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Electron microscopic evidence of two stalks linking the F_1 and F_0 parts of the *Escherichia coli* ATP synthase

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Abstract

The structure of monodisperse ATP synthase from *Escherichia coli* (ECF_1F_0) has been examined by electron microscopy after negative staining of specimens. The F_1 part is seen to be connected by two stalks. One is more centrally located and includes the γ and ϵ subunits. The second stalk, observed here in ECF_1F_0, is arranged peripherally. It probably contains the δ and b subunits which, in addition to γ and ϵ, are required for binding of the F_1 and F_0 parts of the complex. Other novel features of the F_1F_0 complex can be discerned. There is a cap at the top of the F_1 part at which the second stalk may bind. This likely includes N-terminal stretches of the three copies of the α subunit and a part of the δ subunit. The F_0 part is clearly asymmetric. The presence of two stalks in the complex has important functional implications. There is good evidence that the more central stalk of γ and ϵ subunits is a mobile domain that rotates to link the three catalytic sites on β subunits in turn, with the proton channel of the F_0 part. The second stalk of δ and b subunits is then the stator which makes this rotation possible. © 1998 Elsevier Science B.V.

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The F_1F_0 type ATP synthase plays a key role in cellular energy metabolism. This enzyme is found in the plasma membrane in bacteria, the thylakoid membrane of chloroplasts and the inner membrane of mitochondria, where it functions both to synthesize and hydrolyze ATP coupled to active transport of protons, or in some bacteria, of sodium ions, across a lipid bilayer [1–3].

The enzyme from *Escherichia coli* (ECF_1F_0) is composed of a total of eight different subunits, five of which constitute the F_1 part (α_3, β_3, γ, δ, ϵ) and three the F_0 part (a, b_2, c_{9-12}). There are three catalytic sites on the F_1 part, each located predominantly on β subunits. These are linked functionally to a proton pore within the F_0 that appears to be formed at the interface between the single copy a subunit and the c subunit ring [1–3].

The entire ATP synthase of *E. coli* has a molecular weight of around 560 000. Low-resolution structural studies, using electron microscopy, have shown that the F_1 and F_0 parts of this complex are separated by a relatively narrow stalk of around 40–45 Å in length [4,5]. A high resolution (2.8 Å) structure of the $\alpha_3\beta_3$ domain of bovine heart mitochondrial F_1 has been obtained by X-ray diffraction studies, which also reveals a part of the γ subunit [6]. The α and β

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subunits have a similar fold and they are arranged as a hexagon surrounding a central cavity within which resides the γ subunit. The structure of both the ϵ subunit and a major part of the δ subunit of ECF_1 have been obtained recently by NMR [7,8]. Features of the F_0 part are less well understood, although it appears that the c subunits, each arranged as an α-helical hairpin [9], form a ring with the a and b subunits attached on the outside [10,11].

Evidence has accumulated that the three catalytic sites are linked sequentially with the proton channel via the rotation of a mobile domain now known to be constituted by both the γ and ϵ subunits [12–15]. The rotation of γ relative to $\alpha_3\beta_3$ was most elegantly shown in recent single-molecule experiments by Noji et al. [14] The enzyme, then, is a rotary motor enzyme.

The γ and ϵ subunits interact at one end with α and β subunits in the F_1 part [6,16], and with the c subunit ring of the F_0 [17,18]. They are the stalk that has been resolved in electron microscopy. If this stalk part is to rotate, there must be a second connection between the F_1 and F_0 that holds the $\alpha_3\beta_3$ domain fixed relative to the a subunit, i.e., that acts as a stator. Two subunits, δ and b, are also necessary for functional interaction of the F_1 and F_0 parts [2]. Therefore, these subunits are strong candidate components of a second stalk. Recent studies indicate the globular δ subunit is bound to the top part of the F_1 above the catalytic site region [19,20]. Covalent attachment of δ to an α subunit by disulfide bond formation had no effect on the functioning of ECF_1F_0 either in ATP hydrolysis or in ATP synthesis, supporting the idea that δ is fixed relative to the $\alpha_3\beta_3$ hexagon, and is a part of the stator [19]. In contrast, covalent cross linking of γ or ϵ to either α or β subunits in all cases fully inhibits activity [19,21]. The two b subunits have a short N-terminal domain within the lipid bilayer and an elongated, mainly α-helical C-terminal part [22], which has recently been shown to bind to the δ subunit [23]. Also, a disulfide bond can be generated between a Cys at the C-terminal amino acid of the b subunit and Cys 90 of the α subunit without affect on ATPase activity (A.J.W. Rodgers and R.A. Capaldi, unpublished studies). These data place both the δ and b subunits at or near the top of the F_1.

While the biochemical evidence supports the idea of a second connection, such a feature remains inferential until observed directly. Earlier electron microscopy studies that identified one central stalk used membranous ECF_1F_0 embedded in a thin layer of ice [4,5]. A narrow second stalk could have been missed in that the density difference between protein and solvent is low leading to noisy images, a large number of which must be averaged to see any feature clearly.

Here, we have examined monodisperse, detergent-solubilized ECF_1F_0 by negative staining to enhance the protein–solvent contrast. Under these conditions, a second stalk can be seen in a large percentage of the ECF_1F_0 complexes observed.

ECF_1F_0 was isolated in a one-step procedure by using a genetic approach to introduce a His tag at the N-terminus of the a subunit, dissolving the *E. coli* membranes in taurodeoxycholate and binding the ECF_1F_0 to a nickel affinity column. The enzyme was then eluted using the novel amphipathic compound amphipol [24] to maintain solubility. It has been demonstrated repeatedly (e.g., Ref. [25]) that heavy metal salts used as negative stains detach the F_1 from the F_0 part. The purification procedure, using the His-tagged protein as above, also caused some release of F_1. To reduce this loss of the F_1 part, membranes were treated with DCCD prior to solubilization and subsequent negative staining. DCCD reacts with Asp-61 of the c subunits of the F_0 part to inhibit enzyme functioning [9]. Fig. 1 shows a field of ECF_1F_0 molecules stained with uranyl acetate. As expected of enzyme treated with DCCD, the ATPase activity was very low, i.e., in the range 0.5–1.0 mol ATP hydrolyzed per min per mg of protein in an ATP-regenerating system. Treatment of the preparation with 0.5% LDAO increased this activity 100-fold by disturbing the interface between the F_1 and F_0 parts, such that ATPase activity was no longer coupled to events in the F_0 part. The very high DCCD sensitivity after purification is clear evidence that the F_1 and F_0 parts remained together with a functional interaction during purification. The fact that almost all of individual molecules in Fig. 1 are dumbbell shaped, confirms that prior DCCD treatment effectively keeps the F_1F_0 complex intact.

Several features of the ECF_1F_0 complex could be observed in individual protein complexes after negative staining, including the presence of two protein

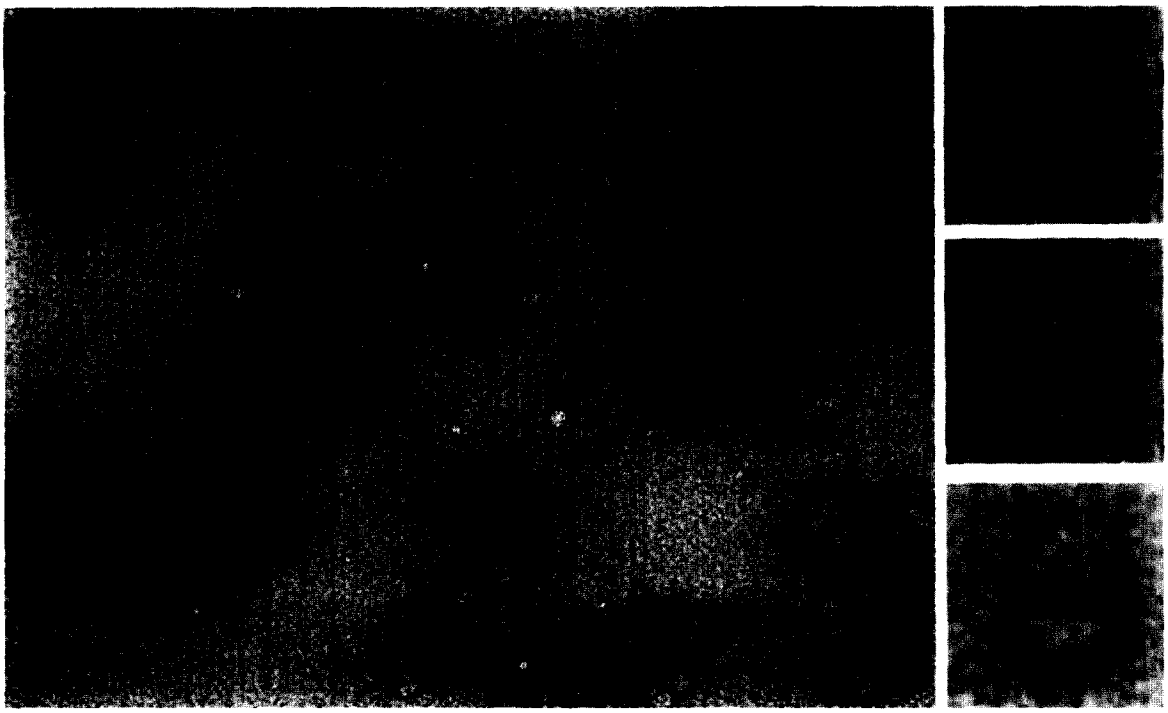

Fig. 1. Electron microscopy of monodisperse ECF_1F_0. Protein was negatively stained with 1% uranyl acetate. Magnification 60 000×. Enlarged F_1F_0 molecules on the right selected from the data set for clear display of the features described in this paper: top, second stalk on the left; middle, symmetric molecule; bottom, second stalk on the right.

densities connecting the F_1 and F_0 parts (right side of Fig. 1). These features become clearer in averages obtained by analyzing a data set of individual images. In the data set described here, 139 randomly selected side views were analyzed by aligning to a symmetrized average of a previous round of alignment, and subjecting these to multivariate statistical analysis, followed by classification into 12 classes. There were two predominant classes identified by the program that together included around 40% of all images (Fig. 2). The remaining images were distributed between the other classes (four to 12 molecules per class). They appeared to be intermediate orientations to those seen in the major classes and, in some cases, a grouping of misaligned molecules. One stalk, more centrally placed in the image on the right panel of Fig. 2, is that seen previously. It is the stalk composed of the γ and ϵ subunits, the former of which is well established to extend from the cavity within the center of the $\alpha_3\beta_3$ hexagon [6], as visualized here. Some substructure of this more central stalk can be seen. It widens near the F_0 part into what is likely the N-terminal domain of ϵ, which interacts with the γ subunit on one side [17] and the c subunit ring at the bottom [18]. This N-terminal domain is a 10-stranded β barrel of dimensions 28×28×22 Å [7].

A second connector is evident extending down from the F_1, while there is a density rising up from the F_0. This second stalk likely includes the δ and b subunits. Density in the middle of the stalk is very weak, as might be expected if this region is provided by one or two α-helices from each of the b subunits. More recently, data sets of side views of ECF_1F_0 dissolved in lauryl maltoside and in lysolecithin have been obtained. Individual images in these detergents are very similar to those described here and, importantly, the percentage of molecules with two stalks is again 35–45% of randomly selected images (S. Wilkens, B. Schulenberg and R.A. Capaldi, unpublished results).

Two other important features are revealed in negatively stained images of ECF_1F_0. There is a 'cap' at the top of the F_1, that extends in the direction of the second, more asymmetrically placed stalk. This feature, as well as indications of a second stalk by an extension of the density downwards from the F_1 part,

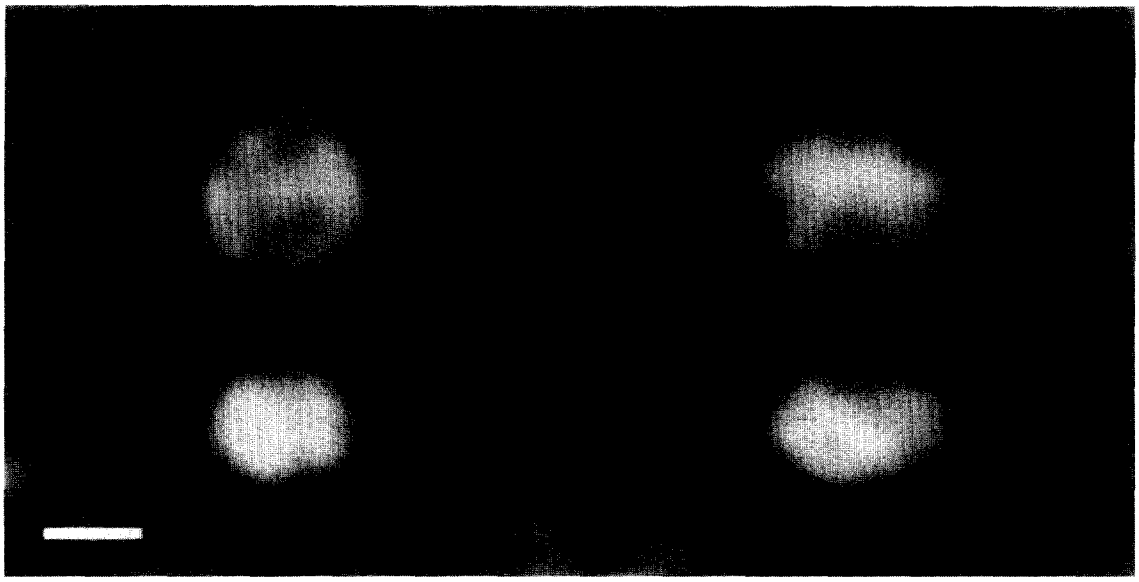

Fig. 2. MSA/classification of single F_1F_0 molecules. Averages show the two most prominent views identified by the MSA/classification procedure. (Modified from ref. [27].)

was seen in cryoelectron microscopy of membranous ECF_1F_0 [4], but not commented on because they were of weak density. It was not evident in the X-ray structure reported for bovine F_1 [6]. It is probable that this cap is made by the N-terminal ~30 residues of the α subunits which were not resolved in the X-ray experiments, along with a part of the δ subunit. The view of ECF_1F_0 in Fig. 2 (right panel) shows a clear asymmetry of the F_0 part. This is consistent with a substructure in which the c subunits form a ring with the a and b subunits.

The image at the left of Fig. 2 could be a projection at approximately 60° to that seen on the right of this figure. Two stalks are also seen, but these are more symmetrically located, and the F_0, instead of being asymmetric, is essentially symmetric.

The presence of a two stalks shown here, and described recently by electron micrographs of the V_1V_0 ATPase from *Clostridium fervidus* [25], has important functional implications. This structure can provide the stator against which the mobile domain of γ and ϵ subunits, probably along with the c subunit ring, rotates relative to a static domain of $\alpha_3\beta_3\delta a_1 b_2$. There is good evidence that the proton channel in F_0 is at the interface between the C-terminal α-helix of the c subunit, including Asp-61, and the C-terminal α-helix of the a subunit [1,2,26]. Taken together, data suggest a model of energy coupling within the F_1F_0 in which ATP hydrolysis in one direction, and ATP synthesis in the other, involves the rotation of the c subunit domain, such that protons are sequentially translocated for individual c subunits to the a subunit and, thereby, across the membrane. This rotation of c subunits is driven by, in one direction, or drives in the other, the rotation of the γ and ϵ subunits between catalytic sites.

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