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The proton pore of the F_0F_1-ATPase of *Escherichia coli*: Ser-206 is not required for proton translocation

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A series of experiments was carried out to investigate the role of some polar amino acids in the *a*-subunit of the ATP synthase of *Escherichia coli*. Site-directed mutagenesis resulted in the amino acid substitutions Ser-199 → Ala, Ser-202 → Ala, Ser-206 → Ala, Arg-61 → Gln or Asp-44 → Asn. None of these amino acid substitutions affected the ability of the cells to carry out oxidative phosphorylation. It was concluded therefore that the effect of the substitution of leucine for Ser-206 reported previously (Cain, B.D. and Simoni, R.D. (1986) J. Biol. Chem. 261, 10043–10050) was due to the presence of the leucine rather than the absence of serine. Even though cells carrying the Asp-44 → Asn substitution were able to carry out oxidative phosphorylation, membranes from such cells remained proton-impermeable after removal of the F_1-ATPase. It appears likely that the proton pore of the F_0 of the ATP synthase of *E. coli* consists of four amino acids, namely Arg-219, Glu-210 and His-245 of the *a*-subunit and Asp-61 of the *c*-subunit.

Introduction

The F_0F_1-ATPase enzyme complex catalyses the terminal step in oxidative phosphorylation and 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 *Escherichia coli* the F_0 comprises the *a*-, *b*- and *c*-subunits, encoded by the *uncB*, *uncF* and *uncE* genes, respectively [2], all of which are required for proton translocation [2,3]. The *a*-, *b*- and *c*-subunits are present in a stoichiometry of 1:2:6–12 and secondary and tertiary structures of these subunits have been proposed [4–6].

The conserved helix 4 of a putative *a*-subunit structure has been suggested as having a major role in proton translocation [5] and a current model of the proton pore includes three amino acids from this helix (Ser-206, Arg-210 and Glu-219) as well as His-245 of helix 5 of the *a*-subunit and Asp-61 of the *c*-subunit [7]. However, some doubt over the role of Ser-206 exists. An *unc* mutant, isolated following random mutagenesis, in which leucine was substituted for Ser-206 resulted in partial loss of ATP-dependent proton translocation [8] whereas mutations affecting the other four amino acids of the proton pore resulted in total loss of proton translocation [7–9]. The substitution of amino acids by those with bulkier side-chains in subunits of the F_0 have been shown

Abbreviation: DCCD, dicyclohexylcarbodiimide.

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to result in impaired F_0 function [10–12]. Therefore, to clarify the role of Ser-206, this amino acid was replaced by alanine which is a similar size to serine.

Two other serine residues on helix 4 are in the region of Ser-206 and one of them is conserved in the homologous subunits from other organisms [8]. To examine their roles, alanine was substituted for Ser-199 and Ser-202.

In our current model [7] of the *a*-subunit, Asp-44 of helix 1 is located in the hydrophobic part of the membrane and Arg-61 lies just outside the hydrophobic portion of the membrane. The high energy cost of burying charged residues in the membrane means that charged residues are unlikely to be present in the membrane unless they are functional. This suggests that either Asp-44 or Arg-61 has a functional role in proton translocation. To test this proposition, Asp-44 was replaced by asparagine and Arg-61 by glutamine.

Materials and Methods

Enzymes and chemicals. All chemicals and enzymes used were of the highest quality available. Oligonucleotides were synthesized by K. Newell, C.S.I.R.O., Division of Plant Industry, Canberra. T4 polynucleotide kinase and T4 DNA ligase were obtained from Amersham (Australia) Pty. Ltd. as was [α-^{32}P]dATP and [α-^{35}S]dATPαS.

Bacterial strains and plasmids. All of the bacterial strains used were derived from *E. coli* K-12 and are described, together with the plasmids used, in Table I.

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

Preparation of plasmids. Plasmid DNA was isolated by the method of Selker et al. [18]. The isolation of single-stranded M13 phage DNA was done by using the method of Schreier and Cortese [19].

DNA sequencing. Nucleotide sequences were determined by the dideoxy chain-terminating method of Sanger et al. [20] using a Bresa Pty. Ltd. (Adelaide, S. Australia) dideoxy nucleotide sequencing kit with [α-^{32}P]dATP or [α-^{35}S]dATP αS.

Media and growth of organisms. The mineral salts minimal medium used and additions were as described previously [21]. Cells for the preparation of membranes were grown in 14-l fermenters as

TABLE I

STRAINS OF *E. COLI* AND PLASMIDS USED

Chromosome nomenclature is that used by Bachman [13]; plasmid nomenclature is that used by Novick et al.[14].

Bacterial strain or plasmid	Relevant genotype	Notes and Refs.
AN727	*uncB402 argH pyrE entA recA*	[15]
AN943	*uncE429 argH pyrE entA recA*	[16]
AN2832	pAN490/*uncB402 argH pyrE entA recA*	AN727 transformed to Cm^r by pAN490
AN2833	pAN491/*uncB402 argH pyrE entA recA*	AN727 transformed to Cm^r by pAN491
AN2834	pAN492/*uncB402 argH pyrE entA recA*	AN727 transformed to Cm^r by pAN492
AN2835	pAN492/*uncB402 argH pyrE entA recA*	AN727 transformed to Cm^r by pAN493
AN2839	pAN494/*uncB402 argH pyrE entA recA*	AN727 transformed to Cm^r by pAN494
AN2840	pAN495/*uncB402 argH pyrE entA recA*	AN727 transformed to Cm^r by pAN495
AN2736	pAN174/*uncB402 argH pyrE entA recA*	AN727 transformed to Cm^r by pAN174
pAN174	$Cm^r Tc^s$	[9]
pAN490	$Cm^r Tc^s$ *uncB593* $E^+ F^+$	This paper
pAN491	$Cm^r Tc^s$ *uncB594* $E^+ F^+$	This paper
pAN492	$Cm^r Tc^s$ *uncB595* $E^+ F^+$	This paper
pAN493	$Cm^r Tc^s$ *uncB596* $E^+ F^+$	This paper
pAN494	$Cm^r Tc^s$ *uncB598* $E^+ F^+$	This paper
pAN495	$Cm^r Tc^s$ *uncB*$^+$ $E^+ F^+$	This paper

described previously [22]. The minimal salts medium in the fermenters was supplemented with 5% (v/v) Luria broth [23].

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 subcellular fractions. The preparation and treatment of subcellular fractions were as previously described [24].

Site-directed mutagenesis. Mutants were obtained as outlined in the Amersham handbook 'Oligonucleotide-directed in vitro mutagenesis system'.

Other methods. ATPase and atebrin fluorescence-quenching activities were assayed as previously described [21]. Dicyclohexylcarbodiimide (DCCD) sensitivity was measured as described by Cox et al. [24]. Protein concentrations were determined using Folin's phenol reagent [25] with bovine serum albumin as standard.

Results

Substitution of alanine for Ser-199, Ser-202 and Ser-206, asparagine for Asp-44 and glutamine for Arg-61

For all nucleotide substitutions, the Amersham 'Oligonucleotide-directed in vitro mutagenesis system' was used. The oligonucleotides carrying the appropriate substitutions are shown in Table II. A 2.8 kb *Hin*dIII-*Eco*RI fragment carrying the *uncB, uncE, uncF* and *uncH* genes was cloned into the multiple cloning site of phage M13mp18 and this plasmid was used as the template for mutagenesis. After mutagenesis, plaques carrying each mutation were identified by DNA sequencing. Sequencing with the appropriate mutant oligonucleotide used as a primer confirmed that the oligonucleotide had annealed to only one site. The replicative form of the vector carrying the mutated insert was digested with restriction endonucleases *Hin*dIII and *Cla*I, mixed with *Hin*dIII-*Cla*I digested vector

TABLE II

OLIGONUCLEOTIDES USED IN THE PREPARATION OF MUTANTS

Underlined nucleotides denote differences from normal sequence [4].

Oligonucleotide	Amino acid substitution
5'-CCTTGAAGGGGTAGCCCTGCTGTCC-3'	Ser-199 → Ala
5'-GCCTGCTGGCCAAACCAGT-3'	Ser-202 → Ala
5'-AACCAGTTGCACTCGGTTT-3'	Ser-206 → Ala
5'-GGTTTTATTCCAAAGCGTAGCC-3'	Arg-61 → Gln
5'-TCAATATTAACTCCATGTT-3'	Asp-44 → Asn

TABLE III

PROPERTIES OF *uncB* MUTANT STRAINS OF *E. COLI*

Bacterial strain (plasmid)	Plasmid-encoded amino acid substitution	Growth on succinate	Growth yield on 5 mM glucose (Klett units)	ATPase activity (μmol/min per mg protein)
AN2839	Ser-199 → Ala	+	223	0.9
AN2835	Ser-202 → Ala	+	224	0.8
AN2833	Ser-206 → Ala	+	228	0.8
AN2834	Asp-44 → Asn	+	232	0.5
AN2832	Arg-61 → Gln	+	234	1.0
AN2736 [a]		−	140	0.2
AN2840 [b]		+	236	0.8

[a] Uncoupled control strain.

[b] Coupled control strain.

pAN174 and then ligated with T4 DNA ligase. The *Hin*dIII-*Cla*I digestion released a 2.2 kb fragment carrying the *uncB, uncE* and *uncF* genes. A similar plasmid, carrying the normal *uncB, uncE* and *uncF* genes, was also made in order to construct a control strain. Ligation mixes were transformed into strain AN943 (*uncE429*) and transformants selected for growth on succinate minimal medium in the presence of chloramphenicol.

Plasmids were isolated from purified transformant colonies for each mutant and were screened for the presence of the insert by gel electrophore-

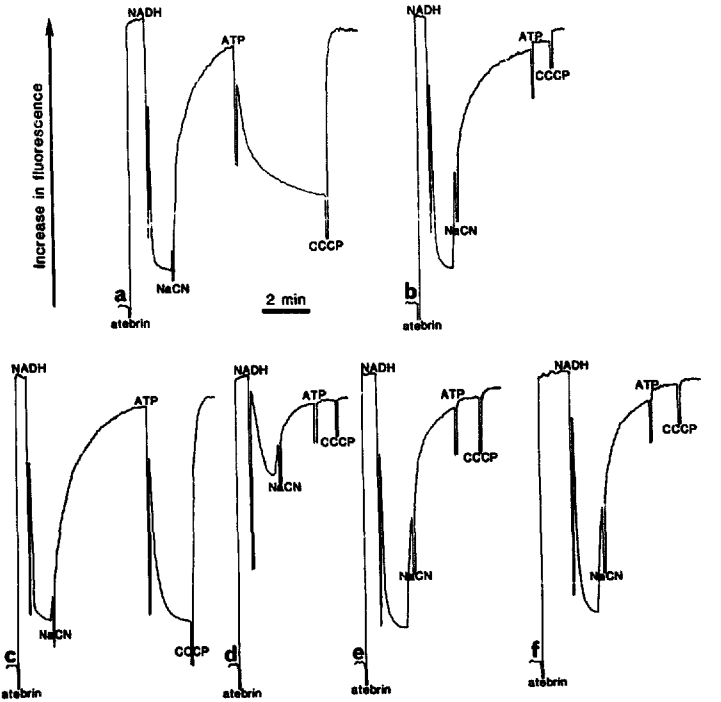

Fig. 1. Atebrin fluorescence-quenching in membranes prepared from various strains of *E. coli*. Atebrin was added to give a final concentration of 4 μM, NADH to 2 mM, NaCN to 2.5 mM, ATP to 1 mM and carbonylcyanide *m*-chlorophenylhydrazone (CCCP) to 2 μM. (a) Membranes from strain AN2834 (Asp-44 → Asn); (b) stripped membranes from strain AN2834; (c) membranes from the coupled control strain, AN2840; (d) stripped membranes from strain AN2840; (e) membranes from the uncoupled control strain, AN2736; (f) stripped membranes from strain AN2736. The results obtained with membranes and stripped membranes from strains AN2839 (Ser-199 → Ala), AN2835 (Ser-202 → Ala), AN2833 (Ser-206 → Ala) and AN2832 (Arg-61 → Gln) were the same as those for the membranes from strain AN2840.

sis after digestion with *Hin*dIII. Plasmids containing the insert were transformed into strain AN727 (*uncB402*). Transformants were selected on rich medium containing chloramphenicol and one transformant from each was purified and retained for further work. These were strains AN2839 (*uncB598*, pAN494), AN2835 (*uncB596*, pAN493), AN2833 (*uncB594*, pAN491) AN2832 (*uncB593*, pAN490) and AN2834 (*uncB595*, pAN492). For clarity, these strains will be referred to as strains AN2839 (Ser-199 → Ala), AN2835 (Ser-202 → Ala), AN2833 (Ser-206 → Ala), AN2834 (Asp-44 → Asn) and AN2832 (Arg-61 → Gln).

Growth properties of strains AN2839 (Ser-199 → Ala), AN2835 (Ser-202 → Ala), AN2833 (Ser-206 → Ala), AN2834 (Asp-44 → Asn) and AN2832 (Arg-61 → Gln)

Strains AN2839 (Ser-199 → Ala), AN2835 (Ser-202 → Ala) AN2833 (Ser-206 → Ala), AN2834 (Asp 44 → Asn) and AN2832 (Arg-61 → Gln) grew normally on succinate minimal medium and had normal growth yields on limiting concentrations of glucose (Table III).

ATPase activities

Subcellular fractions were prepared from the mutant strains, a coupled control strain and an uncoupled control. The ATPase activities of the membranes from all strains were measured and strains AN2839 (Ser-199 → Ala), AN2835 (Ser-202 → Ala), AN2833 (Ser-206 → Ala) and AN2832 (Arg-61 → Gln) were indistinguishable from the coupled control strain, while strain AN2834 (Asp-44 → Asn) had about two thirds the activity of the coupled control strain (Table III). All five strains showed normal sensitivity to DCCD (results not shown).

Atebrin fluorescence-quenching

The membrane preparations from the mutant and control strains were assayed for ATP-dependent and NADH-dependent atebrin fluorescence-quenching activities both before and after removal of the F_1-ATPase (Fig. 1).

Strains AN2839 (Ser-199 → Ala), AN2835 (Ser-202 → Ala), An2833 (Ser-206 → Ala) and AN2832 (Arg-61 → Gln) were not significantly different from the coupled control strain. However, strain An2834 (Asp-44 → Asn) had a slightly lower ATP-dependent atebrin fluorescence-quench than the coupled control strain and retained full NADH-dependent quenching after removal of the F_1-ATPase.

Discussion

Strains AN2839 (Ser-199 → Ala), AN2835 (Ser-202 → Ala) and AN2833 (Ser-206 → Ala) are wild type in all respects, indicating that Ser-199, Ser-202 and Ser-206 are not part of the proton pore. The mutation reported by Cain and Simoni [8] resulting in Ser-206 being replaced by leucine, which partially disrupts proton translocation, was probably due to some conformational change in the F_0 resulting from the larger side-chain of leucine. In strain AN2833, Ser-206 was changed to alanine which is similar in size to serine and no disruption due to size alone would be expected.

A previous model of the proton pore consisted of five amino acids, including Ser-206, each separated by about one turn of the helix [7]. This resulted in a membrane-spanning helix of 25 amino acids. However, the width of the hydrophobic portion of the membrane is about 3.0 nm, which is equivalent to a helix of about 20 amino acids [26]. Furthermore, the side-chains of the amino acids involved in the proton pore should remain buried in the hydrophobic portion of the membrane to be functional and therefore would be unable to reach the hydrophilic phospholipid head group region. This means that the outer amino acids of the proton pore should be at least one turn of the helix away from the phospholipid head group region. Given these constraints, only four amino acids may be required to form a proton pore traversing the hydrophobic region of the membrane (Fig. 2).

All four amino acids in the proposed proton pore are charged. Theoretical models of hydrogen-bonded chains allowing proton translocation have been suggested. Around 20 uncharged, polar amino acids have been postulated to form a chain of hydrogen bonds which would allow passage of protons across the membrane in a similar manner to the movement of protons through ice [27,28]. The proposed proton pore of the

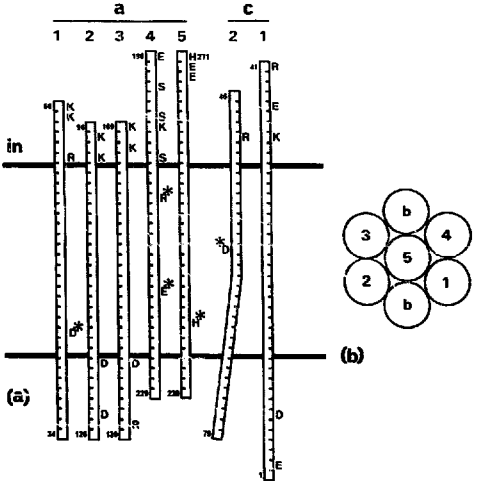

Fig. 2. (a) Proposed transmembrane helices of the *a*- and *c*-subunits from *E. coli* F_0-ATPase. The N-terminal and C-terminal residue numbers for each helix are indicated. All charged residues are indicated, as are the three serine residues altered in this study: D, aspartic acid; E, glutamic acid; H, histidine; K, lysine; R, arginine; S, serine. The residues involved in the proton pore are indicated by a star (*). (b) Proposed packing of the transmembrane helices of the *a*- and *b*-subunits of the F_0-ATPase of *E. coli*. These helices have been proposed to be packed within a ring of *c*-subunits [5].

F_1F_0-ATPase, involving only four charged amino acids, is clearly not consistent with this model.

Strain AN2832 (Arg-61 → Gln) was normal in all respects. It is concluded, therefore, that Arg-61 is not involved in proton translocation and, given the difficulty of burying charged residues in the membrane, is unlikely to be located in the hydrophobic portion of the membrane. This would mean that Asp-44 must be located within the membrane (Fig. 2).

Strain AN2834 (Asp-44 → Asn) has membranes which are impermeable to bulk phase protons, as indicated by the retention of NADH-dependent atebrin fluorescence-quenching after removal of the F_1-ATPase. However, it is able to grow on succinate and has a normal growth yield, indicating that it is able to carry out oxidative phosphorylation. Other mutants have similar properties and it has been suggested that protons from within the membrane or at the surface of the membrane, rather than bulk phase protons, are used for oxidative phosphorylation [24,29,30]. Strain AN2834 (Asp-44 → Asn) has only a small reduction in ATPase activity and ATP-dependent fluorescence-quenching and the ATPase activity is sensitive to DCCD, suggesting that no gross structural or assembly defects exist. This is in contrast to previously described mutants with similar properties (see above): properties which were attributed to the structural effects of the particular amino acid substitutions [24,29,30]. The substitution of asparagine for Asp-44 appears to specifically affect movement of bulk phase protons through the proton pore. Thus, two pathways feeding protons into the pore may exist, as has been suggested for chloroplast thylakoid membranes [31–33]. Protons from the membrane surface [34] may feed directly into His-245, while

Asp-44 may be required to pass protons from the bulk phase to His-245. However the possibility also exists that the Asp-44 → Asn conversion results in instability of the F_0 structure on removal of the F_1-ATPase such that the proton pore is inactivated.

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