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The proton pore in the *Escherichia coli* F_0F_1-ATPase: substitution of glutamate by glutamine at position 219 of the *a*-subunit prevents F_0-mediated proton permeability

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Three mutations in the *uncB* gene encoding the *a*-subunit of the F_0 portion of the F_0F_1-ATPase of *Escherichia coli* were produced by site-directed mutagenesis. These mutations directed the substitution of Glu-219 by Gln, or of Lys-203 by Ile, or of Glu-196 by Ala. Strains carrying either the Lys-203 or Glu-196 substitutions showed growth characteristics indistinguishable from the coupled control strain. Properties of membrane preparations from these strains were also similar to those from the coupled control strain. The substitution of Glu-219 by Gln resulted in a strain which was unable to utilise succinate as sole carbon source and had a growth-yield characteristic of an uncoupled strain. Membrane preparations of the Glu-219 mutant were proton impermeable and the F_1-ATPase activity was inhibited by about 50% when membrane-bound. The results are discussed with reference to a previously proposed intramembranous proton pore involving subunits *a* and *c*.

Introduction

The F_0F_1-ATPase enzyme complex catalyses the terminal step in oxidative phosphorylation and is located in mitochondrial, chloroplast and bacterial membranes [1]. 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. 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]. Secondary and tertiary structures of the *a*-, *b*- and *c*-subunits have been proposed [4–8]. Mutations in the *uncB* and *uncE* genes have resulted in the identification of key amino acids in the *a*- and *c*-subunits, with respect to proton translocation by the assembled F_0 complex. Those amino acid substitutions in the *c*-subunit affecting proton translocation through the F_0 resulting in uncoupling of ATP synthesis are Asp-61 by either Gly or Asn [9,10], Pro-64 by Leu [11], Ala-21 by Val [12] and Ala-25 by Thr [13]. In the *a*-subunit, the amino-acid substitutions affecting proton translocation are His-245 by either Tyr [14] or Leu [15], Arg-210 by Gln [15], and Ser-206 by Leu [14]. It has been suggested that an amphipathic, transmembranous helix exists in the *a*-subunit (helix IV), and that this plays a central role in proton translocation through the

Abbreviation: DCCD, *N,N'*-dicyclohexylcarbodiimide.

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F_0 [7]. Evidence has been presented suggesting that Arg-210 and Ser-206, both in helix IV, are components of the proton pore [14,15]. In the present paper, three further residues of helix IV, Glu-196, Lys-203 and Glu-219, were altered by site-directed mutagenesis and the properties of the resultant mutant strains were examined. The results obtained have been used to rationalise a previously proposed model [7,15] of the F_0 structure.

Materials and Methods

Enzymes and chemicals. All chemicals and enzymes used were of the highest quality available. Restriction endonucleases were obtained from Bethesda Research Laboratories (U.S.A.). T_4-Polynucleotide kinase and T_4-DNA ligase were obtained from Amersham (Australia)Pty. Ltd. as was [α-^{32}P]dATP and [γ-^{32}P]ATP. All dideoxynucleotides and deoxynucleotides were obtained from Boehringer Mannheim (Australia). Oligonucleotides were either synthesised by K. Newell. C.S.I.R.O. Division of Plant Industry, Canberra or kindly provided by A.E. Senior.

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 [18].

Preparation of plasmids. Plasmid DNA was isolated by the alkaline lysis method of Silhavy et al. [22] except that for the isolation of the replicative form of phage M13, cells were grown to a Klett value of 160 (Klett 100 = 0.24 mg dry weight/ml) in glucose minimal medium and then infected with phage M13 mp9 to give initially about 10^{10} plaque forming units/ml. The isolation of single-

TABLE I

STRAINS OF *ESCHERICHIA COLI* AND PLASMIDS USED

Bacterial strain or plasmid [b]	Relevant genotype [a]	Notes and Refs.
AN727	*uncB402 argH pyrE entA recA*	[25]
AN2709	pAN436/*uncB402 argH pyrE entA recA*	AN727 transformed to Cm^r by pAN436
AN2712	pAN437/*uncB402 argH pyrE entA recA*	AN727 transformed to Cm^r by pAN437
AN2736	pAN174/*uncB402 argH pyrE entA recA*	AN727 transformed to Cm^r by pAN174
AN2768	pAN413/*uncB402 argH pyrE entA recA*	AN727 transformed to Cm^r by pAN413
AN2774	pAN45/*uncB402 argH pyrE entA recA*	AN727 transformed to Cm^r by pAN45
AN2402	pAN51/*uncB402 argH pyrE entA recA*	AN727 transformed to Cm^r by pAN51
AN2792	pAN467/*uncB402 argH pyrE entA recA*	AN727 transformed to Cm^r by pAN467
AN1273	*uncG428 argH pyrE entA recA*	[18]
AN2015	*uncH241 argH pyrE entA recA*	[19]
K37	Hfr *SupD*	
JM101	*Δlac-pro thiF supE traDΔ36 proAB, LacI^{Q}ZΔ*M15	[20]
pAN174	Cm^r Tc^r	derived from pAN51 [18]
pACYC184	Cm^r Tc^r	[21]
pAN436	Cm^r Tc^s *uncB$^+$E$^+$F$^+$H$^+$A$^+$G$^+$D$^+$C$^+$*	vector pAN174
pAN437	Cm^r Tc^s *uncB573E$^+$F$^+$H$^+$A$^+$G$^+$D$^+$C$^+$*	vector pAN174
pAN413	Cm^r Tc^s *uncB566E$^+$F$^+$H$^+$A$^+$G$^+$D$^+$C$^+$*	vector pACYC184
pAN467	Cm^r Tc^s *uncB587E$^+$F$^+$H$^+$A$^+$G$^+$D$^+$C$^+$*	vector pAN174
pAN45	Cm^r Tc^s *uncB$^+$E$^+$F$^+$H$^+$A$^+$G$^+$D$^+$C$^+$*	[18]
pAN36	Cm^r Tc^s *uncD$^+$C$^+$*	[18]
pAN51	Cm^r Tc^s *uncB$^+$E$^+$F$^+$H$^+$A$^+$*	[18]
pAN461	*uncB$^+$E$^+$F$^+$H$^+$A$^+$*	phage M13 RF carrying a *Hin*dIII endonuclease generated 4.3 Kb fragment coding for the ATPase subunits *a*, *c*, *b*, α and δ

[a] Chromosome nomenclature is that used by Bachmann [16].

[b] Plasmid nomenclature is that used by Novick [17].

stranded M13 phage DNA was performed essentially by the method of Schreier and Cortese [23].

DNA sequencing. Nucleotide sequences were determined by the dideoxy chain-terminating method of Sanger et al. [24] using [α-^{32}P]dATP.

Media and growth of organisms. The mineral-salts minimal medium used and the additions were as described previously [25]. Cells for the preparation of membranes were grown in 14-1 fermenters essentially as described previously [26]. The mineral-salts media in the fermenters were supplemented with 5% (v/v) Luria broth [27]. The ATPase activity of membranes from strain AN2774 was higher than that described previously [15]. This was due to increased efficiency of aeration of the culture medium during growth. Turbidities of cultures were measured with a Klett-Summerson colorimeter. Growth yields were measured as turbidities after growth ceased in media containing limiting (5 mM) glucose (Klett 100 = 0.24 mg dry wt./ml).

Preparation of subcellular fractions. The preparation and treatment of subcellular fractions were as previously described [28].

Site-directed mutagenesis. The techniques used for site-directed mutagenesis were essentially as those of Zoller and Smith [29]. The oligonucleotides carrying the appropriate base substitutions were annealed to single-stranded preparations of plasmid pAN461 and extended using DNA polymerase 1 large fragment (Klenow) and ligated using T_4 DNA ligase. Strain K37 was transformed with the ligation mixes and the resultant plaques were screened for the mutant sequences using the relevant (γ-^{32}P)-labelled oligonucleotides under conditions favouring hybridization of the probe to the particular mutated nucleotide sequence of the phage DNA. After purification of plaques, confirmation of each of the expected mutations was made by DNA sequence analysis. Single site incorporation of each mutant oligonucleotide was confirmed by sequencing using that oligonucleotide as primer.

Other methods. ATPase and atebrin fluorescence-quenching activities were assayed as previously described [25].

Dicyclohexylcarbodiimide sensitivity of ATPase activity was measured as described by Cox et al. [30]. Protein concentrations were determined using Folins phenol reagent [31] with bovine serum albumin as standard.

Results

Production of a-subunit mutants carrying the amino acid substitutions Glu-219 → Gln, Glu-196 → Ala and Lys-203 → Ile

The three oligonucleotides carrying the appropriate base substitutions (Table II) were used to generate the corresponding mutant derivatives of plasmid pAN461. Previous results [15] involving the two *a*-subunit substitutions of Arg-210 → Gln and His-245 → Leu indicated that successful subcloning of the 4.3 Kb *Hin*dIII endonuclease-generated fragment may require pAN174 as a suitable vector. This vector carries a small deletion which reduces the levels of expression of genes cloned into its unique *Hin*dIII restriction site. Thus, two of the mutant derivatives of pAN461, Lys-203 → Ile, and Glu-196 → Ala were each mixed with the vector pAN174 together with the plasmid pAN36, treated with the endonuclease *Hin*dIII and ligated with T_4-DNA ligase. The liga-

TABLE II

SYNTHETIC PRIMERS USED IN THE PREPARATION OF MUTANTS AND AS SEQUENCING PRIMERS FOR SEQUENCING THE *uncB* GENE

Underlined nucleotides denote differences from normal sequence [5].

Synthetic oligonucleotide	Relevant amino acid substitution	Notes
5'-CTGCTGTCCATACCAGTTTCA-3'	Lys-203→Ile	
5'-TTAATCCTTGCAGGGGTAAG-3'	Glu-196→Ala	
5'-CATGTATGCCGGTCAGCTGATTTTCATTCTG-3'	Glu-219→Gln	
5'-ATGGCACTGGGCGTATTTA-3'	–	sequencing primer used for sequencing from nucleotide 475 in the *uncB* gene

tion mixtures were used to transform strain AN1273 (*uncG428*). Transformants were selected for the presence of plasmids carrying all the genes coding for the F_0F_1-ATPase by plating out on succinate minimal medium supplemented with chloramphenicol. Colonies from the two transformation experiments were purified and plasmid preparations were screened both for equivalence in size to plasmid pAN45 and for similar *Hin*dIII-generated restiction fragments. Such plasmids carried all the genes encoding the subunits of the F_0F_1-ATPase. Of the plasmid preparations screened, those carrying the Glu-196 → Ala substitution proved to be constructs containing either the deleted vector pAN174, or the undeleted vector pACYC184 obtained from the *Hin*dIII digested plasmid pAN36, whilst all 12 plasmids carrying the Lys-203 → Ile substitution proved to be constructs containing pAN174. One colony carrying a plasmid with the Glu-196 → Ala substitution and incorporating the undeleted vector pACYC184 was purified, the plasmid isolated and designated pAN413 (*uncB566*). One colony carrying the Lys-203 → Ile substitution and incorporating the deleted vector pAN174 was also purified, the plasmid isolated and designated pAN437 (*uncB573*). The mutant derivative of pAN461 carrying Glu-219 → Gln was mixed with the vector pAN174, treated with restriction endonuclease *Hin*dIII and then T_4-DNA ligase, and the ligated mixture used to transform strain AN2015 (*uncH243*). Transformants were selected on succinate minimal medium containing chloramphenicol. Transformant colonies were purified and their plasmids were examined. An isolate which contained a plasmid similar in size to pAN51 (carrying the genes encoding *uncB, E, F, H* and *A*) was purified, the plasmid was isolated and designated pAN467 (*uncB587*). The three mutant plasmids, pAN413 (*uncB566*), pAN437 (*uncB573*) and pAN467 (*uncB587*) were each used to transform strain AN727 (*uncB402*). Transformants were selected on rich medium containing chloramphenicol and one transformant colony from each of the three transformations was purified and retained for further work. For clarity, these strains will be further referred to as AN2792 (Glu-219 → Gln), AN2768 (Glu-196 → Ala) and AN2712 (Lys-203 → Ile).

Growth properties of strains AN2792 (Glu-219 → Gln) AN2712 (Lys-203 → Ile) and AN2768 (Glu-196 → Ala)

Strains AN2792, AN2712 and AN2768 were examined for their ability to grow on succinate minimal medium and for their growth yields on limiting levels of glucose (Table III). Strains AN2712 (Lys-203 → Ile), and AN2768 (Glu-196 → Ala) grew on solid media with succinate as the carbon source, whereas strain AN2792 (Glu-219 → Gln) was unable to grow under these conditions. Growth yields for both strains AN2712 (Lys-203 → Ile) and AN2768 (Glu-196 → Ala) were identical to their respective coupled control strains AN2709 and AN2774. In contrast, the growth yield of strain AN2792 (Glu-219 → Gln) was the same as the uncoupled control strain AN2736.

ATPase activities

Sub-cellular fractions were prepared from the mutant strains AN2768 (Glu-196 → Ala), AN2792 (Glu-219 → Gln) and AN2712 (Lys-203 → Ile), together with the relevant control strains AN2774, AN2402 and AN2709, respectively, and the uncoupled control strain AN2736. The ATPase activities of the cytoplasmic fractions from the three mutant strains were negligible (Table III). The ATPase activities of the membrane fractions from both mutant strains AN2768 (Glu-196 → Ala) and AN2712 (Lys-203 → Ile) were the same as their respective coupled control strains AN2774 and AN2709. However, the ATPase activity of the membrane fraction from the mutant strain AN2792 (Glu-219 → Gln) was only approx. 40% of the coupled control strain AN2402 (Table III). If the membrane fraction from the mutant strain was dialysed against low ionic strength buffer in the absence of *p*-aminobenzamidine and reassayed, the ATPase activity increased to about 80% of the control value. The ATPase activities of the coupled and uncoupled control strains were unaffected by this treatment. The dialysis procedure causes the F_1-ATPase to be released from the membranes. This was confirmed for the mutant strain AN2792 (Glu-219 → Gln) by the lack of any appreciable ATPase activity on assaying the membranes after centrifugation of the dialysed preparation (data not shown). Dialysis treatment

TABLE III

PROPERTIES OF *uncB* MUTANT STRAINS OF *Escherichia coli*

Bacterial strain (plasmid)	Plasmid-encoded amino acid substitution	Growth on succinate	Growth yield on 5 mM glucose (Klett units)	ATPase activities (μmol per min per mg protein)		
				cytoplasmic	membrane	
					pre-dialysis	post-dialysis
AN2768 (pAN413)	Glu-196 → Ala	+	228	< 0.1	2.9	2.8
AN2774 (pAN45) [a]	+	+	235	< 0.1	3.1	2.9
AN2712 (pAN437)	Lys-203 → Ile	+	226	0.1	0.7	0.7
AN2709 (pAN436) [b]	+	+	228	0.1	0.8	0.8
AN2792 (pAN467)	Glu-219 → Gln	−	148	< 0.1	0.5	0.9
AN2402 (pAN51) [c]	+	+	234	0.1	1.3	1.2
AN2736 (pAN174) [d]	−	−	144	0.2	0.2	0.2

[a] Coupled control strain for strain AN2768.
[b] Coupled control strain for strain AN2712.
[c] Coupled control strain for strain AN2792.
[d] Uncoupled control strain.

did not affect the levels of ATPase activities in preparations assayed from the two mutant strains AN2768 (Glu-196 → Ala) and AN2712(Lys203 → Ile) (Table III). The sensitivities to the inhibitor DCCD of the ATPase activities in the membrane preparations from all strains were measured (Fig. 1). Membrane-bound ATPase activities from mutant strains AN2768 (Glu-196 → Ala) and AN2712 (Lys-203 → Ile) were inhibited by about 60%, similar to the coupled control strains. The activity in membranes from the mutant strain AN2792 (Glu-219 → Gln) was relatively insensitive to the inhibitor, exhibiting a maximal inhibition of about 25:

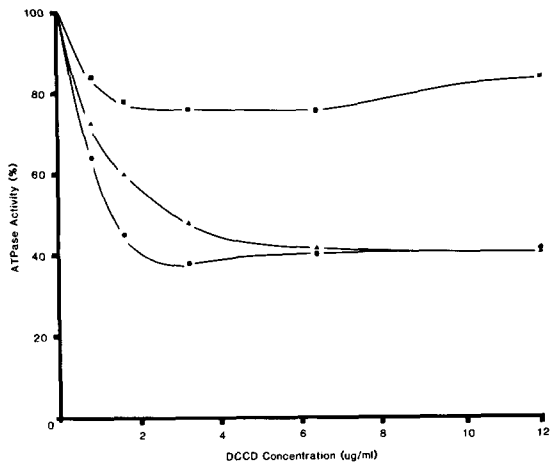

Fig. 1. Inhibition of ATPase activity by DCCD. Membranes (0.3 mg of protein) were incubated at 30 °C in 5 ml of the ATPase assay mixture together with the indicated amount of DCCD. The mixture was sampled at intervals, the rate was determined for each DCCD concentration and the percent inhibition was calculated. Membranes from control strain AN2402 (●), membranes from strain AN2768 (Glu-196 → Ala) (▲), membranes from strain AN2792 (Glu-219 → Gln) (■). Membranes from the control strains AN2709 and AN2774 and from the mutant strain AN2712 (Lys-203 → Ile) gave results similar to those acquired from the inhibitions shown for membranes from strain AN2402.

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. 2). The fluorescence-quenching activities of membranes from the mutant strain AN2712 (Lys-203 → Ile) and the coupled control strain AN2709 were identical and similar to the activities shown for strain AN2402 (Fig. 2c and d), with both NADH- and ATP-dependent fluorescence-quenching responses being lost after removal of the F_1 moiety. ATP-dependent fluorescence-quenching activity in membranes from the mutant strain AN2768(Glu-196 → Ala) (Fig. 2e and f) was similar to the activity in the membranes from the coupled control strains AN2774 and AN2402 (Fig. 2c and d). NADH-dependent quenching activities were identical in in-

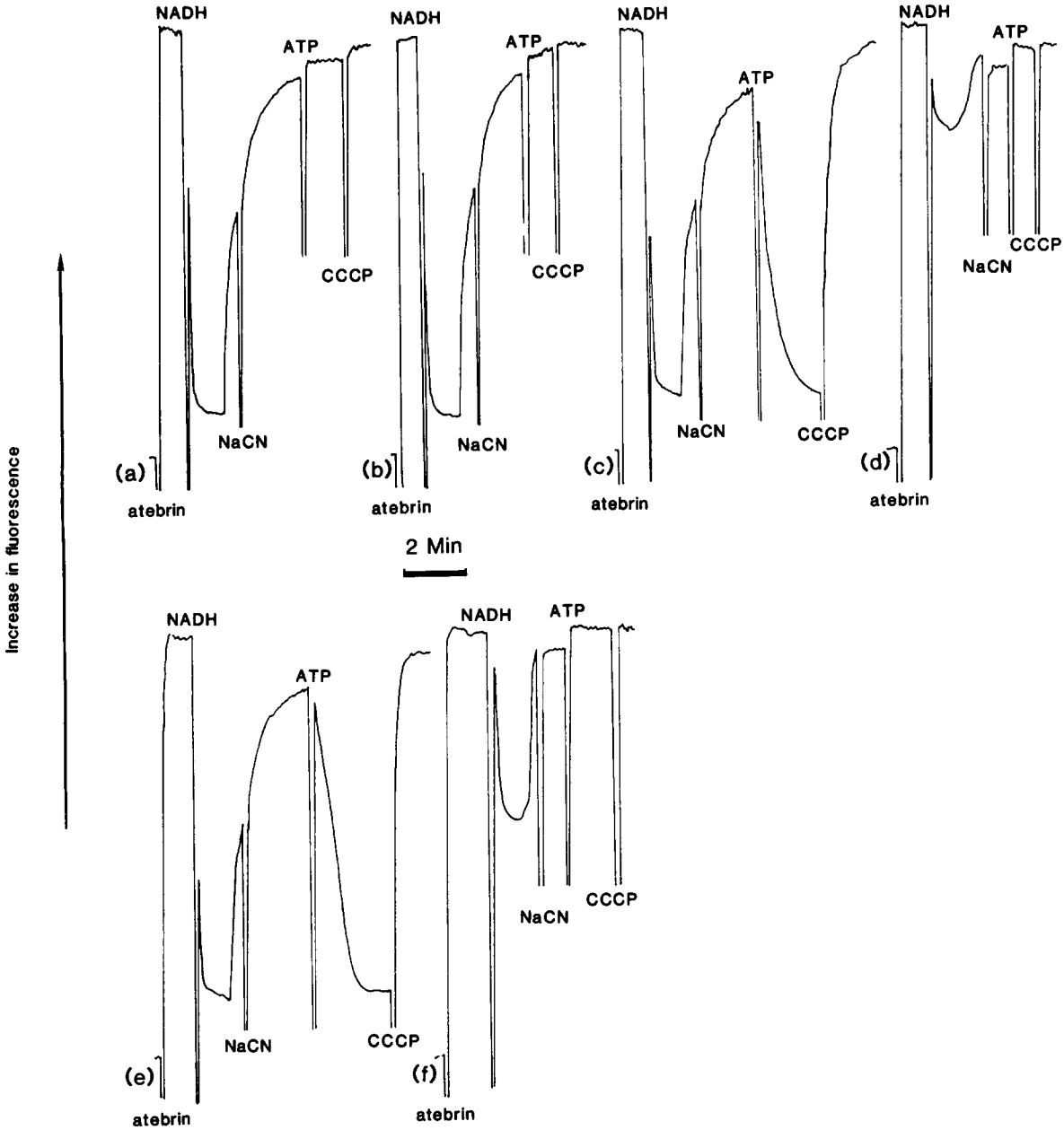

Fig. 2. Atebrin fluorescence-quenching in membranes prepared from strains of *E. coli*. Atebrin was added to give a final concentration of 4 M, NADH to give 2 mM, NaCN to give 2.5 mM, ATP to give 1 mM and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to give 2 μM. (a) Membranes from strain AN2792 (Glu-219 → Gln), (b) stripped membranes from strain AN2792, (c) membranes from strain AN2712 (Lys-203 → Ile), (d) stripped membranes from strain AN2712, (e) membranes from strain AN2768 (Glu-196 → Ala) and (f) stripped membranes from strain AN2712. The results obtained for membranes and stripped membranes from all coupled control strains were similar to the results for the stripped and unstripped membranes of strain AN2712. Results obtained for membranes from the uncoupled control strain AN2736 were identical to the results obtained for membranes from strain AN2792.

tact membranes from these strains. However, some NADH-dependent quenching activity appeared to be retained in membranes from the mutant strain AN2768 (Glu-196 → Ala) after removal of the F_1 moiety. Fluorescence-quenching activities in membranes from the mutant strain AN2792 (Glu-219 → Gln) (Fig. 2a and b) were identical to those in membranes from the uncoupled control strain AN2736. Thus, the membranes lacked ATP-dependent atebrin fluorescence-quenching activity, whilst the NADH-dependent activity was high and this high activity was retained after removal of the F_1-ATPase.

Discussion

Strain AN2712 (Lys-203 → Ile) had growth characteristics and membrane enzymic activities similar to those of the normal control strain AN2709 indicating that the residue Lys-203 of the *a*-subunit is not essential for coupled function of the F_0F_1-ATPase. Similarly, strain AN2768 (Glu-196 → Ala) grew on succinate minimal medium and had a normal growth yield. Membranes from strain AN2768 (Glu-196 → Gln) had a level of ATPase activity equivalent to the appropriate control strain and this activity showed normal sensitivity to the inhibitor DCCD. The ATP-dependent atebrin fluorescence-quenching activity of these membranes was also normal and NADH-dependent atebrin fluorescence-quenching activity was reduced on removal of the F_1-ATPase indicating normal F_0 function.

Given the difficulty of burying charged residues in membranes [33], the assumption is made that such residues will not exist in transmembrane helical segments unless functionally important [15]. It is, therefore, necessary to modify the previously proposed model [7] for the *a*-subunit structure with regard to helix IV so that Glu-196 and Lys-203 are not located in the membrane (Fig. 3). This modification results in another charged residue, Glu-219, of helix IV being positioned in the membrane.

Substitution of Glu-219 by Gln (strain AN2792) resulted in a low growth yield on limiting concentrations of glucose and the inability of the mutant strain to grow on succinate minimal medium. These properties are consistent with a

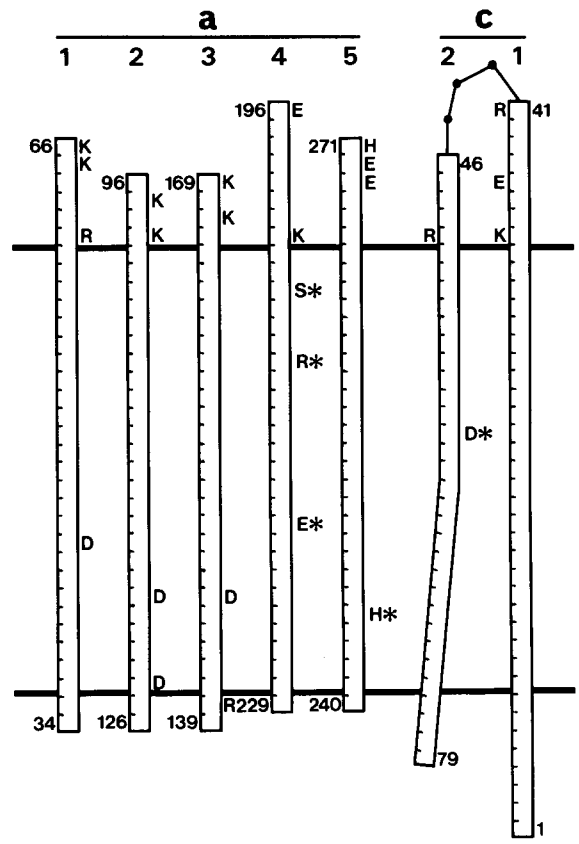

Fig. 3. Proposed alignment of transmembrane helices from subunits *a* and *c* of the *E. coli* F_0 moiety from the F_0F_1-ATPase. The N-terminal and C-terminal residues for each helix are numbered, and all ionisable residues within those helices are indicated. The residues involved in the proton pore are denoted *. Single letter abbreviations for residues are; D, aspartic acid; E, glutamic acid; H, histidine; K, lysine; R, arginine and S, serine.

mutant strain in which oxidative phosphorylation is uncoupled from electron transport. The membranes lacked ATP-dependent atebrin fluorescence-quenching activity and remained proton impermeable after removal of the F_1-ATPase. The ATPase activity of the F_1-ATPase was inhibited by almost 50% when bound to the mutant membranes. The membrane-bound ATPase activity was only marginally sensitive to the inhibitor DCCD. These properties closely resemble those of strains carrying the *a*-subunit mutations Arg-210 → Gln [15] or His-245 → Leu [15] and both Arg-210 and His-245 have been proposed to be components of the F_0 proton pore [7,14]. It is, therefore, con-

cluded that Glu-219 is also a component of the F_0 proton pore (Fig. 3).

It is now possible to define a proton pore through the F_0, involving a series of discrete amino-acid residues (His-245, Glu-219, Asp-61, Arg-210, Ser-206), in the *a*- and *c*-subunits, separated by approximately one turn of the α-helix (Fig. 3). Some doubt has been expressed concerning the direct involvement of Ser-206 in the proton pore [14] and further work is required to establish the involvement of this residue. Three residues, His-322, Glu-325 and Arg-302, have been implicated in another proton pore, that of the membrane protein, lac permease [34]. If the position in the membrane of helix X of the lac permease model is adjusted so that Lys-319, which has been shown not to be involved in the proton pore, is not in the membrane, then the sequence of residues from the periplasmic side of the membrane (His → Glu → Arg) is very similar to that proposed above for the F_0.

It is of particular interest that in the F_0 the proton pore is shared between two subunits of different stoichiometries. The *a*-subunit is present in the F_0F_1-ATPase as one copy, whereas the *c*-subunit is present in from six to ten copies [35]. It is likely that all *c*-subunits are functionally important [36,37], which would require the amino acids involved in the proton pore of the *a*-subunit to interact with the Asp-61 of each *c*-subunit. This is a fundamental feature of a rotational catalysis model proposed for ATP synthesis by the F_0F_1-ATPase [7].

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