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Structure and arrangement of the δ subunit in the *E. coli* ATP synthase (ECF_1F_0)

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Abstract

F_1F_0 type ATPases are made up of two parts, an F_1, which contains three catalytic sites on β subunits, and an F_0 which contains the proton channel. These two domains have been visualized in electron microscopy as linked by a narrow stalk of around 45 Å in length. Biochemical studies have provided clear evidence that the γ and ϵ subunits are components of this stalk. There is an emerging consensus that the γ and ϵ subunits rotate relative to the $\alpha_3\beta_3$ domain as part of the cooperativity and energy coupling within the complex. Two other subunits are required to link the F_1 to F_0 in the *E. coli* enzyme, and these are the δ and b subunits. The structure of a major part of the δ subunit (residues 1–134) has now been obtained by NMR spectroscopy. The main feature is a six α-helix bundle, which provides the N-terminal domain of the δ subunit. This domain interacts with the F_1 core via the N-terminal part of the α subunit. The C-terminal domain of δ is less well defined. This part is required for binding to the F_0 part by direct interaction with the b subunits. It is argued that δ and the two copies of the b subunit are components of a second stalk linking the F_1 and F_0 parts, which acts as a stator to allow the energy-linked rotational movements of γ and ϵ subunits. © 1997 Elsevier Science B.V.

Keywords: *E coli*; ATPase; NMR spectroscopy; Subunits

1. Introduction

A H^+-translocating F_1F_0-ATPase (ATP synthase) directs the synthesis of ATP from ADP and inorganic phosphate (P_i) in bacteria, mitochondria, and chloroplasts during oxidative- or photo-phosphorylation. In the reverse direction, this enzyme also generates an ATP hydrolysis-driven proton gradient for use in ion transport processes. The F_1F_0 from *E. coli* (ECF_1F_0), which we study, is a multi-subunit complex of molecular weight 530000 Da. The F_1 part is composed of five subunits (α, β, γ, δ, and ϵ in the stoichiometry 3:3:1:1:1), while the F_0 part is composed of three different subunits (a, b, and c, in the ratio 1:2:10–12) [1–3].

Electron microscopy first showed that the α and β subunits are arranged hexagonally in F_1, and alternate around a central cavity in which the γ subunit is located [4,5]. The recently-published high resolution structure of a major part of the beef heart F_1 molecule (MF_1) [6] confirms the above-described arrangement of the α, β and γ subunits and adds important details. In particular, it shows the γ subunit arranged with a long C-terminal α-helix passing from the top of the α and β subunits through the cavity within the F_1 molecule and extending from the bottom of the structure. A closely aligned shorter N-terminal

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α-helix runs from the nucleotide binding site region out from the bottom of the F_1, and there is a third short α helix of γ which runs roughly at 45 Å to the two larger helices and along the bottom of the F_1. Approximately half of the γ subunit is unresolved in the structure, presumably because it is disordered in the crystal form.

Electron microscopy studies have shown that the F_1 and F_0 parts are linked by a relatively narrow stalk region, ca. 45 Å in length [7,8]. The N- and C-terminal α helices, as well as yet unresolved parts of the γ subunit, contribute to this stalk, and this subunit binds directly to the c subunit oligomer of the F_0 part via a region around Tyr205 [9]. The stalk also contains the ε subunit which is a two-domain protein [10,11]. The C-terminal helix–loop–helix domain of ε interacts with the α and β subunits [12,13]. The N-terminal 10-stranded β sandwich domain of this subunit interacts for much of its length with the γ subunit [14], with the bottom of the structure linked to the c subunit oligomer [15].

There are two other subunits involved in linking ECF_1 to the F_0 part, namely the δ and b subunits [16,17]. Most models place these subunits in the stalk along with the γ and ε subunits [e.g., [1,2]]. However, this seems unlikely based on the dimensions of the stalk (there does not appear to be enough room for γ, δ, ε and the two copies of the b subunit). The implication is that the δ and the b subunits form a separate connection between F_1 and F_0, which was not clearly visualized in electron microscopy studies. To assess this possibility, we are characterizing the as-yet poorly-defined δ and b subunits. Here, we describe results of a structure determination of the δ subunit using 2D and 3D heteronuclear NMR spectroscopy. The interaction of the δ with both the F_1 part and with the C terminal domain of the b subunits is also described, and a model of ECF_1F_0 presented.

1.1. Structure of the δ subunit

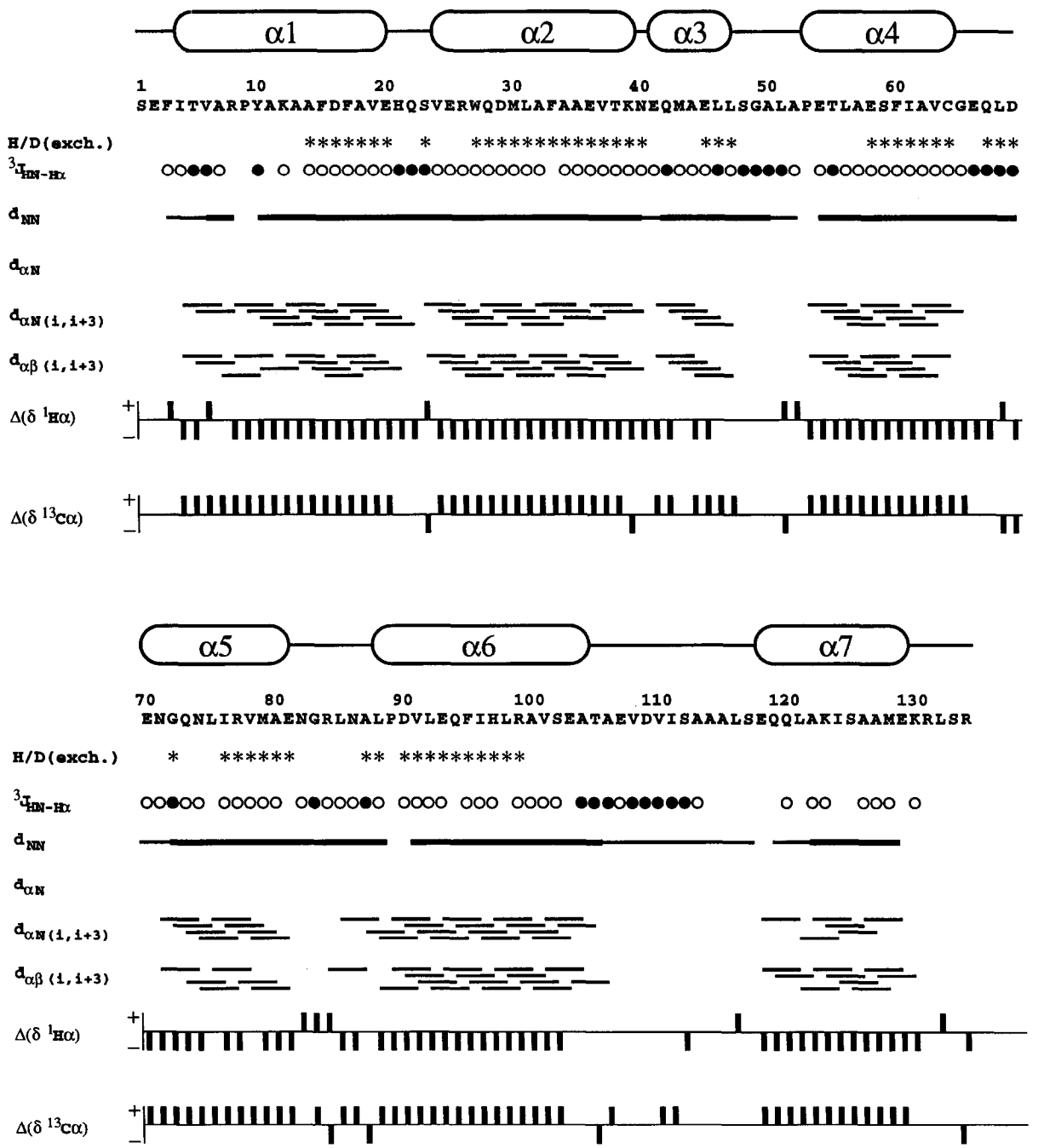
The structure determination described here used δ subunit purified from the bacterial strains MM294 or 594 harboring the overexpression vector pJCI, which contains the δ subunit (*unc*H) gene (J. Chandler and Dunn, unpublished studies). During the isolation procedure, it was found that variable amounts of a fragment of the δ subunit were produced by a bacterial protease. N-terminal sequencing and mass spectroscopy revealed that the fragment consisted of the N-terminal 134 amino acids of the δ subunit. This fragment is present in many preparations of ECF_1.

Fig. 1 summarizes the NMR spectral analysis of the δ fragment. The three-dimensional structure was calculated from a total of 1430 NOE based distance restraints, and 83 backbone dihedral angle restraints. In the initial phase of the structure determination, it became clear that the N-terminal 105 residues form a compact globular domain, while the region from 106–134 is mostly disordered except for one short α helix. The structure of these two regions was determined separately. A starting set of 30 structures was calculated for the N-terminal domain (1–105), which converged to give the same 3D fold. After subsequent rounds of refinement, these 30 structures were averaged, and 44 hydrogen bond donor-acceptor pairs were then included in the distance restraints to provide the final model of the N-terminal part [18], shown here in two different views (Fig. 2).

The N-terminal part of δ is arranged as a six α-helix bundle with helices 1 (residues 4–20), 2 (24–38), 5 (70–81) and 6 (88–104) organized as two intercalating V-shaped pairs that form the core. He-

Fig. 1. Summary of NMR spectral data used to identify the secondary structure of the δ^{1-134} fragment: Sequential and medium range NOE connectivities from the 3D ^{15}N–NOESY–HSMQC and 3D ^{13}C/^{15}N–NOESY–HSQC are drawn as horizontal lines with thick lines indicating strong, and thin lines indicating medium to weak NOE crosspeak intensities; amide protons, which were still observable 20 min. after dissolving the protein in D2 at 22°C and pH 7.2 (uncorrected) are marked with a star. These amides were considered as a hydrogen bond donor in structure calculations; vicinal $3J_{HN-H\alpha}$ coupling constants are indicated by filled circles ($J < 6$ Hz), shaded circles (6 Hz $< J <$ 7 Hz) or empty circles ($J < 6$ Hz). An absence of a circle indicates that data could not be obtained because of spectral overlap or exchange broadening of the corresponding amide proton; chemical shift deviations of the α-^{13}C carbon and α-proton are from the corresponding random coil values according to the standard chemical shift index algorithm. A chemical shift index of +1 or −1 is drawn if the chemical shift deviation of the Hα is larger than 0.1 ppm down- or up-field, respectively. The corresponding values for the ^{13}Cα are +0.5 and −0.8, respectively.

lices 3 (41–47) and 4 (53–64) are packed tightly against this four-helix core. The dimensions of this N-terminal part are $45 \times 20 \times 30$ Å. C-terminal of residue 105 there is a loop region followed by a seventh α-helix (residues 118–129). A comparison of spectral data for the intact δ subunit with that for



the N-terminal fragment shows the same spectral shift of almost all of the resonances attributed to residues 1–104. However, the spectral shift of most of the resolved amide resonances from residues 105–134 are very different in the two spectra, indicating that the structure of this region is affected by the presence of the C-terminal 42 residues which are missing in the δ fragment. Unfortunately, so far, it has not been possible to obtain a three-dimensional structure of this very C-terminal part because the intact subunit aggregates at the protein concentrations necessary for good spectral data collection. A secondary structure analysis based on the amide correlation spectra indicates some α-helical and β structure in this C-terminal part, in agreement with predictions using secondary structure algorithms.

Taken together, the NMR data indicate a two-domain protein, an N-terminal domain of around 100 residues and a C-terminal domain of around 70 residues. As there are some NOEs between α-helix 7 and the N-terminal domain, there is likely a close interaction between the N- and C-terminal domains in the full-length polypeptide. This interaction brings Cys64 close to Cys140, as these two residues form a disulfide bond under oxidizing conditions in isolated δ (Wilkens and Capaldi, unpublished results), as well as in ECF_1 and ECF_1F_0 [19].

1.2. Function studies support a two-domain structure of the δ subunit

The δ subunit of bacteria such as ECF_1 and the thermophile TF_1 shows significant sequence homology to the δ subunit in the plant enzyme (CF_1) [20]. In the mitochondrial ATP synthase, MF_1F_0, the equivalent polypeptide is called the oligomycin sensitivity conferring protein (OSCP) [20,21]. The truncated δ subunit (residues 1–134) retains F_1 binding properties, although the affinity of the shortened polypeptide is not as high as that of the intact δ subunit based on binding competition experiments. Deletion of as few as 4 residues from the C terminus

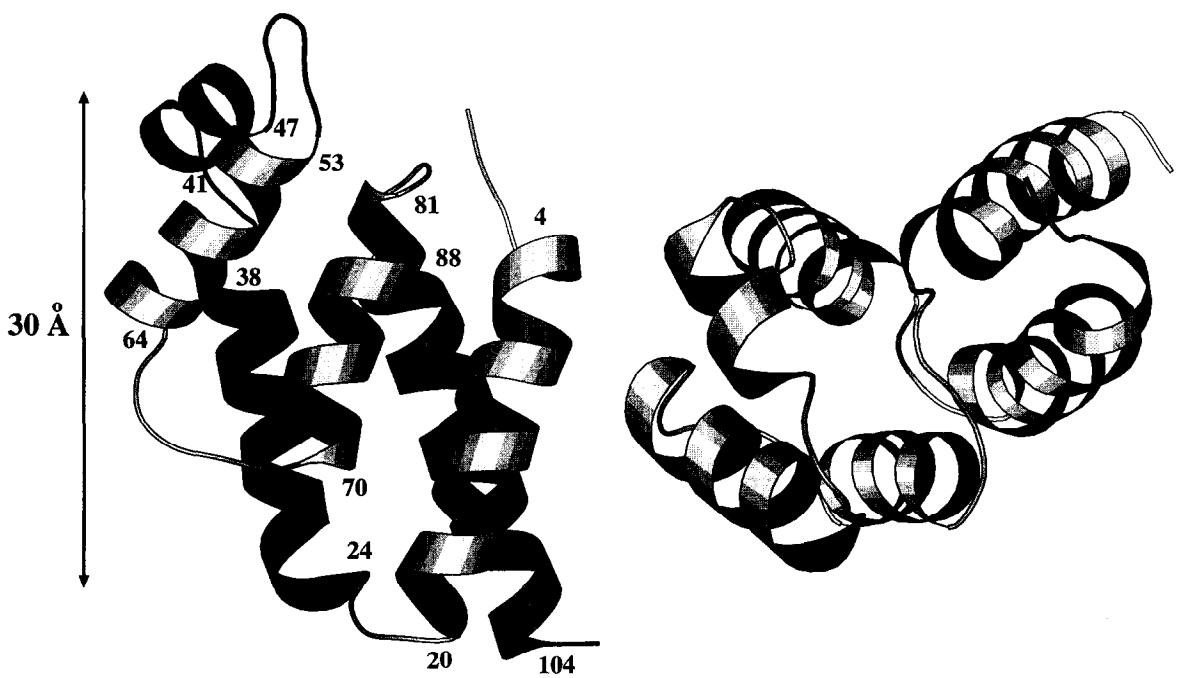


Fig. 2. Two views of the minimized average of 30 calculated structures for the N-terminal domain (residues 1–105) of the δ structure. The dimensions of this domain are 45 × 25 × 20 Å.

of δ [22], or 5 residues from the C terminus of OSCP [23], does not affect the binding of δ (or OSCP) to F_1, but these truncations prevent reconstitution of a coupled F_1F_0 (i.e., the enzyme is an active ATPase, but does not pump protons or synthesize ATP). Taken together, these studies indicate that the N-terminal domain is predominantly involved in binding to F_1, while the C-terminal domain is involved in binding to the F_0 part.

1.3. The δ subunit binding site on F_1

Several earlier studies had implicated the α subunit in binding δ to the core F_1 complex. These include proteolysis [24,25] and genetic studies of α [26], which had demonstrated the importance of the N-terminal approximately 30 residues in δ binding. Also, cross-linking studies have shown that α and δ form a disulfide bond in the presence of oxidizing conditions [27–29]. We are presently exploring the interaction of δ with F_1 in some detail. We have introduced a Cys residue in the N-terminal region of α (by the mutation αQ2C), and this has proved to cross link in essentially 100% yield with the δ subunit in ECF_1F_0 in the presence of low levels of $CuCl_2$ (Ogilvie and Capaldi, unpublished results). Significantly, this cross linking has no effect on either ATP hydrolysis rates or on coupled proton translocation. Under standard conditions, the cross link from α Cys2 is with Cys140 of the δ subunit. However, an α–δ disulfide bond is still made in ECF_1F_0 in which Cys140 has been blocked by N-ethylmaleimide, showing that it is possible to link from α Cys2 to δ Cys64 when Cys140 is not available. This is expected because Cys64 and Cys140 are very close themselves, as described.

In other experiments, we have established the α–δ cross link reported by others [27–29] for wild-type ECF_1 is between Cys90 or Cys47 of α (which are close and readily form a disulfide bond between themselves) and Cys140 of δ (Ogilvie and Capaldi, unpublished results). Taken together, these cross-linking data place the very N terminus of α (whose position is not revealed by the high resolution structure determination of MF_1), Cys47 and Cys90 of α, and both Cys64 and Cys140 of δ, all in close proximity. They indicate that the interaction of δ with the F_1 core is with the N-terminal domain of α, which is at the top of the molecule and away from the stalk seen in the electron microscopy studies.

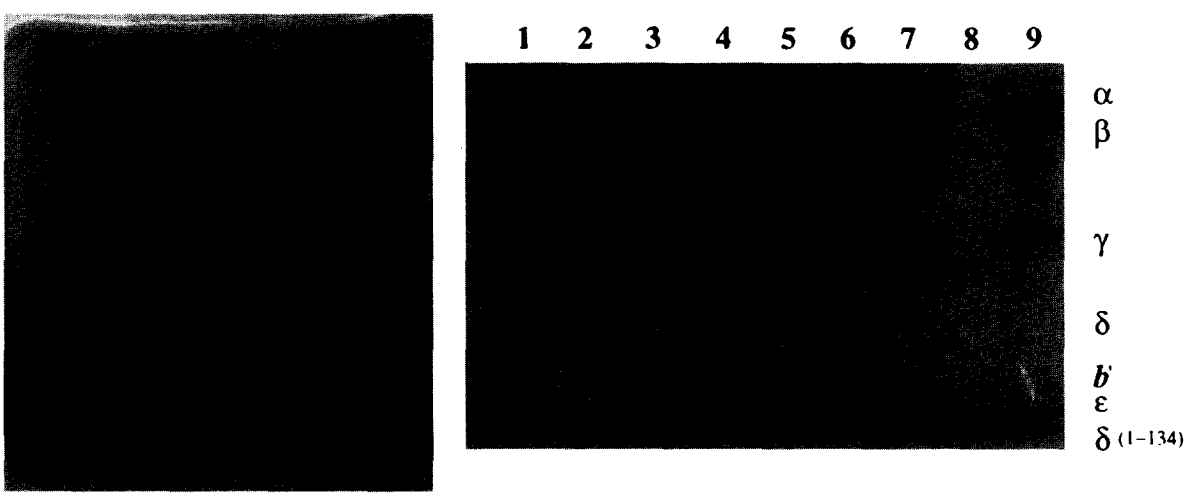


Fig. 3. Binding of the soluble portion of the b subunit (b'_2) to ECF_1 and ECF_1 (−δ) analyzed by two-dimensional gel electrophoresis. Samples were first analyzed by native agarose gel electrophoresis (left). Lanes from left to right: ECF_1 (−δ), b'_2, δ, b'_2 + δ, b'_2 + δ (1–134), b'_2 + ECF_1 (−δ) δ + ECF_1 (−δ), b'_2 + δ + ECF_1 (−δ), δ (1–134) + ECF_1 (−δ), b'_2 + δ (1–134), δ (1–134). Bands were excised from the agarose gel and separated on a 10–18% gradient of polyacrylamide in sodium dodecyl sulfate (right). The individual lanes 1–9 in the polyacrylamide gel are for the bands identified in the agarose gel.

Recent studies of Lill et al. [30] have independently pointed to a position of the δ subunit on the outside of F_1, and near the top of the molecule, and, according to these authors, at the interface between an α- and a β subunit. Briefly, Lill et al. [30] have introduced Cys residues at various positions in the δ of CF_1, and used heterobifunctional cross linkers to find residues close to these sites on other subunits. They obtained cross linking from a Cys at position 10 in the δ of CF_1 to a site within the N-terminal 62 residues of a β subunit, as well as cross linking from Cys residues at 57, 82 and 166 to sites in the N-terminal 192 residues of an α subunit.

1.4. The δ subunit links to the F_0 part via the b subunits

There are two copies of the b subunit in ECF_1F_0. This polypeptide has an N-terminal hydrophobic region, shown to be bilayer intercalated, and a long hydrophilic C-terminal part which extends from the membrane on the same side as the F_1 part [1,2]. In CF_1F_0, there are two polypeptides homologous to the b subunits, called I and II, which are present in one copy of each [31]. The homologue of subunit b in MF_1F_0 is also called subunit b [32].

Evidence for the interaction of the δ with the b

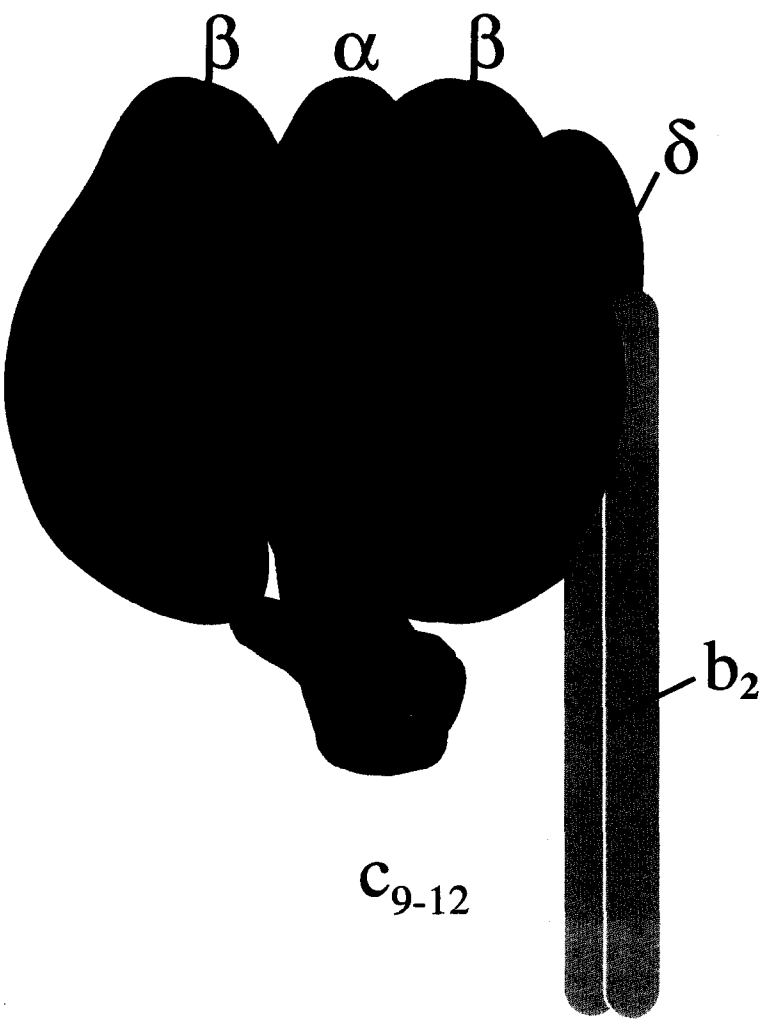


Fig. 4. Model of the arrangement of subunits in ECF_1F_0.

subunit has come from cross-linking studies in CF_1F_0 and MF_1F_0 [33,34]. We have taken advantage of the overexpression and purification of the δ and b subunits from ECF_1F_0 to study their association. Fig. 3 shows the results of binding studies involving ECF_1 and δ, along with a fragment of subunit b truncated genetically at the N terminus to remove the bilayer intercalated domain, thereby providing a water soluble C-terminal part (residues 22 to 156, and here called b'_2). As shown by the native gel and second dimensional SDS polyacrylamide gel, ECF_1 containing the δ subunit binds the C-terminal domain of b (which is present in solution as a dimer), while δ-free ECF_1 does not. ECF_1 reconstituted with δ′ (residues 1–134) does not bind the b subunit, and δ and b subunits do not form a stable complex in the absence of F_1 ($\alpha_3\beta_3\gamma\epsilon$).

These results show that the b subunits require δ for binding to the F_1 part and that the C terminus of δ is the site of b subunit binding. The fact that the F_1 core complex is required for δ–b binding could indicate that interaction of δ with F_1 stabilizes the C-terminal domain of δ in the correct structure for b subunit binding.

1.5. A two-stalk model of ECF_1F_0 and functional significance

ATP hydrolysis and ATP synthesis by F_1F_0-type ATPases is a cooperative process in which reactions at three catalytic sites are linked to one another, as well as to proton translocation through the F_0 part. The model of energy coupling in enzymes of this type which has gained the most general support is called the binding change mechanism [35,36]. In this proposed mechanism, the major energy-requiring step is not synthesis of ATP, but rather its release from catalytic sites once made. All three catalytic sites are thought to participate so that at any one time, one site is open (for binding new substrate, ATP or ADP + P_i, depending on the direction of the reaction), one site is closed (having just made ATP, or hydrolyzed ATP to ADP · P_i) and the third site is partly open (as product is getting ready to be released). With enzyme turnover, each of the (three) sites would then switch between the (three) different states each with a different affinity for nucleotide. The available structural data suggest that the catalytic site affinities are determined by the different interactions of the γ and ϵ subunits with the three α–β subunit pairs (e.g. [6]), and this is supported by direct nucleotide binding measurements [37].

It had been speculated (as early as 1981) that the binding changes required by the above described mechanism could be brought about by rotation of the small single copy subunits (γ, δ and ϵ) [38]. Recent studies provide strong support for rotation of both the γ [39–42] and ϵ subunits [43,44] between at least two, and probably three, α–β pairs. Moreover, there is evidence that the γ subunit can be cross linked in essentially 100% yield via disulfide bonds to the c subunit oligomer, without disrupting ATP hydrolysis in catalytic sites [9]. This result implies that the c subunit ring is moving along with the γ and ϵ subunits relative to the $\alpha_3\beta_3$, δ, a, b_2 sub-complex. The model of ECF_1F_0 based on the data for δ and b subunits presented here (Fig. 4) shows how this could be possible. The arrangement of δ and the b subunits as a second stalk would provide a stator that fixes the $\alpha_3\beta_3$ domain to the a subunit. In such an arrangement, rotation of the movable γ, ϵ and c subunit domain, not only alternates catalytic sites, but switches which of the c subunits is interacting with the a subunit. Such a rotation of c subunits is a key part of a model of proton translocation proposed recently by Vik and Antonio [45], in which the proton channel itself is at the c subunit-a subunit interface. It is important to point out that the evidence for rotation of a γ–ϵ–c oligomer domain is for the most part based on static or steady-state type experiments. What is required now are real time, dynamic measurements to confirm the functional significance of the subunit movements in energy coupling within the ATP synthase. These studies are ongoing in our laboratory.

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