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Subunit Rotation in F_0F_1-ATP Synthases as a Means of Coupling Proton Transport Through F_0 to the Binding Changes in F_1

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The rotation of an asymmetric core of subunits in F_0F_1-ATP synthases has been proposed as a means of coupling the exergonic transport of protons through F_0 to the endergonic conformational changes in F_1 required for substrate binding and product release. Here we review earlier evidence both for and against subunit rotation and then discuss our most recent studies using reversible intersubunit disulfide cross-links to test for rotation. We conclude that the γ subunit of F_1 rotates relative to the surrounding catalytic subunits during catalytic turnover by both soluble F_1 and membrane-bound F_0F_1. Furthermore, the inhibition of this rotation by the modification of F_0 with DCCD suggests that rotation in F_1 is obligatorily coupled to rotation in F_0 as an integral part of the coupling mechanism.

KEY WORDS: Binding change mechanism; subunit rotation; oxidative phosphorylation; F_0F_1-ATP synthase; rotary mechanism.

F_0F_1-ATP synthases are found embedded in the membranes of mitochondria, chloroplasts, and bacteria, and are responsible for the production of most cellular ATP from ADP and P_i. To accomplish this critical task, F_0F_1 must be able to extract energy from a transmembrane, electrochemical gradient of protons produced by photosynthetic or respiratory chain complexes. F_0 is composed of membrane-spanning subunits and catalyzes the transport of protons across the bilayer. F_1 is an extrinsic complex that contains the catalytic sites for ATP synthesis and hydrolysis, and can be removed from the membrane in a water-soluble form that catalyzes hydrolysis of ATP. The mechanism by which F_0F_1 is able to achieve efficient, reversible energy coupling between a vectorial gradient of protons and the chemical energy of the terminal phosphoryl bond of ATP has been a major focus of research in bioenergetics for many years (for reviews see Fillingame, 1990; Senior, 1990; Cross, 1992; Boyer, 1993; Pedersen and Amzel, 1993).

The model for energy coupling by F_0F_1-ATP synthases that has gained the most general support is called the binding change mechanism (Boyer, 1993, 1989). It has two features that are widely accepted (Boyer *et al.*, 1973; Kayalar *et al.*, 1977). The first is that the major energy-requiring step is not the synthesis of ATP at the catalytic site (Fig. 1a, step 2) but rather its release from the site (Fig. 1a, step 1). Second, the tight binding of substrates and release of product occur simultaneously at separate but interacting sites (Fig. 1a, step 1). A third premise has remained speculative (Boyer and Kohlbrenner, 1981). That is that the required binding changes are coupled to proton transport by the rotation of a complex of subunits that extends through F_0F_1 (Fig. 1b). F_1 has the subunit composition $\alpha_3\beta_3\gamma\delta\varepsilon$, in which a hexamer of alternating α and β subunits surrounds the central γ subunit. The catalytic nucleotide sites are located on the β subunits at α/β-subunit interfaces. Rotation of the γ subunit in the center of F_1 is thought to deform the surrounding catalytic subunits to give the binding changes (Fig. 1a). In F_0, with

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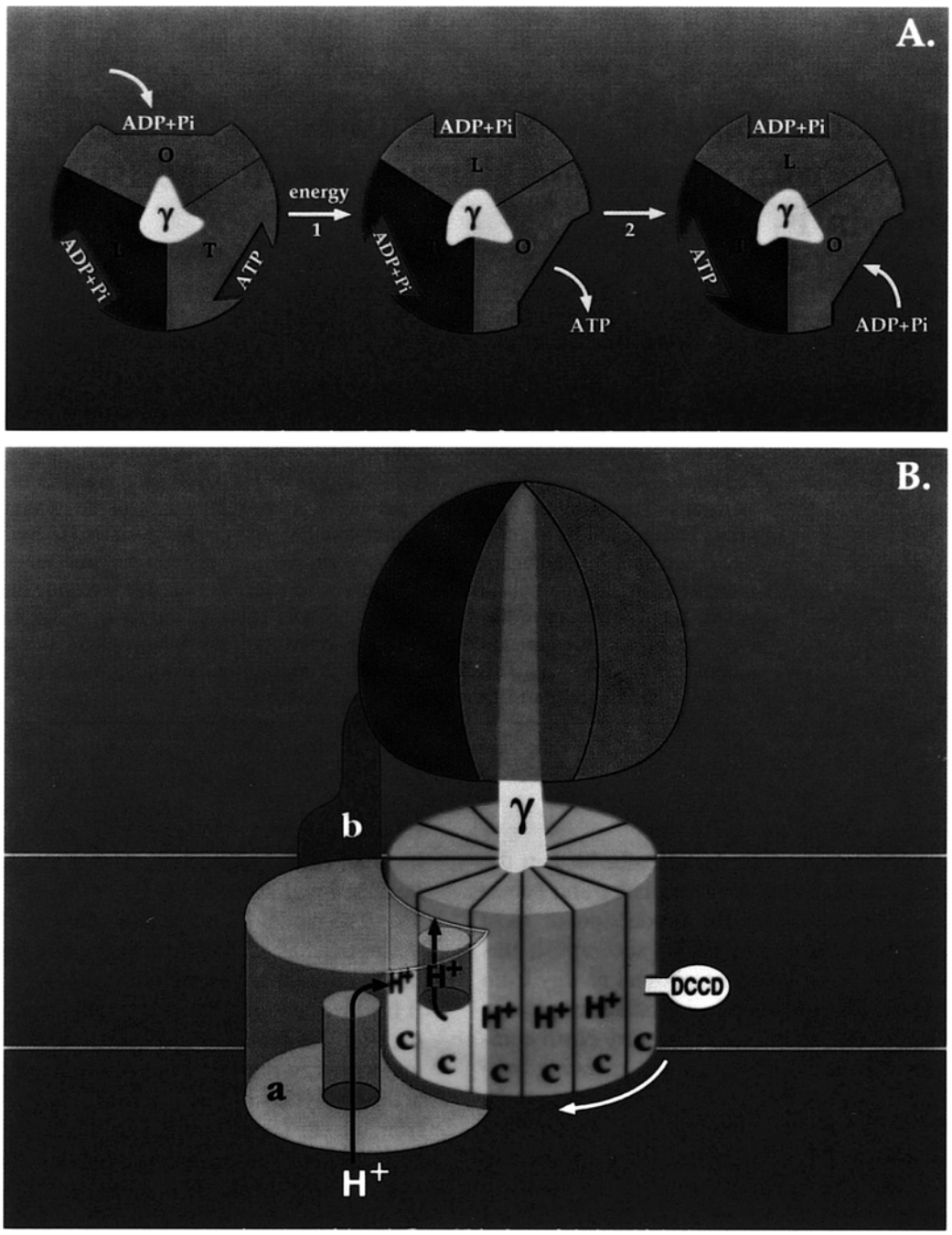

Fig. 1. A model for the binding change mechanism (adapted from Duncan *et al.*, 1995a). Panel a: View from the top of F_1. In step 1, rotation of the asymmetric γ subunit forces conformational changes in the three catalytic subunits which result in affinity changes for substrates and product at the catalytic sites. T, L, and O stand for tight, loose, and open conformations and refer to decreasing affinities of catalytic sites for ligand. Each of the three blue/green areas represents a pair of α and β subunits, where the catalytic sites are interfacial but mostly on β. In this illustration, the α/β pairs remain stationary. In step 2, ATP forms spontaneously from tightly-bound ADP and P_i. Panel b: View from the side of F_0F_1. Subunit *a* contains two partial channels, each in contact with a different side of the membrane. In order for a H^+ to traverse the membrane, it enters one channel, moves to the center of the membrane, transfers to the DCCD-reactive carboxyl on one of the *c* subunits, and is then carried to the other partial channel by rotation of the complex of *c* subunits. The *c* subunits are anchored directly to γ, whereas subunit *a* is anchored indirectly through subunit *b* to the catalytic subunits. Hence, the rotation of *c* subunits relative to subunit *a* in F_0 will drive the rotation of γ relative to the catalytic subunits in F_1. The modification of a single *c* subunit per F_0 by DCCD, as shown, is sufficient to inhibit energy coupling.

subunit stoichiometry ab_2c_{9-12}, residues of both *a* and *c* subunits are thought to participate in proton transport, and rotation of the *c* subunits relative to the *a* subunit is believed to be required for completion of the proton pathway (Fig. 1b).

The idea of subunit rotation as a means of coupling the exergonic transport of protons through F_0 to the endergonic conformational changes required for net synthesis of ATP by F_1 arose as the result of two technical advancements. For the first 20 years of its application, the use of ^{18}O-exchange methodology to follow the path of oxygen during oxidative- and photophosphorylation involved conversion of the oxygens of P_i to CO_2. Mass spectral analysis of the ^{18}O content of the CO_2 gave the average enrichment of ^{18}O in the original P_i pool but provided no information regarding the ^{18}O content of individual molecules of P_i. However, by the late 1970's, methods for esterifying the oxygens of P_i allowed P_i to be volatilized and analyzed directly. The ability to measure the distribution of individual species of P_i having zero to four ^{18}O atoms provides a unique signature of the reaction pathway, one that reflects both the rate of reversible cleavage of ATP at the catalytic site and the rates of substrate binding and product release.

Since it was known that F_1 contained multiple copies of the catalytic subunit but only single copies of the three smallest subunits, $\gamma\delta\varepsilon$, it was assumed that the small subunits must have unique interactions with the catalytic subunits. It seemed reasonable to expect that this intrinsic asymmetry might give rise to sufficient differences in the rate constants at each of the three catalytic sites to allow detection of multiple catalytic pathways by ^{18}O-exchange measurements. However, attempts to detect multiple pathways for enzyme from different sources failed (Hutton and Boyer, 1979; Hackney *et al.*, 1979; Kohlbrenner and Boyer, 1983).

One suggested solution to this apparent paradox was that the small subunits might possess tripartite structure so that their interactions with each catalytic subunit would be similar. This notion was dispelled by a second technical advancement. In 1981, sequences deduced from DNA were published for the EcF_1 subunits (Saraste *et al.*, 1981; Kanazawa *et al.*, 1981; Nielsen *et al.*, 1981). The small subunits did not show repeat sequences.

These results led Paul Boyer to propose a second solution, namely that the asymmetric core of F_1 rotates relative to the surrounding catalytic subunits (Fig. 1a, step 1). Hence in one complete cycle, each catalytic subunit would have identical interactions with the asymmetric core, except that these interactions would be 120° out of phase (Boyer and Kohlbrenner, 1981; Boyer, 1983). However, a number of investigators in the field did not find the data compelling. It was argued that the asymmetric core of F_1 might not cause a sufficient perturbation of the rate constants at the individual sites to allow detection of multiple catalytic pathways. In other words, structural asymmetry does not exclude the possibility of functional symmetry (Kironde and Cross, 1987; Musier and Hammes, 1987; Boyer, 1987).

As a test for rotation, the effects of cross-linking F_1 subunits on ATP hydrolysis activity were measured. The results were mixed. Musier and Hammes (1987) reported that cross-linking the γ subunit of CF_1 to an α or β subunit did not correlate to a proportional loss in enzymatic activity. In light of more recent studies with EcF_1 (Duncan *et al.*, 1995b; Aggeler *et al.*, 1995), these results are difficult to explain except to suggest that with soluble CF_1, rotation may be uncoupled from ATP hydrolysis (Cross, 1992). Using EcF_1, Kandpal and Boyer (1987) found that cross-linking α to the small subunits did inhibit activity. Although these results are consistent with rotation, they do not prove it since intersubunit cross-links may simply prevent required conformational changes. The most clear cut experiment at this time was reported by Tozer and Dunn (1986). They found that an intersubunit disulfide bridge formed spontaneously between δ and one of the α subunits upon passage of EcF_1 through a centrifuge column. No activity was lost. At the time, structural models of F_1 placed all three of the single-copied subunits in the center of F_1, and the authors concluded that the asymmetric core could not rotate relative to the surrounding large subunits. However, we now know from a high-resolution structure (Abrahams *et al.*, 1994) that γ alone fills the center of the molecule. Therefore, δ may be stationary relative to the large subunits.

Two additional approaches to test for subunit rotation were tried that were innovative in their design but flawed in theory. Moradi-Ameli and Godinot (1988) attached antibodies to the α subunit of membrane-bound MF_1, increasing the mass of the F_1 sector about twofold. There was no change in the rate of ATP synthesis, and the authors concluded that subunit rotation was unlikely. However, considering how rapidly protein molecules move at ambient temperature, rotation due to catalytic turnover on a millisecond time scale was far too slow to be affected by the increased mass. Musier-Forsyth and Hammes (1990) measured

the rotation of CF_0F_1 in membranes using the time-resolved phosphorescence emission anisotropy of a covalently incorporated fluorescent probe. At 4°C the correlation time was 100–180 μs. The value obtained under conditions for ATP synthesis was within the same range, and it was concluded that rotation has no role in catalysis. However, any rotation due to catalysis (on a sec time scale under the conditions used in this study) would have been orders of magnitude slower than the rate of rotation of CF_0F_1 in the membrane.

Evidence implicating a rotary-type mechanism also came from studies of F_0. Subunit *c* had long been known to contain a DCCD-reactive carboxyl group essential for proton transport (Hoppe and Sebald, 1984). This residue is predicted to be in the middle of a transmembrane α-helix (Fig. 1b). In an attempt to define the path of the proton through the membrane, several laboratories mutated polar residues of the other two EcF_0 subunits, *a* and *b*, predicted to be within the membrane phase. Several residues of subunit *a* proved essential (Cox *et al.*, 1986; Cain and Simoni, 1986; Lightowlers *et al.*, 1988; Paule and Fillingame, 1989; Vik and Antonio, 1994), and the view emerged that the *a* and *c* subunits together form a proton channel. However, EcF_0 has only one copy of subunit *a* but 9–12 copies of subunit *c* (Foster and Fillingame, 1982). All of the *c* subunits are required since modifying just one inhibits transport (Hermolin and Fillingame, 1989). This would appear to present a problem. How does a single *a* subunit coordinate with 9–12 *c* subunits to transport protons? One potential solution is that the *a* and *c* subunits rotate relative to each other (Cox *et al.*, 1986; Vik and Antonio, 1994; Hatch *et al.*, 1995). As each *c* subunit moves past the *a* subunit, a proton is transported (see caption to Fig. 1b).

The most supportive evidence for subunit rotation has come from studies of F_1 structure. Cryoelectron microscopy by Capaldi and coworkers (Gogol *et al.*, 1990) showed that the central mass in EcF_1 had an altered pattern of interactions with individual β subunits following catalytic turnover. The central mass is now known to be γ, and the results are consistent with its rotation. However, the resolution of the method used in this study was not sufficient to rule out the possibility that γ had simply wobbled rather than rotated. In contrast, the 2.8-Å resolution crystal structure for bovine MF_1 (Abrahams *et al.*, 1994) shows γ to have unique interactions with each of the 3 catalytic subunits. In fact, it appears that the nature of the contact determines the conformational state of each catalytic site as predicted by the third premise of the binding change mechanism (Boyer and Kohlbrenner, 1981).

In addition to providing supportive evidence for subunit rotation, the high-resolution structure also allowed a critical test of the concept. The structure identifies points of contact between γ and the β subunits. One of these includes the positioning of the bovine homolog of *E. coli* γ-subunit C87 close to one of the β-subunit $_{380}$DELSEED$_{386}$ sequences. We mutated several residues in the DELSEED sequence, one at a time to Cys. The rationale was that the ability to form a reversible disulfide cross-link between γ and β could prove useful in assessing the role of subunit rotation in catalysis. Of the residues tested with both soluble and membrane-bound F_1, βD380C reacted most readily with γC87 to form an intersubunit disulfide bridge in the presence of an oxidant (Duncan *et al.*, 1995a). Based on the high-resolution structure of MF_1, the relative orientation of γC87 and the three βD380C's is shown in Fig. 2 (yellow atoms). Designations for the β subunits refer to different conformational states (Abrahams *et al.*, 1994) where the catalytic site is empty (E) or occupied by either an ADP (DP) or a nucleoside triphosphate (TP). The model shows that the Cys of β_E is far too distant (28 Å, sulfur–sulfur) to react with γC87 and, although the Cys on β_{TP} is only ~10 Å from γC87, it points away from γC87 and is sterically blocked by intervening atoms of γ. This suggests that the disulfide forms between γC87 and the Cys on β_{DP} which are ~10 Å apart through unobstructed space.

With soluble βD380C-F_1, we demonstrated a direct correlation between the cross-linking of γ to β and a loss of ATP hydrolysis activity. These results are consistent with, but not proof of, a need for γ to rotate in the center of F_1. As a direct test for rotation, we first cross-linked γ to one of the β subunits in βD380C-F_1 and then, using a subunit dissociation/reassembly approach, we exchanged the two noncross-linked β subunits for radiolabeled β's (Duncan *et al.*, 1995b). The β–γ disulfide was then reduced and the enzyme incubated under different conditions before reoxidation. The question we asked was whether γC87 re-formed a cross-link to its original, nonradioactive partner or, if γ rotated during the incubation, could it now cross-link to radiolabeled β. In the absence of catalytic turnover, the majority of F_1 molecules re-formed a disulfide between γ and the nonradioactive β subunit. However, following a brief episode of catalytic turnover, the level of radiolabel in the cross-linked product was consistent with the ability of γC87 to

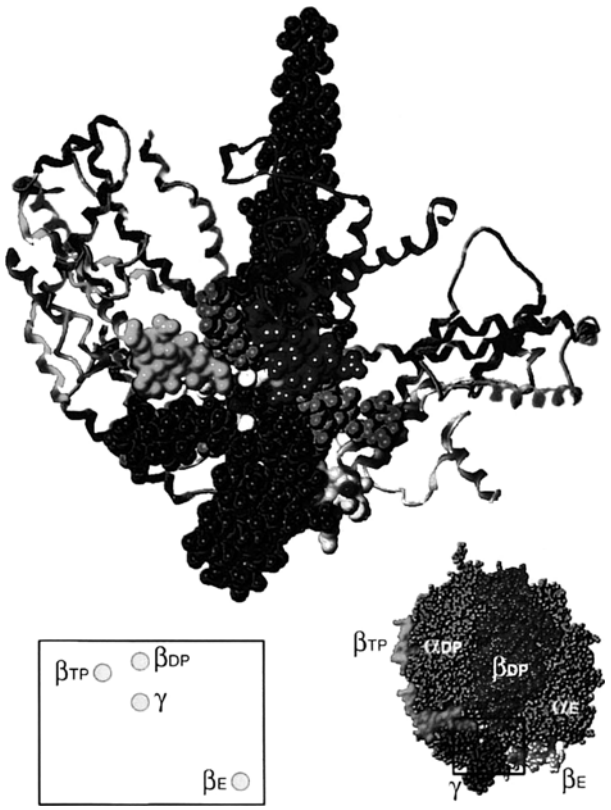

Fig. 2. Putative orientation of βD380C residues relative to γC87 in EcF_1. The molecular model is based on the crystal structure of bovine F_1 (Abrahams *et al.*, 1994), but *E. coli* residue numbers are used here. The large representation shows the following residues in space-filling mode: all defined residues of γ (green), β_{376}ILGMDELSEEDK$_{387}$ for β_{TP} (red), and β_{DP} (purple), β_{380}DELSEEDK$_{387}$ for β_E (orange), and the bovine residues analogous to β_{379}MDELSEEDK$_{387}$ for each α subunit (blue). The above sequences for γ, β_{TP}, β_{DP}, and α_E include all residues of the known F_1 structure within 15 Å of γC87's sulfur. For each α and β subunit, the remainder of the C-terminal helical-bundle domain is represented by ribbons (α-helices) and tubes. The sidechain of βD380 was changed to cysteine for each β in the model structure, and these three residues plus γC87 are colored by atom type (C = gray, N = dark blue, S = yellow). The inset at lower right shows the complete space-filling model of F_1, to provide a frame of reference for the orientation of the molecule. Note that the small helix of γ (containing γC87) extends to the left, and the "top" of F_1 is tilted back through the plane of the page to make the desired cysteines visible. The subunits are labeled according to Abrahams *et al.* (1994). The box on the inset encloses the 4 cysteines. At lower left, a blow-up of this box shows the sulfur atoms in their proper orientation and identifies their subunit locations. Note that, in the model, the sulfur on β_{TP} is mostly obscured and that on β_E is partially obscured by the cysteine's C_β atom.

interact equivalently with each of the three β subunits. Since γC87 cannot achieve equivalent interactions with each β subunit without rotation of the entire γ subunit within the core of F_1 (see Fig. 2), the results provide compelling evidence for rotation.

To determine whether such rotation has a physiological role, it was important to extend these studies to membrane-bound F_0F_1. Hybrid F_1 containing radiolabeled or epitope-tagged β subunits in the two noncross-linked positions was found to bind to F_1-depleted membranes and to restore coupled functions upon reduction of the disulfide bond. Again, an ATP-hydrolysis-dependent rotation of γ was observed. Furthermore, rotation of γ within F_1 was blocked when F_0 was modified by DCCD (Zhou *et al.*, 1996). This suggests that rotation in F_1 is obligatorily coupled to rotation in F_0 as an integral part of the coupling mechanism.

Many interesting details of rotational coupling need further clarification and confirmation. It will be of interest to determine whether rotation is strickly sequential, and whether the direction of rotation reverses when changing from ATP synthesis to ATP hydrolysis. It will be important to measure the rate of subunit rotation in order to confirm that it is fast enough to be an intermediate in the coupling process. Also, a direct demonstration of rotation in the F_0 sector would provide additional compelling evidence for a rotary-type mechanism (Fig. 1b). Finally, a satisfactory understanding of the overall process will likely require a high-resolution structure for F_0.

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