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Invited review

The molecular mechanism of ATP synthesis by F_1F_0-ATP synthase

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Abstract

ATP synthesis by oxidative phosphorylation and photophosphorylation, catalyzed by F_1F_0-ATP synthase, is the fundamental means of cell energy production. Earlier mutagenesis studies had gone some way to describing the mechanism. More recently, several X-ray structures at atomic resolution have pictured the catalytic sites, and real-time video recordings of subunit rotation have left no doubt of the nature of energy coupling between the transmembrane proton gradient and the catalytic sites in this extraordinary molecular motor. Nonetheless, the molecular events that are required to accomplish the chemical synthesis of ATP remain undefined. In this review we summarize current state of knowledge and present a hypothesis for the molecular mechanism of ATP synthesis.

Keywords: ATP synthesis; Oxidative phosphorylation; F_1F_0-ATP synthase; Molecular mechanism; Catalytic site; Subunit rotation

1. Introduction and goals

ATP synthesis by oxidative phosphorylation is the fundamental means of cell energy production in animals, plants and almost all micro-organisms. A typical 70 kg human with a relatively sedentary lifestyle will generate around 2.0 million kg of ATP from ADP and Pi in a 75-year lifespan. Photosynthesis requires ATP, which is generated by photophosphorylation. ATP synthesis by both photophosphorylation and oxidative phosphorylation occurs on the F_1F_0-ATP synthase enzyme, and is one of the most frequent enzyme reactions in biology.

The direct source of energy for ATP synthesis is an electrochemical gradient of protons (Δp) generated initially by electron transfer complexes across the mitochondrial, chloroplast or bacterial membrane. In some microorganisms a sodium ion gradient substitutes for Δp. Functionally, ATP synthase is tripartite, consisting of (1) a motor in the membrane that converts electrochemical ion gradient energy into subunit rotation, (2) a rotating transmission device, the 'rotor stalk', which transmits the energy over a distance of greater than 100 Å to the catalytic sites, and (3) the catalytic sites, three in number, where the mechanical energy of rotation is converted into the chemical bond between the ADP-O and Pi. ATP synthesis occurs at a maximal rate of the order of 100 s^{-1}, and sustains a concentration of around 3 mM ATP in *Escherichia coli* cells, higher in mitochondria and chloroplasts, without noticeable product inhibition. Unsurprisingly, ATP synthase is considered an extraordinary enzyme. Three special series of reviews may be found in [1–3], and other recent reviews may be found in [4–9].

Most experimental work in this field has studied

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not the ATP synthesis reaction, however, but rather the ATP hydrolysis (ATPase) reaction. This is due to the fact that one sector of the enzyme, the F_1 sector, can be isolated in pure, soluble, form with relative ease, and then studied by a wide variety of techniques. Unfortunately F_1, while providing an excellent experimental model of ATP hydrolysis, does not perform net ATP synthesis. For ATP synthesis, either intact membrane vesicles, or detergent-solubilized, purified F_1F_0 that has been reconstituted into proteoliposomes, must be used. Both systems present technical limitations and difficulties. Knowledge of the ATP hydrolysis mechanism is consequently further advanced. Based on older work, the assumption has entered the literature that ATP synthesis and hydrolysis are reversible by a common pathway, and in considering the mechanism of ATP synthesis, properties of the hydrolysis reaction have often been inferred to be common to the synthesis pathway. However, newer work suggests there may be differences as well [10].

The goal of this article is to summarize the present state of knowledge of the molecular mechanism of ATP synthesis. We draw upon recent high-resolution structural evidence, the emerging body of information on subunit rotation, the extensive mutagenesis literature, and information on energy coupling in other systems. We critically appraise conclusions drawn from ATP hydrolysis experiments in terms of their applicability to the synthesis reaction. Finally we present a hypothesis for the molecular mechanism of ATP synthesis.

2. Overall structure of ATP synthase

F_1F_0-ATP synthases are basically similar whatever the source. In their simplest form in prokaryotes they contain eight different subunits, with stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon ab_2c_{10-14}$ [11,12]. The total molecular size is $\sim$530 kDa. In chloroplasts the structure is the same, except there are two isoforms of *b*. In mitochondria there are 7–9 additional 'supernumerary' subunits, depending on source, but in toto they contribute only a small fraction of additional mass, and may have regulatory roles [13–15]. In former times the structure was described in terms of two sectors, a membranous F_0 (ab_2c_{10-14}) and a membrane-extrinsic F_1 ($\alpha_3\beta_3\gamma\delta\varepsilon$). New data from several laboratories, notably that of Capaldi and colleagues, have refined this model, and a current, now familiar view of the structure is shown in Fig. 1 (based on [16–18]). Electron microscopy and protein cross-linking techniques have figured prominently in deduction of this model. The catalytic core of the enzyme is $\alpha_3\beta_3\gamma$, consisting of a hexagon of alternating α and β subunits with helices of γ in the center. ATP synthesis and hydrolysis reactions occur at three catalytic sites [7]. Proton transport is effected by the *a* and *c* subunits. Current models envisage *c*Asp61 and *a*Arg210 as critical residues,[1] with the *c*-subunit ring rotating relative to the *a* subunit [18–20]. The 'rotor stalk' is composed of $\gamma\varepsilon$, connected firmly to the *c*-ring at the base, and interacting with α and β at the top [6,21,22]. The 'stator stalk' is composed of $b_2\delta$, with δ binding to α-subunit at the top of the molecule [16,23], and b_2, anchored in the membrane by the N-terminal transmembrane helices [24], interacting there with the *a* subunit [25,26].

At the time of writing there remains uncertainty as to the stoichiometry of the *c*-subunits in the complex, with values of 10–14 being reported [21,27–32]. There have been suggestions that different species may differ in *c*-subunit stoichiometry, and that *c*-subunit stoichiometry may be variable depending on metabolic demands [33]. This remains one major unresolved issue regarding overall structure of F_1F_0, others being the structure of subunit *a* and the role and location of the supernumerary subunits in the mitochondrial enzyme.

3. High-resolution structure analysis

3.1. X-ray structures

The first atomic-level (2.8 Å) resolution structure was of bovine mitochondrial F_1 in 1994, and it resolved the $\alpha_3\beta_3$ hexagon together with about half of the γ residues [34]. This structure will be referred to here as 'two-nucleotide' F_1. The α and β subunits each had similar three-domain structure, with an N-terminal β-barrel furthest away from the mem-

[1] *E. coli* residue numbers are used throughout.

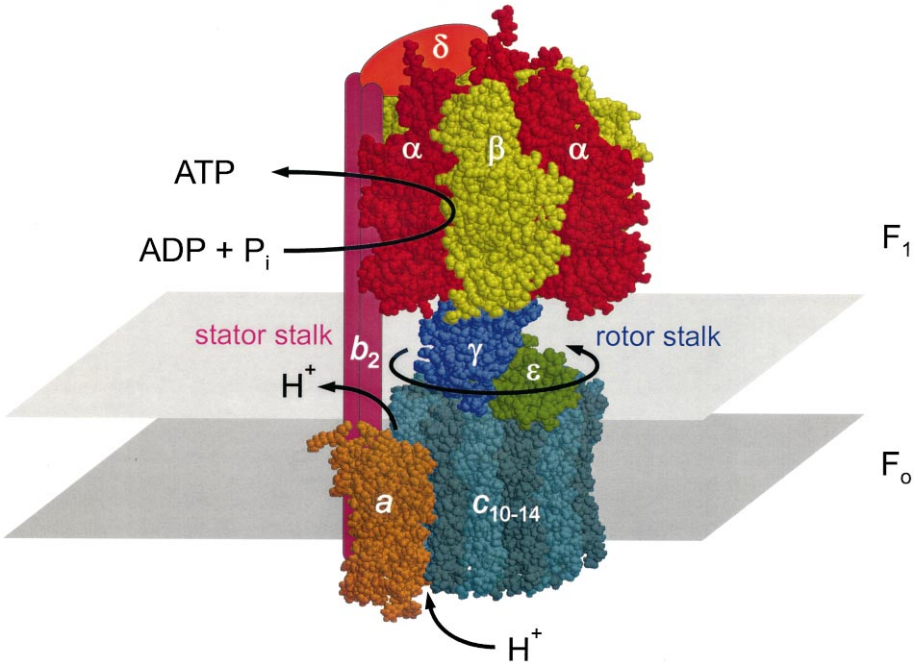

Fig. 1. Structural model of ATP synthase (F_1F_0) from *E. coli*. $\alpha_3\beta_3\gamma\varepsilon$ structure from [41]; ac_{10-14} structure from [20] (note *a* is hypothetical).

brane surface, a central nucleotide-binding domain, and a C-terminal helical domain. The three catalytic sites were located at α/β interfaces, and contained MgADP (the 'βDP site'), MgAMPPNP ('βTP') and no nucleotide ('βE'), respectively. The βDP and βTP subunits appeared in a 'closed' conformation, whereas βE was in an 'open' conformation, in which the C-terminal domain and the lower half of the nucleotide-binding domain were displaced downward, opening up the nucleotide site. Most side-chain ligands from protein to nucleotide in catalytic sites are contributed by β-subunits, with some contribution from α. The three α subunits were designated 'αDP', 'αTP', and 'αE', and share the catalytic site α/β interfaces with βDP, βTP, and βE, respectively. The region of γ resolved consisted mainly of two long helices in a coiled coil. It was evident that the idea of rotation of the γ-subunit within $\alpha_3\beta_3$ was credible, thus the sequential interconversion of the three catalytic sites between different nucleotide-binding conformations induced by subunit rotation, as proposed by Boyer [35,36] was at least possible, and as subsequent experiments have shown, it is real. In addition to stimulating studies of subunit rotation, this structure, by revealing the α- and β-subunit side-chains located close to the nucleotides in catalytic sites, facilitated mutagenesis and mechanistic studies of rate acceleration in catalysis and elucidation of the catalytic transition state.

Subsequently, essentially the same overall structure, with the same arrangement of catalytic sites, was seen in X-ray studies of bovine F_1 inhibited by efrapeptin, aurovertin, and NBD-Cl, and of bovine F_1 containing 1 mol MgADP–trifluoroaluminate complex per molecule [37–40]. The nucleotide content of the catalytic sites in these later structures mirrored that in the original 1994 report, except in the last case, where the βDP site contained MgADP.AlF_3 instead of MgADP. More recently a 'DCCD-inhibited' bovine F_1 structure was resolved at significantly higher resolution (2.4 Å) [41], revealing all of γ-subunit, and all of δ and ε subunits (bovine δ is equivalent to prokaryote and chloroplast ε, bovine ε has no counterpart in prokaryote or chloroplast enzymes). The previously unresolved half of γ was seen to form a substantial protrusion below the $\alpha_3\beta_3$ hexagon, containing a Rossmann fold. δ is bound to one side of this protrusion. Bovine ε has

an elongated structure, and interacts extensively with γ and bovine δ, appearing to be designed to hold them together. DCCD-inhibited enzyme contained MgADP in both βDP and βTP catalytic sites, with βE empty. The conformations of the three sites were the same as in the previous reports, establishing that there is no requirement for occupation of the βTP site by nucleoside triphosphate to produce this asymmetric structure.

An X-ray structure of *E. coli* $\alpha_3\beta_3\gamma\varepsilon$ complex has been obtained [42] at low resolution, which is thought to show an asymmetric arrangement of catalytic sites similar to that of the bovine enzyme, although the bound nucleotides could not be defined. It is hoped that progress can be achieved to higher resolution, to allow structural definition of the many *E. coli* mutant forms that have proved so useful in mechanistic studies.

The 1994 X-ray structure of bovine F_1 and the subsequent similar structures from Walker, Leslie, and colleagues have proved enormously valuable to the field, obviously. Nevertheless it has been puzzling that only two of the three catalytic sites are occupied by nucleotide. There is a large body of biochemical literature, from numerous laboratories, using a variety of techniques, which establishes unequivocally that all three catalytic sites of F_1 are readily filled on addition of mM concentration of adenine nucleotides [4,11]. Our laboratory suggested that use of subsaturating nucleotide concentration during crystallization might be the reason why one catalytic site (βE) remained empty [43]. However, this has now been disproven by the demonstration [44] that addition of saturating concentrations of nucleotide during crystallization does not change the situation: the βE site remains empty. One must surmise that the form of the enzyme that preferentially crystallizes in the presence of saturating adenine nucleotide is the one corresponding to the conformationally immobile ground-state intermediate in the catalytic cycle that occurs immediately after release of product, and thus has one site empty [10].

In the most recent study of bovine F_1 by the Walker group [45], MgADP and aluminum fluoride were present throughout the crystallization procedure, and in the resulting structure, named '$(ADP.AlF_4^-)_2F_1$', all three catalytic sites are filled by nucleotide. The asymmetric arrangement of catalytic sites is retained in this structure, which at 2.0 Å resolution gives the most detailed views to date of the catalytic sites. Both βTP and βDP catalytic sites contain $MgADP.AlF_4^-$ in a transition-state-like complex, and both βTP and βDP subunits adopt a closed conformation. The other catalytic site, called 'βADP+Pi' and corresponding to the position of βE in former structures, contains β-subunit in a 'half-closed' conformation. This site contains MgADP and a sulfate ion which is thought to mimic bound Pi. The $(ADP.AlF_4^-)_2F_1$ structure is thought to mimic a post-hydrolysis, pre-product release state on the hydrolysis pathway, with βADP+Pi ready to open and release products when it changes to βE. Other, significant, conformational changes have occurred in this structure as compared to two-nucleotide F_1, notably in the αE and βDP subunits, and those parts of the γ subunit that are in contact with the C-terminal domains of α and β have rotated by 20°.

Contemporaneously with these X-ray studies, equilibrium nucleotide binding studies of the three catalytic sites have been carried out in our laboratory, using *E. coli* F_1 containing engineered Trp probes, and have revealed four different nucleotide-binding conformations, which we have named 'high affinity', 'medium affinity', 'low affinity', and 'open' [10]. MgATP binds to catalytic sites with three very different binding affinities (K_d), of ~1 nM, 1 μM, and 30 μM [46,47]. MgTNP-ATP binds with three different affinities, of <1 nM, 23 nM and 1.4 μM [48], and in recent work MgADP binding to catalytic sites has also been seen to be satisfactorily accounted by a three-site model, with affinities of 40 nM, 1.8 μM and 35 μM [49]. MgITP binds with K_d values of 0.33 μM, 62 μM and 1.4 mM [50]. In the open site, which can be trapped by reaction with NBD-Cl [39], the affinity for MgATP was very low indeed ($K_d > 10$ mM) [10,47]. Therefore the X-ray structure and functional data concur in showing that the three catalytic sites are strongly asymmetric in presence of Mg-nucleotide. The γ-subunit shows three different faces, one to each α/β catalytic site interface, and is widely assumed to thereby impose a different conformation on each catalytic site.

It is not possible to assess binding affinities from X-ray structures directly. However, the buried surface area at the three catalytic α/β interfaces in

two-nucleotide F_1 is 3030 Å^2 (βDP site), 2200 Å^2 (βTP site) and 1760 Å^2 (βE site) [7], suggesting that these correspond to the high-affinity, medium-affinity and open sites, respectively. The conclusion that the βDP site is more occluded than the βTP site, with functional catalytic groups closer to the nucleotide, supports the notion that βDP is of higher affinity than βTP [45], as does the more optimal Mg^{2+} coordination in βDP [51]. Direct binding studies of *E. coli* F_1 inhibited by MgADP–fluoroaluminate demonstrated that the presence of fluoroaluminate increased affinity for MgADP at the high and medium-affinity sites, but not at the low-affinity site [52]. The half-closed βADP+Pi site in $(ADP.AlF_4^-)_2F_1$ most likely corresponds therefore to the low-affinity site.

Symmetrical forms of F_1 have also been obtained by X-ray crystallography. The $\alpha_3\beta_3$ complex from *Bacillus* PS3 lacking any bound nucleotide showed a symmetrical arrangement of catalytic sites, all similar in structure to βE in bovine enzyme [53]. Rat liver F_1 ($\alpha_3\beta_3\gamma\delta\varepsilon$) was crystallized in absence of Mg^{2+} but with 5 mM ATP [54], and appeared symmetrical in structure. All three catalytic sites contained ADP, two of them also with bound Pi, and all were in a closed conformation. Chloroplast $\alpha_3\beta_3\gamma\varepsilon$ complex, crystallized in absence of Mg^{2+} but with ADP and AMPPNP, also showed a symmetrical structure, with apparently no nucleotide in the catalytic sites, yet with all three sites in a closed conformation [55]. These structures demonstrate the high degree of conformational flexibility of F_1. We suggest that Mg^{2+} ion is a crucial determinant of asymmetry of the catalytic sites. Lack of Mg^{2+} seems to be the common factor in the symmetrical structures described above, and we have previously shown that ATP, ADP, ITP, and TNP-ATP (i.e., uncomplexed with Mg^{2+}) bind to all three catalytic sites in *E. coli* F_1 ($\alpha_3\beta_3\gamma\delta\varepsilon$) with essentially the same affinity, i.e., symmetrically [47,48,50]. Presence of Mg^{2+} appears to enhance catalysis in two separate ways in F_1, by promoting asymmetry of catalytic sites, and by acting as a required cofactor in the chemistry of catalysis. However, it may be noted that in binding measurements made in the absence of Mg^{2+}, the affinity of the three catalytic sites for uncomplexed nucleotide always appeared equal to that of the low-affinity site for Mg-nucleotide, thus the closed conformation of the three sites in the symmetrical rat liver and chloroplast structures is puzzling.

X-ray structures of isolated *E. coli* ε-subunit [56], and of *E. coli* γε complex [57] have also been published. The isolated *E. coli* ε structure was mirrored in the analogous δ subunit in DCCD-inhibited bovine F_1 [41] and in the $\alpha_3\beta_3\gamma\delta\varepsilon c_{10}$ subcomplex from yeast mitochondria [21]. It consists of an N-terminal β-sandwich domain, and a C-terminal helix-turn-helix domain with two antiparallel helices in a hairpin. However, in the γε complex structure, the ε has assumed a markedly different conformation, in which the two helices in the C-terminal domain are wrapped around γ. This conformational switch has been suggested to be relevant to a role of ε as a selective inhibitor of ATP hydrolysis and directional regulator of rotational catalysis [58].

3.2. NMR structures

An NMR structure of isolated *E. coli* ε subunit [59] is in good agreement with the X-ray structure [56]. The structure of the N-terminal domain of *E. coli* δ subunit consisting of residues 1 through 134 was solved also by NMR [60]. Completion of this structure is highly desirable, since functional regions of δ identified by mutagenesis as necessary for energy-coupling [61], and regions of δ located spatially close to subunits *b* and α as identified by cross-linking studies [23,62], unfortunately lie in the C-terminal region between residues 140 and the C-terminal 177. The NMR structure of the *E. coli* *c*-subunit in organic solvent [63] was one of the earliest high-resolution structures of a transmembrane helical protein. *c*-subunit consists of two antiparallel helices connected by a 'polar loop', which interacts with γ and ε [22,64]. Further NMR studies of *E. coli* *c*-subunit have identified two distinct conformations, dependent on pH, and differing drastically in orientation of the C-terminal helix [20]. Modeling of the individual *c*-subunits into a 12-member (or 10-member) ring has been described [65]. This area is not without its controversy, however, since it may be noted that a rather different structure of *Propionigenium modestum* *c*-subunit was determined by NMR in SDS-micelles [66]. The X-ray structure of $\alpha_3\beta_3\gamma\delta\varepsilon c_{10}$ subcomplex from yeast mitochondria [21] is more compatible with the NMR structure of

the *E. coli c*-subunit. Finally the structure of *b*-subunit residues 1–34 has been resolved by NMR, and with supplementary cross-linking data, a model for the transmembrane arrangement of the two N-terminal helices of the b_2 dimer was presented [67].

3.3. Summary of higher resolution structures: what is not yet known?

Progress in atomic resolution structure of F_1F_0 has moved fast in recent years. The structure of F_1 subunits ($\alpha_3\beta_3\gamma\delta\varepsilon$) is now known at atomic level resolution, except for the C-terminal 43 residues (135–177) of δ. There are views of different conformations of ε, and of the three catalytic sites in various conformations, including the transition state. From the point of view of mechanistic studies of ATP synthesis and hydrolysis, getting more structural data on defined catalytic intermediate states appears to be an important next step. As well as the standard enzymological intermediates along the catalytic pathway such as ground-state and transition-state complexes, in ATP synthase we now have also the challenge of defining spatially specific intermediates. We need to know what is going on enzymologically when the γ is in a certain position of its arc. In an intriguing report [68], Grüber and Capaldi demonstrated that cross-linking the γ-subunit to the α-subunit trapped F_1 in a previously unrecognized conformation, in which the high-affinity catalytic site was occluded. Presumably this conformation occurs naturally, but transiently, as γ rotates. Structures in which such transient species are trapped and resolved should prove most valuable in understanding the rotational mechanism.

In regard to F_0, the *a* subunit remains a challenge. Its membrane topology has been defined [69–71], but there is no high-resolution structure yet. Here, atomic level resolution of the interactive surface between *a* and *c*, thought to be the critical region for proton or Na^+ pumping, will be the region most eagerly anticipated. As noted earlier, the number of *c* subunits remains to be defined. In regard to the structure of the 'stator stalk', most of the extramembranous residues of subunit *b* (residues 35–156) have not yet been seen at high resolution, although they are strongly predicted to be mostly helical and functional subdomains have been defined [17,72,73]. The structure of the stator stalk, the nature of its possible elasticity [74,75], and its interaction with F_1 subunits α and β both 'on top' and 'at the sides', remain to be determined by structural studies. However, given the rate of progress in recent years, an atomic level structure of complete ATP synthase may well be just around the corner.

4. Catalytic pathways and general mechanism

4.1. The binding change mechanism

Boyer predicted [35,36] that catalysis requires sequential involvement of the three catalytic sites, each of which changes its binding affinity for substrates and products as it proceeds through a cyclical mechanism, the 'binding change mechanism'. As applied to ATP synthesis, Boyer hypothesized that each site first acts to bind ADP and Pi, then acts to chemically synthesize ATP, then opens to release the ATP, and that all three activities are ongoing simultaneously at the three different sites. The mechanism includes proposals that (a) only one site is catalytically active at any one moment in time, (b) the chemical reaction occurs reversibly ($K_{eq} \sim 1$) at this site, (c) energy input is utilized to bind substrates ADP and Pi into the catalytic sites and to achieve release of ATP, *not* for actual ADP-O to P bond formation. When the X-ray structures first showed catalytic sites asymmetry, and revolving actin filament videos established rotation at least during ATP hydrolysis, there was an initial rush to conclude that Boyer's mechanism was proven. However, it is apparent that we have as yet no real understanding of how rotation is integrated with enzyme catalysis and chemistry, that several of Boyer's proposals lack strong experimental foundation, and, most importantly from the standpoint of this review, that the mechanism has not yet been framed in molecular terms, which will present a true test of its viability. In the sections below we critically discuss various aspects of mechanism.

4.2. Catalytic pathways

Potentially, given that there are three catalytic sites, ATP synthesis and hydrolysis could occur via pathways involving a single occupied site operating alone ('unisite catalysis'), two sites operating together

with one out of the three sites always unoccupied ('bisite catalysis'), or all three sites occupied and working together ('trisite catalysis'). Unisite catalysis does occur on addition of stoichiometric ATP, but accounts for just a single turnover of hydrolysis [76,77]. Unisite ATP synthesis on addition of ADP and Pi does not occur. Studies of unisite catalysis have been empirically useful in identifying catalytic side-chains by mutational analysis [4,78,79], and by demonstrating that Pi binding to F_1 is highly unfavorable in absence of Δp [80,81]. For steady-state turnover, however, bisite and trisite catalysis are the candidate pathways. Boyer's mechanism employs a bisite pathway [82].

For much of its history this field has been hampered by lack of direct assays for determination of the occupancy of catalytic sites during turnover. Hundreds of papers have been published in which velocity measurements have been made as a function of ATP concentration in the hydrolysis direction, or as a function of ADP and Pi concentration in synthesis, and from the resultant kinetic parameters (K_m, K_i, etc.), attempts have been made to infer occupancy of catalytic sites. This led, not unnaturally, to a great deal of controversy and uncertainty, since, in retrospect, the meaning of such kinetic parameters in an enzyme where three catalytic sites are constantly interchanging their identities is not straightforward. At least in the hydrolysis direction, direct and highly specific fluorescent Trp probes of occupancy of catalytic sites by ATP, ADP, and a range of other nucleotide analogs, have now been devised [46,83,84].

Several recent papers discuss the catalytic pathway of ATP hydrolysis in light of the new data [6,10,45,54,85]. The evidence now strongly demonstrates that occupancy of all three catalytic sites by ATP (trisite catalysis) is the required mode of operation to achieve physiological turnover. Indeed, at physiological (mM) concentrations of ATP, which are easily sufficient to fill all three sites, the enzyme could not operate in any other mode. In time average, during steady-state ATP hydrolysis at V_{max}, the population of molecules with even one site unoccupied is very small. From earlier work it was not clear whether occupation of just two sites by ATP led to a small rate of ATP hydrolysis or not [46,86]. This question has now been answered experimentally by using MgITP as substrate. MgITP is hydrolyzed and synthesized rapidly by F_1F_0 [87], drives subunit rotation [88], and has advantages for discriminating between bisite and trisite catalysis due to its lower binding affinity in comparison to MgATP. The new experimental data indicate that bisite catalysis essentially does not exist [50]. As we and others have noted [10,89], the original bisite hydrolysis mechanism [90,91] actually incorporates a serious flaw. More recent theoretical analyses further reveal that bisite catalysis depends on enzyme species that occur with low frequency, and utilizes the same direction of rotation for both synthesis and hydrolysis [50], emphasizing its unlikelihood.

In trisite mechanisms, bound nucleotide persists on the enzyme through more than one turnover and subunit rotation step. For example, in the mechanism that we have proposed for hydrolysis [10], after ATP binding the derived ADP comes off in the third subsequent turnover/rotation step.

When one considers ATP synthesis, however, the situation in regard to the catalytic pathway remains unresolved. There are literally *no* existing experimental measurements of catalytic site occupancy by ATP, ADP, or Pi, made using an equilibrium binding technique at physiological rates of ATP synthesis in presence of a proton gradient. Thus *all* current models of the catalytic pathway of ATP synthesis are speculation and no more. We have proposed and diagrammatically described a trisite mechanism [10], which we favor based on the view that if hydrolysis requires a trisite mechanism to achieve physiological rates, then synthesis likely requires it too. Menz et al. [45] also favor a three-site mechanism for synthesis, based on the fact that the $(ADP.AlF_4^-)_2F_1$ structure contains all three sites occupied and is viewed as a valid intermediate for synthesis as well as hydrolysis. Other workers likewise favor a trisite mechanism [6,85,89] but Boyer still favors a bisite mechanism [82]. Until probes are developed to directly monitor catalytic sites occupancy during steady-state ATP synthesis, the question cannot be resolved.

The original bisite scheme of ATP hydrolysis and synthesis envisaged just three different conformations of the catalytic sites, named 'tight', 'loose' and 'open' [90,91]. Trisite hydrolysis schemes require, minimally, an additional fourth conformation, and as noted above from our functional studies we have desig-

nated the catalytic sites as high affinity (H), medium (M), low (L), and open (O, of very low affinity). In our scheme [10] the order of interconversion of sites in hydrolysis is $O \rightarrow L \rightarrow H \rightarrow M \rightarrow L \rightarrow O$. For a tri-site ATP synthesis scheme it is necessary to incorporate an additional fifth conformation, in which the low-affinity site (L*) has enhanced affinity for Pi and concomitantly lowered affinity for ATP, the net result favoring Pi plus ADP binding to an empty site [10]. In our synthesis scheme the order of sites' conformation change is $O \rightarrow L^* \rightarrow M \rightarrow H \rightarrow L \rightarrow O$. (Note that we have not included the short-lived transition state as a separate conformational state).

From the $(ADP.AlF_4^-)_2F_1$ X-ray structure [45] the following sequence of conformation changes of the catalytic sites in ATP hydrolysis was suggested:

$$\beta E \rightarrow \sim \beta ADP + \Pi \rightarrow \beta TP \rightarrow \beta DP \rightarrow$$

$$\beta DP(ADP.AlF_4^-)_2 \rightarrow \beta ADP + \Pi \rightarrow \beta E$$

βE is the unoccupied open site seen in the two-nucleotide F_1 structure. ∼βADP+Pi is a postulated half-closed site containing bound ATP, not yet seen in a structure, presumably of low affinity, which has preference for binding ATP over ADP plus Pi. It is proposed to be structurally similar but not identical to the βADP+Pi site in $(ADP.AlF_4^-)_2F_1$. βTP and βDP are the closed conformations seen in two-nucleotide F_1; $\beta DP(ADP.AlF_4^-)_2$ is the catalytic transition state. βADP+Pi is the half-closed site seen in $(ADP.AlF_4^-)_2F_1$, thought to have enhanced affinity for Pi + ADP over ATP. As is apparent this sequence is reversible for ATP synthesis because it incorporates a species which preferentially binds ADP+Pi. Excluding the transition state, this sequence incorporates five different conformational states for both synthesis and hydrolysis. Thus both functional and structural approaches now show that (at least) five different catalytic site conformations are required for ATP synthesis.

It should be noted that since Menz et al. believe the βDP site is the site of highest affinity and therefore βTP is thought to be of medium affinity, this dictates that the order of binding affinity changes in their scheme of hydrolysis and synthesis (Fig. 4 in [45]) is opposite to what we have proposed (above). A further difference is that the scheme of Menz et al. envisages that, in time average, two ATP molecules and one ADP would be bound at catalytic sites during steady-state ATP hydrolysis, whereas we have measured two ADP and one ATP in fluorescence experiments [83].

4.3. Is there, in time average, just one catalytic site carrying out the chemical reaction?

The idea that only one catalytic site is carrying out the chemical reaction at any one time has entered the lore of the field, despite the fact that there is little evidence in support of it. Boyer has argued for the idea based on the ^{18}O isotope exchange data that manifested the characteristics of only one catalytic pathway (reviewed in [36]). In truth, however, what this shows is that there is only one type of catalytic site existing in time average, it by no means rules out the possibility of two sites simultaneously carrying out the same reaction pathway. ADP–fluoroaluminate complexes are known to mimic the catalytic transition state in a variety of ATP- and GTP-hydrolyzing enzymes (reviewed in [92]). Earlier work showed that MgADP–fluoroaluminate complexes potently inhibit and bind to F_1 with a 2/1 stoichiometry [93,94]. Our laboratory recently reported that ADP–fluoroaluminate (and ADP–fluoroscandium) complexes bind at two catalytic sites simultaneously in F_1 [49,52], increasing the affinity for MgADP significantly at both sites. The $(ADP.AlF_4^-)_2F_1$ X-ray structure shows two ADP–fluoroaluminate complexes bound, with both showing features of a transition state. The most straightforward interpretation of these data is that two sites are active in the chemical reaction simultaneously. This would seriously challenge current dogma, and if confirmed, require radical revisions of current catalytic pathways. A second interpretation is that only one site (with highest affinity, likely equivalent to βDP) is fully catalytically active, whereas the other site (with lower binding affinity, likely equivalent to βTP) is preparing to become a fully active site at the next binding change. It may have partial activity. It may be noted that when ADP–vanadate complex was used as a probe of the transition state of F_1, photocleavage led to only one β-subunit being cleaved, suggesting that only one catalytic site was in a tight transition state-like conformation [95].

4.4. Is there a catalytic site that shows reversible ATP synthesis/hydrolysis with an equilibrium constant near one that is responsible for the chemical synthesis step during steady-state ATP synthesis?

Another item that has entered the dogma of the field is the idea that during steady-state synthesis of ATP, even at physiological rate, the site that is carrying out the chemical reaction does so in a highly reversible manner, with $K_{eq} \sim 1$. This means, of course, that formation and collapse of the chemical transition state is not an energy-linked step in ATP synthase. This concept has greatly influenced the interpretation of rotational measurements and of catalytic mechanism generally. But is it true? Two lines of experimental evidence have been cited in support of the idea, namely unisite experiments and ^{18}O exchange data.

4.4.1. Unisite experiments

Unisite catalysis, discovered by Penefsky and colleagues [76], is a single turnover event of ATP hydrolysis which occurs when just a single catalytic site is filled by ATP. It follows the pathway:

$$F_1 + MgATP \underset{k_{-1}}{\overset{k_{+1}}{\leftrightarrow}} F_1.MgATP \underset{k_{-2}}{\overset{k_{+2}}{\leftrightarrow}} F_1.MgADP.Pi \underset{k_{-3}}{\overset{k_{+3}}{\leftrightarrow}}$$

$$F_1.MgADP + Pi \underset{k_{-4}}{\overset{k_{+4}}{\leftrightarrow}} F_1 + MgADP$$

It is slow, for example the chemical step ATP → ADP + Pi (k_{+2}) occurs at the rate of only 0.1 s^{-1} in *E. coli* F_1, as compared to at least 100 s^{-1} at V_{max} [79–81]. Subsequently the bound ADP and Pi are released, but only very slowly (in *E. coli* F_1, k_{+3}, k_{+4}, ≤ 0.001 s^{-1}) [80,81]. Intriguingly, as the bound ATP is hydrolyzed in this slow reaction, resynthesis of ATP also occurs from the bound products ADP and Pi, also at a rate (k_{-2}) of 0.1 s^{-1} in *E. coli* enzyme, giving therefore a $K_{eq} \sim 1$ for the reversible reaction. The question then is, is this relevant to physiological ATP synthesis?

On addition of sufficient 'chase' ATP to fill the other two catalytic sites and elicit steady-state turnover, bound unisite ATP is immediately hydrolyzed at V_{max} rate [79–81,96–98]. This has been cited as evidence that the unisite is a 'normal' site. But one can equally argue that the enzyme molecules undergoing unisite catalysis are recruited into the normal pathway by the addition of excess ATP, at which point their unisite behavior goes away. Also, whereas the rate acceleration of ATP hydrolysis (k_{+2}) from 0.1 s^{-1} up to 100 s^{-1} (V_{max}) is shown by this type of experiment, there has been no parallel demonstration that the rate of ATP synthesis (k_{-2}) is also accelerated. This would be necessary both to achieve physiological rate of ATP synthesis, and to retain the equilibrium constant at unity. Lack of such a demonstration greatly weakens the idea that during steady-state ATP synthesis a catalytic site exists in which the equilibrium constant is unity.

There are other difficulties also. In [99] it appeared that *E. coli* F_1 that was replete in δ-subunit was unable to show the chase of unisite catalysis by excess ATP, yet the enzyme had V_{max} ATP hydrolysis activity in steady-state assays. In [100] it was shown that the entire process of unisite catalysis occurred in an enzyme constrained from subunit rotation by cross-linking, suggesting unisite catalysis is not part of the normal catalytic pathway. Although mutations that impair unisite catalysis generally do impair steady-state catalysis, an important exception has now been found in the form of mutations at the αArg376 residue, which do not impair unisite catalysis [101], but are highly detrimental to steady-state ATP synthesis and hydrolysis [101,102]. Residue αArg376 is critical for the transition state structure in steady-state catalysis, as discussed later, but is obviously not required for unisite catalysis.

Therefore, the conclusion is that unisite catalysis is a special type of catalysis that occurs when only one catalytic site is occupied by ATP, but the behavior of this site is not representative of physiological catalysis, particularly in ATP synthesis.

4.4.2. ^{18}O exchange experiments

Boyer's laboratory has carried out extensive experiments using measurements of ^{18}O isotope exchange in chloroplast or mitochondrial membranes, and purified F_1 from chloroplasts, mitochondria, *Bacillus* PS3 or *E. coli*, which demonstrate that reversible reaction of ATP hydrolysis or synthesis can occur at one or more catalytic sites on F_1 [5,36]. However, while there is no doubt that extensive ^{18}O exchange occurs at low concentrations of nucleotide, or in the absence of a proton gradient and hence rotation, in experiments where ^{18}O exchange

was measured at rates of ATP hydrolysis or synthesis approaching (but not actually reaching) the physiological, the amount of exchange decreased very substantially [103–109]. The small residual exchange can plausibly be ascribed to F_1 or F_1F_0 molecules that were not actively engaged in rapid turnover. There are further difficulties in the interpretation of the ^{18}O experiments that arise from the fact that Boyer and colleagues assumed that a bisite mechanism pertained, such that ^{18}O exchange involving nucleotide bound in the site undergoing chemical catalysis was modulated by binding of a second nucleotide at just one other site [103–106]. Whereas, in a trisite mechanism, which is now shown to be the operative mode at least in hydrolysis, modulation may occur by nucleotide binding at two other sites, introducing significant extra complexity into interpretation of the results. Also, if as discussed above, two sites are capable of entering the transition state simultaneously during catalysis, interpretation of the ^{18}O exchange data becomes even more uncertain. Thus it seems clear that the ^{18}O exchange technique as used previously is at best qualitative; it cannot access, for example, a true reaction equilibrium constant (as was indeed acknowledged in [106]). Like unisite catalysis, the ^{18}O exchange experiments are probably monitoring a special activity that occurs only at low nucleotide occupancy of catalytic sites, or in absence of rotation, but is not reflective of physiological steady-state catalysis.

4.4.3. Other considerations

It has been recognized for some time that a reaction K_{eq} close to unity leads to the problem that the catalytic site would contain ADP and ATP in equal proportion, leading to the inefficient situation where the binding change would release ATP only half of the time [10,36,54]. No satisfactory solution to this problem that does not, in our view, in effect bias the equilibrium constant toward ATP, and thus ultimately require energy input, has yet been devised. This is a serious shortcoming. Also, it is generally conceded that in order to achieve a situation where the ATP synthesis reaction can occur with free reversal, a very highly sequestered site is required. Earlier measurements of a K_d of 1 pM for MgATP binding to the high-affinity catalytic site in mitochondrial F_1 [76] were consistent with such a scenario; however, recent data show that the K_d for MgITP in the high-affinity site in *E. coli* F_1 is only 0.33 μM [50], and yet MgITP is synthesized well by *E. coli* ATP synthase [87].

4.5. Are binding and release of substrates and products energy-linked?

Using Trp probes we find that Pi does not bind to catalytic sites of F_1 or detergent-solubilized F_1F_0 at physiological concentration in absence of a proton gradient ($K_d(Pi) \geq 10$ mM [4,86]).[2] Since $K_m(Pi)$ is around 1 mM for ATP synthesis, the proton gradient clearly induces formation of a Pi binding pocket (reviewed in [10]). Thus, Pi binding is energy-linked, and rotation is linked to formation and disappearance of a Pi binding site(s). Earlier work from Boyer's laboratory had adumbrated the energy-linked nature of Pi binding [111].

MgADP binding to all three catalytic sites occurs readily in F_1 and F_1F_0 in absence of a proton gradient [47,86]. MgADP is a competitive inhibitor of ATP hydrolysis activity,[3] and K_m(MgADP) during ATP synthesis is similar to K_d(MgADP) for binding

[2] It should be noted that there are numerous reports in the literature of various effects on F_1 or F_1F_0 activities, induced by the presence of Pi, which have been ascribed to Pi binding. However, specific evidence that Pi binding to catalytic sites was responsible for such effects is lacking. As we pointed out earlier [10], experiments with radioactive Pi can be confounded by the presence of radioactive PPi or PPPi in commercial [^{32}P]Pi preparations. Both PPi and PPPi bind to noncatalytic sites [10]. In our hands, when care was taken to remove such contaminants, no binding of radioactive Pi to *E. coli* F_1 occurred [110].

[3] ADP shows two kinds of inhibition of ATPase activity in F_1F_0. In addition to standard competitive inhibition, it also shows a different type of inhibition caused by tenacious trapping of MgADP in a catalytic site during turnover. This latter effect has been documented in mitochondrial, chloroplast, and *Bacillus* PS3 F_1 [4,36,85,112]. In mitochondria, chloroplasts, and *Bacillus* PS3 cells, the sole function of ATP synthase is to synthesize ATP, thus the tight MgADP inhibition could potentially be beneficial in preventing unwanted ATP hydrolysis, although it has not been shown yet to be a true physiological regulatory effect. However, in *E. coli* (and in *Enterococcus* species lacking respiratory chains) such an inhibition would be physiologically harmful, since these organisms use F_1F_0 to generate the membrane potential from ATP hydrolysis. We have not seen this type of inhibition in *E. coli* F_1. However, others have described such an effect [113,114] albeit under specific conditions including high Mg^{2+} and/or very low substrate (MgATP) concentrations.

at the low-affinity site (reviewed in [10]). Therefore there is no evidence that binding of MgADP requires the proton gradient or rotation.

MgATP binding to all three sites of F_1 or F_1F_0 occurs readily in absence of a proton gradient [46,86]. However, MgATP is only a weak inhibitor of proton-gradient-driven ATP synthesis (apparent $K_i = 5$ mM, [115]). This is in concurrence with Boyer's postulated binding change mechanism – release of ATP is an energy-linked function, and rotation in some way opens the catalytic site that has just synthesized ATP and renders it unable to rebind the dissociated molecule.[4] Since the ATP concentration in *E. coli* cells is around 3 mM (higher in mitochondria), this is a necessary property of the enzyme.

4.6. Summary of the current status of catalytic pathways and general mechanism

The binding change mechanism remains the overall guiding mechanism for ATP synthase, strongly supported by demonstration of subunit rotation, widely different binding affinities for substrates ATP or ADP at the three catalytic sites, and asymmetric X-ray structures. Nevertheless, as a result of recent data, and of re-evaluation of older data, details of the original hypothesis clearly require revision. Trisite catalysis is the only mode of operation of ATP hydrolysis, and is likely to be operative also for synthesis, although this is still an open question. The catalytic sites must interconvert between at least five different conformations in ATP synthesis, six if one includes the catalytic transition state. There is no compelling evidence at this time that the chemical reaction steps in ATP synthase occur at only one site in time average, and there is some evidence suggesting that two sites may be active simultaneously. Nevertheless, because conceptualization of the mechanism is greatly simplified by postulating only one site active in time average, we presume this will remain the preferred model until conclusively disproven. The concept that there is a site or sites at which rapid reversible catalysis without free energy input occurs during steady-state ATP synthesis at physiological rates is seen to be unsupported or at best only weakly supported. Pi binding and ATP release are clearly energy-linked, i.e., dependent upon rotation, whereas ADP binding is not. A further question is, how is newly synthesized ATP protected from hydrolysis before release? In proposing a molecular mechanism of ATP synthesis, each of these items must be taken into account.

[4] Earlier data indicated that induction of a proton-gradient by respiratory chain oxidation caused energy-linked release of unisite-bound ATP [116] although this occurred in the absence of nucleotide bound at sites 2 and 3, and may not therefore be representative of steady-state turnover.

5. Reaction in the catalytic sites: critical catalytic side-chains and the catalytic transition state

5.1. Mutational analysis of critical catalytic site residues

5.1.1. General

Mutagenesis of catalytic sites of ATP synthase have proceeded extensively in the *E. coli* enzyme, somewhat less so in *Saccharomyces cerevisiae* and *Bacillus* PS3, and to far lesser extent in other species. Almost without exception, detailed in vitro biochemical analyses of purified mutant enzymes have been limited to the ATP hydrolysis reaction. However, in both yeast and *E. coli*, growth tests on nonfermentable substrates (succinate, glycerol, etc.) provide sensitive if qualitative assays of ATP synthesis in the cell. In all cases, mutation of catalytic site residues has led to similar effects on ATP synthesis and hydrolysis Thus at least as far as catalytic sites are concerned, no 'one-way mutations' indicating a different pathway for synthesis versus hydrolysis have been seen.

5.1.2. Mg^{2+}-coordination

Ligands to the Mg^{2+} cation have been studied in detail by mutagenesis and functional analysis [51,117]. We deduced that, in the high-affinity site, the critical ligands were an O of the hydroxyl of βThr156 (in the Walker A sequence), a water molecule H-bonded to the Walker B Asp (βAsp242) and two water molecules H-bonded to βGlu185 [51]. The X-ray structures of the two-nucleotide and $(ADP.AlF_4^-)_2F_1$ structures agree with these assignments, and showed that β- and γ-P oxygens of bound ATP completed the octahedral coordina-

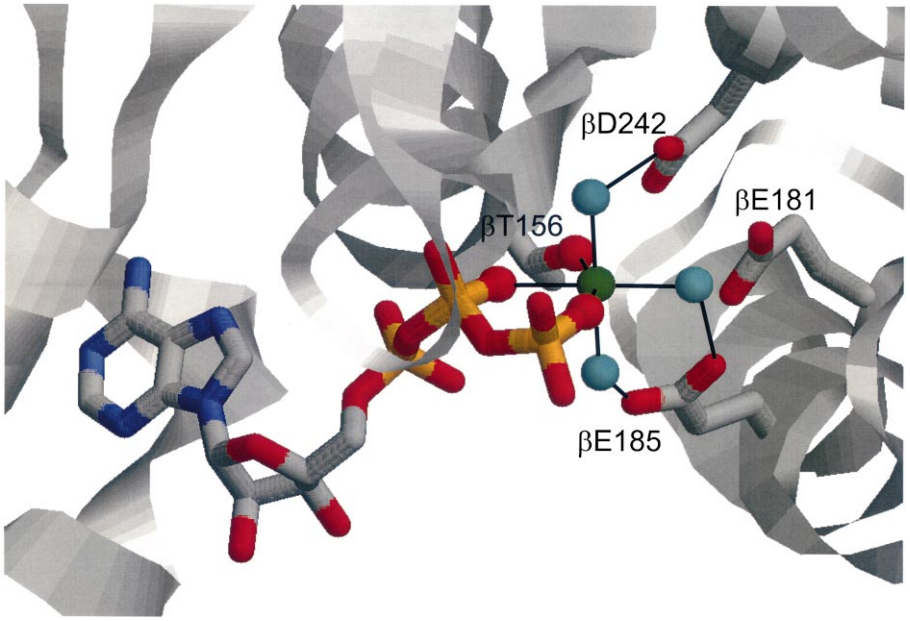

Fig. 2. Octahedral coordination of Mg^{2+} in the catalytic site of ATP synthase. ATP is shown as stick model, Mg^{2+} is shown as green sphere, water molecules in the first coordination shell as cyan spheres. Residues involved in Mg^{2+}-coordination are βThr156, βGlu185 and βAsp242. Also shown is the 'catalytic carboxylate', βGlu181, which is not involved in Mg^{2+} or nucleotide binding.

tion around the Mg^{2+}. This configuration is shown in Fig. 2.

5.1.3. Ligands to bound MgATP or MgADP

Extensive mutational studies coupled with functional assays have documented the quantitative role of catalytic site residues in binding MgATP and MgADP, and their roles in rate acceleration of catalysis. Several reviews of this work have been published [78,79,85,118]. Here we emphasize only salient points. βTyr331 was found to be intimately involved in binding the purine ring of bound nucleotide [119]; in the X-ray structures it is stacked against the adenine ring. Other aromatic residues (βPhe404, βPhe410) were seen in X-ray structures to line the adenine-binding pocket, and mutagenesis of these residues has confirmed a role in substrate binding [84]. Positively charged residues required for MgATP binding and catalysis are βLys155 (the Walker A Lys) and βArg182 [120–123]. Both residues are functionally involved in binding the γ-P; in X-ray structures they lie close to the phosphates of bound nucleotide. The residue that appears to bind the attacking water as seen in X-ray structures, βGlu181, had been known for some time to be critical for catalysis [123–125]. Further studies indicated it was not involved in nucleotide binding nor in Mg^{2+}-coordination [51,120]. Two other critical residues, βArg246 and βMet209 [124], appear from both functional studies and X-ray structures to be important in stereochemically aligning βGlu181 such that the attacking water is correctly aligned with the γ-P. Because Pi binding to catalytic sites cannot yet be directly assayed, and ATP synthesis measurements have been rare, we have little functional information from mutagenesis studies regarding residues specifically involved in Pi binding. Frasch [126] has suggested that βTyr297 is important for Pi and MgADP binding in ATP synthesis.

5.2. The catalytic transition state in phosphoryl transfer enzymes

Transition state structures in phosphoryl transfer enzymes have been discussed [127–130] and the most widely supported chemical intermediate is the pentacovalent phosphorus with two apical and three equatorial bonds, of the type shown diagrammatically in Fig. 3. Such a structure results from an associative mechanism, in F_1 in the hydrolysis direction resulting from attack of a water molecule upon the γ-P of ATP. Studies of the stereochemical course of the

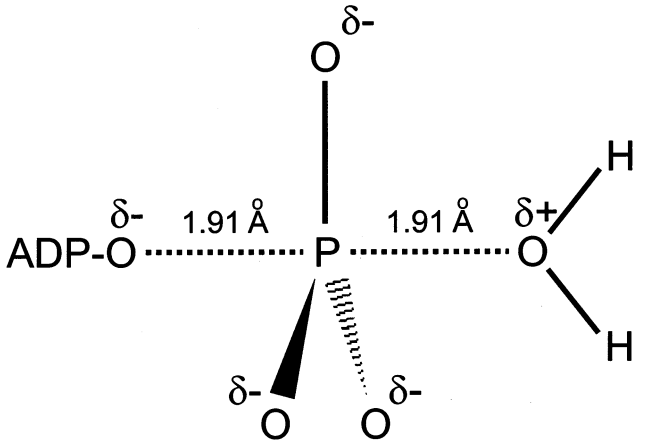

Fig. 3. Features of an anticipated pentacovalent phosphorus transition state in ATP synthase. Anticipated pentacovalent phosphorus transition state complex for a 50% associative phosphoryl transfer reaction [129,130].

F_1-ATPase reaction are consistent with such a mechanism [131,132]. Each of the (planar) equatorial oxygens will become negatively charged, and require compensation by close positively charged side-chains. Mg^{2+} ion also provides charge compensation. Immobilization, polarization, and orientation of the attacking water is required. The ADP-O to P distance, normally 1.55 Å in ATP will lengthen to ~2 Å in the transition state, then further as the transition state collapses and the negative Pi and ADP move apart. The ATP synthesis reaction is expected to utilize the same pentacovalent intermediate, here the problems facing the enzyme are to bring the negative Pi and ADP together, to shorten the ADP-O to P bond after the transition state has formed, and to prevent reformation of the transition state that could lead to hydrolysis by removing the product water, by removing functional groups that support the transition state, or both.

Transition states have been mimicked in a wide variety of phosphoryl transfer enzymes by use of potently inhibitory analogs, notably ADP–vanadate and ADP–fluoroaluminate. Multiple X-ray structures of enzymes containing these inhibitors bound in catalytic sites are available. As noted above, ADP–fluoroaluminate has been visualized bound at the catalytic sites of bovine F_1 either as AlF_3 [40] or as AlF_4^- [45]. In the former case the F atoms were planar around the Al, however the ADP-O to Al distance (2.4 Å) and Al to water distance (3.1 Å) were both somewhat long, suggesting a 'late transition state' or 'early ground-state' (ADP + Pi) structure. In the latter case, the bound analog, in two catalytic sites, had the characteristics of a true transition state-like structure. No X-ray structure of F_1 containing bound ADP–vanadate has been obtained yet.

5.3. The catalytic transition state in ATP synthase

The $(ADP.AlF_4^-)_2F_1$ X-ray structure gives a detailed view of the catalytic transition state [45], which conforms to the expected pentacovalent phosphorus intermediate, although it should be noted that since AlF_4^- is the form of fluoroaluminate bound, it cannot exactly mimic the true transition state. Notably the apical bonds from Al to ADP-O and water oxygen are of length 2.1 and 2.2–2.3 Å, respectively, and the fluorine atoms are planar around the Al. Functional studies using a combination of mutagenesis and quantitative ADP–fluoroaluminate or ADP–fluoroscandium binding are in full agreement with this structure [49], and a model of the transition state was proposed from such studies [92]. A diagram of the proposed catalytic transition state is shown in Fig. 4.

A major finding that emerged from the functional studies was that residue αArg376 is critical for formation of the transition state, yet it is not involved in ground-state binding of MgATP or MgADP [102], despite the fact that in X-ray structures it appears to lie close to the phosphates of bound nucleotide. We have named this residue the 'arginine finger'. Positive catalytic cooperativity results from insertion of this arginine finger into the transition state complex, across the α/β interface, reminiscent of the catalytic activation of G-proteins by their cognate GAPs. Insertion of αArg376 into a catalytic site that contains bound ATP is the key feature in making that site fully catalytically active. Functional studies also established that residues βLys155 and βArg182 are two other positively charged residues supporting formation of the transition state [52,121]. The fact that residue βGlu181 was critical for ADP–fluoroaluminate binding [52] provided important evidence for an associative mechanism, involving bond formation between the attacking water and the γ-P.

Further evidence for a trigonal bipyramidal geometry comes from the fact that ADP–vanadate appears to mimic the transition state in rat liver mitochon-

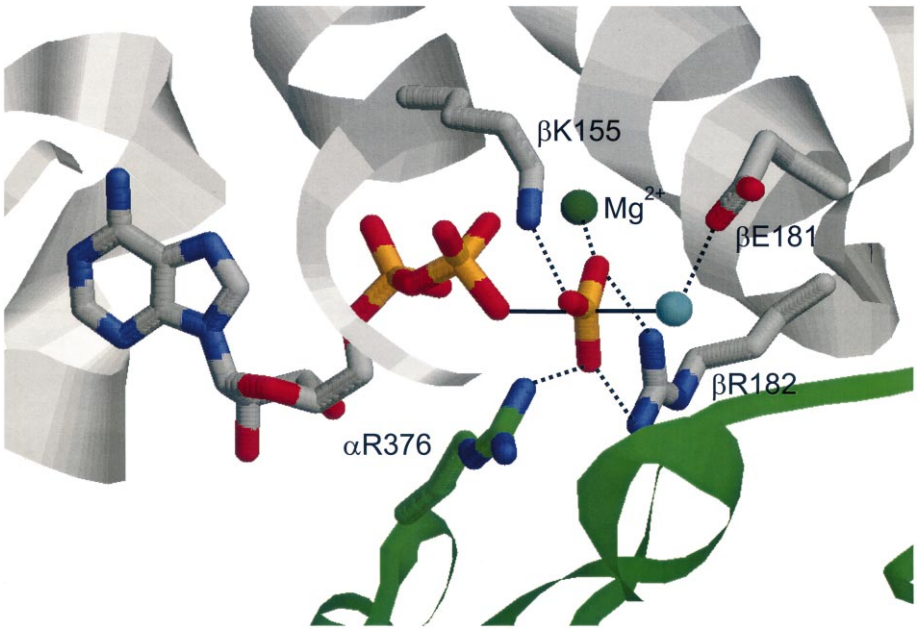

Fig. 4. Proposed catalytic transition state of ATP synthase. Mg^{2+} is shown in green, the reactant water molecule in cyan, the latter bonded to residue βGlu181. Ligands to Mg^{2+} (see Fig. 2) are omitted here for clarity. α-Subunit in green, β in gray.

drial F_1 [95]. From their photocleavage experiments using ADP–vanadate, Ko et al. conclude that residue βAla151 lies close to the phosphate oxygens in the transition state [95]; it may help to provide a hydrophobic, anhydrous environment, predicted from earlier studies to be an essential factor in catalysis [110].

6. Rotational catalysis

6.1. Experimental observations of rotation of subunits

What makes F_1F_0-ATP synthase an extraordinary enzyme is that energy coupling between catalytic sites and proton transport machinery is accomplished by rapid rotation of certain subunits. Suggestive evidence was obtained by photobleaching experiments [133]. Then, direct, visually striking fluorescence microscopy experiments established rotation of subunits γ [134], ε [135] and *c* [136,137] in response to ATP hydrolysis, at the same speed and in the same direction, indicating that these three subunits together form the central rotary transmission device of a molecular motor. As viewed from the membrane, the rotation was anticlockwise. While not yet demonstrated, and this point should be given due emphasis, it is generally believed that Δ*p*-driven proton transport generates rotation, in the opposite direction to that driven by ATP hydrolysis.

A fluorescent actin filament attached to γ subunit was used by Yoshida and colleagues initially to visualize rotation [134]. Single F_1 subcomplexes ($\alpha_3\beta_3\gamma$) were immobilized to an Ni-coated support through His-tags engineered at the N-termini of the β-subunits, and the actin filament was attached to γ via a Cys–biotin–streptavidin–biotin linkage. ATP-driven rotation of γ has now been seen in *Bacillus* PS3, *E. coli* and chloroplast enzymes using this type of setup [134,138–140]. Initially the Ni-coated support was a glass plate, subsequently polystyrene beads [141] and 200 nm high polymethylmethacrylate pillars [142] have been used, to minimize physical interruption of rotation of the actin filament (or of the artificial polymethylmethacrylate rods used instead of actin filaments in [142]). Attachment of actin filaments to the ε-subunit in PS3 F_1, and to the *c*-subunits in *E. coli* F_1F_0 was used to demonstrate rotation of these two subunits [135–137]. The experiment with the *c*-subunits was further validated by reversing the setup, i.e., by immobilizing the *c*-subunits to the Ni-coated support, and attaching the actin filament to the α or to the β-subunits [143].

It was clear that the actin filament impeded rotation through hydrodynamic friction at higher con-

centrations of ATP, because maximal rotation rates were of the order of only 4 s^{-1}, as compared to ATP hydrolysis rates of 52 s^{-1} [134]. Further refinements of the technique included use of angle-resolved imaging by polarized fluorescence of the fluorophore Cy3-maleimide attached specifically to the γ at a Cys residue [144], and most recently by laser dark-field microscopy imaging of 40 nm diameter gold beads attached to the γ-subunit through a biotin–streptavidin–biotin–BSA linkage [145]. Large increases in rotation rate at saturating ATP concentration were produced. In [145] maximal rotation rate seen (130 s^{-1}) was close to that of V_{max} of ATP hydrolysis turnover divided by three, consistent with the view that one complete 360° rotation consumes three ATP molecules. The apparent K_m(ATP) for rotation also increased drastically, from 0.8 μM using the actin filament technique [141], to 15 μM with the gold bead technique [145], this being explicable simply because apparent V_{max} of rotation was much higher using gold beads. The data using gold beads demonstrate that there is only a single rotary mechanism at all ATP concentrations tested (20 nM to 5 mM), as judged by the Michaelis–Menten dependence of rotation on ATP concentration and other arguments [145].

Yoshida, Kinosita and colleagues interpret these data to mean that a bisite mechanism of ATP hydrolysis drives subunit rotation [145]. However, we disagree on the following grounds. First, the existence of bisite catalysis has now been seriously questioned as noted above [50]. Second, it has been unambiguously established that ATP hydrolysis at saturating (mM) ATP concentration requires filling of all three catalytic sites, both in *E. coli* and PS3 enzymes [46,85,146,147]; together with the Michaelis–Menten dependence of rotation upon ATP concentration, this provides convincing evidence that only a three-site mechanism of ATP hydrolysis can drive rotation. Although evidence from K_m measurements alone can give no indication as to the number of sites occupied, it is relevant that the apparent K_m(ATP) of 15 μM for rotation of the PS3 enzyme [145] is similar to the value of 21–35 μM for K_{d3}(ATP) in PS3 F_1 as measured by a direct equilibrium binding method [147,148]. In contrast values of K_{d2} reported in PS3 F_1 (0.25–0.9 μM) are far below the apparent K_m for rotation [147,148]. Even at very low ATP concentrations (20 nM) where rotation was still seen, a few molecules with three sites occupied would occur, and would be selected by the rotation assay. Therefore, we argue that a molecule with two sites occupied does not rotate, and that binding of ATP to the third site, of low affinity, in a molecule already containing two bound ATP is the event that initiates rotation.[5]

The torque generated by the revolving γ- or *c*-subunits is around 40 pN.nm [136,139,141,145], at all ATP concentrations. This is unexpectedly high, certainly in comparison with other biological motors, and approximately equivalent to the phosphorylation potential within the cell, implying that the motor is remarkably efficient in transducing chemical bond energy of ATP hydrolysis into mechanical energy [141,142].

Rotation of the γ subunit occurs in 120° steps, separated by pauses which are most visible at low ATP concentration [141,145]. Each 120° rotation step has been broken down further into an initial 90° substep, lasting <0.25 ms, followed by a stationary interval of a few ms, followed by a 30° substep, also lasting <0.25 ms. The stationary interval was suggested to consist of two separate components of around 1 ms each [145]. In Fig. 5A we have shown this pattern of rotation, and have included the interpretation of the associated enzymatic events given in [145] (Fig. 5B), together with an alternative interpretation of the enzymatic events which we favor (Fig. 5C).

The length of the pauses between 120° steps is highly dependent on ATP concentration, varying from a few μs at high ATP concentration to >20 ms at low ATP; the conclusion that pauses occur as the enzyme waits to bind ATP to initiate a single turnover of hydrolysis [145] seems firm, and thus during the pauses the enzyme would have two sites filled with nucleotide with one site empty. Fluorescence experiments indicate that one site would contain ATP and one ADP [83]. The 30° substep that terminates the 120° rotation step must be due to product release. While the 90° substep is clearly ini-

[5] In a recent abstract [149], rotational catalysis driven by a fluorescent ATP molecule was seen to operate by a trisite mechanism, with the fluorescent nucleotide remaining bound through two rotation steps and being released at the third step.

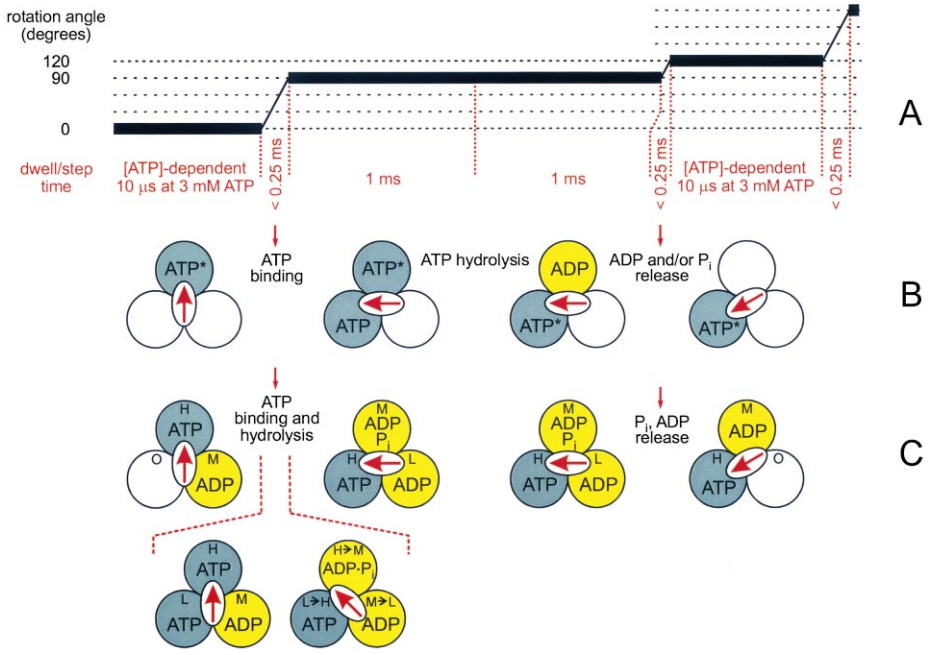

Fig. 5. Substeps in ATP synthase subunit rotation: possible correlations to enzyme catalytic pathway intermediates. (A) The series of substeps of γ rotation as determined by Yasuda et al. [145]. (B) Correlation of substeps of γ rotation with enzymological intermediates, as postulated by Yasuda et al. [145]. ATP with asterisk represents ATP or ADP+Pi [145]. (C) Correlation of substeps of γ rotation with enzymological intermediates, as postulated by the authors of this review. H, M, L refer to high-, medium- and low-affinity catalytic sites; O indicates an empty site.

tiated by ATP binding, this does not necessarily mean that it is driven only by ATP binding. In F_1 undergoing steady-state catalysis, binding of ATP promotes immediately and obligatorily the hydrolysis of ATP already bound at a high-affinity site, the process known as positive catalytic cooperativity [76]. Hence the 90° substep likely is driven by both ATP binding to an empty site and ATP hydrolysis at a high-affinity site, and ends with ADP and Pi products in the site that has undergone hydrolysis. In our view, therefore, at the beginning of the stationary interval between 90° and 30° substeps, the enzyme likely has already completed the hydrolysis step (Fig. 5C). This interpretation is also the one favored by Menz et al. [45] from their analysis of the $(ADP.AlF_4^-)_2F_1$ X-ray structure. However, Yasuda et al. [145] feel the 90° step is resultant only on ATP binding and that hydrolysis follows during the stationary interval, i.e., that hydrolysis per se is not rotation- or energy-linked (Fig. 5B). At the present time the cause of the two 1 ms components of the stationary interval is unclear. They may reflect rate-limiting intrinsic conformational changes.

6.2. *Speculative mechanisms of ATP-driven subunit rotation*

How the enzymatic process of ATP hydrolysis can drive rotation is clearly a crucial and highly topical question, about which little is yet known. It should be emphasized that we cannot be sure that rotation-driven ATP synthesis will exactly mirror hydrolysis-driven rotation in sub-steps or mechanism. Nevertheless a discussion of current ideas regarding ATP hydrolysis-driven rotation is valuable in providing clues to the molecular mechanism of ATP synthesis.

The catalytic nucleotide binding site is ≥20 Å away from γ, thus ATP-driven conformational changes in α and/or β must be transmitted to γ through γ/α or γ/β interaction sites. While speculative proposals have been advanced to describe ATP-driven subunit rotation [8,150,151], actual experimental information linking known enzymology and mechanics is minimal. Presently there are two steps in the ATP hydrolysis pathway which are known to involve protein movements. The first is the ATP binding step. X-ray structures [7,45] predict that binding of

ATP will partly close the βE-subunit by motion of the C-terminal domain, producing a catalytic site similar in conformation to βADP+Pi, and mutagenesis of the 'hinge region' in β subunit supports this [112]. The second is the hydrolysis step. Steady-state ATP hydrolysis is known from mutational analysis (reviewed in [4]) to require conformational movement at the catalytic α/β interface. Either or both of these steps could in principle trigger protein movements that are transmitted, through α or β, to γ, although one could argue that ATP binding cannot alone provide sufficient energy to drive full rotation, on the basis that binding of MgATP to site three occurs only with $K_d \sim 30$ μM, and rotation is dependent upon ATP binding to site three in a trisite mechanism. The argument may be academic, since under conditions where rotation occurs, binding of ATP to an empty catalytic site will immediately trigger hydrolysis of ATP already bound at a high-affinity site. In some models, it is argued that ATP binding is primarily if not solely responsible for driving rotation. One such model has been developed in detail [150,151]. In contrast, a hypothesis from our laboratory [152], invokes ATP binding and ATP hydrolysis acting in sequence to drive rotation. The recent $(ADP.AlF_4^-)_2F_1$ X-ray structure, in which γ is rotated relative to its position in two-nucleotide F_1, indicates that movements of γ are linked to movements of β and/or α subunits [45]. These authors also favor the view that both binding and hydrolysis steps drive rotation.

In myosin, G-proteins, and nitrogenase, small spatial movements of catalytic site ligands occurring at the steps of formation and collapse of the catalytic transition state are amplified into long-range domain movements, which are involved in energy transduction or signaling (reviewed in [92]). The conclusion that energy coupling occurs at the stage of attainment of the transition state is also well-supported in the case of P-glycoprotein [92]. Thus there are precedents for the idea that in ATP synthase small spatial changes in the catalytic sites involving ligands that stabilize the transition state might be amplified into larger domain movements, including subunit rotation. There are further reasons to consider such a model. (a) The catalytic transition state of ATP hydrolysis forms at the α/β interface [45]. (b) Early mutational studies (reviewed in [4]) demonstrated that conformational changes at the α/β interface are critical for positive catalytic cooperativity and attainment of steady-state catalysis. Rotation is clearly interdependent upon both features. (c) Recent mutational studies of residues αArg376 and βArg182, two important contributors to the catalytic transition state (Fig. 4), uncovered the fact that mutations Arg to Lys still supported formation of the transition state, but nevertheless abolished steady-state catalysis [102,121]. These results indicated that these two Arg residues not only help to form the transition state, but perform an additional function. Our proposed explanation is that in both cases the Arg residue is linked to α-subunit, either directly (αArg376) or by hydrogen-bonds (βArg182, αArg376), and normally has the dual function of forming the transition state complex, then eliciting movement of α versus β subunits in the catalytic site as the transition state collapses and products MgADP and Pi separate. We suggest that a Lys residue at position β182 or α376 can support formation of the transition state, but is not long enough, or lacks sufficient H-bonding capability, to elicit movement of α versus β. (d) The αS373F mutation, at the α/β interface, abolishes transition state formation [152], probably by preventing αArg376 from reaching into the catalytic site. This mutation abolishes steady-state catalysis and positive catalytic cooperativity but does not affect MgATP binding parameters [47].

The hypothesis that we presented for ATP-driven rotation (described in detail in [152]) is based on this evidence and incorporates the following key features. (i) ATP binding to an open site partly closes that site. A conformational change is transmitted to the neighboring high-affinity site containing bound ATP, by partial rotation of γ. Insertion of the arginine finger αArg376 into this neighboring site triggers hydrolysis by forming the transition state. (ii) As the transition state collapses, Pi separates from MgADP by mutual repulsion, and αArg376 and βArg182 move with the Pi. This results in movement of α, which is communicated to γ, resulting in rotation, i.e., the actual hydrolysis step is an energy-linked step. (iii) Further rotation of γ enables Pi release, as αArg376 withdraws from the catalytic interface and the Pi binding pocket disappears. Based on the $(ADP.AlF_4^-)_2F_1$ structure, Menz et al. [45] have also

proposed a mechanism of ATP-driven rotation of γ-subunit. Common features between the two models [45,152] are as follows. Binding of MgATP to an empty site is suggested to initiate partial rotation of γ (indeed in [45] it is argued that formation of the half-closed MgATP-containing site requires rotation of γ). The subsequent ATP hydrolysis step is seen as directly responsible for the major fraction of the 120° rotation, and the mobility of residue αArg376 as critical for transition state formation. This residue is also seen to play a structural role in the Pi binding pocket, which from [45] is seen to be transitory, present in the βADP+Pi conformation but not in βTP, βDP or βE.

As opposed to α/γ interactions, Menz et al. [45] suggest that interaction of the C-terminal domains of two β-subunits with the γ-subunit provides the propulsion system to move γ. In their proposal, binding of MgATP to βE forms a half-closed site (thought to be similar to βADP+Pi in structure). Catalytic cooperativity, transmitted through αE to βDP, causes βDP to become actively catalytic. Concerted movement of the C-terminal domains of the two β-subunits, βADP+Pi and βDP, drives rotation, in a 'push–pull' fashion. While the C-terminal domain of one β moves away from γ, that of the other moves towards it. Thus in this mechanism, the α-subunit is involved primarily in signal transmission (cooperativity) between two β-subunits, coordinating their movements and activities, rather than being directly in mechanical contact with γ. Additionally, while separation of the negative ADP and Pi and liganded groups within the catalytic sites is seen as the primary spatial movement that is amplified into larger domain movements, Menz et al. feel this occurs not by mechanical coupling to α across the α/β interface, but by mechanical coupling to the C-terminal region of the β-subunit via movement of residue βTyr297 in the catalytic site. βTyr297 occupies the space of the Pi site in the closed βTP and βDP sites but moves to accommodate Pi in βADP+Pi.

Based on the original two-nucleotide F_1 structure the prime candidate for a mechanical transmission site between β and γ that might cause rotation appeared to be a conserved loop of mainly acidic residues (DELSEED, residues βAsp380 to βAsp386) in the C-terminal domain of the β subunit. Mutational analyses initially seemed to support this notion, as an extra hydrogen bond in the γM23K mutant *E. coli* enzyme was found to cause a loss in coupling efficiency [6,153]. However, a subsequent study cast doubt 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 (AAL-SAAA), ATP hydrolysis generated the same torque of γ as in the wild-type [154]. Now that the $(ADP.AlF_4^-)_2F_1$ structure has redirected attention to the C-terminal domains of β-subunits as major players in rotation, further work seems necessary. In regard to the role of the α-subunit in rotation, there is no doubt that α is critical for signal transmission between adjacent β-subunits, as earlier mutagenesis studies had uncovered [155,156]. Menz et al. [45] report significant conformational movement of αE in the transition state structure, and there may well be transitory species present during rotation in which significant α/γ interactions occur. Further studies of this aspect also seem justified.

7. Hypothesis: the molecular mechanism of ATP synthesis

Several conditions appear prerequisite for the ATP synthesis reaction. Binding of Pi, being energy-linked, will require at least partial rotation of γ and movement of catalytic site residues to form the binding site for Pi. The negatively charged Pi and ADP must somehow be squeezed together to form the transition state for ATP synthesis, a process that likely requires energy in the form of rotation. Hydrolysis of the newly formed ATP must be prevented; this could be achieved by removing, at the appropriate time, catalytic groups necessary for transition state re-formation. Finally, the high-affinity, occluded site at which ATP has been made must be opened such that its affinity for ATP becomes very low indeed ($K_d \geq 5$ mM). Of course, proton-gradient-driven rotation is the ultimate energy source for all of this. A hypothesis to explain linkage between proton-gradient rotation and the molecular details of ATP synthesis is as follows.

In Fig. 6A the substrates binding step is shown. It requires binding of substrates ADP and Pi (likely $H_2PO_4^-$, [80]) into the empty (βE) catalytic site. Because the concentration ratio of ATP/ADP in cells is

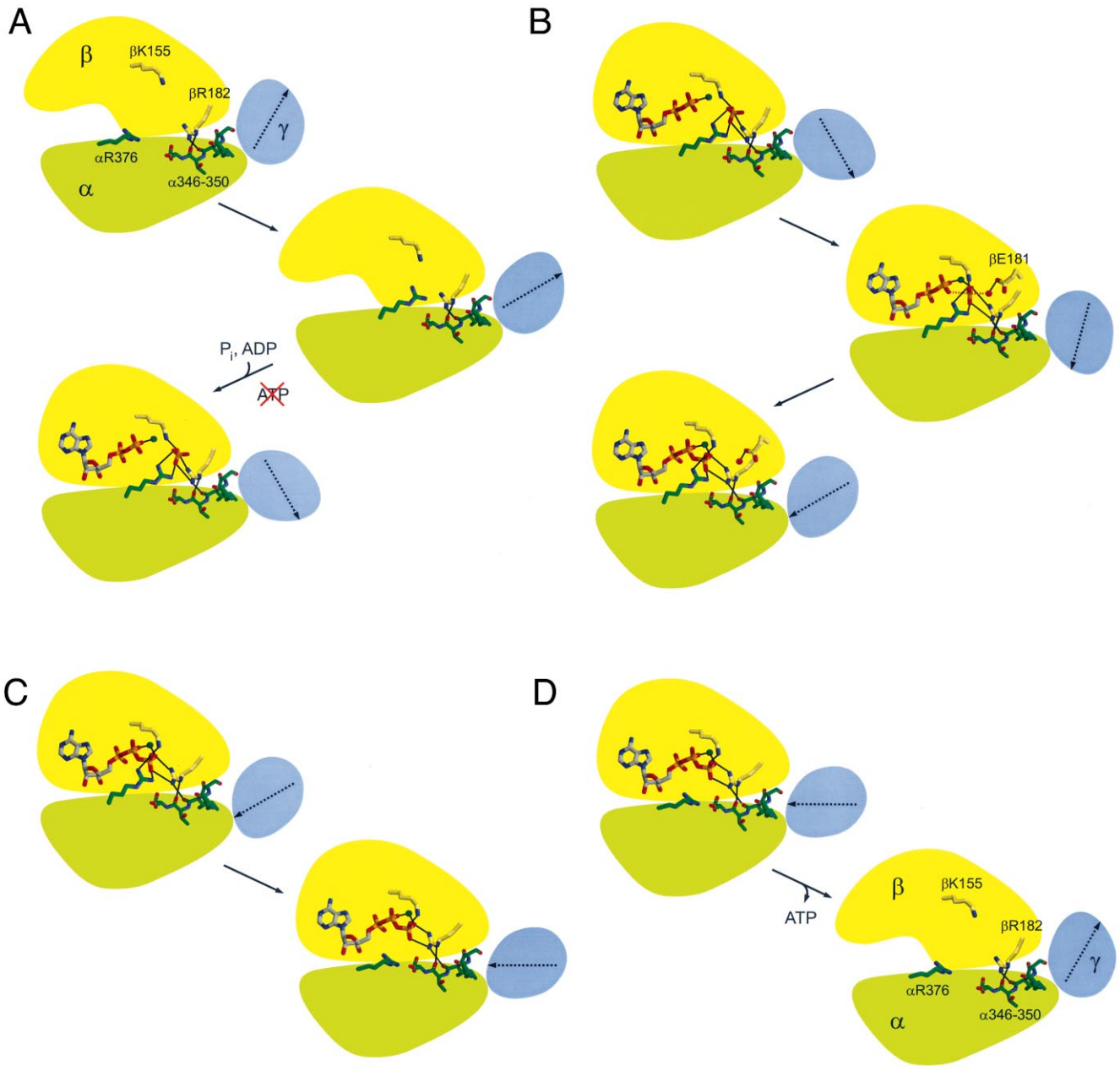

Fig. 6. Proposed molecular mechanism for ATP synthesis by ATP synthase. (A) Binding of Pi and ADP to an empty site. In this step a Pi binding pocket is created by the movement of αArg376 ('arginine finger') into the catalytic site. ATP binding is prevented, only ADP can bind with Pi. α-Subunit is in green, β in yellow, γ in blue. The arrow in γ points to the location of the high-affinity catalytic site (in the pause position of rotation [145]. H-Bonds or Mg^{2+}-coordination ligands are shown by solid lines. Mg^{2+} ion is shown in dark green. Note that most of the Mg^{2+}-coordination ligands (see Fig. 2) are not shown here. Residues α346–350 lie at the catalytic site α/β interface. βArg182 is shown H-bonded to these residues. H-Bonds involving these residues with αArg376 are omitted for clarity. (B) Formation of the catalytic transition state and then of bound ATP. In this diagram, the transition state forms as the catalytic α/β interface closes and αArg376 and βArg182 carry the Pi toward the ADP-O. Apical bonds are shown by the dotted red lines. The red sphere represents product water. Next, the ADP-O to P bond is shortened as the α/β interface closes further, compressing the reactants. As the transition state collapses in this hydrophobic pocket, βGlu181 binds the product water. ATP is formed in a highly sequestered site. (C) Prevention of hydrolysis of newly synthesized ATP by removal of the arginine finger. Rotation of γ results in rearrangement of the catalytic α/β interface such that αArg376 is withdrawn. Consequently the catalytic transition state which requires this residue cannot re-form and bound ATP cannot hydrolyze. (D) Opening of the catalytic site releases bound ATP.

in excess of unity, a means of excluding ATP binding is necessary. We suggest that this is achieved by partial rotation of γ which causes insertion of the arginine finger, αArg376, into the catalytic site. The positive charge of αArg376, together with those of βArg182 and βLys155, forms a Pi binding pocket. This is consistent with the data of Menz et al. [45] who conclude that Pi binding in the βADP+Pi site will only occur after a partial rotation of γ and that αArg376 acts 'as a discriminator to ensure that the appropriate substrates are bound' (in synthesis versus hydrolysis). Once Pi has bound, it will prevent ATP binding by steric and electrostatic hindrance. Additionally, model building [45] showed that movement of αArg376 into the site would itself prevent accommodation of ATP within the site. The βE nucleotide binding site is largely disordered and solvent accessible, whereas upon formation of the half-closed site (βADP+Pi) after partial γ rotation, the movement of the C-terminal domain, and of the lower half of the nucleotide binding domain that moves with the C-terminal domain, significantly re-positions a number of β-subunit catalytic side-chains (e.g., βGlu181, βArg182) [45]. Thus, at the end of this first step a site with bound Pi and ADP primed for catalysis is present.

Fig. 6B shows formation of the transition state from ADP plus Pi, then of ATP. Pi has to be brought close to the ADP-O, overcoming charge repulsion. Such a reaction can only occur in a tightly constrained situation, with the ADP, Mg^{2+}, and Pi firmly anchored and exquisitely oriented. We propose that the rotation of γ changes the conformation of the α/β catalytic interface, pushing the α-subunit surface toward β, and moving αArg376 and βArg182 (H-bonded to residues α346–α350) toward the ADP. In effect Pi is literally crashed into the ADP, forming the pentacovalent transition state and concomitantly an Mg^{2+} coordination between a β-phosphate oxygen of ADP and an equatorial oxygen of the (to be) γ-P. Charge neutralization is achieved by αArg376, βArg182, βLys155, and Mg^{2+}. Then the bond between γ-P and the ADP-O has to be shortened from around ∼2 Å in the transition state down to 1.55 Å in ATP. Again, we propose a mechanical mechanism, with γ rotation acting to close the α/β interface, and squeeze the γ-P toward the ADP-O. Concomitantly, βGlu181 must be in position to accept the product water. ATP is now formed but is potentially unstable. As shown in Fig. 6C, a further rotational movement of γ causes αArg376 to be withdrawn from the catalytic site, meaning that now the catalytic transition state cannot re-form, thus hydrolysis cannot occur. ATP, and *only* ATP, will be released as the high-affinity site opens (Fig. 6D).

The molecular mechanism of ATP synthesis hypothesized in Fig. 6 requires energy input in the form of rotation and mechanical work at the chemical step of condensation of ADP and Pi. Therefore, it contradicts Boyer's mechanism. This issue has yet to be settled. However, the chemical reaction of ATP synthesis, the structure of the transition state, and the roles of catalytic side-chains, are all now reasonably well established. Thus, no matter what the energetics, the final reaction is surely going to look similar to Fig. 6.

During a single 120° rotation step of γ, each catalytic site is expected to be carrying out a different part of the catalytic pathway. It is not yet clear exactly how the various parts of the reaction in Fig. 6 are distributed among the three catalytic sites during synthesis of ATP, and how they correspond temporally to steps in the rotation cycle. As noted earlier at least five conformations of the catalytic sites, not including the transition state, are required for ATP synthesis, so that at least two of the sites must undergo changes between three conformations during a single 120° step of rotation. One attempt to correlate the reaction steps with rotation would be as follows. In Fig. 6A the catalytic site starts out open (βE) and changes via low affinity (half-closed, βADP+Pi) to medium affinity (closed conformation, βTP-like but with ADP and Pi bound) during one 120° step. Simultaneously during the same step, at a second site undergoing the reactions in Fig. 6B and C, the medium-affinity site changes transiently to the short-lived, occluded, transition state, and then to high affinity (closed conformation, with ATP bound, βDP-like). In the third site, during this same step, the high-affinity site changes from closed to open (βE) (Fig. 6D). We emphasize that these ideas are speculative. The obvious conclusion is that one very challenging future research area is to determine exactly what each of the three different sites is doing during a single step of rotation.

As a corollary it is worth remarking that, although

the idea of elasticity of the γ-subunit or of the stator stalk has been discussed [74,75], this might not be mandatory. The idea came about from the necessity to couple the transmembrane movement of 3–4 protons to release of a single ATP in one rotational step [157], implying the need to 'store' the energy of several protons. However, if proton-gradient-driven γ rotation proves to be composed of several substeps, each driving a partial reaction of catalysis (e.g., binding of Pi, formation of the transition state, compression of the transition state, removal of the arginine finger, opening of the site), one can imagine that protons could be used incrementally. One transported proton could drive one substep of rotation to drive one intermediate step of catalysis.

How does rotation of γ bring about conformational changes of catalytic sites during ATP synthesis? The first step (Fig. 6A) starts with the empty βE site and ends with ADP and Pi bound in the medium-affinity βTP-like site. Taking the suggestion of Menz et al. for the hydrolysis direction and applying it in reverse, rotation of γ could act on β to cause upward hinging of the C-terminal domain, moving with it the lower part of the nucleotide domain to which it is 'geared'. However, there has to be a change in α-subunit conformation also to cause the catalytic α/β interface to close and insert αArg376 into the catalytic site. This might be a direct effect on α, or indirect through first γ/β then β/α interactions. Forming the catalytic transition state and tightly bound ATP (Fig. 6B,C) will require the same types of movement, to further close the α/β interface. Catalytic groups on β, notably βGlu181, βArg182, βLys155, will be brought into correct position as the site begins to assume a closed conformation [45]. Insertion of βTyr297 into the Pi binding space could occur upon forced closure of the β-subunit, if it were coupled to movement of the C-terminal domain as proposed [45], and this would enhance formation of tightly bound ATP. Opening the high-affinity site to release ATP (Fig. 6D) will require opposite movement of the C-terminal domain of β-subunit, producing βE. Clearly the study of linkage between rotation, conformation of catalytic sites, and enzyme catalysis, is another experimental area ripe for development. At present we have the static X-ray structures that tell us what different conformations are possible, the functional studies that tell us rates and final outcomes in terms of different properties of the sites, and the visual images of γ showing us the fine details of its angular trajectory. The future need is for real-time integration of the three approaches.

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