Hypothesis

ATP-driven rotation of the γ subunit in F_1-ATPase

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Abstract We present a mechanism for F_1-ATPase in which hydrolysis of MgATP in the high-affinity catalytic site at the α/β interface drives rotation of the γ subunit via conformational changes in the α subunit. During hydrolysis, transition state formation and separation of P_i from MgADP causes movement of portions of α, transmitted via two Arg residues which are hydrogen-bonded to the γ-phosphate of MgATP, αArg376 and βArg182; the latter is also hydrogen-bonded to interfacial α residues between α346 and α349. Changes in α conformation then push on γ, resulting in rotation. Supporting evidence from the literature and from new data is discussed.

Key words: ATP synthase; ATP synthesis; ATP hydrolysis; Rotation; Nucleotide binding

1. Introduction

F_1F_o-ATP synthase catalyzes synthesis of ATP from ADP and P_i, driven by the energy contained in a transmembrane proton gradient. In bacteria, the enzyme also serves to generate a transmembrane proton gradient, fuelled by energy from ATP hydrolysis. ATP synthase consists of two sectors, the membrane-embedded F_o and the peripheral F_1. In the *Escherichia coli* enzyme, F_o has a subunit composition of ab_2c_{10-12}, F_1 has a subunit composition of $\alpha_3\beta_3\gamma\delta\varepsilon$. F_o contains the proton channel, consisting of residues of a and c subunits, F_1 carries the three catalytic nucleotide binding sites where ATP synthesis and hydrolysis occur, located at α/β interfaces (for reviews, see [1–3]; recent short reviews on individual topics can be found in [4,5]). The phosphate binding subdomain of the catalytic sites is about 100 Å distant from the center of the membrane [6].

Coupling between ATP synthesis/hydrolysis and proton translocation over such a long distance occurs via a rotational mechanism. In a series of elegant experiments it was shown (a) that ATP hydrolysis leads to rotation of the central γ subunit within the pseudohexagon formed by alternating α and β subunits [7], (b) that rotation occurs in 120° steps, probably at the expense of one molecule of ATP per step [8], and (c) that, in addition to γ, the 'rotor' consists of ε and the ring of c subunits [9–11]. The $\alpha_3\beta_3$ hexagon seems to be connected to the a subunit via δ and b_2, forming the 'stator', so that ATP-driven rotation of $\gamma\varepsilon c_{10-12}$ leads to sliding of c subunits across a and to proton translocation [12–14]. Conversely, it is assumed that in ATP synthesis, proton translocation across the a/c interface generates rotation of $\gamma\varepsilon c_{10-12}$, and rotation of γ forces the β subunit to open and release ATP pre-formed at the catalytic site [2]. However, due to the more complex set-up required under ATP synthesis conditions, none of these assumed features has yet been demonstrated experimentally [15].

Even in hydrolysis direction, the molecular basis of the rotary mechanism is not yet understood. Of special interest are the subunit interactions at the two coupling sites where chemical energy (ATP hydrolysis) is converted into mechanical energy (torque) and back into chemical energy (transmembrane proton gradient). As the catalytic nucleotide binding site is more than 20 Å away from γ, energy transformation at the first coupling site requires ATP-driven conformational changes in the α and/or β subunits, which are then transmitted to γ. Based on the crystal structure [16], one prime candidate for such a transmission site between α/β and γ appeared to be a conserved loop of mainly acidic amino acid residues (DELSEED) in the C-terminal domain of the β subunit, residues βAsp380 to βAsp386, which can form a number of hydrogen bonds with residues in γ. Mutational analyses seemed to support this notion, as an extra hydrogen bond in the γM23K enzyme was found to cause a loss in coupling efficiency [17]. However, a more recent study casts serious doubts on the functional importance of the interaction between this β loop and γ. In a mutant where all five acidic residues were replaced by alanine (AALSAAA), ATP hydrolysis generated the same torque of γ as in the wild-type enzyme [18].

In the present paper, we present the hypothesis that ATP hydrolysis causes conformational changes in the α subunit at the catalytic site α/β interface. These conformational changes in α are then transmitted to γ, resulting in rotation. Bases for this hypothesis are: (a) mutational analyses of residues at the catalytic α/β interface, most notably αArg376 [19,20], βArg182 [21], and αSer373 (this paper); (b) the fact that the catalytic transition state forms at this interface, and there are clear indications from other enzyme systems, e.g. myosin, G proteins, P glycoprotein, that protein movements engendered by transition state formation and collapse can generate long-range conformational effects (reviewed in [22]); (c) the consideration that energy realized when MgATP binds to the low-affinity catalytic site (K_{d3} = 100 μM) is insufficient by itself to drive full rotation.

2. The catalytic mechanism and the structure of the catalytic site

The three catalytic binding sites show large differences in

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their affinity for MgATP, with $K_{d1} \leq 1$ nM, $K_{d2} = 1$ μM, and $K_{d3} = 100$ μM [1]. If only high-affinity site 1 is occupied by substrate MgATP, both hydrolysis and product release are very slow ('unisite' catalysis). To achieve physiologically relevant hydrolysis rates, all three catalytic sites have to be filled ('multisite' catalysis) [1], although it is generally assumed that catalysis itself occurs only in the high-affinity site. In multisite catalysis, at one step of the catalytic process the three sites switch their affinities in a synchronized manner ('binding change mechanism' [23]). This affinity switch appears to coincide with the rotation of γ. In contrast, unisite catalysis can occur without rotation of γ [24].

Mutational analysis has identified a number of residues in the environment of the γ-phosphate of bound MgATP that are crucial for catalysis (reviewed in [25]). βThr156, βGlu185, and βAsp242 coordinate the essential Mg^{2+} ion in MgATP and MgADP complexes [26]. βGlu181 aligns and polarizes the hydrolytic water molecule for the in-line nucleophilic attack on the γ-phosphate and assists in stabilization of the catalytic transition state [25,27]. βLys155 and βArg182 contribute to binding of the γ-phosphate of MgATP and to stabilization of the catalytic transition state [21,27]. Although in the crystal structures [16,28] the guanidinium group of αArg376 is close to the phosphates of bound nucleotide, this residue does not contribute to nucleotide binding in the ground state [19], only to transition state stabilization, and this only in multisite [19], but not in unisite catalysis [20]. αArg376 acts as an 'arginine finger', stretching across the α/β interface to complete the multisite transition state, and thus bringing about positive catalytic cooperativity [22].

3. Hypothesis

Fig. 1 illustrates our hypothesis for ATP-driven rotation of γ. The figure shows the events that occur as catalysis proceeds at the high-affinity catalytic site one at the α/β interface. Starting at *State 1*, with β in pink, α in green and γ in blue, the site is occupied by MgATP, and several interfacial amino acid residues of functional importance are highlighted, namely αArg376, βArg182, and the stretch from αIle346 to αAsp350. As mentioned above, αArg376 is not involved in MgATP binding at this stage, but βArg182 is. βArg182 not only hydrogen-bonds the γ-phosphate of MgATP, but is also capable of forming several hydrogen bonds with α residues, especially with the main-chain oxygens of residues αIle348 and αThr349 [21,22].

Binding of MgATP to the low-affinity site three (which is not depicted in Fig. 1) 'promotes' catalysis in site one (positive catalytic cooperativity [15]). We suggest it does so by causing conformational changes in the α subunit at the high-affinity site. Such changes could be triggered by a partial rotation of γ upon MgATP binding at site three, as suggested in *State 2* of Fig. 1. (Alternatively, they might be transmitted directly from the low-affinity site to the high-affinity site around the α/β ring.) An important result of the conformational movement of α is a close approach of the side chain of αArg376 towards the high-affinity site-bound MgATP. This enables hydrogen bond formation between the guanidinium group and γ-phosphate oxygen (*State 2*), which in turn allows hydrolysis to proceed at a physiological multisite rate.

State 3 shows the catalytic transition state, where the γ-phosphate has started to separate from the β-phosphate and to move towards the attacking water molecule (red sphere). Conformational changes associated with this movement are transmitted to the α subunit, by (at least) two mechanisms, namely directly to αArg376, and indirectly, via βArg182, to α346–350. The resulting conformational changes in α 'push' γ, causing its rotation. Several close and possible functional contact regions between the α and γ subunits are evident from the X-ray structure [16], examples being around residues αArg283/αGlu284, αAsp336, αGlu356/αAsn358, αArg401/αGlu402 and αSer411/αAsp402. High-yield crosslinking between the α and γ subunits was observed using the αS411C mutant [29].

Continuation of the hydrolysis reaction results in amplification of the described changes, until *State 4* is reached, where reaction products MgADP and P_i are fully separated. The relevant residues of α are still hydrogen-bonded to P_i and are thus dragged further along the α/β interface to their 'end' position. γ has been pushed further across the final energy barrier, so that it can now complete the rotation step on its own. The conformation in *State 4* (as in *State 2* and *State 3*) is unstable and transient, and P_i is rapidly released. In *State 5*, γ has completed a 120° rotation, and the involved portions of the α subunit have returned to their 'relaxed' positions. The 'binding change' has occurred and has altered the affinities of each of the catalytic sites. Thus, the depicted catalytic site in Fig. 1, which began as the high-affinity site in *State 1*, has now become the medium-affinity site in *State 5* ($k_{off} \approx 1\ s^{-1}$); the bound MgADP will be released after the next 120° rotation step, when this site becomes the low-affinity site ($k_{off} \approx 100\ s^{-1}$). The dwell times seen between 120° rotation steps at low ATP concentration [8] occur in *State 1*, as the empty 'promoting' site waits for MgATP to bind. It may be noted that without rotation of γ, acceleration of the chemical reaction step and of product dissociation steps cannot occur. γ rotation and positive catalytic cooperativity are therefore interdependent.

4. Supporting evidence

4.1. αR376K mutant F_1

αR376Q and αR376K mutant F_1 are both strongly impaired in multisite catalysis. However, in contrast to Gln, Lys is able to stabilize the catalytic transition state, as evidenced by high-affinity binding of MgADP.fluoroaluminate [19]. Thus the Lys mutant can reach *State 3* of Fig. 1, but is unable to support the next step, the transition from *State 3* to *State 4*. In the crystal structures of ground as well as transition states [16,30], the wild-type αArg376 residue is in an elongated conformation; in *State 4*, it will be fully extended. Because Lys is shorter than Arg, the hydrogen bond(s) with P_i in the Lys mutant will break before *State 4* is reached. Thus, the conformational changes in α necessary to push γ over the energy barrier cannot be finalized, and no rotation occurs.

4.2. βR182K mutant F_1

Both βR182Q and βR182K mutant enzymes are also strongly impaired in multisite catalysis, and, while the Gln mutant cannot support formation of the catalytic transition state, the Lys mutant can [21]. Apparently, it is again at the switch from *State 3* to *State 4* where Lys is unable to perform the functions of the wild-type βArg182 residue. As indicated

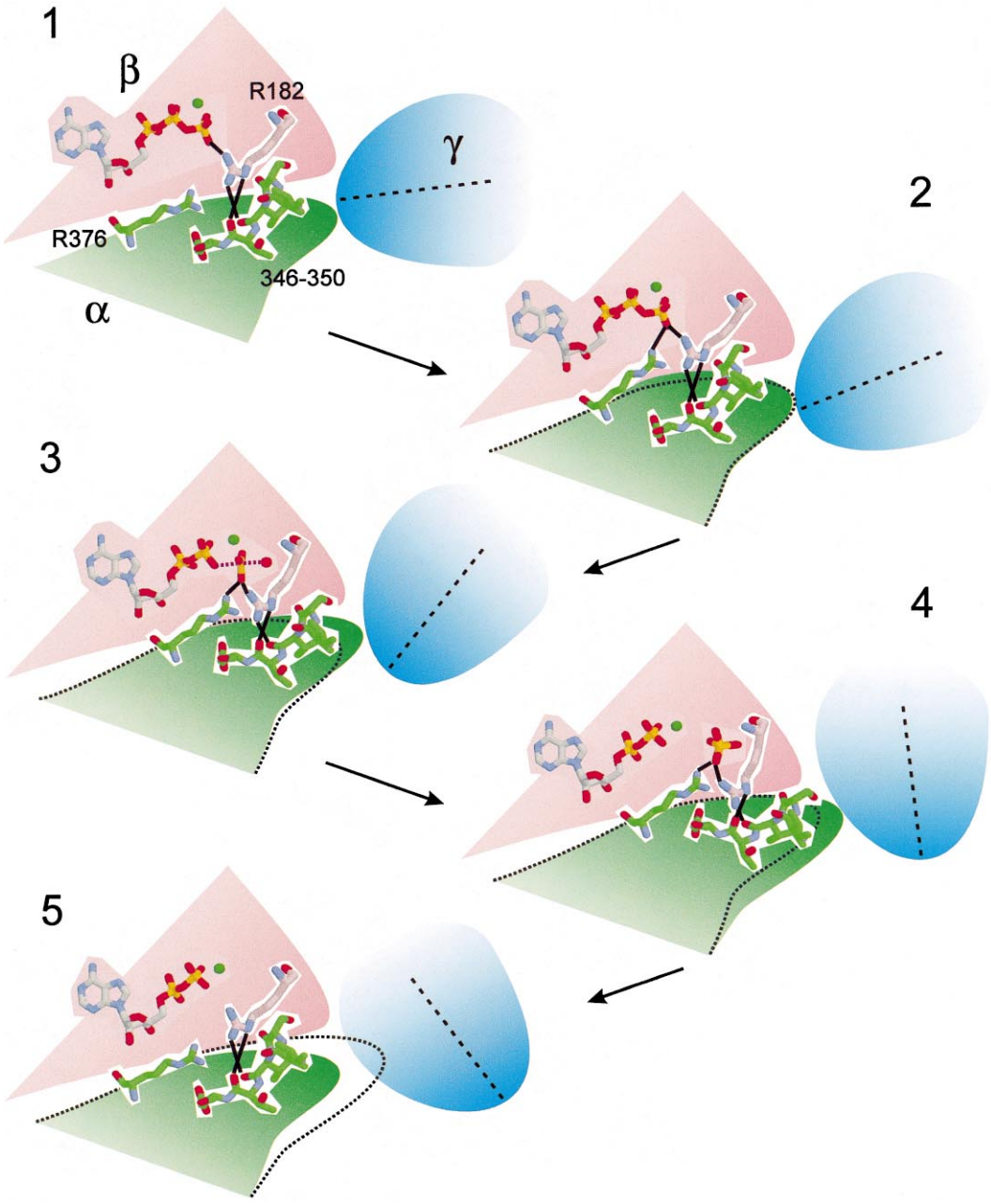

Fig. 1. Hypothesis for ATP hydrolysis-driven rotation of γ subunit in F_1-ATPase. *State 1* shows the high-affinity catalytic site one, with MgATP bound. α subunit is green, β subunit is pink, γ subunit is blue, the Mg^{2+} cofactor is a green sphere. Black solid lines indicate hydrogen bonds relevant to the mechanism. To indicate that α and γ change their relative positions as catalysis proceeds, the black dotted lines in *States 2–5* demarcate the position of α subunit in the immediately preceding *States 1–4*, respectively. The black dashed line indicates the relative rotation of γ. In *State 3* the red sphere is the attacking water. The figure is not drawn to scale. The progression of the MgATP hydrolysis reaction through *States 1–5* is described in Section 3.

above, βArg182 is at the center of a hydrogen-bonding network between the γ-phosphate of MgATP and backbone oxygens of α346–349. Lys has less hydrogen-bonding capacity than Arg (three instead of five from the side chain), and, with one nitrogen atom in the side chain instead of three in the guanidinium group of Arg, its hydrogen bonds cannot cover the same distance. Thus, in βR182K mutant enzyme, the α/β connection is broken at this point, and the α subunit cannot undergo the conformational changes necessary to enforce a 120° rotation of γ. Results obtained with the αR376K and βR182K mutants emphasize that both these α/β interaction points must be preserved to achieve rotation and multisite catalysis. It is very possible that there are points of interaction which have not been identified so far.

4.3. Unisite catalysis experiments

At low MgATP concentrations, when the medium- and low-affinity sites are empty, overall hydrolysis is very slow [1]. In our hypothesis, under such unisite conditions, αArg376 is prevented from assuming its role in stabilization of the catalytic transition state and transmission of conformational changes to α and, subsequently, to γ. This is in agree-

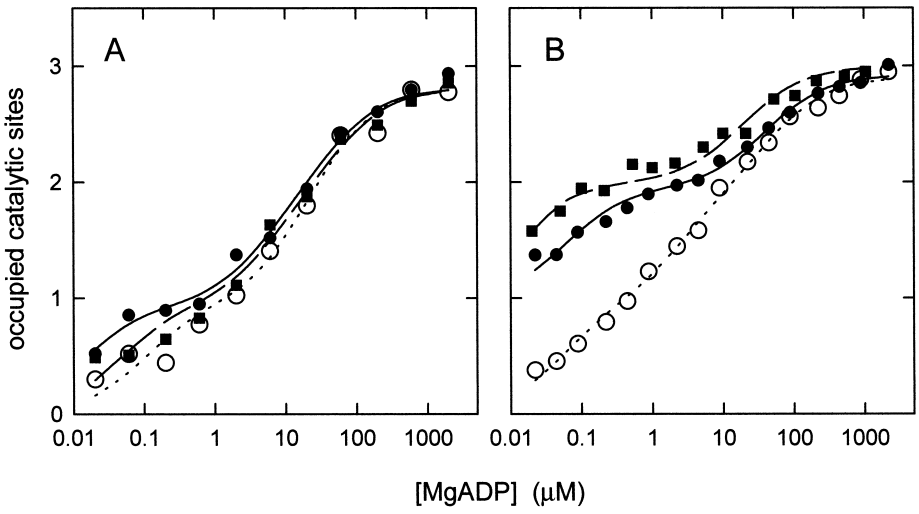

Fig. 2. Binding of MgADP.fluoroaluminate and MgADP.fluoroscandium to F_1 catalytic sites. A: αS373F/βY331W mutant F_1. αS373F/βY331W mutant F_1 is described in [33]. Binding was measured in a buffer containing 50 mM Tris/SO_4, 2.5 mM $MgSO_4$, pH 8.0, using βTrp331 fluorescence as signal [31,32]. ○, MgADP binding in the absence of Al^{3+}, Sc^{3+}, and F^-. ●, MgADP binding in the presence of 1 mM $AlCl_3$ and 10 mM NaF; ■, MgADP binding in the presence of 1 mM $ScCl_3$ and 10 mM NaF. Lines are fits of theoretical curves to the data points, assuming three sites of different affinity. For MgADP alone (dotted line), $K_{d1} = 0.10$ μM, $K_{d2} = 13$ μM, $K_{d3} = 37$ μM; for MgADP.fluoroaluminate (solid line), $K_{d1} = 0.014$ μM, $K_{d2} = 4.9$ μM, $K_{d3} = 41$ μM; for MgADP.fluoroscandium (dashed line), $K_{d1} = 0.044$ μM, $K_{d2} = 6.1$ μM, $K_{d3} = 51$ μM. B: βY331W mutant F_1. βY331W mutant F_1 is described in [32]. These data are taken from [27,31]. Symbols and lines are as in A above. For MgADP alone (dotted line), $K_{d1} = 0.05$ μM, $K_{d2} = 2.5$ μM, $K_{d3} = 47$ μM; for MgADP.fluoroaluminate (solid line), $K_{d1} < 0.001$ μM, $K_{d2} = 0.06$ μM, $K_{d3} = 40$ μM; for MgADP.fluoroscandium (dashed line), $K_{d1} < 0.001$ μM, $K_{d2} < 0.01$ μM, $K_{d3} = 21$ μM.

ment with two recent findings. (A) Replacement of αArg376 by Ala has no significant effect on the hydrolysis step in unisite catalysis; apparently, αArg376 does not participate in transition state stabilization under these conditions [20], making the reaction step much less efficient. (B) Unisite catalysis can occur in an enzyme where β and γ are cross-linked so that rotation is prohibited [24].

4.4. αS373F mutant F_1

αS373F is an example of a group of mutations in α, at the catalytic α/β interface, which allow unisite catalysis while abolishing or strongly impairing multisite catalysis. Other mutations in this group are αG351D and αS375F (see Fig. 9 in [1]). In contrast to the proximate αArg376, none of these affected residues is directly involved in catalysis. What these mutations have in common is that in each case a small amino acid side chain has been replaced by a larger one. Investigation of potential steric clashes indicates that the bulkier side chains could prevent formation of the proper α/β interface necessary to support rotation and multisite catalysis. To deduce at what point α/β interactions might be affected, we investigated the effect of two transition state analogues, fluoroaluminate and fluoroscandium [27,31], on MgADP binding in αS373F/βY331W mutant F_1. The βY331W mutation is present to allow fluorometric determination of binding affinities [1,32,33]. As Fig. 2A shows, fluoroaluminate and fluoroscandium have relatively little effect on MgADP binding affinity in αS373F/βY331W F_1, whereas in βY331W F_1 they have large effects (Fig. 2B). In αS373F/βY331W, affinity at site one is increased about seven-fold by fluoroaluminate, and two-fold by fluoroscandium. With βY331W, binding of MgADP.fluoroaluminate and MgADP.fluoroscandium at site one was so tight that it could not be accurately measured ($K_{d1} < 0.001$ μM), indicating an affinity increase of at least two orders of magnitude, probably much greater. The results show that the αS373F mutation weakens interactions necessary to stabilize the catalytic transition state in multisite catalysis. As unisite catalysis is not impaired [34], this shows that the bulky Phe side chain prevents αArg376 from moving toward the γ-phosphate to optimize transition state stabilization. The mutation αS373F therefore blocks the *State 1* to *State 2* progression in Fig. 1.

4.5. P_i affinity

Under MgATP hydrolysis conditions in the absence of a proton gradient, the affinity of the catalytic site(s) for P_i is very low [1,15]. This is in agreement with Fig. 1, because the only state in which bound P_i is present as a separate species is the transient, unstable *State 4*. In the ground states (*State 1* and *State 5*) at least one of the residues responsible for P_i binding, namely αArg376, is too far removed to fulfill this function. It is important to note that in the presence of a proton gradient, the affinity for P_i is greatly increased [1,15]. One mechanism to achieve this affinity increase would be to bring αArg376 into a position where it is able to support P_i binding. Thus, presence of a proton gradient, by generating partial rotation of γ, could stabilize the catalytic site in a conformation similar to that shown in *State 4*, enabling the enzyme to bind P_i.

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