An F₁Fₒ-type ATP synthase is found in the inner mitochondrial membrane, the inner membrane of bacteria and the thylakoid membrane of chloroplasts, where it functions to convert the free energy of the proton-motive force into the chemical energy source ATP (for reviews, see Boyer, 1993; Junge et al., 1997; Weber and Senior, 1997). This large enzyme complex is composed of two major parts, a water-soluble F₁ sector made up of three α subunits, three β subunits and one copy of each of the γ, δ and ε subunits, and a membrane-embedded Fₒ sector consisting of one α, two β and 12 c subunits. The overall molecular mass of the complex is 520 kDa. There are three catalytic nucleotide-binding sites located on the β subunits of the F₁ and one proton channel formed by the α and c subunits in the membrane-embedded Fₒ sector.

The available evidence suggests that the coupling of catalytic sites to proton translocation in both the ATP synthesis and hydrolysis modes is conformational and that this enzyme works as a rotary molecular motor. The key to its functioning is in the nature of the interaction between the F₁ and Fₒ sectors. These interactions have been worked out largely by a combination of electron microscopy and cross-linking studies.

**ECF₁ is linked to the Fₒ sector by two stalks**

The bipartite nature of the F₁Fₒ complex had been observed in early electron micrographs using negative staining to optimize the contrast between protein and solvent, and such pictures appeared to show a stalk linking the two parts (Fernandez-Moran, 1962; Soper et al., 1979; Telford et al., 1984). However, it was uncertain whether this stalk was an artifact of the staining procedure until our cryoelectron microscopy images confirmed its presence in buffered solutions without heavy atoms added (Gogol et al., 1987; Lücken et al., 1990). In these first cryoelectron microscopy pictures of ECF₁Fₒ, F₁ appeared to extend to Fₒ at one side in some of the images, as though there were two linkages between the two parts.

More recently, we have obtained electron micrographs of ECF₁Fₒ in a monodisperse, detergent-solubilized form (Wilkens and Capaldi, 1998a,b). Under these conditions, the overlap of molecules seen in membranous material is absent, simplifying the analysis of images. These new data establish conclusively that there are two stalks in ECF₁Fₒ (Fig. 1) and, in concert with recent data for chloroplast F₁Fₒ (Böttcher et al., 1998) and mitochondrial F₁Fₒ (J. E. Walker, personal communication), indicate that this is a common feature of F₁Fₒ-type proton-pumping ATPases as well (Boekema et al., 1997).

**The central stalk contains the γ and ε subunits**

Key evidence that the γ subunit contributes to the central stalk came from the high-resolution structure of mitochondrial F₁α₃β₃γ subcomplex of Abrahams et al. (1994). This structure showed the γ subunit passing through a cavity within the α₃β₃ hexagon and extending from the bottom of the structure as expected of a stalk component. Our finding that the γ subunit can be cross-linked in high yield to the c subunit ring (Watts et al., 1996) established that the γ subunit extends the full length of the stalk. Also present in the central stalk is the ε subunit. This polypeptide has a two-domain structure (Wilkens...
et al., 1995; Uhlin et al., 1997). There is a C-terminal pair of α-helices that fits below F₁ and interacts with either the α or β subunit, depending on nucleotide binding in catalytic sites (Aggeler et al., 1995; Aggeler and Capaldi, 1996; Wilkens and Capaldi, 1998c). The N-terminal domain is a 10-stranded β sandwich that binds on one face to the γ subunit (Tang and Capaldi, 1996; Watts et al., 1996) and by the bottom of the sandwich to the c subunit ring (Zhang and Fillingame, 1995).

The peripheral stalk contains the δ and b subunits

Obvious candidates for the second stalk in the bacterial enzyme are the δ and b subunits. Our nuclear magnetic resonance (NMR) structure determination of the δ subunit shows a largely globular α-helical protein (Wilkens et al., 1997) which cross-linking studies have established is attached near the top of F₁ by interactions with an α subunit (Lill et al., 1996; Ogilvie et al., 1997). Along with N-terminal segments of the three α subunits, it probably generates the cap seen at the top of ECF₁F₀ in recent electron micrographs (Wilkens and Capaldi, 1998a,b).

Cross-linking studies indicate that the C-terminal part of the b subunits interacts with the C-terminal region of the δ subunit and with the α subunit (Rodgers et al., 1997; Rodgers and Capaldi, 1998) and that these interactions are close to the top of the F₁ sector. The two b subunits extend as paired α helices through the length of F₁F₀, with interactions inside the lipid bilayer (Dmitriev et al., 1999).

Two stalks, a central rotor and a peripheral stator

F₁ is unusual in being a trimeric enzyme (three α–β pairs). During functioning, three catalytic sites must be coupled alternately to one proton channel. To explain how this coupling might occur, both Cox and colleagues (e.g. Cox et al., 1984) and Boyer (1993) independently proposed that, during ATP synthesis, a central element in the enzyme rotated in one direction between catalytic sites, driven by a proton gradient. During ATP hydrolysis in the three catalytic sites, the central element rotates in the opposite direction. Electron microscopy provided the first indication that this central element of the F₁ sector was the γ subunit (Gogol et al., 1989, 1990). Furthermore, we were able to show that the γ subunit was not fixed in the cavity within the αβ hexagon, but could be found at each of the three α–β pairs (Gogol et al., 1990). Additional evidence that the γ subunit could rotate within the F₁ sector was obtained from cross-linking studies (Duncan et al., 1995) and by polarized absorption relaxation after photobleaching (PARAP) measurements (Sabbert et al., 1996). Conclusive evidence of ATP-hydrolysis-driven rotation of the γ subunit came with the video microscopy of single particles by Yoshida, Kinosita and their colleagues (Noji et al., 1997). In our earlier cryoelectron microscopy studies, we had observed movements of the γ subunit relative to the ε subunit. We now know that the ε subunit moves in concert with the γ subunit, as shown by cross-linking experiments in which ε can be covalently linked to γ without alteration of function (Aggeler et al., 1992; Tang and Capaldi, 1996; Schulenberg et al., 1997). More recently, rotation of the ε subunit has been observed directly by video microscopy (Kato-Yamada et al., 1998). In our earlier experiments, the interaction of ε with γ had been perturbed by the monoclonal antibody used to tag it, resulting in this subunit remaining fixed at one β subunit.

The rotation of the γ–ε central stalk, two subunits that react with the c subunit ring, suggests a model in which γ–ε and the c subunits form a rotor, which moves relative to the rest of the complex, αβδεδγε. In such a scheme, the second stalk of δε should act as a stator positioning the αβδε subunits in a fixed disposition relative to the a subunit. Rotation of the c subunit ring relative to the a subunit then provides the proton-translocation mechanism. The evidence that the γ–ε–c₁₂ subcomplex is moving relative to the rest of the complex is so far based only on cross-linking studies. The cross-linking of γ to ε, or of ε to c, does not block ATP-driven proton translocation in ECF₁F₀, whereas the cross-linking of either γ or ε to α or β does (Aggeler and Capaldi, 1992, 1996; Aggeler et al., 1992, 1993, 1995).

Cross-linking of δ or b to α also has no effect on the functioning of the ATP synthase (Ogilvie et al., 1997; Rodgers and Capaldi, 1998), as expected if these two subunits are the stator element. A summary of our cross-linking experiments to date in ECF₁F₀ is shown in Fig. 2. Additional evidence for the rotation of γ and ε during ATP hydrolysis in ECF₁F₀ (Aggeler et al., 1997; Zhou et al., 1997) and of γ during ATP synthesis (Bulygin et al., 1998) has been provided by cross-linking experiments, and rotation of the c subunit ring relative to subunit a is implied by recent cross-linking studies of Fillingame and coworkers (Jones et al., 1998). However, convincing evidence

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Fig. 1. Electron microscopy images of detergent-dispersed F₁F₀-ATPase of Escherichia coli (ECF₁F₀). Parts A–C show three different classes of images resolved. A and C are mirror images of one another and are side views that clearly separate a central and peripheral stalk. Part B is a view more end-on, in which the F₀ sector is more symmetrical. The scale bar in C is 5 nm. The figure is reproduced from Wilkens and Capaldi (1998a) with permission.
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of c subunit rotation from visualizing it in real time, as in the video microscopy experiments, has not yet been provided.

Elasticity of the F₁Fₒ complex during rotation

Most studies of the rotary motor model have focused on the torque generated in relation to energy conservation during coupling within the enzyme complex. However, as pointed out by Cherepanov et al. (1999), there is likely to be an elasticity of the transmission between F₀ and F₁ when four small steps of rotation of c relative to a are coupled to one 120° rotation of γ-ε. We have been looking for conformational changes in ECF₁Fₒ that could correspond to this storage and release of elastic energy. We have recently found that there are significant nucleotide-dependent alterations in the relationship between F₁ and F₀ that should be part of the functioning of the enzyme. Thus, single particles of F₁Fₒ show the now characteristic pattern of F₁ separated by two stalks when Mg²⁺ADP is bound, but the picture is altered with ATP in catalytic sites (in the presence of Mg²⁺AMP-PNP). There is a contraction of the complex in the ATP state such that the F₁ and F₀ sectors are closer together and the two stalks are only partly discerned. As

Fig. 2. A summary of the cross-links generated so far between subunits of F₁Fₒ-ATPase of Escherichia coli (ECF₁Fₒ). Red bow-ties show cross links that inhibit both ATP hydrolysis and ATP synthesis. The blue bow-tie differentiates the cross link between γ and c from others because, in this case, ATP synthesis is affected but not ATP hydrolysis. Yellow bow-ties show cross links that have no effect on functioning of the enzyme.

Fig. 3. Size distribution of F₁Fₒ-ATPase of Escherichia coli (ECF₁Fₒ) single particles when examined in side view. Cross-hatched bars are molecules in Mg²⁺ADP. Open bars are for enzyme in Mg²⁺AMP-PNP.
shown in Fig. 3, the average particle length of side views is 10% shorter in the presence of ATP than in the presence of ADP, a contraction of 1.5–2.0 nm. Such a conformational change would require alterations in both stalks. If the conformational changes described above represent energy storage by elastic strain, the uncoupling of catalytic site and proton-translocation events seen in many different mutants of ECFιFο may be due to perturbation of rotation or of the up–down movements as well as to direct disruption of the proton channel (Fig. 4).

Epilogue

As reviewed here, electron microscopy and cross-linking studies of the E. coli ATP synthase have contributed significantly to our understanding of the structure and functioning of FιFο-type ATPases. The dynamic nature of the functioning of the enzyme means that a full understanding of its mechanism will require high-resolution structural data for the intact complex trapped in different conformations. Cross-linking to fix subunits in position and, thereby, trap specific conformations can continue to provide new insights into the workings of the ATP synthase, particularly if ECFι and ECFιFο can be crystallized in a variety of trapped states. Our efforts to obtain such crystals continue.

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