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

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A site-directed mutation in the gene which codes for the *c*-subunit of the F_1F_0-ATPase, resulting in the substitution of Ala-25 by Tyr, has been constructed and characterized. A plasmid carrying the mutation was used to transform strain AN943 (*uncE429*). The resulting strain is unable to grow on succinate as sole carbon source and possesses an uncoupled growth yield. Membranes prepared from the mutant possess low levels of ATPase activity and are proton-impermeable. The F_1-ATPase activity was found to be inhibited by 80% when bound to the membrane. When carried on a plasmid, the mutation is dominant in complementation tests with all mutant *unc* alleles tested and when transformed into wild-type strain AN346, the mutation results in an uncoupled phenotype. A mutant which overcomes this dominance was isolated and found to possess an 11-amino-acid deletion extending from Ile-55 to Met-65 within the *c*-subunit. These results are discussed in relation to the previously isolated Ala-25 to Thr mutant (Fimmel, A.L., Jans, D.A., Hatch, L., James, L.B., Gibson, F. and Cox, G.B. (1985) Biochim. Biophys. Acta 808, 252–258) and in relation to a previously proposed model for the F_0 (Cox, G.B., Fimmel, A.L., Gibson, F. and Hatch, L. (1986) Biochim. Biophys. Acta 849, 62–69).

Introduction

The membrane-bound energy-transducing F_1F_0-ATPase has a remarkably similar structure in mitochondria, chloroplasts and bacteria. The enzyme complex can be readily dissociated into two portions, the F_1-ATPase and the membrane-bound F_0 sector, which forms a proton pore. The F_0 sector 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. Each of the proteins consists of two hydrophobic segments separated by a central polar region. Within the C-terminal hydrophobic segment there exists a conserved acidic residue, Asp-61 in *Escherichia coli*, which reacts with DCCD resulting in inhibition of ATPase activity [1].

In *E. coli* the proteolipid or *c*-subunit is coded for by the *uncE* gene [2]. Analysis of DCCD-resistant mutants has indicated that the *c*-subunit is folded in a hairpin-like structure in the membrane, such that isoleucine at position 28 is close to the aspartic acid at position 61 [3]. Nuclear magnetic resonance studies of the *c*-subunit indicate that the molecule has extensive α-helical segments and support a hairpin structure for the molecule [4].

In this paper we further investigate the effect of amino-acid substitutions at position Ala-25 in the *c*-subunit. Strains carrying the previously isolated Ala-25 to Thr mutation possess both a growth yield and a level of oxidative phosphorylation intermediate between that of an uncoupled strain and a wild-type strain [5]. In order to investigate the effect of substituting an amino acid larger than threonine at position 25 in the *c*-subunit, site-directed mutagenesis was used to construct the substitution Ala-25 to Tyr. The characteristics of strains carrying this mutation are described.

Abbreviations: DCCD, dicyclohexylcarbodiimide; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

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Materials and Methods

Enzymes and chemicals. All chemicals and enzymes used were of the highest quality available. Restriction endonucleases were obtained from Pharmacia. T4-DNA ligase, polynucleotide kinase, alkaline phosphatase, DNA polymerase (large fragment, Klenow enzyme), dideoxynucleotides and deoxynucleotides were obtained from Boehringer Mannheim Australia. [α-^{32}P]dCTP and [γ-^{32}P]ATP were obtained from Amersham Australia. Oligonucleotides were synthesized on an Applied Biosystems DNA Synthesizer. A Bio-Rad Muta-Gene in vitro Mutagenesis Kit was a generous gift from K.C. Reed.

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.

TABLE I

Strains of E. coli and plasmids used

Chromosome nomenclature is that used by Bachmann [19]; plasmid nomenclature is that used by Novick [20].

Bacterial strain or plasmid	Relevant genotype	Source or reference
Bacterial strains		
AN346	*ilvC* [a]	F. Gibson
CJ236	pCJ105/*dut1 ung1 thi1 relA*	Bio-Rad
MV1190	F′ *traD proAB lacI*^{q}*ZΔM15*/ *Δ(lac-proAB) thi supE Δ(srl-recA)*306::Tn10	Bio-Rad
K37	Hfr *supD*	N. Dixon
JM101	F′ *traD36 proAB lacI*^{q}*ZΔM15*/ *Δ(lac-pro) supE thi*	F. Gibson
AN727	*uncB402 recA* [a]	F. Gibson
AN943	*uncE429 recA* [a]	F. Gibson
AN1440	*uncF469 recA* [a]	F. Gibson
AN2015	*uncH241 recA* [a]	F. Gibson
AN1124	*uncA450 recA* [a]	F. Gibson
AF55	pAN174/*uncE429 recA* [a]	this work
AF59	pAN174/*ilvC* [a]	this work
AF61	pAN51/*uncE429 recA* [a]	this work
AF72	pAF14/*uncB402 recA* [a]	this work
AF73	pAF14/*uncE429 recA* [a]	this work
AF74	pAF14/*uncF469 recA* [a]	this work
AF75	pAF14/*uncH241 recA* [a]	this work
AF76	pAF14/*uncA450 recA* [a]	this work
AF77	pAF14/*ilvC* [a]	this work
AF78	pAF16/*uncE429 recA* [a]	this work
AN2188	pAN258/*unc416::MuB*$^+$ *EFHAGDC recA* [a]	[5]
Plasmids		
pCJ105	Cm^r	Bio-Rad
pAN51	Cm^r Tc^s *uncB*$^+$ *E*$^+$*F*$^+$*H*$^+$*A*$^+$	F. Gibson
pAN174	Cm^r Tc^s	F. Gibson
pAF9	M13mp18 *uncB*$^+$ *E*$^+$*F*$^+$*H*$^+$*A*$^+$	this work
pAF14	Cm^{r}Tc^s *uncB*$^+$ *E1001 F*$^+$*H*$^+$*A*$^+$	this work
pAF16	Cm^r Tc^s *uncB*$^+$ *ΔE*$^-$*F*$^+$*H*$^+$*A*$^+$	this work
pAN258	Cm^r Tc^s *uncB*$^+$ *E513 F*$^+$*H*$^+$*A*$^+$*G*$^+$*D*$^+$*C*$^+$	[5]

[a] Other markers, *argH pyrE entA*.

TABLE II

Oligonucleotides used in site-directed mutagenesis and as sequencing primers for sequencing the uncE gene

Underlined nucleotides denote differences from the wild-type sequence [22].

Oligonucleotide	Amino-acid substitution
5′-TCGGTGCTTACATCGGTATC-3′	Ala-25 → Tyr
5′-AACACTACTACGTTTTAACT-3′ [a]	–

[a] Sequencing primer used for sequencing from the *uncB–uncE* intergenic region.

Media and growth of organisms. 2YT broth was prepared as described by Miller [6]. The mineral-salts minimal medium used and additions were as described previously [7]. Cells for the preparation of membranes were grown in 14-l fermenters essentially as described previously [8]. The media in the fermenter vessels were supplemented with 5% (v/v) Luria broth [9]. Turbidities of cultures were measured with a Klett-Summerson colorimeter. Growth yields were measured as turbidities after growth ceased in minimal media containing limiting (5 mM) glucose [5].

Genetic techniques. The techniques used for genetic experiments were as outlined previously [7,10,11].

Preparation of plasmid and phage M13 DNA. Plasmid DNA was prepared as described by Selker et al. [14]. The replicative form of phage M13 was prepared from a 250 ml 2YT broth culture inoculated with strain K37 and phage M13 at a multiplicity of infection of 1.0. Late-exponential phase cells were centrifuged and replicative form DNA prepared as described for plasmid DNA.

The isolation of single-stranded phage M13 DNA was carried out as described by Messing [15].

Site-directed mutagenesis. Site-directed mutants were constructed using a Bio-Rad Muta-Gene in vitro Mutagenesis Kit, which is based on the method of Kunkel [12]. Single-stranded uracil containing plasmid pAF9 was prepared by growth on strain CJ236. The oligonucleotide carrying the appropriate base substitution (see Table II) was annealed to this single-stranded preparation and extended using DNA polymerase and ligated using T4-DNA ligase. Strain MV1190 was transformed with this preparation and the plaques so obtained were screened, using the appropriate γ-^{32}P-labelled oligonucleotide as probe, under conditions favouring hybridization of the probe to mutant plaques. Confirmation of the expected mutation was made by DNA sequence analysis. Single-site incorporation of each mutant oligonucleotide was confirmed by sequencing using that oligonucleotide as primer. To confirm that the site-directed mutation was the only mutation in the *uncE* gene, the entire *uncE* gene was sequenced.

DNA sequencing. Nucleotide sequences were determined by the dideoxy chain-terminating method of Sanger et al. [13] using [α-^{32}P]dCTP.

Isolation and characterization of revertants. Cells were spread on solid succinate minimal medium supplemented with 0.05% casamino acids and incubated at 37°C. After 3 days colonies formed and revertant strains were purified and plasmids isolated. The plasmids were re-transformed into the original recipient strain and those which conferred growth on succinate were isolated. The plasmid DNA from the revertant strains was sequenced after subcloning into the *Hin*dIII restriction site of phage M13mp18.

Preparation of cell membranes. The preparation and treatment of membranes were as described previously [16].

ATPase activity. ATPase activity was measured by using the method described by Gibson et al. [17].

Atebrin-fluorescence quenching. Atebrin-fluorescence quenching was measured as described by Gibson et al. [17].

Protein determination. Protein concentrations were determined using Folin's phenol reagent [18].

Results

Isolation of amino-acid substitution Ala-25 to Tyr within the c-subunit

It was found that a phage M13mp9 derivative carrying the *uncB, E, F, H* and *A* genes [21] was unable to form plaques on Bio-Rad strains CJ236 and MV1190. However, phage M13mp18 does form plaques on these strains. Therefore, the 4.3 kb fragment in plasmid pAN51 carrying the *uncB, E, F, H* and *A* genes was subcloned into the *Hin*dIII restriction site in phage M13mp18, using restriction endonuclease *Hin*dIII and T4-DNA ligase. Strain JM101 was then transformed with the ligation mixture. The resulting plaques were screened for clones carrying the 4.3 kb *Hin*dIII fragment in the correct orientation by hybridization at room temperature with the γ-^{32}P-labelled oligonucleotide listed at the top of Table II. A small number of plaques were found to hybridize to the oligonucleotide probe. Replicative form phage DNA was prepared from one such plaque and analysis by restriction endonuclease *Hin*dIII digestion confirmed the presence of the 4.3 kb fragment. This phage was designated pAF9 and was used in the construction of the site-directed mutant.

The site-directed mutation resulting in the amino acid substitution Ala-25 to Tyr was constructed using the mutant oligonucleotide indicated in Table II. Plasmid pAF9 was digested with *Hin*dIII and the 4.3 kb fragments carrying the site-directed mutation were subcloned into the *Hin*dIII site of alkaline phosphatase-treated vector pAN174. Following ligation, strain AN2015 (*uncH241*) was transformed with the ligation mix and transformants were selected on rich medium containing chloramphenicol. Unexpectedly, when tested, all the transformants failed to grow on succinate as the sole carbon source. In order to isolate a mutant plasmid with an equivalent structure to plasmid pAN51, plasmid DNA was prepared from a small number of transformants carrying the mutation and analysed by digestion with restriction endonuclease *Hin*dIII and *Bam*HI. A restriction pattern identical to that of plasmid pAN51 was observed in approximately half the plasmids examined confirming the presence and orientation of the 4.3 kb fragment carrying the site-directed mutation. One such plasmid carrying the Ala-25 to Tyr mutation was designated pAF14. The mutant allele, resulting in the substitution of Ala-25 by Tyr in the *c*-subunit, was designated *uncE1001*. It was concluded that the presence of the *uncE1001* allele on plasmid pAF14 (Ala-25 → Tyr) prevented the complementation of the *uncH241* allele in strain AN2015 on succinate minimal medium containing chloramphenicol.

Growth properties of strains carrying the Ala-25 to Tyr mutation

Strain AN943 (*uncE429*) was used to examine the properties of the mutation carried on plasmid pAF14 (Ala-25 → Tyr), since the chromosomally encoded *uncE429* gene product does not assemble into the membrane [23]. Plasmid pAF14 was transformed into strain AN943 (*uncE429*) and transformants were selected on rich medium containing chloramphenicol. One such transformant carrying plasmid pAF14 was designated strain AF73 (Ala-25 → Tyr). Strain AF73 (Ala-25 → Tyr) is unable to grow on succinate as the sole carbon source and possesses a growth yield similar to the uncoupled control strain AF55 (pAN174/*uncE429*) (Table III).

The dominance or lack of complementation displayed by plasmid pAF14 (Ala-25 → Tyr) in complementation tests with strain AN2015 (*uncH241*) was investigated further. Plasmid pAF14 (Ala-25 → Tyr) was

TABLE III

Growth properties of uncE mutant strains of E. coli carrying plasmid-encoded amino-acid substitution Ala-25 → Tyr

Bacterial strain	Growth on succinate	Growth yield (Klett units)
AF72 (pAF14/*uncB402*)	–	159
AF73 (pAF14/*uncE429*)	–	156
AF74 (pAF14/*uncF469*)	–	157
AF75 (pAF14/*uncH241*)	–	168
AF76 (pAF14/*uncA450*)	–	162
AF77 (pAF14/*unc*$^+$)	–	170
AF55 (pAN174/*uncE429*) [a]	–	159
AF59 (pAN174/*unc*$^+$) [b]	+	210

[a] Uncoupled control strain.
[b] Coupled control strain.

transformed into the following series of strains each carrying a mutation in one of the remaining *unc* genes carried on plasmid pAN51: strain AN727 (*uncB402*), strain AN1440 (*uncF469*) and strain AN1124 (*uncA450*). The wild-type strain AN346 was also transformed. Transformants were selected on nutrient medium containing chloramphenicol and their growth properties were examined. Interestingly, transformants from all strains, including the wild-type, failed to grow on minimal medium containing succinate as the sole carbon source and possessed growth yields similar to the uncoupled control strain AF55 (pAN174/*uncE429*) (see Table III).

Isolation and characterization of revertants

It was observed that strain AF75 (pAF14/*uncH241*) carrying the Ala-25 to Tyr mutation produced revertants which grew well on succinate minimal medium. One such revertant colony was isolated and characterized. Plasmid DNA, designated pAF16, was isolated from this revertant and shown to carry the reversion mutation by complementation tests. Plasmid pAF16 was used to transform strains AN727 (*uncB402*), AN943 (*uncE429*), AN1440 (*uncF469*), AN2015 (*uncH241*), AN1124 (*uncA450*) and AN346 (*unc*$^+$). Transformants were selected on rich medium containing chloramphenicol and their phenotype on succinate minimal medium was examined. All transformants, except for those derived from strain AN943 (*uncE429*), were able to grow on minimal medium containing succinate as the sole carbon source. Thus, the reversion mutation carried on plasmid pAF16 overcomes the dominant phenotype of the Ala-25 to Tyr mutation on plasmid pAF14 (Ala-25 → Tyr). Thus, it was likely that the *uncB*, *uncF*, *uncH* and *uncA* genes on revertant plasmid pAF16 were wild-type, but that the *uncE* gene on this plasmid contained the reversion mutation. To investigate this possibility, the *uncE* gene on plasmid pAF16 was sequenced and found to contain an in-frame 33 bp deletion starting at nucleotide 163 within the *uncE* gene, in addition to the original Ala-25 to Tyr mutation. This deletion results in the removal of 11 amino acids from Ile-55 to Met-65 inclusive from the *c*-subunit encoded by plasmid pAF16.

ATPase activities

Cell membranes were prepared from mutant strain AF73 (Ala-25 → Tyr) together with coupled control strain AF61 (pAN51/*uncE429*) and revertant strain AF78 (pAF16/*uncE429*). Strain AF78, carrying the *uncE429* allele on the chromosome and a 33 bp deletion in the *uncE* gene on plasmid pAF16, also acts as an uncoupled control strain.

The ATPase activity of membranes from mutant strain AF73 (Ala-25 → Tyr) was similar to the uncoupled strain AF78 (pAF16/*uncE429*) and were about 20% of the activity of membranes from the coupled control strain AF61 (pAN51/*uncE429*) (Table IV). When the membrane fractions were dialysed against low ionic strength buffer in the absence of *p*-aminobenzamidine and reassayed, the ATPase activity of strain AF73 (Ala-25 → Tyr) increased to 85% of the coupled control strain AF61 (pAN51/*uncE429*) (Table IV). The ATPase activity of uncoupled strain AF78 (pAF16/*unc*-

TABLE IV

Membrane properties of uncE mutant strains of E. coli

Bacterial strain	Plasmid-encoded amino-acid substitution	ATPase activity (μmol/min per mg protein)	
		pre-dialysis	post-dialysis
AF61 (pAN51/*uncE429*) [a]		0.84	0.57
AF73 (pAF14/*uncE429*)	Ala-25 → Tyr	0.17	0.48
AF78 (pAF16/*uncE429*)	Ala-25 → Tyr Δ(Ile-55–Met-65)	0.17	0.17

[a] Coupled control strain.

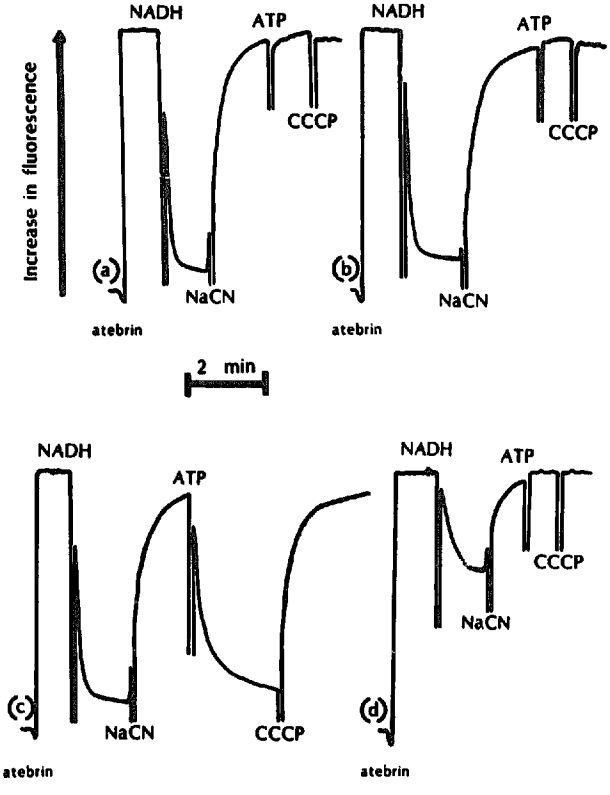

Fig. 1. Atebrin fluorescence-quenching in membranes prepared from strains of *E. coli*. Atebrin was added to give a final concentration of 4 μM, NADH to 2 mM, NaCN to 2.5 mM, ATP to 1 mM and carbonylcyanide *m*-chlorophenylhydrazone (CCCP) to 2 μM. (a) Membranes (0.9 mg of protein) from strain AF73 (Ala-25 → Tyr); (b) stripped membranes (1.4 mg of protein) from strain AF73; (c) membranes (0.8 mg of protein from coupled control strain AF61; (d) stripped membranes (2.1 mg of protein) from coupled control strain AF61.

E429) was unchanged (Table IV). The dialysis treatment causes the F_1-ATPase to be released from the membranes [24]. Two-dimensional gel electrophoresis of membranes from strain AF73 (Ala-25 → Tyr) showed that the F_1-ATPase assembled normally on the membrane (Fimmel, A., unpublished data).

Atebrin fluorescence quenching

Membrane preparations from the mutant and control strains were assayed for ATP-dependent and NADH-dependent atebrin fluorescence-quenching activities before and after removal of the F_1-ATPase (Fig. 1). Membranes from the mutant strain AF73 (Ala-25 → Tyr) retained NADH-dependent atebrin fluorescence-quenching activity after removal of the F_1-ATPase while unstripped membranes from this strain lacked ATP-dependent atebrin fluorescence-quenching activity (Fig. 1).

Discussion

The mutant allele *uncE1001* codes for a *c*-subunit of the F_1F_0-ATPase in which Ala-25 is replaced by Tyr. Strain AF73, carrying the Ala-25 to Tyr substitution, possesses a low growth yield on limiting concentrations of glucose and is unable to grow on succinate as the sole carbon source. When carried on plasmid pAF14 (Ala-25 → Tyr) the mutation is dominant in complementation tests with strains carrying a mutation in either *uncB, uncF, uncH* or *uncA* genes, as well as with the wild-type strain AN346. These results indicate that the mutant *c*-subunit assembles into what would otherwise be a normal F_1F_0-ATPase complex and disrupts enzyme function in these strains.

A revertant which overcame the dominance of the Ala-25 to Tyr mutation was isolated in strains AN2015 (*uncH241*). Plasmid pAF16, carrying the reversion mutation, was found to possess an in-frame 33 bp deletion in the *uncE* gene and complemented all mutant *unc* alleles tested except the *uncE429* allele. This result indicates that the expression of the *c*-subunit carrying the amino-acid substitution Ala-25 to Tyr results in the dominant phenotype observed in complementation tests, since the introduction of this 11-amino-acid deletion, which includes Asp-61, would make the *c*-subunit nonfunctional.

Mutant membranes prepared from strain AF73 (Ala-25 → Tyr) possessed low levels of ATPase activity, similar to the uncoupled strain AF78 (pAF16/*uncE429*). Dialysis of membranes from strain AF73 (Ala-25 → Tyr) in the absence of *p*-aminobenzamidine resulted in the ATPase activity increasing to a level near that of the coupled control strain AF61 (pAN51/*uncE429*). This indicates that the ATPase activity in strain AF73 (Ala-25 → Tyr) is inhibited by approx. 80% when membrane-bound. Two-dimensional gel electrophoresis indicated that the F_1-ATPase was assembled normally. Membranes from strain AF73 (Ala-25 → Tyr) lack ATP-dependent atebrin fluorescence-quenching activity and are proton-impermeable even when the F_1-ATPase is removed.

A comparison of the properties of strain AF73 (Ala-25 → Tyr) with those of strain AN2188 carrying the previously isolated Ala-25 to Thr mutation [5] can be made. Strain AN2188 (Ala-25 → Thr) possesses a growth yield intermediate between uncoupled and wild-type and an ability to grow slowly on minimal media containing succinate as the sole carbon source [5]. Membranes from strain AN2188 (Ala-25 → Thr) were found to possess an ATPase activity about 80% of that of wild-type membranes [5]. Also, the *uncE513* allele carried by strain AN2188 (Ala-25 → Thr) is not dominant in complementation tests.

The above results suggest that small amino acids are preferred at position 25 for ATPase function. Thus, an inverse relationship appears to exist between the size of amino acids at position 25 in the *c*-subunit and the level of ATPase activity. This suggests that steric hindrance may be responsible for the 80% inhibition of membrane-bound ATPase activity observed in membranes from strain AF73 (Ala-25 → Tyr).

Another *c*-subunit mutation has been described which results in the substitution of Ala-21 by Val [25]. If the α-helical hairpin structure of the *c*-subunit is correct, this mutation at Ala-21 occurs one turn of an α-helix away from Ala-25 and both these amino acid residues lie on a helical arm of uniformly small amino acids consisting of Gly-18, Ala-21, Ala-25, Gly-29 and Gly-32. A property common to all mutations so far described at position 21 and position 25 is that they result in an increase in size of the amino acid substituted at these positions.

These observations provide support for a proposed mechanism for oxidative phosphorylation in which rotating *a*- and *b*-subunits interact with a ring of *c*-subunits [26]. The increased size of particular amino acids may thus cause loss of activity due to steric hindrance of rotational catalysis [27].

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