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The chloroplast CF_0I subunit can replace the *b*-subunit of the F_0F_1-ATPase in a mutant strain of *Escherichia coli* K12

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The amino acid sequence of the CF_0I subunit from the chloroplast F_0F_1-ATPase has only a low similarity to the amino acid sequence of the *b*-subunit of the *E. coli* F_0F_1-ATPase. However, secondary and tertiary structure predictions plus the distribution of hydrophobic and hydrophilic amino acids have indicated that these two subunits serve a similar function. This proposition was investigated directly. A *cDNA* clone for the chloroplast *atpF* gene, encoding the CF_0I subunit, was altered by site-diected mutagenesis such that the translation start site corresponded to the N-terminus of the mature protein. An *E. coli* mutant strain carrying a chain-terminating mutation in the *uncF* gene, encoding the *b*-subunit, was transformed with the plasmid carrying the altered *atpF* gene. The resultant transformant was able to grow on succinate and gave a growth yield similar to that of a wild-type control. Assays on membrane preparations from the transformant also clearly indicated that the mature CF_0I subunit from spinach chloroplasts was able to replace the *E. coli b*-subunit in the *E. coli* F_0F_1-ATPase.

Introduction

The F_0F_1-ATPase enzyme complex catalyses the terminal step in oxidative phosphorylation or photophosphorylation and is located in mitochondrial, chloroplast and bacterial membranes. The structure is highly conserved and the complex can be readily dissociated into two portions, the water-soluble F_1-ATPase and the membrane-bound F_0 portion which forms a proton pore [1]. In both chloroplasts and *E. coli*, the F_1 complex is composed of five subunits, α, β, γ, δ and ε, with the stoichiometry of α_3, β_3, γ_1, δ_1 and ε_1. The F_0 complex of *E. coli* (ECF_0) contains three polypeptide species (*a*, *b* and *c*) in the ratio of a_1, b_2, c_{6-12}, while chloroplast F_0 (CF_0) appears to contain four polypeptide species (I, II, III and IV) of unknown stoichiometry [2]. The subunits I, III and IV are thought to be equivalent to the *E. coli* subunits *b*, *c* and *a*, respectively, [4,5,6], on the basis of comparison of predicted secondary and tertiary structures.

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In *E. coli* the F_0F_1-ATPase subunits are encoded in a single operon located at about 83 min on the *E. coli* chromosome with the genes in the order *unc* IBEFHAGDC (see Ref. 3). The *uncI* gene product does not appear to be part of the F_0F_1-ATPase complex and the remaining genes encode the subunits *a*, *c*, *b*, δ, α, γ, β and ε, respectively. In spinach chloroplasts, six of the nine subunits of the F_0F_1-ATPase are encoded by genes in the chloroplast genome. The six chloroplast genes are arranged in two operons, but in each case the order is similar to that of the *E. coli unc* genes. Thus one operon carries the genes coding for the CF_0IV-, CF_0III-, CF_0I- and $CF_1\alpha$-subunits [4–6], and the other carries the genes for the $CF_1\beta$- and $CF_1\varepsilon$-subunits [7,8]. The *atpF* gene for CF_0I is split by a single intron [4–6]. Table I summarises the equivalent polypeptides and genes for the F_0F_1-ATPases of *E. coli* and chloroplasts.

The amino acid sequences of the ECF_0 *b*-subunit and the CF_0I-subunit have only a 19% similarity [6], but share the unusual feature of a short hydrophobic N-terminal sequence with the remainder of the molecule being hydrophilic. The hydrophilic regions of both subunits have been predicted to be largely α-helical with relatively long α-helices stabilised by ion-pair formation [6]. The CF_0I subunit has particular features in that the mature subunit appears to be 17 amino acids shorter

TABLE I

Subunits and corresponding genes of the E. coli and spinach chloroplast F_0F_1-ATPases

Subunit		Molecular mass (kDa)		Gene	
E. coli	chloro-plast	*E. coli*	chloro-plast	*E. coli*	chloro-plast
α	α	55	56	*uncA*	*atpA*
β	β	50	54	*uncD*	*atpB*
γ	γ	31	36	*uncG*	*atpC*
δ	δ	19	20	*uncH*	*atpD*
ε	ε	15	15	*uncC*	*atpE*
a	CF_0IV	30	25	*uncB*	*atpI*
b	CF_0I	17	19	*uncF*	*atpF*
–	CF_0II	–	16 *	–	*atpG*
c	CF_0III	8	8	*uncE*	*atpH*

* Molecular mass estimated after SDS-gel electrophoresis.

than the *atpF*-encoded protein and the hydrophobic and hydrophilic domains are encoded in separate exons. In the present paper we demonstrate that the ECF_0 *b*-subunit and the CF_0I-subunits are indeed analogous in that the CF_0I-subunit can replace the *b*-subunit in an appropriate mutant strain of *E. coli*.

Materials and Methods

Enzymes and chemicals

All chemicals and enzymes used were of the highest quality available. Restriction endonucleases, T4-polynucleotide kinase and T4 DNA ligase were obtained from Pharmacia (Australia). [α-^{35}S]dATP was obtained from Amersham (Australia). The nucleotide sequencing kit was obtained from USB (Cleveland, OH). The site-directed mutagenesis kit was obtained from Amersham (Australia). Oligonucleotides were synthesised by G. Mayo, division of Biochemistry and Molecular Biology, John Curtin School of Medical Research.

TABLE II

Strains of E. coli and plasmids used

Genotypes are according to Bachmann [21]. Plasmid nomenclature is that used by Novick et al. [22]. Ap, ampicillin; Cm, chloramphenicol; Tc, tetracycline.

Bacterial strain or plasmid	Relevant genotype [References]
AN1440	*uncF469, argH, pyrE, entA, recAsrl::Tn10* [17]
AN2704	pAN423, *uncF469, argH, pyrE, entA, recAsrl::Tn10*
AN2936	pAN573, *uncF469, argH, pyrE, entA, recAsrl::Tn10*
AN2401	pAN51, *uncF469, argH, pyrE, entA, recAsrl::Tn10*
AN2987	pUC18, *uncF469, argH, pyrE, entA, recAsrl::Tn10*
pAN423	Ap^r, *atpF*cDNA
pAN573	Ap^r, *atpF406*
pAN51	Cm^r, Tc^s, *uncBEFHA* [9]

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 II.

Genetic techniques

The techniques used for genetic experiments were as outlined previously [9].

Preparation of plasmids

Plasmid DNA was prepared as described by Selker et al. [10].

DNA sequencing

Nucleotide sequences were determined by the dideoxy chain-terminating method of Sanger et al. [11], using α-[^{35}S]dATP as described by Biggin et al. [11a].

Site-directed mutagenesis

The method used was that outlined in the Amersham handbook 'Oligonucleotide-directed in vitro mutagenesis system'.

Preparation of CF_0I cDNA

The method used was that described by Hudson et al. [6].

Media and growth of organisms

The mineral salts minimal medium used and additions were as described previously [12]. Cells for the preparation of membranes were grown in 14-litre fermenters as described previously [13]. The mineral salts medium in the fermenters was supplemented with 5% (v/v) Luria broth [14].

Turbidities of cultures were measured with a Klett-Summerson colorimeter. Growth yields were measured as turbidities after growth had ceased in medium containing limiting (5 mM) glucose.

Preparation of membranes

Membranes were prepared as described previously [15], although some preparations did not proceed beyond the 50 mM Tes buffer-wash. Briefly, in this modified preparation, washed cells were suspended in a 0.1 M Tes buffer system (pH 7.0), containing magnesium acetate, sucrose, EGTA, 6-aminohexanoic acid and *p*-aminobenzamidine. The cells were disintegrated by using a Sorvall Ribi cell-fractionator, cell debris was removed by centrifugation and the membranes were separated by ultracentrifugation. The buffer system described above was used in the first wash and in the second wash the buffer system used contained Tes (50 mM), glycerol (15%, v/v), 6-aminohexanoic acid (40 mM) and *p*-aminobenzamidine (6 mM). The washed membrane preparation was suspended in the second buffer system. The F_1-ATPase was removed from the membranes by

dialysing against a buffer system that contained Tes (5 mM), glycerol (15% v/v), dithiothreitol (0.5 mM) and 6-aminohexanoic acid (40 mM).

Other methods

ATPase and atebrin fluorescence-quenching activities were assayed as previously described [12]. Protein concentrations were determined using Folin's phenol reagent [16], with bovine serum albumin as standard.

Results

Preparation of plasmids and strains

The *uncF469* allele carries a chain-terminating codon instead of the codon for tryptophan at position 25 of the *b*-subunit of the ECF_0 [17]. A strain (ANI440) carrying this allele is therefore suitable as a host for testing the ability of CF_0I, expressed from an introduced plasmid, to replace the *b*-subunit without its having to compete with a mutant *E. coli* subunit.

The *atpF* gene includes two exons and a cDNA clone of the *atpF* region was prepared as described previously [6]. The *atpF* gene is located on a *Hin*dIII-*Eco*RI fragment. Such a fragment was ligated into the vector pUC8 and the resultant plasmid (pAN423) used to transform strain AN1440 (*uncF469*) to ampicillin resistance to give strain AN2704. This strain will be referred to as AN2704 (*atpFcDNA*).

The mature CF_0I protein appears to have had 17 amino acids removed from the N-terminal end [18]. As this processing is unlikely to be carried out in the *E. coli* cell, it was necessary to mutate the *atpF cDNA* such that translation commenced at the eighteenth residue. An oligonucleotide of the following sequence was prepared: 5′-CGTTTTCTAGGGTCACTGAGGATCCGCCATGAGTTTCG. This varied from the normal sequence of the *atpF* gene in the regions of those nucleotides underlined. The underlined TAG is a chain-terminating triplet replacing the eleventh codon of the *atpF* gene. The GAGGA region forms a Shine-Dalgarno sequence six nucleotides upstream from the introduced ATG translation start-site. The ATG codon replaces the codon for glycine-18 in the *atpF* gene. The *Hin*dIII-*Eco*RI fragment carrying the *atpF* gene was ligated into the M13 vector mp18 and site-directed mutagenesis was carried out using the mutant oligonucleotide. Nucleotide sequencing was used to confirm successful mutagenesis. The replicative form of the M13 mp18 carrying the mutant *atpF* gene was prepared and the *Hin*dIII-*Eco*RI fragment ligated into the vector pUC18. The resultant plasmid (pAN573), was used to transform strain AN1440 to ampicillin resistance to give strain AN2936. This strain will be referred to as AN2936 ($mutCF_0I$).

Two control strains were prepared. Strain AN2987 was prepared by transforming AN1440 to ampicillin resistance with the vector pUC18 and will be referred to as AN2987 (pUC18). Strain AN2401 was prepared by transforming strain AN1440 to chloramphenicol resistance with the plasmid pAN51, which carries a normal *uncF* allele. Strain AN2401 will be referred to as AN2401 ($uncF^+$).

Growth characteristics of strains AN2704 (atpFcDNA), AN2936 (mut CF_0I), AN2987 (pUC18) and AN2401 ($uncF^+$)

Mutant strains of *E. coli* which have lost the ability to carry out oxidative phosphorylation are unable to grow on media in which succinate is the sole source of carbon [19]. Strain AN2987 (pUC18) was unable to grow on succinate-minimal medium, whereas strain AN2401 ($uncF^+$) was able to grow. Strain AN2704 (*atpFcDNA*) was also unable to grow on this medium, but strain AN2936 ($mutCF_0I$) grew as well as the positive control strain AN2401. Strains AN2936 and AN2401 both grew with a doubling time of 1.3 h.

The cell mass produced from a limiting concentration of glucose under aerobic growth conditions is less for an *E. coli* mutant strain unable to carry out oxidative phosphorylation than for a wild-type strain [19]. Such growth yields were determined for each of the four strains (Table III), with the results supporting those obtained for growth on succinate minimal medium. Thus, strains AN2987 (pUC18) and AN2704 (*atpFcDNA*) gave growth yields characteristic of uncoupled strains, whereas strain AN2936 ($mutCF_0I$) gave a growth yield similar to that of the wild-type control strain AN2401 ($uncF^+$).

TABLE III

Effects on cell and membrane preparations of the presence of the plasmid-encoded chloroplast atpF gene in an uncF mutant strain of E. coli

Bacterial strain	Growth on succinate	Growth yield on 5 mM glucose (Klett units)	ATPase activity (μmol/min) per mg protein	Atebrin fluorescence quenching (%)	
				NADH dependent	ATP dependent
AN2987(pUC18)	−	134	0.20	87	15
AN2704(*atpFcDNA*)	−	150	0.13	82	19
AN2936($mutCF_0I$)	+	207	0.44	83	54
AN2401(pAN51)	+	218	0.45	80	81

Enzymic activities of membrane preparations

Membranes were prepared from strains AN2987 (pUC18), AN2704 (*atpFcDNA*), AN2936 (mutCF_0I) and AN2401 (*uncF*$^+$). The procedure normally used in our laboratory for the preparation of membranes involves washing with low ionic strength (5 mM) buffer and using 4-aminobenzamidine to specifically maintain the integrity of the F_0F_1-ATPase. When this procedure was used in the preparation of membranes from strain AN2936 (mutCF_0I), the membranes were somewhat proton-permeable and had only a low level of ATP-dependent atebrin fluorescence-quenching activity (data not given). Since this activity could be reconstituted by the addition of purified ECF_1-ATPase, it was concluded that the 4-aminobenzamidine was not maintaining the integrity of the F_0F_1-ATPase in strain AN2936 membranes in the 5 mM Tes buffer normally used. The comparison of the various activities was therefore carried out using membrane preparations from each of the four strains which had been washed in a 50 mM Tes buffer system.

The ATPase activities and fluorescence-quenching activities (Table III), clearly indicate that the CF_0I formed in strain AN2936, was able to replace the *E. coli* *b*-subunit to give activities similar to that of the wild-type control. The unprocessed CF_0I formed in strain AN2704 gave only marginal increases on the activities obtained for the mutant control strain AN2987.

Fig. 2. Comparison of predicted α-helical regions of the *E. coli* EC*b*-subunit and the chloroplast CF_0I-subunit. Hydrophobic residues are accentuated and conserved hydrophobic residues are marked by a star. The amino acid sequences are arranged in the periodicity required for a coiled-coil, with the left-hand column for each sequence required to comprise hydrophobic residues.

Discussion

Despite efforts in a number of laboratories, only two missense mutations in the *uncF* gene, affecting the *b*-subunit of the F_0F_1-ATPase, have been reported [23,24,25]. These mutations result in Gly-9 or Gly-131 being replaced by Asp with both substitutions affecting assembly of the F_0F_1-ATPase complex. It would appear then that very few or no specific amino acid residues of the *b*-subunit are essential for F_0F_1-ATPase function. Furthermore, it would seem that the *b*-subunit is not involved in any close-packing such that an increase in size of any amino-acid residue results in loss of function. This view is now supported by the observation that the CF_0I subunit essentially completely replaces the EC*b*-subunit in an appropriate mutant strain. Despite the suggestion that the secondary and tertiary structures are similar, the best amino acid sequence-match between CF_0I and EC*b* is 17 residues out of 156, or 11%

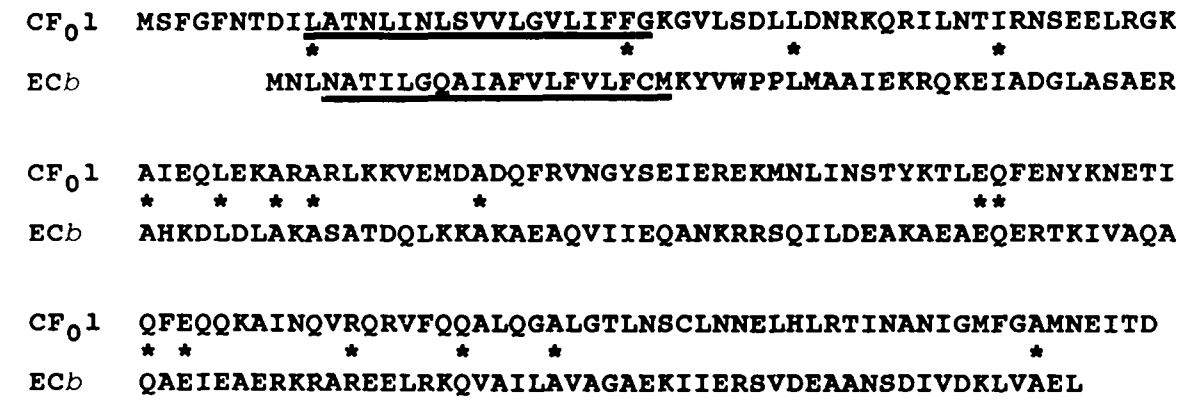

Fig. 1. Comparison of amino acid sequences of *E. coli* *b*-subunit (EC*b*) and chloroplast CF_0I-subunit (CF_0I). The lines indicate the putative N-terminal transmembrane helices. Homologous amino acids are indicated by stars.

(Fig. 1). This value can be increased if some gaps are allowed, but such manipulations may not be relevant when considering replacement of one subunit with another. Of the 17 matched residues, only 3 are charged; Glu-95, Glu-108 and Arg-117 (using ECb residue numbers). Glu-108 had previously been implicated in the binding of the F_1-ATPase minor subunits [20]. Of the eleven matched hydrophobic residues, seven occur in the postulated α-helix, extending from residues 29 to 77. Six of these matched residues are arranged in a periodicity appropriate for formation of a coiled coil [26] (Fig. 2). There are two *b*-subunits in each F_0F_1-ATPase and it may be that a parallel coiled coil forms between the postulated extended α-helices (from about residues 40 to 70), from each of the pair of subunits.

We conclude that the *E. coli uncF* and chloroplast *atpF* genes encode proteins of homologous structure and function. Remarkably, a higher plant chloroplast polypeptide can substitute for a subunit in the assembly and function of a multimeric membrane complex of *E. coli*, despite the low level of sequence identity and more than 10^9 years of evolution between bacteria and chloroplasts [27].

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