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The proton pore in the *Escherichia coli* F_0F_1-ATPase: a requirement for arginine at position 210 of the *a*-subunit

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Site-directed mutagenesis was used to generate three mutations in the *uncB* gene encoding the *a*-subunit of the F_0 portion of the F_0F_1-ATPase of *Escherichia coli*. These mutations directed the substitution of Arg-210 by Gln, or of His-245 by Leu, or of both Lys-167 and Lys-169 by Gln. The mutations were incorporated into plasmids carrying all the structural genes encoding the F_0F_1-ATPase complex and these plasmids were used to transform strain AN727 (*uncB402*). Strains carrying either the Arg-210 or His-245 substitutions were unable to grow on succinate as sole carbon source and had uncoupled growth yields. The substitution of Lys-167 and Lys-169 by Gln resulted in a strain with growth characteristics indistinguishable from a normal strain. The properties of the membranes from the Arg-210 or His-245 mutants were essentially identical, both being proton impermeable and both having ATPase activities resistant to the inhibitor DCCD. Furthermore, in both mutants, the F_1-ATPase activities were inhibited by about 50% when bound to the membranes. The membrane activities of the mutant with the double lysine change were the same as for a normal strain. The results are discussed 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 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] and 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 are Asp-61 by either Gly or Asn [9,10], Pro-64 by Leu [11], Leu-31 by Phe [12], Ala-21 by Val [13] and Ala-25 by Thr [14]. In the *a*-subunit the amino-acid substitutions affecting proton translocation are His-245 by Tyr and Ser-206 by Leu [15].

It has previously been suggested that an amphi-

Abbreviation: DCCD, dicyclohexylcarbodiimide.

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pathic helix exists in the *a*-subunit and that Arg-210 in this helix plays an important role by interacting with the Asp-61 of subunit *c* [7]. In the present paper, evidence regarding the possible participation of Arg-210 in a proton channel has been sought by site-directed mutagenesis. In addition, His-245 has been changed to Leu to investigate whether the reported effects of the change of His-245 to Tyr [15] were due to the Tyr residue per se, or the lack of His. Finally, further information has been sought about the structure of subunit *a* by changing both Lys-167 and Lys-169 to Gln. The previously proposed model of the F_0 structure [7] has been rationalized with respect to the properties of these mutant strains.

Materials and Methods

Enzymes and chemicals. All chemicals and enzymes used were of the highest quality available. The synthetic oligonucleotides were a generous gift from A.E. Senior. Restriction endonucleases were obtained from Bethesda Research Laboratories (U.S.A.). T4-Polynucleotide kinase and T4-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, Australia.

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 alkaline lysis method of Silhavy et al. [23] or the method described by Selker et al. [24], except that, for the isolation of the replicative

TABLE I

STRAINS OF *ESCHERICHIA COLI* AND PLASMIDS USED

Chromosome nomenclature is that used by Bachmann [21]; plasmid nomenclature is that used by Novick [22].

Bacterial strain or plasmid	Relevant genotype	Notes and References
AN727	*uncB402/argH pyrE entA recA*	16
AN2709	pAN436/*uncB402 pyrE entA recA*	AN727 transformed to Cm^r by pAN436
AN2734	pAN433/*uncB402 pyrE entA recA*	AN727 transformed to Cm^r by pAN433
AN2735	pAN378/*uncB402 pyrE entA recA*	AN727 transformed to Cm^r by pAN378
AN2736	pAN174/*uncB402 pyrE entA recA*	AN727 transformed to Cm^r by pAN174
AN2757	pAN451/*uncB402 pyrE entA recA*	AN727 transformed to Cm^r by pAN451
AN2770	pAN385/*uncB402 pyrE entA recA*	AN727 transformed to Cm^r by pAN385
AN2774	pAN45/*uncB402 pyrE entA recA*	AN727 transformed to Cm^r by pAN45
AN1273	*uncG428 argH pyrE entA recA*	17
AN2015	*uncH241 argH pyrE entA recA*	18
K37	Hfr *SupD*	
JM101	*Δlac-pro thl supE traD36 proAB, LacI*^{Q}ZΔM15	19
pAN378	Cm^r Tc^s *uncB540* $E^+F^+H^+A^+G^+D^+C^+$	vector pAN174
pAN174	Cm^r Tc^s	derived from pAN51: Ref. 17 and this paper
pACYC184	Cm^r Tc^s	20
pAN436	Cm^{r}Tc^s *uncB*$^+$ $E^+F^+H^+A^+G^+D^+C^+$	vector pAN174, otherwise identical to pAN45
pAN385	Cm^r Tc^s *uncB546* $E^+F^+H^+A^+G^+D^+C^+$	
pAN451	Cm^r Tc^s *uncB577* $E^+F^+H^+A^+G^+D^+C^+$	vector pAN174
pAN45	Cm^r Tc^s *uncB*$^+$ $E^+F^+H^+A^+G^+D^+C^+$	17
pAN36	Cm^r Tc^s *uncD*$^+$ C^+	17
pAN51	Cm^{r}Tc^s *uncB*$^+$ $E^+F^+H^+A^+$	17
pAN461	*uncB*$^+$ $E^+F^+H^+A^+$	phage M13 RF carrying a *Hind*III endonuclease generated 4.3 kb fragment-coding for the ATPase subunits *a*, *c*, *b*, α and δ.

form of phage M13, cells were grown to a Klett value of 160 in glucose minimal medium and then infected with phage M13 mp9 added to give initially about 10^{10} plaque-forming units/ml. The isolation of single stranded M13 phage DNA was performed essentially by the method of Schreier and Cortese [25].

DNA sequencing. Nucleotide sequences were determined by the dideoxy chain-terminating method of Sanger et al. [26] using [α-^{32}P]dATP.

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 several 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 M13 mp9.

Media and growth of organisms. The mineral-salts minimal medium used and additions were as described previously [27]. Cells for the preparation of membranes were grown in 14-1 fermenters essentially as described previously [28]. The mineral-salts media in the fermenters were supplemented with 5% (v/v) Luria broth [29].

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.

Preparation of subcellular fractions. The preparation and treatment of subcellular fractions were as previously described [30].

Site-directed mutagenesis. The techniques used for site-directed mutagenesis were that of Zoller and Smith [31]. 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 T4 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 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 [27]. Dicyclohexylcarbodiimide sensitivity of ATPase activity was measured as described by Cox et al. [30]. Protein concentrations were determined using Folins phenol reagent [32] with bovine serum albumin as standard.

Results

Production of a-subunit mutants carrying the amino acid substitutions Arg-210 → Gln, His-245 → Leu and the double mutant Lys-167 → Gln, Lys-169 → Gln

The *uncB, E, F, H* and *A* genes are carried on a 4.3 kb fragment in plasmid pAN51 [17]. Plasmid pAN51 was mixed with the M13 mp9 replicative form, treated with the restriction endonuclease *Hin*dIII and ligated with T4 DNA ligase. The ligation mixture was then used to transform strain JM101. Phage carrying DNA inserted into the *Hin*dIII site were indicated by the appearance of colourless plaques [19]. The DNA of the replicative form was purified from several clear plaques and analyzed by restriction endonuclease digestion using either *Hin*dIII or *Cla*I endonuclease. One phage DNA preparation which showed patterns consistent with insertion of the relevant 4.3 kb fragment was designated pAN461.

The three oligonucleotides carrying the appropriate base substitutions (Table II) were used to generate the corresponding mutant derivatives of plasmid pAN461. The three mutant derivatives of pAN461 were each mixed with the vector pACYC184, treated with the restriction endonuclease *Hin*dIII and then T4 DNA ligase and the ligated mixture used to transform strain AN2015 (*uncH243*). Transformants were selected on succinate minimal medium containing chloramphenicol. However, transformants were obtained only from the mutant plasmid carrying the double Lys-167, Lys-169 → Gln change. The subcloning was therefore repeated for the Arg-210 → Gln and His-245 → Leu mutant plasmids but using the vector pAN174 instead of pACYC184. The

TABLE II

SYNTHETIC OLIGONUCLEOTIDES 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
5′-CAGCATCCAAATGCAAGGCAT-3′	Lys-167 → Gln, Lys-169 → Gln
5′-CTCGGTTTGCAACTGTTCGG-3′	Arg-210 → Gln
5′-CCATTTTCCTCATCCTGAT-3′	His-245 → Leu
5′-GGGTCTGTTGTTCCTGGTTT-3′ [a]	–
5′-ATGGCACTGGGCGTATTTA-3′ [b]	–

[a] Sequencing primer used for sequencing from nucleotide 300 in the *uncB* gene.
[b] Sequencing primer used for sequencing from nucleotide 475 in the *uncB* gene.

the vector pAN174 instead of pACYC184. The vector pAN174 carries a small deletion which reduces the expression of genes cloned into the *Hin*dIII restriction site (see Table III). Transformant colonies were obtained for both of the mutant types on the selective medium. Colonies from the three transformation experiments were purified, plasmids were isolated and then screened for equivalence in size and restriction pattern to plasmid pAN51. A plasmid from each of the transformations was selected and partially digested with the restriction endonuclease *Hin*dIII. The partial digest was mixed with *Hin*dIII digested pAN36, ligated with T4 DNA ligase, and the ligation mixture used to transform strain AN1273 (*uncG428*). Transformants were selected on succinate minimal medium containing chloramphenicol. This medium selects for the presence of plasmids carrying all the genes coding for the F_0F_1-ATPase. Plasmids were purified from colonies of transformants and screened for equivalence in size to plasmid pAN45. A plasmid was selected from each of the three transformations (corresponding to each of the three site-directed mutations) and used to transform strain AN727 (*uncB402*). Transformants were selected on rich medium containing chloramphenicol and one transformant from each of the three transformations purified and retained for further work. These strains were AN2735 (*uncB540*, carried on plasmid pAN378), AN2757 (*uncB577*, carried on plasmid pAN451) and AN2770 (*uncB546*, carried

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
AN2770 (pAN385)	Lys-167 → Gln Lys-169 → Gln	+	225	< 0.1	1.6	1.5
AN2774 (pAN45) [a]		+	230	< 0.1	1.5	1.4
AN2735 (pAN378)	Arg-210 → Gln	–	141	0.1	0.3	0.6
AN2757 (pAN451)	His-245 → Leu	–	143	0.2	0.4	0.6
AN2736 (pAN174) [b]		–	144	0.2	0.2	0.2
AN2709 (pAN436) [c]		+	228	0.1	0.7	0.7

[a] Coupled control strain for strain AN2770.
[b] Uncoupled control strain.
[c] Coupled control strain for strains AN2735 and AN2757.

on plasmid pAN385). For clarity, these strains will be further referred to as AN2735 (Arg-210 → Gln), AN2757 (His-245 → Gln) and AN2770 (Lys 167 → Gln, Lys-169 → Gln).

Growth properties of strains AN2735 (Arg-210 → Gln) AN2757 (his-245 → Leu) and AN2770 (Lys-167 → Gln, Lys-169 → Gln)

Strains AN2735, AN2757 and AN2770 were examined for their ability to grow on succinate minimal medium and for their growth yields on limiting levels of glucose (Table III). Strains AN2735 (Arg-210 → Gln) and AN2757 (His-245 → Gln) were unable to grow on solid media with succinate as the carbon source, whereas strain AN2770 (lys-167 → Gln, Lys-169 → Gln) showed apparently normal growth. Growth yields for both strains AN2735 and AN2757 were the same as the uncoupled strain AN2736. In contrast, the growth yield of strain AN2770 (Lys-167 → Gln, Lys-169 → Gln) was the same as the coupled control strain AN2774.

Isolation and characterization of revertants

Revertants were isolated from the two uncoupled strains AN2735 (Arg-210 → Gln) and AN2757 (His-245 → Gln) following culture on succinate minimal medium. The number of revertant colonies obtained ranged from about 200 to 400 colonies per 10^8 mutant cells. Plasmids were isolated from the revertants and used to transform strain AN727 (*uncB402*) selecting for growth on succinate minimal medium containing chloramphenicol. Subcloning into the phage M13 mp9 and DNA sequence analysis as outlined above showed single base reversions to the wild-type sequence. These full revertants showed identical properties to the appropriate coupled control strain AN2709 (not shown).

ATPase activities

Subcellular fractions were prepared from the mutant strains AN2735 (Arg-210 → Gln), AN2757 (His-245 → Leu) and AN2770 (Lys-167 → Gln, Lys-169 → Gln) along with the coupled control strains AN2774 (pAN45/*uncB402*) and AN2709 (ΔpAN45/*uncB402*) and the uncoupled control strain AN2736 (pAN174/*uncB402*). Strain AN2774 is the appropriate coupled control strain for strain AN2770, whereas strain AN2709 is the appropriate coupled control strain for strains AN2735 and AN2757 (see Table I). The ATPase activities of the cytoplasmic fractions from the three mutant strains were negligible. The ATPase activity of the membrane fraction from the double mutant strain AN2770 (Lys-167 → Gln, Lys-169 → Gln) was the same as that for the membranes from the coupled control strain AN2774 (pAN45, *uncB402*). However, the ATPase activities of the membrane fractions from the two mutant strains AN2735 (Arg-210 → Gln) and AN2757 (His-245 → Leu) were about 50% of the activity of the membranes from the coupled control strain AN2709. If the membrane fractions from the two mutant strains were dialysed against low ionic strength buffer in the absence of *p*-aminobenzamidine and re-assayed, the ATPase activities of the preparations increased to about the same level as in the control strain AN2709. The ATPase activity of the uncoupled control strain AN2736 was unchanged by this treatment. The dialysis treatment causes the F_1-ATPase to be released from the membranes [11]. This was confirmed for the mutant strains by testing for lack of ATPase activity in the membrane fraction after centrifugation of the dialysed preparations (not shown).

The sensitivities to the inhibitor DCCD of the ATPase activities of the membrane preparations from all strains were measured (Fig. 1). The ATPase activities of membranes from strains AN2735 (Arg-210 → Gln) and AN2757 (His-245 → Leu) were relatively insensitive to lower concentrations of DCCD but were stimulated by about 20% at higher concentrations. The ATPase activity of the coupled control strain (AN2709) was maximally inhibited by about 60% (Fig. 1). ATPase activities of the membranes from the double mutant strain AN2770 (Lys-167 → Gln, Lys-169 → Gln) and the coupled control strain AN2774 were equally sensitive to DCCD (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. 2). The membranes from the double mutant strain AN2770 (Lys-167 →

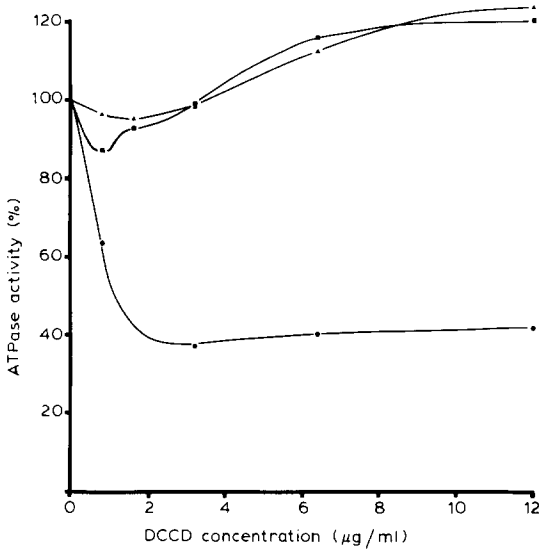

Fig. 1. Inhibition of ATPase activity by DCCD. Membranes (0.3 mg of protein) were incubated at 30 °C in 5 ml of the ATPase assay mixture together with the indicated amount of DCCD. The mixture was sampled at intervals, the rate was determined for each DCCD concentration, and the per cent inhibition was calculated. Symbols: ●, membranes from control strain AN2709; ■, membranes from strain AN2735 (Arg-210 → Gln); ▲, membranes from strain AN2757 (His-245 → Leu).

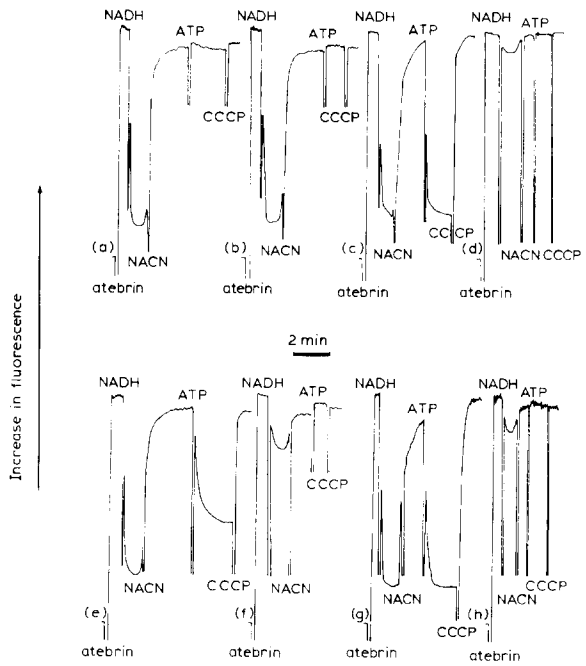

Fig. 2. Atebrin fluorescence quenching in membranes prepared from strains of *E. coli*. Atebrin fluorescence quenching was measured as described previously [16]. Atebrin was added to give a final concentration of 4 μM, NADH to 2 mM, NaCN to 2.5 mM, ATP to 1 mM and carbonylcyanide *m*-chlorophenylhydrazone (CCCP) to 2 μM. (a) Membranes from strain AN2735 (Arg-210 → Gln); (b) stripped membranes from strain AN2735; (c) membranes from strain AN2770 (Lys-167 → Gln, Lys-169 → Gln); (d) stripped membranes from strain AN2770; (e) membranes from the coupled control strain AN2709; (f) stripped membranes from the coupled control strain AN2709; (g) membranes from the coupled control strain AN2774; (h) stripped membranes from the coupled control strain AN2774. The results obtained with membranes and stripped membranes from strain AN2757 (His-245 → Leu) were the same as those for the membranes from strain AN2735. Strain AN2709 is the control strain for mutant strains AN2735 and AN2757 and strain AN2774 is the control strain for strain AN2770 (see subsection Atebrin fluorescence quenching).

Gln, Lys-169 → Gln) and the coupled control strain had similar NADH-dependent and ATP-dependent atebrin fluorescence-quenching activities which were lost after removal of the F_1-ATPase. In contrast, membranes from the two mutant strains AN2735 (Arg-210 → Gln) and AN2757 (His-245 → Leu) retained NADH-dependent atebrin fluorescence-quenching activity after removal of the F_1-ATPase and the unstripped membranes from both mutants lacked ATP-dependent atebrin fluorescence-quenching activity (Fig. 2).

Discussion

The growth characteristics and membrane enzymic activities of strain AN2770 (Lys-167 → Gln, Lys-169 → Gln) indicate that the residues Lys-167 and Lys-169 of the *a*-subunit are not involved in the function of the F_0F_1-ATPase. A structure of the *a*-subunit has been proposed [7] in which there are five transmembrane helices. The residues Lys-167 and Lys-169 were in transmembrane helix 3 of that structure. Given the difficulty of burying charged residues in membranes [34], the assumption is made that such residues will not exist in transmembrane helical segments unless functionally important. It is therefore necessary to modify the previously proposed model for the *a*-subunit structure [7] with regard to helices 2 and 3 (Fig. 3).

The substitution of Arg-210 by Gln in the proposed helix 4 of the *a*-subunit results in a low

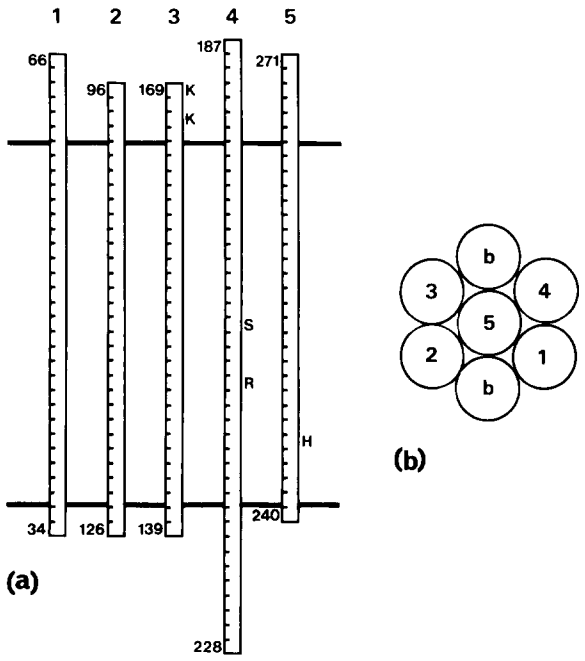

Fig. 3. (a) Proposed transmembrane helices of the *a*-subunit from *E. coli* F_0-ATPase. The N-terminal and C-terminal residue numbers for each helix are indicated, as are lysines -167 and -169 (K). The residues involved in the proton pore are shown; H, histidine; R, arginine; S, serine. (b) Proposed packing of the transmembrane helices of the *a*- and *b*-subunits of the F_0-ATPase of *E. coli*.

growth yield of the mutant strain on limiting concentrations of glucose and the inability of the mutant strain to grow on succinate minimal medium. Both properties are typical of a mutant strain in which oxidative phosphorylation is uncoupled from electron transport [35]. The membranes lack ATP-dependent atebrin fluorescence-quenching activity and are proton impermeable even when the F_1-ATPase is removed. The ATPase activity of the F_1-ATPase is inhibited by about 50% when bound to the membranes of the mutant and is insensitive to the inhibitor DCCD. Higher DCCD concentrations caused a stimulation of ATPase activity and this may have been due to release of some of the F_1-ATPase from the membranes as was reported for a *c*-subunit mutant [14] but this possibility was not tested. In the original model, Arg-210 was proposed to be the residue that interacted with Asp-61 of the *c*-subunit. The properties of strain AN2735 (Arg-210 → Gln) are consistent with this proposition.

The properties of strain AN2757 (His-245 → Leu) are similar to those of strain AN2735 (Arg-210 → Gln) and are consistent with the conclusion of Cain and Simoni [15] that the histidine residue at position 245 in the *a*-subunit is required for the translocation of protons through the F_0 and for oxidative phosphorylation. However, there are two characteristics of the membranes from strain AN2757 (His-245 → Leu) which apparently differ from those of the His-245 → Tyr mutant described by Cain and Simoni. These are the inhibition of ATPase activity when the F_1-ATPase is bound to the membrane and the insensitivity of the ATPase activity to DCCD; both properties are shared with membranes from strain AN2735 (Arg-210 → Gln). An analogous situation exists for the substitution of Asp-61 in the *c*-subunit by two different amino acids. Thus ATPase is not inhibited when membrane-bound, following the Asp-61 → Gly substitution, but is inhibited about 50% as a result of the Asp-61 → Asn substitution [36].

From the effects of various amino acid substitutions in the F_0-subunits, it would appear that the proton channel through the F_0 involves residues in helices 4 (Arg-210, Ser-206) (see above; see also Ref. 15) and 5 (His-245) [15] of the *a*-subunit as well as helix 2 (Asp-61) of the *c*-subunit [9,10]. In addition, amino acid substitutions at position 240 of the *a*-subunit (helix 5) have been shown to suppress the effects of a Gly-9 → Asp mutation in the *b*-subunit [37]. As there are two *b*-subunits per F_0 this interaction between the *b*-subunits and helix 5 suggests a more central positioning of helix 5 (Fig. 3) than previously proposed. Further experiments utilizing site-directed mutagenesis should allow continued refinement of the model.

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