Hoppe *et al.*, 1980a).

Amino acid analysis

Proteins or peptides were hydrolysed in 6 M-HCl at 100°C for 22 h. The acid was removed by rotary evaporation and the amino acids dissolved in 0.2 M-sodium citrate buffer, pH 2.2. Amino acid analysis was carried out with a Beckman 120B analyser as described by Spackman *et al.* (1958) and ninhydrin reagent prepared with titanous chloride (James, 1978), or using a Beckman System 6300 high-performance analyser.

CNBr treatment and peptide analysis

The purified *c*-subunit (about 15 mg) was dissolved in 2 ml of 99% formic acid and 1 g of CNBr was added. After most of the CNBr had dissolved, 0.5 ml of water was added and the solution left at room temperature for 20 h. The reaction mixture was freeze-dried and redissolved in about 0.2 ml of 99% formic acid, after which 10 ml of water was added and the mixture again freeze-dried. The peptide mixture was then extracted with 1 ml of 0.1% phosphoric acid for 1 h at 65°C and centrifuged. The supernatant was filtered through a Millipore filter (0.22 μ m pore size). The filtrate was 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 0–50% acetonitrile in aq. 0.1% phosphoric acid over a 50 min period (Fullmer & Wasserman, 1979). 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 as described above.

The material insoluble in 0.1% phosphoric acid, which was separated from the soluble material by centrifugation, was dissolved in chloroform/methanol (2:1, v/v) and chromatographed on thin-layer cellulose sheets (Merck t.l.c. aluminium/cellulose-coated sheets) with chloroform/methanol/water (25:75:1, by vol.) as the developing solvent. Fragment B6 chromatographed with an R_F value of 0.8 and was detected by weak colour development after spraying part of the chromatogram with ninhydrin. The purified B6 fragment was dissolved in

6 M-HCl, hydrolysed and analysed for amino acids as described above.

Other methods

Media and growth of organisms (Gibson *et al.*, 1977b), assay of ATPase activity (Gibson *et al.*, 1977a) and determination of protein concentrations (Lowry *et al.*, 1951) were as described previously. Membranes enriched in ATPase activity by washing in low-ionic-strength buffer in the presence of *p*-aminobenzamidine were prepared as described previously (Senior *et al.*, 1979).

Results

Three mutations in the *uncE* gene have been described previously (Downie *et al.*, 1981) that result in the non-incorporation into membranes of the *c*-subunit of the F_1F_0 -ATPase, namely the *uncE429*, *uncE408* and *uncE463* alleles. Increasing the gene dosage of the *uncE408* and *uncE463* alleles resulted in some assembly of the mutant proteins into the membrane (see below). A similar gene dosage effect was not seen with the *uncE429* allele. The nature of the mutation in the *uncE429* allele was therefore determined by sequencing the mutant gene.

Cloning and sequencing of the *uncE429* allele

Chromosomal DNA was prepared from strain AN936 (*uncE429*) and digested with the endonuclease *Hind*III. This DNA was mixed with *Hind*III-digested pHC79 plasmid DNA and treated with T4-virus DNA ligase. Strain AN1453 (*uncB402*) was then transformed with the ligated DNA mixture and the selection made for growth on succinate/ampicillin/minimal medium (Gibson *et al.*, 1977b; Downie *et al.*, 1980). One colony was selected at random, purified, and this strain (AN1905) was shown (Fig. 1) to carry a plasmid (pAN149) with an insert corresponding in size to that of pAN51 (Downie *et al.*, 1980). The plasmid pAN149 was used to transform a series of strains, each carrying one of the *unc* alleles *uncB402*, *uncE429*, *uncF469* or *uncA401*. Selection was made on ampicillin medium and the ampicillin-resistant transformants tested for the ability to grow on succinate. Complementation, as judged by growth on the succinate medium, was obtained between the plasmid pAN149 and the *uncB402*, *uncF469* or *uncA401* alleles, but not the *uncE429* allele. The *Hinf*I- and *Bam*HI-restriction-endonuclease-generated DNA fragments of plasmid pAN149 were used to derive the nucleotide sequence of the *uncE429* allele as delineated in Fig. 2. The corresponding normal DNA sequence was also determined by using analogous fragments from plasmid pAN51. The DNA sequence of the *uncE429* allele differed from normal in that a G→A base change occurred at nucleotide 68 of the *uncE* gene, resulting in glycine

Fig. 1. Electrophoresis of *Hind*III-restriction-endo-nuclease-cleavage fragments of plasmid DNA

(a) pAN51; (b) and (g) pACYC184; (c) pAN146; (d) pHC79; (e) pAN149; (f) pAN160.

Fig. 2. Restriction fragments from the plasmids used for DNA sequencing

Arrows indicate the direction and approximate length of the DNA fragments sequenced from the *Bam*HI or *Hin*FI restriction sites. The location of the DNA segment coding for the c-subunit (DCCD-binding protein) is indicated.

being replaced by aspartic acid at position 23 in the c-subunit (Fig. 3). Both pAN51 and pAN149 DNA sequences differed from the published sequence, which was determined by using DNA cloned into λ phage (Gay & Walker, 1981; Kanazawa *et al.*,

1981; Nielsen *et al.*, 1981) at nucleotides 106 and 117; however, neither change results in any alteration to the amino acid sequence.

Effects of increased gene dosage of the uncE408 or uncE463 alleles

Mutant strains carrying the *uncE408* or *uncE463* alleles are unable to grow on succinate/minimal media and have growth yields on limiting concentrations of glucose characteristic of uncoupled mutant strains (Cox & Gibson, 1974; Downie *et al.*, 1981). The mutant alleles *uncE408* and *uncE463* were transferred to an F-plasmid by the method described previously (Gibson *et al.*, 1977b) to give F-plasmids pAN10 (*uncE408*) and pAN34 (*uncE463*). Strains carrying these plasmids were used as donor strains in complementation tests with a series of female strains each carrying one of the *unc* alleles *uncA401*, *uncB402*, *uncC424*, *uncD409*, *uncE429*, *uncF469* or *uncG428*. Complementation was judged as occurring if 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 *uncE408* and *uncE463* alleles gave positive complementation tests with each of the mutant alleles except *uncE429*. However, when the partial-diploid strains AN1330 (*uncE463/uncE463*) and AN1332 (*uncE408/uncE408*) were prepared, it was found that these strains were able to grow on succinate/minimal medium. Similarly strains AN1331 (*uncE463/uncE408*) and AN1333 (*uncE408/uncE463*) were able to grow on succinate/minimal medium. The growth yields of all four strains were also similar (Table 2) and indicated that the partial-diploid strains had regained some capacity for oxidative phosphorylation as compared with the haploid controls. The ATPase activities (Table 2) of membrane preparations from these four strains also indicated that some assembly of the complete F_1F_0 -ATPase had occurred compared with the interrupted assembly (Cox *et al.*, 1981a) that occurs in the haploid strains.

*Amino acid substitutions in the c-subunits of the F_1F_0 ATPases from strains AN1330 (*uncE463/uncE463*) and AN1332 (*uncE408/uncE408*)*

The c-subunits of the F_1F_0 -ATPase were extracted from membranes of strains AN1330 (*uncE463/uncE463*) and AN1332 (*uncE408/uncE408*) and purified by chromatography on CM-cellulose. The purified proteins were solubilized in formic acid and cleaved with CNBr. Cleavage of the ATPase c-subunit with CNBr produces nine fragments (Fig. 4). 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 pro-

Fig. 3. 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 base changes and the corresponding amino acid changes due to the *uncE429* allele (glycine-23 $\rightarrow$ aspartic acid) and the *uncE408*, *uncE463* alleles (leucine-31 $\rightarrow$ phenylalanine) are indicated. The bases shown in bold typeface differ from those previously reported (Gay & Walker, 1981), but the amino acid sequence is unchanged.

file obtained by measuring A_{210} was the same for both mutant proteins and for the normal protein (Fig. 5). The eluate corresponding to each of the peaks of absorption was collected, freeze-dried and the amino acid compositions were determined. The analyses for each of the soluble fragments (B2, B3, B4, B7, B8, B9) from each protein indicated that the amino acid compositions were normal (Table 3). The material insoluble in 0.1% phosphoric acid was dissolved in chloroform/methanol and the B6 fragments purified by t.l.c. on cellulose. Amino acid analyses indicated that the B6 fragments from strains AN1330 (*uncE463/uncE463*) and AN1332 (*uncE408/uncE408*) contained one less leucine residue and one more phenylalanine residue than the B6 fragment from the normal strain (Table 3).

A spontaneous revertant strain (AN1897) of strain AN795 (*uncE408*) was obtained from succinate/minimal medium. Strain AN1897 was indistinguishable from a normal strain both in growth yield and membrane ATPase activity (see Table 2). The *c*-subunit of the F_1F_0 -ATPase was extracted from membranes of strain AN1897 and purified by chromatography on CM-cellulose. The purified protein was fragmented with CNBr and each of the fragments purified as described above. The amino acid analyses of each of the purified fragments indicated that the *c*-subunit from strain AN1897 was the same as that from a normal strain (Table 3).

Cloning and sequencing of the *uncE408* and *uncE463* alleles

There are six leucine residues in the B6 fragment of the *c*-subunit and to determine which of these residues was altered as a result of the *uncE408* or *uncE463* alleles, the DNA coding for the mutant

Table 2. Growth yields and membrane ATPase activities

Growth yields were measured as turbidities (Klett units) after growth in 5 mM-glucose/mineral-salts medium with the addition of 5% (v/v) Luria broth. The values shown are averages for at least three determinations. For the partial-diploid strains, the *unc* alleles shown first in parentheses are those on the plasmid and those shown second are on the chromosome.

Strain	Growth yields	ATPase activity (μ mol/min per mg of protein)
AN1330 (<i>uncE463/uncE463</i>)	179	0.34
AN1332 (<i>uncE408/uncE408</i>)	184	0.46
AN1331 (<i>uncE463/uncE408</i>)	188	0.57
AN1333 (<i>uncE408/uncE463</i>)	186	0.58
AN795 (<i>uncE408</i>)	138	0
AN1897 (<i>unc</i> ⁺)	208	1.4
AN248 (<i>unc</i> ⁺)	211	1.6

Fig. 4. Amino acid sequence of the *c*-subunit of the F_1F_0 -ATPase from *E. coli*, indicating the fragments generated by CNBr treatment

The designations of the fragments (B1–B9) are those used by Wachter *et al.* (1980). Abbreviation used: f-Met, formylmethionine.

c-subunits was sequenced and compared with the DNA sequence for a normal *uncE* gene.

The *uncE408* allele was cloned by repair of the deletion in plasmid pAN92. Plasmid pAN92 was prepared from plasmid pAN45 after digestion with the restriction endonuclease *Pst*I and re-ligation with T4-virus DNA ligase (Fig. 6). The *uncE408* allele was transferred to plasmid pAN92 by recombination repair in strain AN795 (*uncE408*, *recA*⁺) to give the plasmid pAN128 (Fig. 6). The plasmid pAN128 was digested with the restriction endonuclease *Hind*III, re-ligated, and this DNA used to transform strain AN727 (*uncB402*, *recA*). A chloramphenicol-resistant strain (AN1875) was isolated and shown to carry a plasmid (pAN146) corresponding in size to the plasmid pAN51 (Figs. 1 and 6).

For cloning of the *uncE463* allele into the vector pACYC184, the F-plasmid pAN34 (*uncE463*) was prepared from strain AN1330 (*uncE463/uncE463*). The F-plasmid pAN34 (*uncE463*) and the vector pACYC184 were digested with the restriction endonuclease *Eco*RI. The digested DNA preparations were mixed and ligated with T4-virus DNA ligase. Strain AN727 (*uncB402*, *recA*) was then transformed with the ligated-DNA mixture selecting for growth on succinate/tetracycline/minimal medium. A transformed strain (AN1915) was purified and the plasmid (pAN159), which was present in this strain, was shown by complementation of the polar *unc* mutant strain AN888 (*unc-416::Mu*) to carry an intact *unc* operon. Restriction-endonuclease analysis indicated an insert consisting of four *Eco*RI fragments (results not shown). The plasmid pAN159 and the vector

pACYC184 were digested with restriction endonuclease *Hind*III, mixed, and the mixture re-ligated with virus-T4 DNA ligase. Strain AN727 (*uncB402*, *recA*) was transformed with the ligation mixture and selection made for growth on succinate/chloramphenicol/minimal medium. One colony was purified, and this strain (AN1916) was shown to carry a plasmid (pAN160) corresponding in size to the plasmid pAN51 (see Fig. 1).

The sequencing strategy used for plasmids pAN146 and pAN160 was the same as that used for plasmid pAN149 (see Fig. 2). The *uncE408* and *uncE463* mutant DNA sequences were identical and differed from normal in that a C→T base change had occurred at nucleotide 91 of the *uncE* gene, resulting in leucine being replaced by phenylalanine at position 31 in the *c*-subunits (see Fig. 3). The variations in DNA sequence from the published sequence at nucleotides 106 and 117, described above for plasmids pAN51 and pAN149, also occurred in the plasmids pAN146 and pAN160.

Discussion

The mutant allele *uncE429* codes for a mutant *c*-subunit of the F_1F_0 -ATPase in which the glycine at position 23 in the normal *c*-subunit is replaced by an aspartic acid residue. The glycine at position 23 is one of the five invariant amino acids found in the proteolipid from seven sources, including mitochondria, chloroplasts and prokaryotes (Sebal & Hoppe, 1981). This mutant *c*-subunit is unable to assemble in the membrane (Downie *et al.*, 1981). The mutant alleles *uncE408* and *uncE463* similarly

Fig. 5. High-pressure liquid chromatography of soluble fragments of the CNBr-treated *c*-subunits from strains AN1460 (*pAN45*, *unc*⁺) (a), AN1330 (*uncE463/uncE463*) (b) and AN1332 (*uncE408/uncE408*) (c). Experimental details are given in the Materials and methods section. The CNBr fragments were identified after amino acid analysis (see Fig. 4 and Table 3). Peaks appearing early in the elution profile did not contain amino acids and those peaks appearing between B7 and B8 were due to combinations of fragments B2, B3 and B4. The amounts of these latter peaks varied depending on the effectiveness of the CNBr treatment. The reverse-phase *C*₁₈ column was repacked for the chromatography of the soluble fragments derived from strain AN1332 (*uncE408/uncE408*) with a resultant improvement in resolution.

Table 3. Amino acid compositions of CNBr fragments of the *c*-subunits from three strains of *Escherichia coli*. Amino acid compositions of the purified CNBr fragments were determined by using a Beckman 120-B amino acid analyser or a Beckman System 6300 high-performance analyser after hydrolysis in 6M-HCl for 22h. The amounts obtained for tyrosine were always low, and the presence or absence of this amino acid in the CNBr fragments was confirmed by monitoring the high-pressure-liquid-chromatography eluant at 280nm as well as at 210nm. Leucine always analysed as seven residues in the B6 fragment of the *c*-subunit from normal strains. The numbers in parentheses are the number 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 concentration of an amino acid known to be in that fragment as a single residue.

Strain	Frag- ment	Amino acid	Molar ratio	Frag- ment	Amino acid	Molar ratio
AN1460 (<i>pAN45</i> , <i>unc</i> ⁺)	B2	Leu	1.0 (1)	B7	Ala	1.0 (1)
		Glu	1.1 (1)		Asp	1.1 (1)
		Asp	2.2 (2)		Pro	1.1 (1)
	B3	Asp	1.0 (1)		Gly	1.0 (1)
		Tyr	0.4 (1)		Val	0.9 (1)
		Leu	1.9 (2)		Ile	0.9 (1)
	B4	Val	1.0 (1)	B8	Leu	1.1 (1)
		Ala	3.2 (3)		Val	2.0 (2)
	B6	Val	0.9 (1)		Gly	2.4 (2)
		Ile	5.7 (6)		Ala	1.0 (1)
		Leu	7.0 (6)		Ile	0.8 (1)
		Phe	2.6 (3)		Leu	2.1 (2)
		Lys	1.0 (1)		Tyr	0.4 (1)
		Arg	2.2 (2)	B9	Val	1.0 (1)
		Asp	1.3 (1)		Phe	1.0 (1)
		Thr	1.0 (1)		Ala	2.0 (2)
AN1330 (<i>uncE463/uncE463</i>)	B2	Glu	1.0 (1)	B7	Val	1.0 (1)
		Asp	2.1 (2)		Asp	1.3 (1)
		Leu	1.1 (1)		Pro	0.9 (1)
	B3	Asp	1.0 (1)		Gly	1.0 (1)
		Leu	1.7 (2)		Ala	1.2 (1)
		Tyr	0.4 (1)		Ile	0.9 (1)
	B4	Val	1.0 (1)	B8	Leu	1.4 (1)
		Ala	3.3 (3)		Ile	1.0 (1)
	B6	Ala	1.2 (1)		Gly	2.0 (2)
		Lys	1.0 (1)		Ala	1.2 (1)
		Arg	2.0 (2)		Val	1.9 (2)
		Asp	1.0 (1)		Leu	2.2 (2)
		Thr	1.0 (1)		Tyr	0.5 (1)
		Glu	3.2 (3)	B9	Val	1.0 (1)
		Pro	2.3 (2)		Ala	2.1 (2)
		Gly	6.8 (7)		Phe	1.1 (1)
		Ala	5.9 (6)			
		Val	1.1 (1)			

code for mutant *c*-subunits that have difficulty in assembling in the membrane. Both mutant alleles code for a mutant *c*-subunit in which the leucine at position 31 in the normal *c*-subunit is replaced by

Table 3—(contd.)

Strain	Frag- ment	Amino acid	Molar ratio	Frag- ment	Amino acid	Molar ratio
AN1332 (<i>uncE408</i> / <i>uncE408</i>)	B2	Ile	5.9 (6)	B7	Ala	1.0 (1)
		Leu	6.3 (6)		Asp	1.1 (1)
		Phe	3.8 (3)		Pro	0.9 (1)
	B3	Glu	1.0 (1)		Glu	1.2 (1)
		Asp	2.0 (2)		Val	1.0 (1)
		Leu	0.9 (1)		Ile	0.9 (1)
	B4	Tyr	1.0 (1)		Leu	1.0 (1)
		Val	1.0 (1)	B8	Ile	1.0 (1)
		Ala	3.2 (3)		Gly	1.8 (2)
	B6	Lys	1.0 (1)		Ala	1.0 (1)
		Arg	2.4 (2)		Val	1.7 (2)
		Asp	1.3 (1)	B9	Leu	2.1 (2)
		Thr	1.0 (1)		Tyr	0.5 (1)
		Glu	3.0 (3)		Phe	1.0 (1)
		Pro	1.9 (2)		Ala	2.1 (2)
		Gly	7.1 (7)		Val	1.0 (1)
		Ala	5.7 (6)			
		Val	1.2 (1)			
		Ile	5.6 (6)			
		Leu	5.8 (6)			
		Phe	4.0 (3)			

phenylalanine. These two mutations were isolated during two different mutant hunts and are therefore not siblings. The assembly of the mutant *c*-subunit is improved by increasing the gene dosage of both the *uncE408* and *uncE463* alleles and, once assembled, the mutant *c*-subunit is at least partially functional.

Engelman & Steitz (1981) have proposed that the initial event in the insertion of a protein into a membrane is the spontaneous penetration of the hydrophobic portion of the membrane by a helical 'hairpin'. There is evidence from the study of DCCD-resistant mutant strains (Hoppe *et al.*, 1980b) that the *c*-subunit of the *E. coli* F_1F_0 -ATPase is present in the membrane as a hairpin structure. The predominantly helical hairpin structure of the *c*-subunit has also been predicted from the amino acid sequence by using a number of prediction methods based on data from water-soluble proteins (see Sebald & Hoppe, 1981) and also using a method modified for membrane proteins (Senior, 1983) (see Fig. 7).

In the *uncE429* mutant the incorporation of the mutant *c*-subunit into the membrane would be affected by an increased energy cost due to the extra charged amino acid required to be inserted into the membrane. Engelman & Steitz (1981) estimate that the insertion of the helical hairpin into the mem-

Fig. 6. Derivation of plasmids bearing the *uncE408* allele

Map of plasmids pAN45, pAN92, pAN128 and pAN146 showing cleavage sites for the restriction endonucleases *Hind*III and *Pst*I and the approximate positions of the *unc* genes. The heavy lines (—) indicate the vector (pACYC184) portions of the plasmids and the 'open' lines (—) the recombinant *unc* regions carrying the *uncE408* allele. The calibrations marked on the plasmids represent 1 kb (kilobase) intervals. The gene order *pstB/phoT* is undetermined (Cox *et al.*, 1981b). Step 1: plasmid pAN45 (Downie *et al.*, 1980) digested with restriction endonuclease *Pst*I and re-ligated to yield the deletion plasmid pAN92. Step 2: the deletion in plasmid pAN92 repaired in strain AN795 (*uncE408*, *rec*⁺) to give plasmid pAN128. Step 3: plasmid pAN128 digested with restriction endonuclease *Hind*III and re-ligated to give the plasmid pAN146 that carried the *uncE408* allele.

Fig. 7. Predicted secondary and tertiary structures of the c -subunit of the F_1F_0 -ATPase of *E. coli*

Structure A represents the form of the c -subunit involved in the initial penetration of the hydrophobic portion of the membrane. Structure B represents the c -subunit when stably integrated into the membrane. The amino acids altered in the mutant c -subunits and which affect assembly are indicated. The conserved acidic residue aspartic acid-61 is also indicated. The secondary structures are as indicated: $\square$, α -helices; $\sim$, β -sheets and β -turns; $\rightarrow$, random coil.

brane is driven by a hydrophobic free energy of about 251–314 J (60–75 kcal) for paired α -helices containing 40–50 amino acids. The free-energy 'cost' of burying the polar and charged amino acids of the c -subunit would be about 217 J (52 kcal) according to the estimates of Engelman & Steitz (1981) and may be as high as 293 J (70 kcal) if those groups not involved in backbone hydrogen bonds around proline-64 are taken into account. It would appear, then, that the energy balance is only just in favour of assembly and the substitution of glycine-23 by aspartic acid, which would increase the free-energy cost by an estimated 33.4 J (8 kcal), might be sufficient to prevent assembly.

Energetically there is no increased barrier to assembly of the *uncE408* or *uncE463* mutant c -subunits (leucine-31 $\rightarrow$ phenylalanine) and they

are in fact assembled provided they are formed in sufficient amounts. The residue leucine-31 occurs in a region of the c -subunit predicted to be involved in β -turns (see Sebald & Hoppe, 1981) and these β -turns may be required to allow the C-terminal and N-terminal ends of the molecule to be adjacent in the structure initially involved in the penetration of the hydrophobic portion of the membrane (see Fig. 7). The substitution of leucine-31 by the bulkier phenylalanine may prevent a sufficient amount of this form of the c -subunit to accumulate in the cytoplasm for assembly to occur. Such an effect should respond to an increased gene dosage.

The c -subunit of the F_1F_0 -ATPase is a relatively simple membrane protein and the further study of mutations in the *uncE* gene, which prevent assembly of the c -subunit, should provide insight into the basic

mechanisms of the incorporation of proteins into membranes.

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