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The mechanism of ATP synthase: a reassessment of the functions of the *b* and *a* subunits

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A model for the mechanism of ATP synthase was proposed previously (Cox, G.B., Jans, D.A., Fimmel, A.L., Gibson, F. and Hatch, L. (1984) Biochim. Biophys. Acta 768, 201–208) in which the *b* subunit of the F_0 of *Escherichia coli* rotated. The driving force was proposed to be an interaction between two charged residues in the membrane, namely, Lys-23 of the *b* subunit and Asp-61 of the *c* subunit. To test this proposal the Lys-23 of the *b* subunit was replaced by threonine using site-directed mutagenesis. The resulting mutant, although it had an impairment in the assembly of the F_1F_0-ATPase, was normal with respect to oxidative phosphorylation. The role of the *a* subunit, which had been previously proposed to be a structural one, was reassessed by examination of the possible secondary and tertiary structure of the analogous proteins from several sources. Not only did these subunits appear to have very similar structures, but in each there was a highly conserved helical arm on one of the transmembrane helices which could form a proton channel if it interacted with the Asp-61 of the *c* subunit. A revised model is therefore presented in which five transmembrane helices from the *a* subunit and two from the *b* subunit are surrounded by a ring of *c* subunits. The highly conserved nature of the structures of the *a*, *b* and *c* subunits from various organisms suggests that the model may have relevance for ATP synthases from bacterial plasma membranes, mitochondria and chloroplasts.

Introduction

The ATP-synthesizing complex (F_1F_0-ATPase) from mitochondria, chloroplasts and bacteria has a number of features in common (see Refs. 1 and 2). As a result of DNA and protein sequencing the amino acid sequences of F_1F_0-ATPase subunits from a variety of sources is now known and in the case of *Escherichia coli* the sequences of all of the subunits of the F_1F_0-ATPase have been determined (see Ref. 2).

The subunits of the proton-translocating adenosine triphosphatase complex of *Escherichia coli* are encoded by the genes of the *unc* operon, mapping at about 84 min on the *E. coli* chromosome [3]. As established by genetic complementation studies [4–6] and the determination of the complete DNA sequence (see Ref. 2) the *unc* operon comprises nine genes transcribed in the order *uncIBEFHAGDC,* with the *uncI* gene promoter proximal. The *uncI* gene appears not to encode a structural component of the F_1F_0-ATPase [7]. The *a*, *c* and *b* subunits of the membrane F_0 portion are coded for by the *uncB, uncE* and *uncF* genes, respectively [5], while the δ, α, γ, β and ε subunits of the soluble F_1-ATPase are encoded by the *uncH, uncA, uncG, uncD* and *uncC* genes, respectively [5,8].

The experimental results obtained with mutants of *Escherichia coli* affected in oxidative phos-

Abbreviation: DCCD, dicyclohexylcarbodiimide.

phorylation, together with proposed secondary and tertiary structures of the components of the F_0 allowed the proposal of a hypothesis [9] for the mechanism of ATP synthesis in *E. coli*. It was suggested that the minor subunits of the F_1-ATPase are attached to a dimer of the *b* subunit and that this complex rotated, driving the conformational changes associated with the three catalytic sites of the F_1F_0-ATPase [10]. Other models, stimulated by the example of the proton-driven flagellar motor or the energy-dependent binding change mechanism hypothesis [10] have been suggested. Such models involved rotation of the β subunit, or α plus β subunits relative to other F_1-ATPase subunits [10–12]. In the model being investigated here [9], the driving force for the rotation of the central core was proposed to be an interaction between the charged amino group in the membrane portion of the *b* subunit (Lys-23) and the carboxyl groups (Asp-61) of a surrounding ring of *c*-subunits. To test the latter part of the hypothesis, site-directed mutagenesis was carried out in which the Lys-23 of the *b* subunit was replaced by threonine. Consideration of the properties of the resulting mutant strain, in which assembly was affected but not function, led to a reassessment of the roles of the *a* and *b* subunits. This involved a critical examination of the probable secondary and tertiary structure of the *a*-subunit and a comparison of the equivalent F_0 subunits from a range of organisms.

Materials and Methods

Enzymes and chemicals

All chemicals and enzymes used were of the highest quality available. The synthetic oligonucleotide was synthesized in the Research School of Biological Sciences, Australian National University.

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 [4,6,14].

Preparation of plasmids. Plasmid DNA was prepared as described by Selker et al. [15].

DNA sequencing. Nucleotide sequences were determined by the method of Maxam and Gilbert [16] with end-labelling of DNA carried out using DNA polymerase and [α-^{32}P] deoxyadenosine triphosphate [16].

Media and growth of organisms. The mineral salts minimal medium used and additions were as described previously [6]. Cells for the preparation of membranes were grown in 14-l fermenters essentially as described previously [17]. The media in the fermenters were supplemented with 5% (v/v) Luria broth [18]. Turbidities of cultures were measured with a Klett-Summerson colorimeter.

Preparation of cell membranes. The preparation and treatment of membranes were as previously described [19].

TABLE I

STRAINS OF *E. COLI* AND PLASMIDS USED

Bacterial strain or plasmid	Relevant genotype	Notes and references
AN888	unc-416::MuB$^+$ EFHAGDC argH pyrE entA recA	43
AN1461	pAN45/unc-416::MuB$^+$ EFHAGDC argH pyrE entA recA	44
AN1363	uncG428 thr-1 leu-6 recA	4
AN1707	uncF476 thr-1 leu-6 recA	45
AN2355	pAN328/uncF476 thr-1 leu-6 recA Cm^r	AN1707 transformed to Cm^r by pAN328
AN2359	pAN330/unc-416::MuB$^+$ EFHAGDC argH pyrE entA recA Cm^r	AN888 transformed to Cm^r by pAN330
pAN51	Cm^{r}Tc^{s}uncB$^+$ E$^+$ F$^+$ H$^+$A$^+$	4
pAN36	Cm^{r}Tc^{s}uncD$^+$ C$^+$	4
pAN45	Cm^{r}Tc^{s}uncB$^+$ E$^+$ F$^+$ H$^+$A$^+$ G$^+$ D$^+$ C$^+$	4
pAN328	Cm^{r}Tc^{s}uncB$^+$ E$^+$ F535H$^+$A$^+$	described in this article
pAN330	Cm^{r}Tc^{s}uncB$^+$ E$^+$ F535H$^+$A$^+$ G$^+$ D$^+$ C$^+$	described in this article

Two-dimensional gel electrophoresis. Membranes washed in 5 mM-Tes buffer containing *p*-aminobenzamidine were examined by 2-dimensional gel electrophoresis as described previously [20]. This technique is essentially that of O'Farrell [21], involving isoelectric focussing in the first dimension and sodium dodecyl sulphate polyacrylamide-gradient-gel electrophoresis in the second dimension.

Site-directed mutagenesis. Site-directed mutagenesis was carried out as described by Wallace et al. [22] as modified by Inouye et al. [23]. The oligonucleotide was synthesised using automated solid-phase phosphite-triester synthesis. Labelling of the oligonucleotide was carried out using T4 polynucleotide kinase and [γ-^{32}P]adenosine triphosphate [16]. Colony hybridisation to detect mutant colonies was carried out at 52°C for 16 h.

Other methods. ATPase and atebrin fluorescence-quenching activities were assayed as described previously [24]. Oxidative phosphorylation and dicyclohexylcarbodiimide (DCCD) sensitivity of ATPase activity were measured as described by Cox et al. [19]. Protein concentrations were determined using Folin's phenol reagent [25].

Results and discussion

Replacement of Lys-23 by threonine in the b subunit by site-directed mutagenesis

Plasmid pAN51, which carries the *uncB, E, F, H* and *A* genes and part of the *uncG* gene, was treated with restriction endonuclease *Eco*R1 in the presence of ethidium bromide causing a site-specific nick at the beginning of the *uncA* gene. The nicked DNA was then treated with exonuclease-III, annealed with the oligonucleotide 5′-TTCTGCATGACGTACGTATG-3′, and the plasmid repaired using DNA polymerase and T4 DNA-ligase. The sequence of the oligonucleotide is identical to the sequence from nucleotides 58 to 77 of the *uncF* gene with the exception of nucleotide 68 where A is replaced by C. The plasmid preparation was then used to transform AN1707 (*uncF476*) to chloramphenicol resistance. Transformant colonies were screened by hybridization with the above ^{32}P-labelled oligonucleotide under conditions in which the probe only hybridized with those colonies containing plasmids carrying the site-directed mutation. Plasmid DNA was prepared from such strains and, in order to purify the mutant plasmid, the preparation was used to transform strain AN1707(*uncF476*) to chloramphenicol resistance. The transformant colonies were probed as above, a positive colony was selected, purified (strain AN2355), and plasmid DNA(pAN328) prepared. The *uncF* gene carried by plasmid pAN328 was sequenced using the strategy described previously [26]. The only change in the *uncF* gene was the expected A → C at position 68 (Fig. 1) which would cause Lys-23 of the *b*-subunit to be replaced by threonine. Plasmid pAN328, carrying the *uncF535* allele was mixed with pAN36 (carrying the *uncD* and *uncC* genes and part of the *uncG* gene), partially digested with the restriction endonuclease *Hin*dIII, religated with T4 DNA ligase and the mixture used to transform

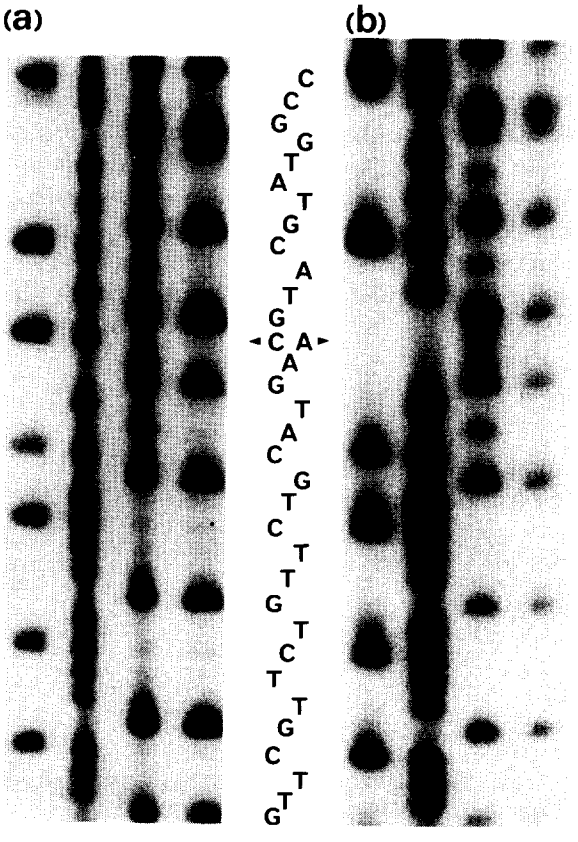

Fig. 1. Portion of DNA-sequencing gels from (a) plasmid AN328 carrying the *uncF535* allele and (b) plasmid pAN51 carrying a normal *uncF* allele. The site-directed A → C change is indicated.

strain AN1363 (*uncG428*) to chloramphenicol resistance on succinate-minimal medium. Transformant colonies were screened for the presence of plasmids equivalent in size to plasmid pAN45 which carries the structural genes for all of the subunits of the F_1F_0-ATPase. One such transformant was selected, the plasmid (pAN330) prepared and used to transform strain AN888 to chloramphenicol resistance. A transformant colony was selected (AN2359) and retained for further work. Strain AN888, which is a Mu-insertion polar mutant affected in all genes promoter distal to the *uncB* gene, was used as the recipient strain because the effects of the *uncF535* allele could then be studied without interference from the chromosomal *uncF* gene product.

Properties of strain AN2359(uncF535)

The growth yields of strain AN2359(*uncF535*) on limiting concentrations of glucose were tested and found to be the same as for the control strain AN1461 [27]. In addition, strain AN2359 was able to grow on succinate as sole source of carbon. These results suggest that replacement of Lys-23 by threonine in the *b* subunit does not cause loss of oxidative phosphorylation. This conclusion was confirmed by the study of membrane preparations. Thus, membranes were prepared from strain AN2359(*uncF535*) grown on either glucose or succinate as carbon source and ATPase, atebrin fluorescence quenching and oxidative phosphorylation activities determined (Table II). The DCCD sensitivity of the ATPase activity was also determined. All activities were found to be similar to those of the control strain AN1461.

The membrane preparations were examined by two-dimensional gel electrophoresis and the amount of β subunit on the membrane appeared to be considerably in excess of the amount of α subunit (Fig. 2a). The F_1-ATPase was removed from the membranes by dialysis against low ionic-strength buffer in the absence of *p*-aminobenzamidine [19], the stripped membranes isolated by centrifugation and then examined by two-dimensional gel electrophoresis. The minor subunits γ, δ, and ε were no longer present on the stripped membranes, but α and β subunits remained (Fig. 2b). Visual inspection suggested a ratio of about $2\beta:1\alpha$ corresponding to one of the previously proposed intermediates in the assembly of the F_1F_0-ATPase [29]. The altered *b* subunit is also present and it would appear that the 2β, 1α assembly intermediate is accumulating in the membrane during cell growth.

A reassessment of the role of the a subunit

In view of the conclusion (see above) that the replacement of Lys-23 by threonine in the *b* sub-

TABLE II

MEMBRANE ACTIVITIES OF *unc*$^+$ AND MUTANT STRAINS

The loss of NADH-dependent atebrin fluorescence quenching in stripped membranes indicates that membranes possess proton translocating activity (see Ref. 28).

	AN1461(*unc*$^+$)		AN2359(*uncF535*)	
	glucose-grown	succinate-grown	glucose-grown	succinate-grown
ATPase activity (μmol/min per mg protein)	1.8	3.6	1.4	2.6
DCCD inhibition of ATPase activity (%)	83		66	
NADH-dependent atebrin fluorescence:				
(% for unstripped membranes)	84	85	88	84
(% for stripped membranes)	6	4	7	8
ATP-dependent atebrin fluorescence:				
(% for unstripped membranes)	72	69	81	68
(% for stripped membranes)	0	0	0	0
P/O ratio	0.19	0.38	0.18	0.23

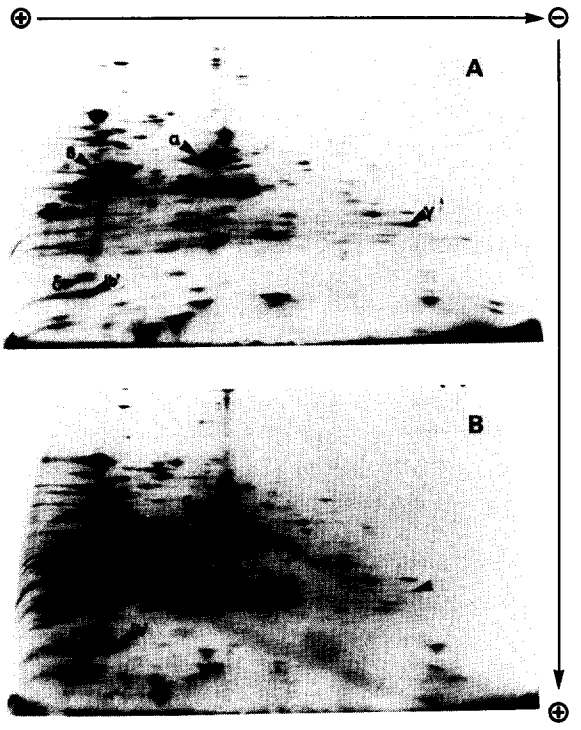

Fig. 2. Two-dimensional gel electrophoresis of membrane preparations. Membranes were prepared from strain *AN2359* (*uncF535*) grown with succinate as carbon source. Samples (1 ml containing about 20 mg of protein) of membranes washed in low-ionic strength buffer in the presence of *p*-aminobenzamidine were extracted twice with 5 ml of cold acetone. The dried residue was solubilized in about 0.8 ml of lysis buffer and 100 µl used for electrophoresis. In the first dimension, ampholines with a pH range from 5 to 7 and from 3.5 to 10 were present at 2.4% and 1.6% (w/v), respectively. In the second dimension, an acrylamide gradient of 10.5 to 24.5% (w/v) was used. The arrows labelled α, β, γ, δ and *b* identify the corresponding subunits of the F_1F_0-ATPase. (A) Membranes washed with low-ionic strength buffer in the presence of *p*-aminobenzamidine, under which conditions the F_1 is retained on the membrane [41]. (B) Membranes dialysed against low-ionic strength buffer in the absence of *p*-aminobenzamidine under conditions which 'strip' the F_1-ATPase from normal membranes [19]

unit does not affect oxidative phosphorylation, the Lys-23 residue cannot be involved in the interaction with the Asp-61 of the *c* subunit. This interaction was previously proposed [9] to be the driving force for the rotation of the *b* subunit complex within the ATPase. The function of the *a* subunit, which was previously proposed to play a largely structural role was therefore reassessed by critical examination of its probable secondary and tertiary structure. A comparison was also made with the

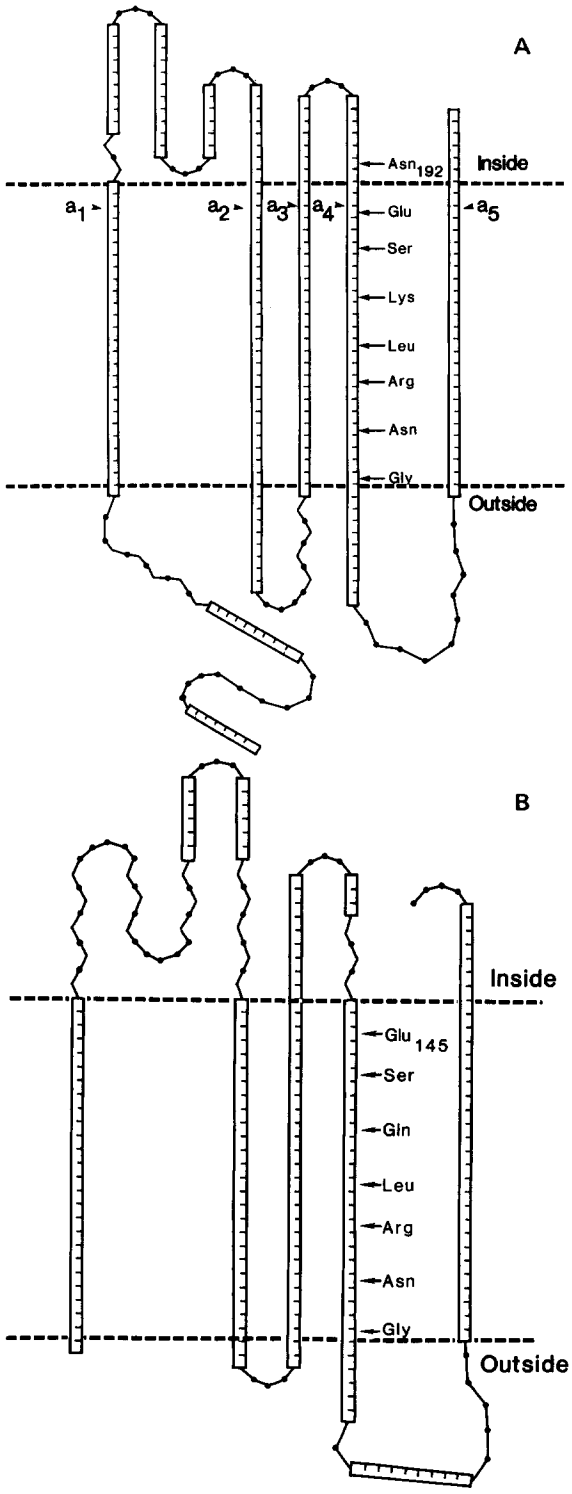

Fig. 3. Proposed secondary and tertiary structures of (A) the *a* subunit from *E. coli* and (B) the ATPase-6 subunit from human mitochondria. The amino acid sequences were derived from DNA sequences [2,42].

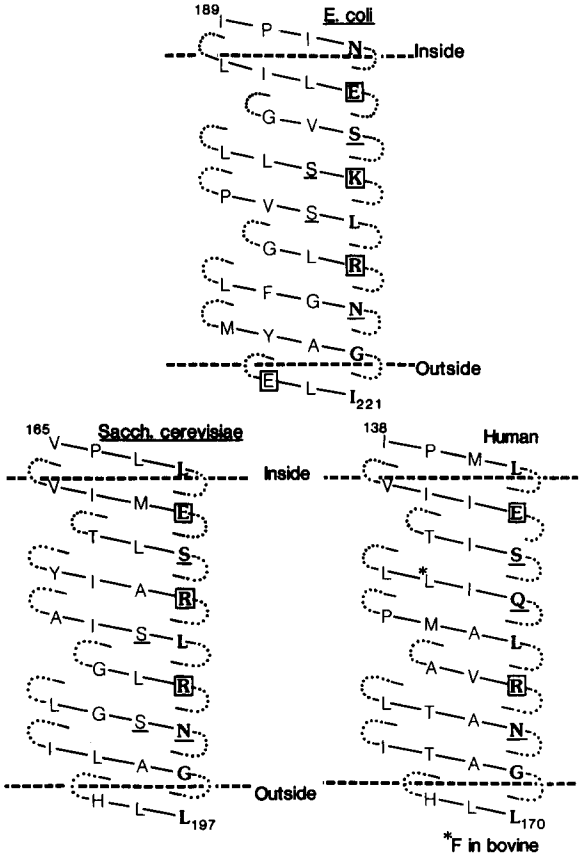

Fig. 4. Conserved polar residues on an amphipathic helical arm of helix 4 (see Fig. 3) of the F_0 subunit from various sources.

analogous subunits from mitochondria from various sources. The proposed structure of the *a* subunit (Fig. 3) was deduced from the amino acid sequence by consideration of the polarity profile, the application of Chou-Fasman [30] rules (with the modification that those portions of proteins located within membranes are α-helical) and with regard for the effect of the membrane potential on the charge distribution of the assembled protein (a preponderance of basic amino acids on the cytoplasmic side of the membrane). The derived structures differ from those previously proposed [2,31,32] which have six or seven transmembrane helices. Two principal features were evident. Firstly, the analogous subunits from various sources (*a* subunit from *E. coli* and subunits 6 from yeast, bovine and human ATPase) all appear to have five transmembrane helices and those from *E. coli* and human mitochondria are depicted in Fig. 3. The second feature is the conserved amphipathic helix 4. The amino acids involved in helix 4 for the *a* subunit from *E. coli* and the subunits 6 from three sources are depicted in Fig. 3. There is conservation of most of the residues on the polar helical arm (see Fig. 4) including the non-polar leucine predicted to be Located at about the middle of the membrane. It would appear likely, in view of the conservation of the *a* subunit structures and particularly helix 4 that the *a* subunit has a more specific role than the general structural role previously suggested [9].

Furthermore with the similarities in the structures of the *c* subunits of the ATPases from all sources [33] and the *b* subunits, at least from *E. coli* and chloroplast ATPases [34] it would appear that the structure of the F_0 sectors of these ATPases are likely to be the same. A previously proposed

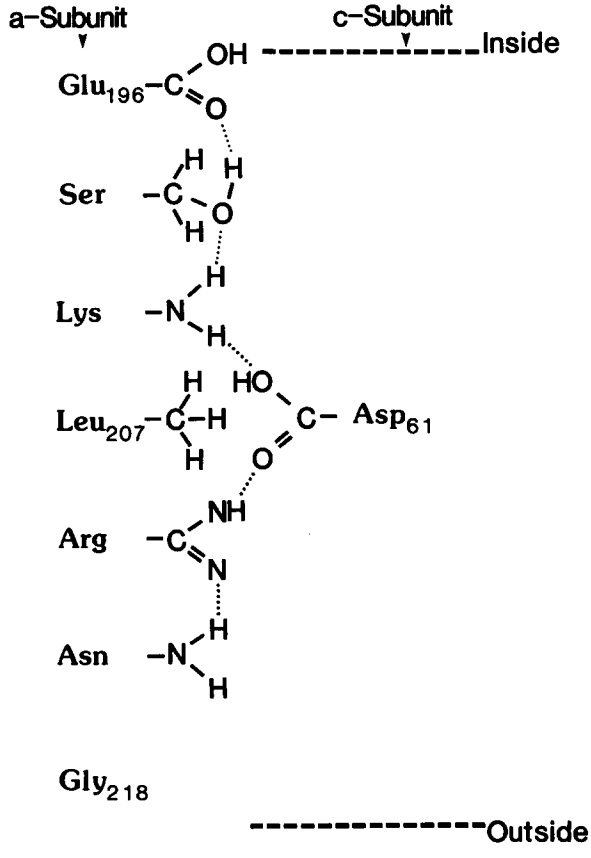

Fig. 5. Proposed formation of a proton pore following interaction of the polar groups on the *a* subunit of *E. coli* F_0-ATPase and the Asp-61 of the *c* subunit.

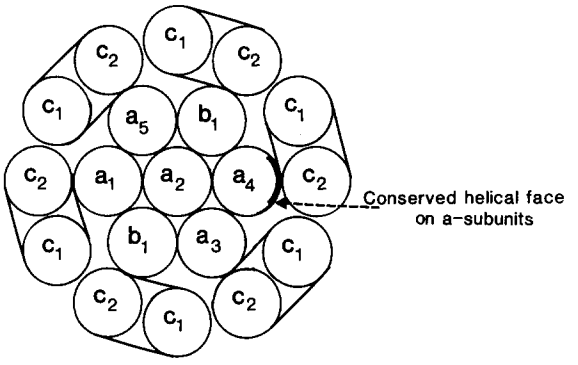

Fig. 6. Possible packing of the transmembrane helices of the *a* and *b* subunits of the F_0-ATPase of *E. coli* within a surrounding ring of *c* subunits, allowing interaction between the *a* and *c* subunits.

structure of the F_1F_0-ATPase in *E. coli* therefore requires modification and the modified structure of the F_0, which may be relevant to ATPases from mitochondria and chloroplasts as well, is depicted in Fig. 5. It is proposed that the conserved acidic residues of the ring of *c* subunits are located at about the middle of the membrane adjacent to the conserved leucine of helix 4 of the *a* subunit. Such an arrangement would constitute a proton pore through the membrane (Fig. 6). The number of *c* subunits shown (Fig. 5) is six, but estimates of the number of *c* subunits in the F_0 from various sources varies from 4 to 14 [35–40] and an increased number could be accommodated in the present model.

Space-filling models of the *c* subunit and helix 4 of the *a* subunit of the ATP synthase of *E. coli* have been constructed using CPK molecular models and show that the proton channel depicted in Fig. 6 is entirely feasible.

In the present modification of the original model, the rotation of the inner complex consisting of the *a*, *b*, γ, δ and ε subunits relative to the outer components consisting of the α, β and *c* subuntis allows successive interactions of helix 4 with the conserved acidic residue of each of the *c* subunits.

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