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Glutamate residues at positions 219 and 252 in the *a*-subunit of the *Escherichia coli* ATP synthase are not functionally equivalent

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Abstract

The role of glutamate-219 in the a-subunit of the *Escherichia coli* F_0F_1-ATPase was examined using site-directed mutagenesis. The replacement of Glu-219 by lysine, alanine or glycine resulted in a partially functional F_0F_1-ATPase. Combining any of these mutations with the substitution of glutamate for Gln-252 did not result in any increase in function. These findings rule out a proposal that glutamate at position 252 can functionally replace glutamate at position 219 [S.B. Vik, B.J. Antonio, J. Biol. Chem. 269 (1994) 30364–30369]. All the single and double mutants grew better at 25°C than at 37°C, suggesting a role for Glu-219 in maintaining the structure of the F_0. © 1998 Elsevier Science B.V.

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1. 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. In *Escherichia coli*, the enzyme comprises eight non-identical subunits, *a*, *b*, *c*, α, β, γ, δ and ε, encoded by the genes *uncB*, *F*, *E*, *A*, *D*, *G*, *H* and *C* respectively [1]. The *a*, *b* and *c* subunits are integral membrane proteins and form the F_0 portion of the complex which can function as a proton pore. The α, β, γ, δ and ε subunits are peripheral membrane proteins forming the F_1-ATPase portion of the complex which retains ATP hydrolytic activity when removed from the membrane. The *a*, *b* and *c* subunits of the proton pore are present in a stoichiometry of 1:2:6–12 [2] and all are required for proton translocation. The *b*-subunit is anchored in the membrane by a single transmembrane helix and appears not to have a direct role in proton translocation. Residues essential for proton translocation have been found in the *a* and *c* subunits [2]. It has been proposed that the proton pore involves four amino acids: Arg-210, Glu-219 and His-245 on the *a*-subunit and Asp-61 on the *c*-subunit [3].

In order to understand the mechanism of proton translocation, information on the positions of these essential residues is required. There is a considerable

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body of evidence indicating that the *c*-subunit forms a helical hairpin structure, placing Asp-61 in the centre of the membrane (reviewed in Ref. [4]). However, the structure of the *a*- subunit is less clear, with a number of different models being proposed [2,5–9]. These models differ in the positioning within the membrane, of the amino-acid residues essential for proton translocation. In particular, there have been different proposals for the role and position of Glu-219. Glu-219 is not strictly conserved and some amino-acid substitutions at this position retain some proton translocating activity [10–13]. Recent experiments found that double mutants involving substitutions at position 219, in combination with the replacement of Gln-252 by glutamic acid, were partially functional [11]. These data were used to suggest that an acidic group at position 252 could substitute for Glu-219, implying that positions 219 and 252 were in close proximity. However, the properties of the single mutations at position 219 corresponding to the partially active double mutations were not reported [11]. In the present study, the previously isolated double mutations [11] were duplicated and the equivalent single mutations were also constructed, allowing direct comparisons to be made.

2. Materials and methods

2.1. Enzymes and chemicals

All chemicals and enzymes used were of the highest quality commercially available. Oligonucleotides were synthesised by the Biomolecular Resource Facility, ANU Canberra. [^{35}S]dATPS was obtained from Amersham (Australia).

2.2. Media and growth of organisms

All bacterial strains were derived from *E. coli* K12 and are described in Table 1. The mineral salts minimal medium used and additions were as described previously [15]. Cells for the preparation of membranes were grown in 10-l fermenters as described previously [16]. The minimal salts medium in the fermenters was supplemented with 5% (v/v) Luria broth [17]. Turbidities of cultures were measured with a Klett-Summerson colorimeter. Growth yields were measured as turbidities after growth had ceased in minimal medium containing limiting (5 mM) glucose and supplemented with 5% Luria broth as well as specific requirements.

2.3. Preparation of plasmid and phage M13 DNA

Single-stranded 'template' DNA was prepared as described in the Amersham handbook 'Oligonucleotide-directed in vitro mutagenesis system'. Replicative form DNA and plasmid DNA were prepared by the alkaline lysis method [18]. Single-stranded DNA for sequencing was prepared as described by Messing [19].

Table 1
Strains used

Strain number	Codon change(s) in plasmid-borne *uncB* gene	Amino-acid change encoded
AN727[a]	N/A	N/A
AN2736[b]	N/A	N/A
AN2840[c]	N/A	Wild type
AN3891	G(655)AGGCA	E219A
AN3898	G(655)AGGCA, C(754)AAGAG	E219A, Q252E
AN3899	C(754)AAGAG	Q252E
AN3907	G(655)AGAAA	E219K
AN3908	G(655)AGGGT	E219G
AN3924	G(655)AGAAA, C(754)AAGAG	E219K, Q252E
AN3925	G(655)AGGGT, C(754)AAGAG	E219G, Q252E

[a] $uncB_{402}$ *argH pyrE entA recA* [14].
[b] AN727 transformed with the vector pAN174.
[c] AN727 transformed with pAN495 ($uncB^+E^+F^+$).

2.4. Site-directed mutagenesis

Mutagenesis was carried out using the Amersham 'Oligonucleotide-directed in vitro mutagenesis system'. The method used was that described in the Amersham handbook. The oligonucleotide primers were designed to be between 17 and 25 nucleotides in length, to contain the mutant sequence approximately in the middle and to have GC-rich N and C termini. The mutant codons are shown in Table 1. Single-stranded template was derived from the replicative form of M13 mp18 in which a 2.4 kb *Hind*III/*Eco*RI fragment carrying the *uncB*, *uncE*, *uncF* and *uncH* genes was cloned into the multiple cloning site. The presence of each mutation was confirmed by DNA sequencing using the dideoxy chain-terminating method of Sanger et al. [20]. The USB T7 sequenase kit version 2.1 and [^{35}S]dATPS were used.

2.5. Construction of plasmids

A 2.2-kb *Hind*III/*Cla*I fragment carrying the mutated *uncB* gene as well as the *uncE* and *uncF* genes was subcloned from the recombinant M13 mp18 replicative form into the vector pAN174 as previously described [3]. The correct plasmid was identified where possible as one which conferred on a strain with a chromosomal mutation in the *b*-subunit AN1440 (*uncF469*) [21] the ability to grow on succinate minimal medium. In all cases, the presence of the correct insert was confirmed by restriction analysis. At least three independent isolates with the correct restriction pattern were characterised in each case. Plasmids containing the correct insert were then used to transform strain AN727 (*uncB402*). Several independent isolates from each transformation were compared for growth characteristics and growth yield on limiting glucose. One typical isolate was retained for biochemical studies. Two control plasmids were also transformed into appropriate background strains: a coupled control in which wild type *uncB*, *uncE* and *uncF* genes were subcloned into pAN174 as described and an uncoupled control consisting of the vector, pAN174. The mutant strains constructed are shown in Table 1.

2.6. Preparation of subcellular membrane fractions

The growth of the cells in 10-l fermenters, fractionation and subsequent washing procedures have all been described previously [22].

2.7. Assays

Mg–ATPase activity was determined as described previously [23]. Atebrin fluorescence quenching activities were measured as previously described [22]. Protein concentrations were determined using Folins phenol reagent with bovine serum albumin as standard [24].

3. Results

3.1. Growth properties of mutants

A previous study had used saturation mutagenesis to isolate a large number of mutants with substitutions at position 219 in combination with the mutation of Gln-252 to glutamic acid [11]. We duplicated some of these mutations and constructed the equivalent single mutations as well, in order to determine the effects of these changes singly and together. Glu-219 was replaced with glycine, lysine and alanine in the presence and absence of the Q252E mutation. It had been previously found that some of these double mutants grew better at lower temperatures [11] so the growth yields on 5 mM glucose of mutants were determined at 25°C and 37°C (Table 2). The ability of the mutants to grow on nutrient plates with succinate as the sole carbon source was also determined at both temperatures. The previously isolated mutants, E219Q and E219H [10,12,13] were also included. Similar results were obtained for the growth of the mutants on succinate and on glucose. In all cases involving mutations at position 219, the growth yields were higher at 25°C than at 37°C. However, the magnitude of the difference varied considerably. For example, at 37°C the single mutants, E219A and E219Q, and the double mutant, E219A/Q252E had growth yields similar to that of the uncoupled control, while at 25°C their growth yields rose to 20–50% of the coupled control. Other

Table 2
Growth properties of mutants

a-subunit mutation	Growth yield at 25°C[a]	Growth yield at 37°C[a]	Growth on succinate at 25°C	Growth on succinate at 37°C
Coupled control	100	100	+ + +	+ + +
Uncoupled control	0	0	−	−
E219K	92 ± 2	70 ± 2	+ + +	+ +
E219A	51 ± 1	0	+	−
E219G	91 ± 3	40 ± 3	+ +	+
E219H	83 ± 1	35 ± 3	+	−
E219Q	20 ± 1	0	−	−
E219K/Q252E	82 ± 1	59 ± 2	+ + +	+ +
E219A/Q252E	27 ± 3	2.5 ± .3	+	−
E219G/Q252E	69 ± 3	13 ± 1	+ +	+
Q252E	93 ± 5	95 ± 2	+ + +	+ + +

[a]Growth yield on 5 mM glucose, measured in Klett units, and expressed as a percentage of the difference between the values obtained for the coupled and uncoupled control strains. Values shown are the mean ± the standard error in the mean for at least four independent experiments. The growth yields of the control strains were similar at 25°C and 37°C, being about 180 Klett units for the coupled control and 120 Klett units for the uncoupled control.

mutants, such as E219K and E219K/Q252E, had growth yields which were intermediate at 37°C but almost as high as that of the coupled control at 25°C. Only Q252E had wild type growth yields at both temperatures.

3.2. Properties of membranes prepared from mutants

Membranes from each of the mutant strains were prepared and the ATPase and atebrin fluorescence quenching activities were measured (Table 3). The three single mutants with substitutions at position 219, E219K, E219A and E219G, had levels of ATPase activity that were similar to, or higher than, the coupled control strain. The ATPase activity of Q252E was somewhat lower, at about half the level of the coupled control. Q252E and E219K had significant levels of ATP-dependent atebrin fluorescence quenching activity (70% and 20% respectively) whereas this activity was very low for the E219G and

Table 3
Properties of membrane preparations from *a*-subunit mutant strains[a]

a-subunit mutation(s) on plasmid	ATPase activity[b]	Atebrin fluorescence quench (%)		
		NADH-dependent		ATP-dependent
		Native	Stripped	
Uncoupled control	0.3	86	89	0
Coupled control	1.1	87	49	87
E219K	1.5	90	89	20
E219A	1.0	86	86	6
E219G	1.7	81	67	4
E219K/Q252E	0.5	95	95	< 2
E219A/Q252E	0.5	89	80	< 2
E219G/Q252E	0.6	85	83	< 2
Q252E	0.6	87	60	20

[a]The values shown are those for a typical experiment. Values from replicate experiments were within 5% of each other.
[b]ATPase activity expressed as μmol per min per mg protein.

E219A mutants. E219K and E219A had proton impermeable membranes after removal of the F_1-ATPase, as indicated by the retention of NADH-dependent atebrin fluorescence quenching activity after dialysis of the membranes against low-ionic-strength buffer. Growth of the mutant strains E219K, E219A and E219G at 25°C, rather than 37°C, did not affect the properties of isolated membranes (results not shown).

The combination of the Q252E mutation with each of the mutations at position 219 was deleterious. All three double mutants had lower ATPase activity on the membranes than the equivalent single mutants. ATP-dependent atebrin fluorescence quenching activity was completely abolished in the double mutants and all three showed retention of NADH-dependent atebrin fluorescence quenching activity after dialysis of the membranes against low-ionic-strength buffer.

4. Discussion

We have shown that lysine, alanine or glycine can substitute for Glu-219 in the *a*-subunit without complete loss of function. Previously, it had been suggested that Glu-219 was an essential component of the proton pathway through the F_0 on the basis that the replacement of Glu-219 by tyrosine, glutamine or leucine resulted in the loss of proton translocation [10,12,13]. Other studies had also found that E219D retained full function [12] and E219H had a very low level of function which could be increased by second site suppressor mutations in which Arg-140 was replaced by histidine or leucine [25]. This suggested that a residue able to protonate and deprotonate was required at position 219 and was consistent with a role for Glu-219 in proton translocation. Of the residues substituted for Glu-219 in the present study, only lysine is able to protonate and deprotonate. However, alanine and glycine are small amino acids and a water molecule may occupy the space normally filled by the much larger glutamate. A water molecule has been found to partially replace an aspartate residue required for proton movement in the D85A mutant of bacteriorhodopsin [26]. A similar effect may be occurring in the E219A and E219G mutants of the *a*-subunit. The results of this study do not, therefore, rule out a role for Glu-219 in the proton pathway.

The finding that all mutants with substitutions for Glu-219 had a higher growth yield at 25°C than at 37°C suggests that Glu-219 may normally have a structural role. The fact that most of the single and double mutants tested had membranes which were impermeable to protons after the F_1-ATPase had been removed is also consistent with a structural role.

The double mutants, E219K/Q252E, E219A/Q252E and E219G/Q252E, had been previously isolated by Vik and Antonio [11]. Our results agree with theirs in that all three double mutants were functional at 25°C, as measured by the growth yield on glucose and ability to grow with succinate as the sole carbon source. However, Vik and Antonio found that these mutants had significant ATP-dependent proton-pumping activity whereas in our hands this activity was negligible. This difference may be due to the different background strains used. Mutations which allow growth on succinate but abolish ATP-dependent proton-pumping activity have been observed before and it has been suggested that they allow the F_0F_1-ATPase to operate in the direction of ATP synthesis only [27].

The interpretation of these results by Vik and Antonio was that a glutamate residue at position 252 could substitute for Glu-219 when this was replaced by other residues, implying that positions 219 and 252 were adjacent. However, in order to draw this conclusion, it is necessary to compare the effects of the mutations at position 219 alone with the double mutants in which the changes at position 219 are combined with Q252E. To our knowledge, the single mutations E219K, E219G and E219A have not previously been reported. We have shown that E219K, E219G and E219A are partially functional. Since Q252E is also functional it is not surprising that the double mutants are functional. There is, therefore, no basis for the conclusion of Vik and Antonio [11] that glutamate at position 252 can functionally replace Glu-219. Instead, it is likely that Gln-252 is adjacent to Arg-210 because function is partially retained when the essential arginine residue is moved from position 210 to position 252 [27].

These results also have implications for the mechanism of proton translocation. In the model proposed by Vik and Antonio, Glu-219, His-245 and Gln-252

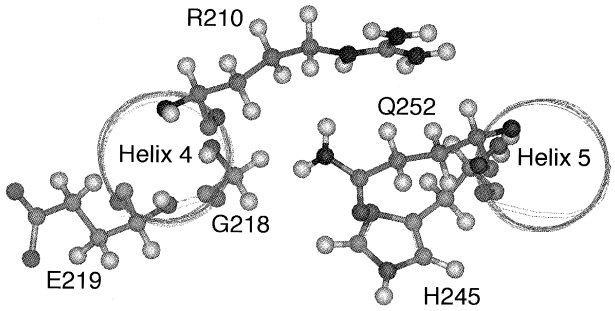

Fig. 1. Computer-generated model of putative transmembrane helices 4 and 5 of the *a*-subunit from *E. coli* F_0-ATPase showing the positions of the side chains of the amino-acid residues discussed in the text.

are located in a water-filled channel which forms a pathway for protons from Asp-61 in the *c*-subunit to the cytoplasmic side of the membrane. However, there are several lines of evidence which make this unlikely. In most models of the *a*-subunit, these residues occur on two transmembrane helices, with the residues from Arg-210 to Glu-219 included in one transmembrane helix and the stretch from His-245 to Gln-252 occurring on another (Fig. 1). It has also been suggested that Gly-218 and His-245 are adjacent as these two residues can be switched and function is retained [28]. This is consistent with the juxtaposition of Arg-210 and Gln-252. Since Glu-219 is on the opposite side of the helix to Arg-210 and Gly-218 (Fig. 1), it would be unable to occupy a water-filled channel which also contained His-245 and Gln-252.

The positioning of the key residues for proton translocation shown in Fig. 1 is consistent with an alternative mechanism [27,29] in which His-245 would feed protons from the periplasm to Asp-61 of the *c*-subunit. This would cause destabilisation of a salt bridge between that Asp-61 residue and Arg-210 of the *a*-subunit, with the energy-released driving rotation of the *a*-subunit relative to a ring of *c*-subunits [29]. Arg-210 would then form a salt bridge with the next Asp-61 residue and a proton would be released from Arg-210 to the cytoplasmic side of the membrane. The location of Glu-219 precludes a direct role in this proton relay system but Glu-219 would be in a position to interact with the Asp-61 residue on the adjacent *c*-subunit.

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