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The Rotary Mechanism of the ATP Synthase

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Abstract

The F_0F_1 ATP synthase is a large complex of at least 22 subunits, more than half of which are in the membranous F_0 sector. This nearly ubiquitous transporter is responsible for the majority of ATP synthesis in oxidative and photo-phosphorylation, and its overall structure and mechanism have remained conserved throughout evolution. Most examples utilize the proton motive force to drive ATP synthesis except for a few bacteria, which use a sodium motive force. A remarkable feature of the complex is the rotary movement of an assembly of subunits that plays essential roles in both transport and catalytic mechanisms. This review addresses the role of rotation in catalysis of ATP synthesis/hydrolysis and the transport of protons or sodium.

Keywords

ATP synthase; kinetic mechanism; rotation; transport

Like many transporters, the F_0F_1 ATP synthase (or F-type ATPase) has been a fascinating subject for the study of a complex membrane-associated process. The ATP synthase is a critically important activity that carries out synthesis of ATP from ADP and P_i driven by a proton motive force, $\Delta\mu_{H^+}$, or sodium motive force, $\Delta\mu_{Na^+}$. This final step of oxidative or photo-phosphorylation provides the vast majority of ATP in the cell. The proton or sodium motive force is also needed to power other membrane processes such as secondary transporters or in the case of bacteria, flagellum rotation. In anaerobic conditions, facultative bacteria use the ATP synthase as an ATP-driven H^+ or Na^+ pump to generate the $\Delta\mu_{H^+}$ or $\Delta\mu_{Na^+}$ (see [1] for a textbook review.) The F_0F_1 complex is nearly ubiquitous in the cell membranes of eubacteria, in the thylakoid membrane of chloroplasts, and the inner membrane of mitochondria. The transporter has remained structurally and mechanistically conserved, except for a few additional domains or subunits in mitochondria, which may play roles in regulation or assembly.

Many years of innovative biochemical, genetic, kinetic, and thermodynamic studies led to the first structural solution of the catalytic F_1 portion of the complex by Walker, Leslie and co-workers [2] in 1994. This landmark structure provided critical information on the catalytic portion of the complex but the subunit arrangement of much of the rest of the complex was still not elucidated. The partial F_1 structure, which at the time was the largest asymmetric unit solved, provided the impetus and the structural information needed to test the notion that the

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transporter was a rotary motor. Boyer earlier hypothesized that rotation of one or more subunits was an intrinsic part of the catalytic mechanism [3] and this idea had been debated for years. Investigators used various biochemical or spectroscopic approaches to address this question (for example, [4,5]). These approaches produced results that were clearly consistent with a rotational model but skepticism remained. The elegant single particle studies by Yoshida, Kinoshita and co-workers first published in 1997 [6] provided direct visual evidence that ATP hydrolysis drove the rotation of the centrally located γ subunit relative to the $\alpha_3\beta_3$ complex (Fig. 1). Finally, the visual evidence of the fluorescent actin filament rapidly spinning in an ATP hydrolysis-dependent manner (see [6] and <http://www.res.titech.ac.jp/~seibutu/> for the experimental set up and video) was powerful enough to convince almost all (see Ref. [7] for an opposing view.)

Suddenly, the quaternary arrangement of the subunits needed to be understood in terms of a rotary machine (see [8–10] for reviews). Investigators hypothesized that the transport mechanism was also rotational and that energy was transferred between the two functions *via* torque on the rotor. This idea implied that there must be rotor and stator elements in both the catalytic F_1 sector and the transport F_O sector. If so, then there must be two linkages between the sectors, one to connect the rotor elements, and the other to connect parts of the stator. There was already structural evidence that the central stalk rotor consisted of the γ , ϵ and c subunits (reviewed in [9]). Wilkens and Capaldi [11] quickly found evidence in electron micrographs of negatively stained *E. coli* complex that there was indeed a “second stalk” at the periphery of the complex, which was then shown to consist of the F_O b subunits and the F_1 δ subunit. At least on a gross level, we finally understood the role of each subunit in the complex.

Because of the large size, multiple subunits many of which are integral membrane proteins, and asymmetry, determination of the subunit stoichiometry and defining subunit interactions has been challenging. There is still some debate about the number of c subunits and whether this number can vary within a complex. The eubacterial ATP synthase contains at least 22 subunits and eight different polypeptides with a total molecular mass around ~530,000 Daltons. Thirteen of the subunits consisting of three different polypeptides are integral membrane proteins, which require non-ionic detergents for solubilization. In *Escherichia coli* ATP synthase, which will be the focus of this review, the F_O contains one a , two b , and about 10 c subunits (Fig. 1). The soluble F_1 sector has three each of α and β subunits, which have the same fold [2], and one copy each of γ , δ and ϵ . The total molecular mass is 380,000 Daltons for the F_1 portion of the complex and it can be easily and reversibly dissociated from the F_O and the membrane. In the case of *E. coli*, all of the subunits must be present to reconstitute F_1 back onto F_O [12]. Of course, the F_1 catalytic domain is coupled to transport and can carry out net ATP synthesis only when bound to F_O . By itself, F_1 in solution is only an ATPase, while the F_O by itself acts as a passive proton carrier.

With consideration of the rotational mechanism of the transporter, the “rotor” assembly in *E. coli* includes the F_1 γ and ϵ subunits, and the F_O c subunits, which form a ring within the plane of the membrane bilayer. In mitochondria, there is an additional subunit in the rotor, which unfortunately is called “ ϵ ” and has no equivalent in bacteria or chloroplasts (Table I). The mitochondrial “ δ ” subunit is the equivalent of the bacterial “ ϵ ”. The “stator” complex includes the three α and β subunits alternating in a pseudo hexamer and the single copy of the F_O subunit a . The catalytic $\alpha_3\beta_3$ complex is connected to the transport mediating subunit a by the “peripheral stalk,” which in most bacteria is made up of two copies of the helical b subunits and the single δ subunit (reviewed in [13]). In some photosynthetic bacteria and chloroplasts, the two b subunits are similar but are the products of two genes. In the mitochondrial complex, there are four additional membranous subunits, e , f , g and A6L associated with subunit a , and two additional subunits in the peripheral stalk, d and F_6 (see [10] for a review.)

The stalk must hold the stator elements together against the torque generated during the rotational catalytic or transport cycles. In *E. coli*, subunit *a* interacts with the *b* subunits mostly through their single transmembrane segments and parts near the membrane [14,15], while the δ subunit (the ortholog of the mitochondrial OSCP or oligomycin sensitivity conferral protein) interacts with the amino termini of the α subunits at the “top” of the complex, which is the side away from the membrane (Fig. 1; [16,17]; reviewed in [10,13]). The affinities between the F_1 and δ subunit, and *a* and *b* subunits appear to be sufficiently strong to withstand the considerable torque that has been measured in the single particle experiments [6]. Interestingly, the strength of association between the carboxyl termini of the *E. coli b* subunits and δ appears much weaker, but the results may be due to the experimental system. Direct association of the *b* subunits with the “side” of the α/β subunit hexamer may contribute to the strength of the peripheral linkage (Fig. 1; reviewed in [13]). In addition, the structure of the helical *b* subunits appears to be flexible. Cain and co-workers found that complexes with limited deletions of up to 11 residues or extensions up to 14 residues retained function [18,19].

Overall, the structure of the ATP synthase is well conserved as is its mechanism. The following sections of this review will highlight features of the structure but also point out what we do not know. Parts of the ATP synthase complex are still lacking high resolution structural information, in particular the membranous F_0 sector. There is no high resolution structure of the complete *a*, *b* or δ subunits, or of the interactions between the two *b* subunits and δ . There are partial structures such as the amino terminal transmembrane segments of *E. coli* subunit *b* by NMR [20]; a crystal structure of the central region of the *E. coli* subunit *b* which is believed to be responsible for homodimerization [21]; the amino terminal half of δ by NMR [22]; and the amino terminal domain of δ in complex with a peptide representing the amino terminal region of α subunit [16,23]. Even though we can piece together these fragments to generate a useful model, there are many reasons to obtain structures of entire complexes from different sources. For example, the vast majority of mutagenic analyses have been done on the *E. coli* complex but there is no high resolution structure of the complex from this species as yet. Although crystals were obtained for the F_1 sector, they only diffracted to 4.4 Å resolution [24]. Moreover, only a few structures representing different states of the catalytic domain have been obtained (see [25] for discussion). Many more must be obtained to understand the molecular features of the rotational mechanism.

Rotation in the catalytic mechanism

The direct observation experiments show that the rotation is always in the same direction with ATP hydrolysis [6]. With only rare reversals, the γ subunit rotates counter-clockwise when observed from the “bottom” (the side closest to the F_0 and the membrane) of the α and β pseudo-hexamer. The obvious corollary is that rotation must be in the opposite, clockwise direction, during ATP synthesis. This conjecture was proven by mechanically forcing a clockwise rotation of an attached magnetic bead with an arrangement of magnets [26]. In this clever experiment, ATP synthesized was monitored by light produced from a luciferin-luciferase system.

These single particle experiments were done with the minimal catalytic unit, $\alpha_3\beta_3\gamma$, whose structure was solved in several different conditions by Walker, Leslie, and co-workers. The first structure [2] of bovine heart mitochondrial enzyme modeled almost all of the α and β subunits but only about one third of the γ subunit was found in the electron density map. A later structure in which the β subunits were modified with dicyclocarbodiimide, resolved the remainder of the γ subunit along with the mitochondrial δ (equivalent of the *E. coli* ϵ subunit) and ϵ [27]. The α and β subunits are homologues and have nearly identical folds [2]. There is a nucleotide binding site within each subunit with some residues contributed from the adjoining subunit (Fig. 2 and 3). The three sites primarily in the β subunits carry out ATP synthesis and

hydrolysis and, as will be discussed below, all three participate in steady state rotational catalysis. The sites primarily in the α subunits are non-catalytic and exchange bound nucleotide very slowly (see [28–30] for reviews). The terminal regions of the γ subunit form a long coiledcoil, which penetrates into the core of the α/β pseudo-hexamer (Fig. 2). The central location the γ subunit and its obvious resemblance to a camshaft stimulated investigators to develop approaches that would demonstrate rotation. The middle portion of the γ subunit complexes with the mitochondrial δ and ε subunits to form the remainder of the F_1 central stalk (or in the case of bacteria, only the ε subunit) and interacts with the c subunit ring to form the complete rotor. Specific interactions between rotor and stator, and among the rotor subunits, γ , ε and c , play important roles in efficient coupling between transport and catalysis (see [31] for a review.)

Studies of the catalytic mechanism have been extensively covered in many excellent reviews (see [9,29,32–34]). Here, we will only discuss the more recent experiments which have brought us to our current understanding of the three site, rotational catalytic mechanism.

A distinct feature of the initial F_1 crystal structure is the different conformations of the β subunits and their catalytic sites (Fig. 2 and 3). Abrahams et al. [2] designated the nomenclature of the sites based on the nucleotide bound to that conformation: the site with the non-hydrolyzable ATP analog, 5'-adenylyl β,γ -imidodiphosphate (AMPPNP) was the β_{TP} site, the site with ADP was the β_{DP} site, and the site devoid of nucleotide was β_E . In a recent 1.9 Å structure from crystals made in the absence of azide, AMPPNP was bound to both β_{TP} and β_{DP} sites [35]. The authors believe that this structure represents the true ground state intermediate of F_1 . In both structures, the β_{TP} and β_{DP} sites are closed while the β_E site is open to solvent (Fig. 3). As suggested by the different nucleotide-bound forms, all F_1 structures have an inherent asymmetry in the β subunit conformations, which are defined by the γ subunit. As the γ subunit rotates, each β subunit switches conformation depending on the face of γ it contacts (Fig. 4). Using single particle experiments, Yasuda et al. [36] showed that the γ subunit rotation tended to dwell at 120° intervals when in conditions of low ATP concentration. One may conjecture that the three dwell positions represent low energy ground states, which may correspond to the conformations observed in the ground state crystal structure reported by Bowler et al. [35]. Each catalytic site would transition through the three conformations during a 360° rotation, and a different site would complete its cycle every 120° rotation. This model implies that three ATP are hydrolyzed or synthesized for each 360° rotation. Furthermore, because the direction of rotation is known, the order of the conformations is $\beta_E \rightarrow \beta_{TP} \rightarrow \beta_{DP}$ in hydrolysis (counter-clockwise rotation) and $\beta_E \rightarrow \beta_{DP} \rightarrow \beta_{TP}$ in the direction of synthesis (clockwise).

Partial reaction and rotation steps in steady state

The next challenge is assigning the conformations to specific partial reactions of the catalytic cycle and how the partial reactions are linked to rotation. Yasuda et al. [37] added high-speed imaging capabilities to their single particle experiments to enable detection of sub-steps of ATP-driven γ subunit rotation. The γ subunit was tagged with a 40 nm-fluorescent bead, which was small enough not to impede motion. With saturating ATP of 2 mM, full speed rotation of about 130 revolutions per second was observed which corresponded to a k_{cat} for ATP hydrolysis of 390 s⁻¹. In addition, the high speed imaging system was able to resolve a short dwell between 90° and 30° partial steps (later refined to 80° and 40°, Ref. [38]). The rates of movement through the individual 80° and 40° steps were fast and appeared to be constant regardless of the ATP concentration. The overall rate of the rotation, and therefore the steady state ATPase activity, was dependent upon the average length of the dwell before the 80° rotation. The length of this dwell was ATP-dependent which suggested that it represented the ATP binding step. In

contrast, the dwell before the 40° rotation was independent of substrate concentration. This point will be further discussed below.

The next question is dissecting how rotation steps are coupled to the enzymatic steps. While the single particle experiments have revealed a tremendous amount of information about the behavior of the rotation, it is difficult to correlate the steps of ATP hydrolysis/synthesis, and the release of products relative to the rotation steps. As described in the previous paragraph, the one step that was accessible to the direct observation was substrate binding. In ATP synthesis, one may assume that energy is transferred to and from transport by rotation of the γ subunit, which drives conformational changes in the catalytic β subunits. Hence, the reaction pathway should be assessed for energy requiring steps that would likely be coupled to conformational changes. The elegant experiments of Boyer and co-workers show that the F_1 catalytic reaction is driven by binding energy [39]. Using ^{18}O isotopic exchange methods, Boyer discovered that the step of chemistry, $\text{ATP} + \text{H}_2\text{O} \leftrightarrow \text{ADP} + \text{Pi} + \text{H}^+$, has very similar rate constants in either direction for hydrolysis and synthesis; therefore, there is little free energy change associated with this step [40]. Rather, the large free energy changes occur with changes of affinity for the binding and release of substrates and products [39]. An extensive analysis of many mutant forms of the *E. coli* F_1 amplified on this model [41]. Replacement of most of the catalytic site residues allowed Al-Shawi, Senior and co-workers to assign the contributions of the relevant amino acids to the energy of binding substrate [42]. Al-Shawi et al. [43] argued that the binding energy gained by the enzyme from the coordination of substrate *via* numerous substrate-protein interactions is the main driving force for catalysis. In ATP synthesis, the energy requiring steps are Pi binding and ATP release, both of which have changes of affinity on the order of 10^6 - 10^7 (see [9] for a review). As will be discussed below, similar changes in affinity are found in the ATP hydrolytic pathway.

The basic catalytic reaction is most easily monitored when using sub-stoichiometric Mg-ATP because this condition avoids some of the complications of the cooperative interactions among the catalytic sites. The first ATP binds with very high affinity, $K_D=0.001$ nM for mitochondrial enzyme and 0.2 nM for *E. coli* F_1 , and is hydrolyzed, but the products, ADP and Pi, are released very slowly [29,44]. Consistent with the ^{18}O experiments, the ratio of the hydrolysis and synthesis rate constants is found to be close to unity [45,46]. This type of catalysis has been termed “uni-site” because only one of the sites is occupied. When excess ATP is added, the rates of product ADP and Pi release are greatly enhanced and the remaining ATP in the high affinity site is completely hydrolyzed and chased off the enzyme. The chase is due to binding of ATP to the other two sites for which the affinities are much lower. This positive cooperative mechanism promotes catalysis 10^5 - 10^6 fold [45,47].

Weber, Senior and co-workers engineered a mutant *E. coli* F_1 in which the β subunit Tyr331 was replaced with Trp [48]. They found that the Trp fluorescence was proportionally quenched as nucleotide bound to the catalytic sites. They used the $\beta\text{Y331W } F_1$ to monitor occupancy of the catalytic sites with the true substrate Mg-ATP and found that the first site was indeed extremely high affinity, the second was intermediate, $K_D \sim 0.5$ μM , and the third was relatively low with a $K_D \sim 25$ μM . Importantly, they found the K_m for steady state ATP hydrolysis was essentially the same as the lowest affinity site indicating that all three catalytic sites must be filled with Mg-ATP in order to promote steady state catalysis. These titrations beautifully show the negative cooperativity of ATP binding by the catalytic sites, which appears to be dependent upon the γ subunit. The enzyme from the Bacillus PS3 strain was found form a stable $\alpha_3\beta_3$ complex lacking the γ subunit, and an x-ray structure showed that this complex was symmetrical [49]. Using the fluorescent nucleotide trinitrophenyl-ATP (TNP-ATP), the $\alpha_3\beta_3$ had a single type of relatively low affinity binding site and very slow steady state activity [50]. Furthermore, the activity was not promoted by binding of nucleotides to the second and

third sites. Therefore, in the absence of the γ subunit, the catalytic sites appeared unable to interact in a cooperative manner to promote steady state catalysis.

Significantly, the K_m for Mg-ATP in the single particle rotation experiments ($\sim 15 \mu\text{M}$; [36]) is also the same as the K_m for steady-state ATP hydrolysis and the K_D for binding to the lowest affinity site on F_1 ($\sim 25 \mu\text{M}$; [48]). Taken together, these results strongly suggest that rotation of the γ subunit only occurs in steady state ATP hydrolysis conditions upon binding of ATP to the low affinity site and all three catalytic sites are occupied with nucleotide. This conclusion is further supported by the lack of effect on uni-site catalysis of a disulfide cross-link connecting β and γ subunits [51]. The rotor-stator cross-linked enzyme has almost no steady state hydrolytic activity, implying that blocking rotation only affects the cooperative steady state turnover.

To assign the partial reactions to the rotation step, Baylis Scanlon et al. [52] used pre-steady state kinetic analysis of the *E. coli* wild-type F_1 to determine each of the resolvable reaction steps. Upon rapid addition of $5 \mu\text{M}$ to $260 \mu\text{M}$ radiolabeled $[\gamma\text{-}^{32}\text{P}]\text{ATP}\cdot\text{Mg}$ to the enzyme, a burst of ATP hydrolysis occurs in the first 10 ms followed by immediate entry into steady state. The extent of the burst is dependent upon the concentration of ATP-Mg added and only occurs in one site. The burst also indicates that the rate limiting step must occur after the step of hydrolysis. Further kinetic constraints are provided by steady state ATPase activities at different substrate concentrations, inhibition of ATPase by ADP, and the rate of ATP-Mg binding determined by fluorescence stopped-flow mixing to monitor the kinetics of ATP association with the three catalytic sites of the $\beta\text{Y331W } F_1$. All of the data are fit with a minimal reaction scheme which is based on the uni-site reaction:

1. $F_1 + \text{ATP} \leftrightarrow F_1 \cdot \text{ATP}$
2. $F_1 \cdot \text{ATP} \leftrightarrow F_1 \cdot \text{ADP} \cdot \text{Pi}$
3. $F_1 \cdot \text{ADP} \cdot \text{Pi} \leftrightarrow F_1' \cdot \text{ADP} \cdot \text{Pi}$
4. $F_1' \cdot \text{ADP} \cdot \text{Pi} \leftrightarrow F_1' \cdot \text{ADP} + \text{Pi}$
5. $F_1' \cdot \text{ADP} \leftrightarrow F_1 + \text{ADP}$

Only one additional step in the kinetic model is needed to fit the data. Step (3) represented the slow rate limiting step which is likely to be a conformational change that follows hydrolysis, Step (2), and precedes Pi release, Step (4). Importantly, the data are accurately fit using the same rate constants for both pre-steady state and steady state phases of the kinetics (see [52] for the full data analysis and derived rate constants).

Several interesting findings become apparent from the kinetic model, which is shown graphically in Fig. 5. First, each of the rate constants is much faster than the uni-site rates, but are consistent with known properties of the enzyme based on previously published experimental data. Second, the apparent rate of ATP binding is slightly slower than diffusion limited. This likely corresponds to the kinetics of the 80° rotation step observed by Yasuda et al. [37], where the length of the dwell before this rotation is found to be ATP-dependent. Third, the ratio of the rate constants for the reversible step of hydrolysis/synthesis, Step (2) $F_1 \cdot \text{ATP} \leftrightarrow F_1 \cdot \text{ADP} \cdot \text{Pi}$, remains close to unity even though the enzyme is in steady state. This is similar to the uni-site mode of the enzyme, and is consistent with the binding change mechanism of Boyer [53,54]. Fourth, the rate limiting step, Step (3), must precede the release of Pi, Step (4). This step, which is termed k_γ , most likely includes the 40° rotation step. The data could not be fit with a model where Pi is released before or at the same time as the rate limiting step, as suggested in the model of Adachi [55] or Ariga [56]. The release of Pi following the rotation step was previously hypothesized by Al-Shawi et al. [44] based on the effects of the uncoupling mutation γM23K [57,58]. Baylis Scanlon et al. [52] argue that one of the effects

of the rotation is to reduce the binding affinity for Pi from a $K_D \sim 1$ mM, to a $K_D > 10$ M. This is clearly one of the energetic steps of the reaction pathway. In the reverse direction of ATP synthesis, the role of the 40° rotation is to create the high affinity Pi binding site. It then follows that the role of the 80° rotation is to reduce the affinity for ATP thus causing its release.

The last question we will address is which catalytic site is carrying out which function. As mentioned above, binding of ATP to the low affinity site ($K_D \sim 25$ μ M) is required for steady state rotational catalysis. Therefore the other sites must also be occupied with nucleotide which is consistent with the observation of Weber et al. [48]. The β Y331W Trp fluorescence reports that the three nucleotide sites are almost completely occupied during steady state catalysis. Using another Trp mutant at position β Phe148, Weber et al. [59] found that the fluorescence characteristics of this Trp was different with bound ADP compared to ATP. They reported that on average, two moles of ADP and one mole of ATP were bound during steady state. This result suggests that the ATP bound during the first 120° rotation, is hydrolyzed to ADP+Pi during the second 120° rotation, and is released during the third rotation. Assuming that the open β_E conformer is the low affinity site and recalling that the order of the conformations in counter-clockwise rotation is $\beta_E \rightarrow \beta_{TP} \rightarrow \beta_{DP}$, then ATP·Mg must bind to β_E . As indicated in Fig. 5 and based on the F_1 structure [25], this conformation appears to be more closed than the β_E conformer and is called half-closed or β_{HC} . With γ rotation, the conformation is changed to β_{TP} where it hydrolyzes ATP; the next rotation changes the conformation to β_{DP} where it holds products; and finally, the site changes back to β_E when it releases Pi and ADP. The site is then ready to bind another ATP·Mg. This model is different from that of Walker and co-workers [25] or Weber et al. [60] where they suggest that reversible hydrolysis/synthesis occurs in the β_{DP} site. They argue that amino acids in this site are closer to bound ATP and are in the proper position to catalyze the reaction, in particular the α subunit residue Arg376 (*E. coli* numbering; see Fig. 3), which has been called the “arginine finger” of the F_1 enzyme [61]. Such an Arg residue is essential for catalysis in the small Ras-like GTPases and is provided by the GTPase activating proteins, or GAPs [62]. The Arg is believed to stabilize the transition state thus greatly accelerating the reaction.

Le et al. [63] also showed that α Arg376 can be replaced and the F_1 enzyme retains the ability to carry out uni-site catalysis; however, the enzyme had essentially no cooperative steady state ATPase activity which involves all three sites. These results suggest that α Arg376 may also be important to create the high affinity Pi binding site as β_E converts to β_{DP} during ATP synthesis (Fig. 5). In doing so, the α Arg376 detects Pi binding and allows γ subunit rotation to occur converting the β_{DP} site with bound ADP+Pi to the β_{TP} site, thus creating the site for the synthesis reaction. In the reverse direction in ATP hydrolysis, α Arg376 maintains the binding of Pi in the β_{DP} site so that it is not released until the catalytic site takes the β_E conformation. This conversion from high to low affinity for Pi is critical because this reaction occurs just after the 40° rotation step which is essentially irreversible in the absence of the F_O and the proton motive force. This model has been reinforced by the findings of Mao and Weber [64] using a fluorescence energy transfer approach. They find that the distances are most consistent with the high affinity site, or the uni-site, being the β_{TP} conformer.

Rotation in the transport mechanism

Using direct observation of single particles, Sambongi et al. [65] and Pänke et al. [66] demonstrated that the c subunit ring rotates with the γ subunit when the $F_O F_1$ complex was anchored by the F_1 α and β subunits. Similarly, the Futai laboratory showed that the α and β subunits rotated when the complex was attached to the substrate through affinity tags on the amino-termini of the F_O c subunits [67]. These results strongly suggest that transport also involves a rotational mechanism and that transport and catalysis are coupled by the rotor subunits. The final proof of rotational coupling is driving rotation by a proton motive force

and observing the synthesis of ATP from ADP and Pi. Zimmermann et al. [68] generated such a result with a sophisticated fluorescence resonance energy transfer system which monitored the position of the γ subunit in the membrane bound complex. When an electrochemical gradient of protons was formed, rotation in the direction opposite that of hydrolysis was detected.

The one part of the F_0 sector for which considerable structural information is available is the c subunit ring. In all examples, the hydrophobic subunit consists of a hairpin of two helical trans-membrane segments connected by a short hydrophilic loop on the cytoplasmic or matrix surface facing the F_1 sector. The essential carboxylic acid (usually a glutamate except in some bacteria which have an aspartate) is near the middle of the carboxyl terminal trans-membrane helix. The c subunit was purified by organic solvent extraction of the membranes, and the first structural information of the *E. coli* subunit was obtained by NMR spectroscopy of the monomeric polypeptide in chloroform:methanol:water [69]. Structures were solved at different pH, which suggested a conformational difference depending on protonation of the conserved carboxylic acid [70].

The quaternary arrangement of the c subunit ring has been observed in examples from chloroplast and some bacteria by atomic force and electron microscopy [71–73] as well as from yeast [74] and the bacterium *Ilyobacter tartaricus* [75] by crystallography. The number of the c subunits in the ring can be variable ranging from 10–15 depending on the source (reviewed in [76]). The number of c subunits is important because it implies the stoichiometry of ions per ATP hydrolyzed or synthesized. Assuming that one ion is carried per c subunit, as was observed in the Na^+ transporting *I. tartaricus* crystal structure [75], then the number of ions transported per 360° rotation is equal to the number of c subunits. Because three ATP are hydrolyzed or synthesized per rotation as discussed above, the number of c subunits divided by three equals the coupling ratio and implies the $\Delta\mu_{\text{H}^+}$ or $\Delta\mu_{\text{Na}^+}$ required for ATP synthesis (see [1]).

There is relatively little structural information on subunit a but the topology of the *E. coli* subunit has been carefully studied by labeling of cysteine mutants [77,78]. There are likely five transmembrane segments, some of which interact with the ring of c subunits to create the pathway for H^+ or Na^+ . In current models of rotational transport [79–82], H^+ are guided to the subunit c carboxylic acid near the center of the bilayer *via* pathways created by the interface between subunit a and the c subunit ring on the cytoplasmic half of the membrane, or within subunit a on the periplasmic half of the membrane [83,84]. In order to couple rotation direction to the direction of proton flow, the pathways from either side of the membrane lead to adjacent c subunits. The pathway from the outside (the space between inner and outer membranes of mitochondria or the periplasm of bacteria) leads to the c subunit that is clockwise (as viewed from the outside; see Fig. 1) of the c subunit that is accessed from the inside (the matrix space in mitochondria or the cytoplasm of bacteria). In the presence of a $\Delta\mu_{\text{H}^+}$, the flow of protons is through the outside pathway to protonate the c subunit carboxylic acid. The acidic group now neutralized is allowed to rotate clockwise (again as viewed from the outside). This c subunit rotates until it again encounters the subunit a interface and deprotonates, releasing the proton to the inside pathway. A critical feature of this model is a positive charge positioned on the stator subunit which forces deprotonation of the c subunit carboxylic acid and prevents short circuiting of protons directly between the two pathways. The conserved $a\text{Arg210}$ (*E. coli* numbering) likely plays this role within the hydrophobic core of the bilayer. This residue is the first and only amino acid in subunit a that is essential for coupled transport [85]. Consistent with this model, the $a\text{Arg210}$ to Ala mutant has no ATP-dependent H^+ pumping even though the F_0 sector still appears to translocate H^+ after the F_1 sector is stripped off the membranes [86].

As discussed above, H⁺ translocation is carried out by the interactions between the ring of *c* subunits and subunit *a*. While there is biochemical data and molecular modeling that indicates how the subunits interact, it is clear that a high resolution structure of the entire sector will be needed to decipher the molecular mechanism of transport.

Perspectives

While genetic diseases involving the ATP synthase are relatively rare (see [87] for a review), there has been tremendous interest in this complex for many years. Its central role in metabolism and bioenergetics has always made it an important research topic and the more recent discovery of its rotary mechanism opened the field to new areas of motor proteins and single particle analyses. The most important question now is elucidation of the transport mechanism. High resolution structures of complete F_O complexes will lead to a molecular understanding of H⁺ translocation, its regulation, the subtleties that lead to differences in stoichiometry, and the molecular determinants for distinguishing H⁺ versus Na⁺. Another question which is very important, but will be much more difficult to dissect, is the matter of coupling efficiency. While the crystallographic structures provide critical information on the interactions of the subunits and suggest the possible dynamics in conformational states, the static structures do not tell the whole story. In such a dynamic system as the ATP synthase complex, extensive conformational studies must be done in a kinetic mode so that different enzymatic and transport states can be correlated to structural states. Some such states have been elucidated for the catalytic F₁ sector [2,25,88] but there is a disturbing lack of differences in the structures. It is likely that the conditions used to achieve crystallization are not able to lock the complex into states that accurately mimic true catalytic intermediates during rotational catalysis, especially the unstable high energy intermediates. Unfortunately, no adequately diffracting crystals of the complete *E. coli* or Bacillus PS3 complex have yet been obtained. The vast majority of mutagenic analyses have been done on these enzymes and high resolution crystal structures would be invaluable in interpreting the effects of amino acid substitutions.

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Fig. 1.

Overall subunit organization and structure of the ATP synthase complex (see text for references). The F_1 is based on the structures of Stock et al. [74] and Gibbons et al. [27] (α yellow, β red, γ blue, ϵ brown, and c subunits (black lines) within the membrane (the surfaces of which are indicated by the green rectangles). The subunits whose structures are not completely known are shown graphically. The transport essential carboxylic acid of the c subunits are indicated by the “-” symbols in red circles. The proposed H^+ or Na^+ channels are indicated by the yellow (outwards) and green (matrix or cytoplasmic) cylinders, with the conserved and essential a subunit Arg residue indicated by the “+” in blue.

Fig. 2. Ribbon diagram of the bovine heart mitochondrial F₁ based on [2]. A. From the “bottom”, or membrane facing side, of the F₁ complex. α subunits are in gray, β_{TP} in yellow, β_{DP} in red, β_E in blue, and γ in the surface model showing electrostatic potential (blue is negative, red is positive and white is apolar). B. From the “side” of the complex showing only the γ subunit in relationship to two of the β subunit conformers, β_{DP} (red) with bound ADP (in CPK colors), and β_E (blue). Note that the lower portion of the β_E subunit is swung outwards which results in an open conformation of the nucleotide binding catalytic site.

Fig. 3.

Key residues contributing to the three catalytic sites. A. Overlay of the residues in β_{TP} (in CPK colors) and β_{DP} sites (in yellow). Bound ATP or ADP and Mg^{2+} are in black. B. The same amino acids in β_E . Note the difference in positions 32 of certain amino acids, in particular α Arg376 (*E. coli* numbering) and α Ser347 (red in β_{TP} , green in β_{DP} and β_E), which indicate the more open β_E conformation [2].

Fig. 4.

The rotation of the β subunit conformers during rotational catalysis. In looking at the F_1 from the “bottom” or membrane oriented side of the complex, rotation of the γ subunit is counter-clockwise for ATP hydrolysis, and clockwise in ATP synthesis (see [9] for a review). Note the order of the conformations in hydrolysis is $\beta_E \rightarrow \beta_{TP} \rightarrow \beta_{DP}$, and in synthesis is $\beta_E \rightarrow \beta_{DP} \rightarrow \beta_{TP}$.

Fig. 5.

Model for partial reaction steps during rotational catalysis based on the kinetic model of Baylis Scanlon et al. [52]. The model explicitly denotes the binding of ATP to the site in the β_{HC} conformer [25], the reversible hydrolysis/synthesis occurring in the β_{TP} site, and the release of product P_i and ADP from β_E . Note that the 80° rotation of the γ subunit (eccentric in the middle of the trimer of the β subunit conformers) is associated with ATP binding or release, and the 40° rotation, k_γ , is the rate limiting step. The central blue arrows indicate the counter-clockwise rotation in hydrolysis. Notice that the “offset” indicates a 120° rotation in the counter-wise direction in hydrolysis, or 120° rotation in the clockwise direction in synthesis.

Table I

Subunit nomenclature for the bacterial, mitochondrial, and chloroplast ATP synthase complexes.

	Bacteria	Mitochondria	Chloroplast
F ₁ Stator	α	α	α
	β	β	β
F ₁ Stator Peripheral Stalk	δ	OSCP ^I	δ
F ₁ Rotor, Central Stalk	γ	γ	γ
	ε	δ	ε
		ε	
F _O Stator	a	$a, e, f, g, A6L$	a
F _O Stator Peripheral Stalk	b_2 , or bb'	$b_2, d, F6$	bb'
F _O Rotor, Central Stalk	c_{10-15}	c_{10}	c_{10-15}

^I OSCP, Oligomycin Sensitivity Conferral Protein