FEBS 27216

FEBS Letters 545 (2003) 61–70

Minireview

ATP synthesis driven by proton transport in F_1F_0-ATP synthase

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Received 3 January 2003; accepted 6 January 2003

First published online 22 April 2003

Edited by Bernard L. Trumpower

Abstract Topical questions in ATP synthase research are: (1) how do protons cause subunit rotation and how does rotation generate ATP synthesis from ADP+Pi? (2) How does hydrolysis of ATP generate subunit rotation and how does rotation bring about uphill transport of protons? The finding that ATP synthase is not just an enzyme but rather a unique nanomotor is attracting a diverse group of researchers keen to find answers. Here we review the most recent work on rapidly developing areas within the field and present proposals for enzymatic and mechanoenzymatic mechanisms.

Key words: ATP synthase; Subunit rotation; ATP synthesis; ATP hydrolysis; Molecular motor; Proton transport

1. Introduction

F_1F_0-ATP synthase catalyzes synthesis of ATP in the terminal step of oxidative phosphorylation and photophosphorylation and is found in organisms from bacteria to man. In prokaryotes, the enzyme also catalyzes ATP hydrolysis when needed to generate the transmembrane proton electrochemical gradient (Δp) required for locomotion, nutrient uptake, and other functions. ATP hydrolysis and synthesis occur on three catalytic sites in the F_1 sector, peripheral to the membrane, whereas proton transport occurs through the membrane-embedded F_0. Direct evidence has been found for ATP hydrolysis-driven rotation of a subset of subunits termed the 'rotor', thus energy transmission from F_1 to F_0 and uphill proton transport are functions of rotation. Proton gradients generated by electron transfer complexes and/or light harvesting proteins provide the energy for ATP synthesis. It is widely anticipated (although not experimentally demonstrated at time of writing) that protons moving down the gradient through F_0 generate rotation of the rotor in the opposite direction to that when ATP hydrolysis is the motor. Thus, contemporary ideas envisage that angular motion of the rotor vis-à-vis the catalytic sites produces net ATP synthesis from ADP and Pi. Recent reviews may be found in [1–9]. Here our two goals are, first, to review recent work on a selected group of rapidly developing topics, and second to discuss mechanistic models of rotation-linked ATP synthesis and hydrolysis.

2. Rotational catalysis

The story began in 1977 with the demonstration by Berg and colleagues that the proton gradient powers bacterial flagellar rotation [10]. Researchers started to assay for rotation in ATP synthase, but it was experimentally difficult, and not until critical X-ray structure information became available [11], was rotation confirmed in 1997 [12]. In the meantime, Boyer developed his binding change principles for ATP synthesis on F_1 [13], and Cox et al. suggested a principle for proton transport in F_0 [14], both invoking rotation of subunits. These concepts are now well-accepted. There is convincing evidence that rotation of a central $\gamma\varepsilon c_{ring}$ rotor relative to the $\alpha_3\beta_3$ hexagon is critical for operation of the catalytic sites, and that rotation of $\gamma\varepsilon c_{ring}$ relative to subunit *a* is critical for proton transport. Subunits *b* and δ form a 'stator' which ensures that subunits *a* and the $\alpha_3\beta_3$ hexagon do not rotate with $\gamma\varepsilon c_{ring}$. Fig. 1 describes the structure of *Escherichia coli* F_1F_0-ATP synthase[1,2].

Visualization of single molecules fixed to a surface proved to be the key to demonstration of rotation. Initial experiments used attached fluorescent actin filaments to visualize ATP hydrolysis-driven rotation of γ- and ε-subunits in immobilized subcomplexes $\alpha_3\beta_3\gamma$ or $\alpha_3\beta_3\gamma\varepsilon$ [7]. Rotation was seen to be unidirectional and anticlockwise as viewed from the membrane. Later experiments included extension of the procedure to detergent-solubilized F_1F_0 [23] or membrane fragments [24], and use of a highly specific, engineered attachment site for the actin filament [25], yielding confirmation of rotation of c_{ring} together with γ and ε. Initially it was found that the rotor advanced in 120° steps, with intervening pauses evident at low MgATP concentration [26]. MgATP binding was shown to initiate each step and pausing molecules were concluded to be awaiting a productive collision with substrate. Replacement of actin filaments by 40-nm-diameter gold beads with

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Abbreviations: NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; DCCD, dicyclohexylcarbodiimide; AMPPNP, 5′-adenylyl-β,γ-imidodiphosphate

[1] *E. coli* enzyme represents the simplest form of ATP synthase, containing eight core subunits ($\alpha_3\beta_3\gamma\delta\varepsilon ab_2c_{ring}$) as in Fig. 1. In higher organisms as many as nine additional subunits are present.

[2] Certain microorganisms, e.g. *Propionigenium modestum* which has been widely studied by Dimroth and colleagues, have an ATP synthase that uses Na^+ ions instead of H^+. This has proven experimentally valuable.

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00394-6

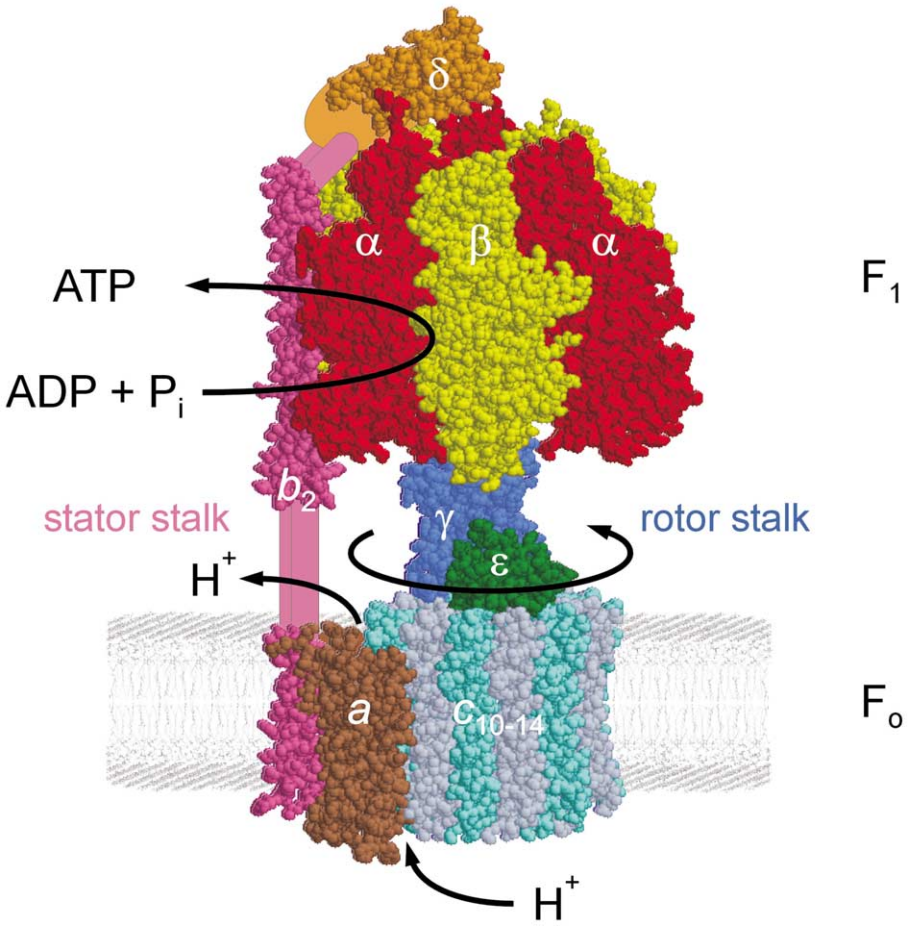

Fig. 1. Structure of *E. coli* F_1F_0-ATP synthase. *E. coli* ATP synthase consists of eight subunits, $\alpha_3\beta_3\gamma\delta\varepsilon ab_2c_n$. F_1 corresponds to $\alpha_3\beta_3\gamma\delta\varepsilon$ and F_0 to ab_2c_n. In recent terminology, the rotor consists of $\gamma\varepsilon c_n$, the stator consists of $b_2\delta$. The *c*-subunits form a ring, with $n = 10$ favored in *E. coli* but values 10–14 reported for various species (see text). *a*-subunit interacts with *c*, and lies outside the *c*-ring. γ consists of a globular foot, which interacts with *c* and ε, plus a long helical coiled-coil (not shown) which penetrates almost to the top of the central cavity within the $\alpha_3\beta_3$ hexagon. δ-subunit is situated on top of F_1, most distant from the membrane. Its N-terminal domain interacts with the N-terminal region of α-subunits, and its C-terminal domain with the C-termini of the b_2 dimer. High-resolution structure has been determined for all subunits except *a*-subunit, C-terminal domain of δ, and portions of the *b*-subunits [15–21]. The *a*-subunit structure shown here is a model [22], not a determined structure. The proton pathway lies between *a*- and *c*-subunits. There are three catalytic sites, situated at interfaces of α- and β-subunits, where ATP is synthesized and hydrolyzed. Catalysis, rotation of $\gamma\varepsilon c_{ring}$, and proton movement occur simultaneously. There are three 'non-catalytic' nucleotide sites (not shown) situated at the three non-catalytic α/β interfaces, which have no known function and may be evolutionary relics.

less drag allowed a major advance in time resolution [27]. Now it was seen that upon MgATP binding there occurred a 90° rotation substep of γ in ≤ 0.25 ms, followed by a stationary interval of around 2 ms, followed by a terminating 30° substep (also ≤ 0.25 ms duration). Most recently fluorescence resonance energy transfer was used to follow MgATP-driven rotation of γ in liposome-reconstituted F_1F_0, with confirmation of rotor stepping [28]. The body of beautiful work described in this paragraph has revolutionized the field. It raises many intriguing questions. The obvious next technical challenge is demonstration of proton gradient-driven rotation, and the nature of its substeps, both eagerly anticipated.

Early calculation found that MgATP hydrolysis achieved a rotor torque of ~40 pN nm [26], later calculation based on a different procedure suggested values of 50–56 pN nm [29]. Thus there is no doubt that this is a highly efficient molecular motor [30]. Whether rotation is driven solely by ATP binding [27,31] or whether it is geared also to the ATP hydrolysis (chemical) step [1] is a currently debated question. Recent reports throw light on this point. First, it was found that MgITP hydrolysis generates the same rotational torque as MgATP [32]. K_m(MgITP) and K_d(MgITP) values are significantly higher than for MgATP, with a difference in binding energy of ~10 kJ/mol [33]. Second, mutagenesis of two Phe residues in the substrate binding pocket was employed to reduce MgATP binding affinity [34]. Turnover of ATP hydrolysis and frequency of rotor steps were diminished, as expected for a slow MgATP association rate, but rotor torque was unaffected by the appreciable resultant loss of substrate binding energy. These experiments support the proposal that the initial 90° substep of rotation involves both binding of MgATP and its hydrolysis. The 30° substep of rotation has been attributed to product release [27], although whether of Pi or ADP, or both, could not be decided. Consideration of reaction chemistry and thermodynamics had led us previously to hypothesize that release of Pi from catalytic sites entailed partial rotation of γ [35]. Recently use of a novel Trp probe revealed a conformational change in β-subunit which corre-

lates specifically with product Pi release, and could well be propagated to γ [36]. Further work along these lines, to clarify the relationship of enzymatic mechanism to rotation, is now a major goal in the field.

Many mutations that affect catalysis have been documented, particularly in *E. coli*, and their effects on rotation are just beginning to be studied. Thus, in [37] it was shown that the mutation βS174F[3] reduced ATP-driven rotor torque to 17 pN nm; suppressor mutations at βIle163 and βIle166 restored normal torque. Between the mutation and suppressor sites is a loop (residues β170–172) suggested in [38] to form the hinge which allows closing and opening of the β-subunit upon substrate binding/release. This work therefore supports the widely considered view that rotation of γ occurs coincident with opening/closing of β, and we return to this point later.

There are three catalytic sites, so for the oft-cited stoichiometry of four protons moving per ATP molecule synthesized or hydrolyzed, one might presume the existence of a ring of 12 *c*-subunits. Contrarily, X-ray crystallography of detergent-solubilized yeast F_1F_0 [39], atomic force microscopy of a *c*-ring from chloroplast [40], or cryoelectronmicroscopy of 2D crystals of an *Ilyobacter tartaricus* *c*-ring [41], yielded values of 10, 14, and 11 *c*-subunits per ring, respectively. Cross-linking studies in *E. coli* suggested a preferred stoichiometry of 10 *c*-subunits [42]. H^+/ATP stoichiometries may vary therefore in different organisms. Lack of three-fold symmetry between *c*-rings and catalytic sites has been discussed as an advantage for a rotational machine, to prevent sinkage into energy minima [39], or to assist elastic power transmission between F_0 and F_1 [43]. Thus, functional consequences of diverse and paradoxical *c*-ring stoichiometries are currently intensely debated.

The idea of elastic power transmission was proposed in explanation of the high efficiency of energy transmission during rotation [44]. In general terms the proposal is that energy can be stored during the rotational step by protein conformational torsion, then released smoothly to be translated into ligand binding affinity changes. Flexibility of the *b*-subunit [45], unwinding of γ (see later), and conformational transitions within β [46] could provide a physical basis for elastic energy storage.

To make rotation feasible requires a stator strong enough to resist rotor torque. Fig. 2 shows established and probable stator interactions in *E. coli* F_1F_0. Binding of δ to the very top of F_1, established by electronmicroscopy [47], involves the N-terminal ~20 residues of α [48], at a single site with K_d of 1 nM [49,50], equivalent to 50 kJ/mol. This region of α is not seen in X-ray structures, but an α-helix is predicted for residues α6–18. Whether all three or just one α is required is unknown. The N-terminal domain of δ-subunit fully suffices for δ binding to F_1 [49] and its structure has been determined by nuclear magnetic resonance (NMR) to consist of a six-helix bundle [17]. One face of this domain shows sequence conservation, and helices 1 and 5, lying on this face, provide binding interactions with F_1 (Weber, J., Wilke-Mounts, S. and Senior, A.E., unpublished data). Interestingly, Mg^{2+} ions enhance δ binding affinity; release of F_1 from F_0 has long been known to require Mg^{2+} chelation. The subunit of mitochondrial ATP synthase that is analogous to *E. coli* δ is called 'OSCP'; its site

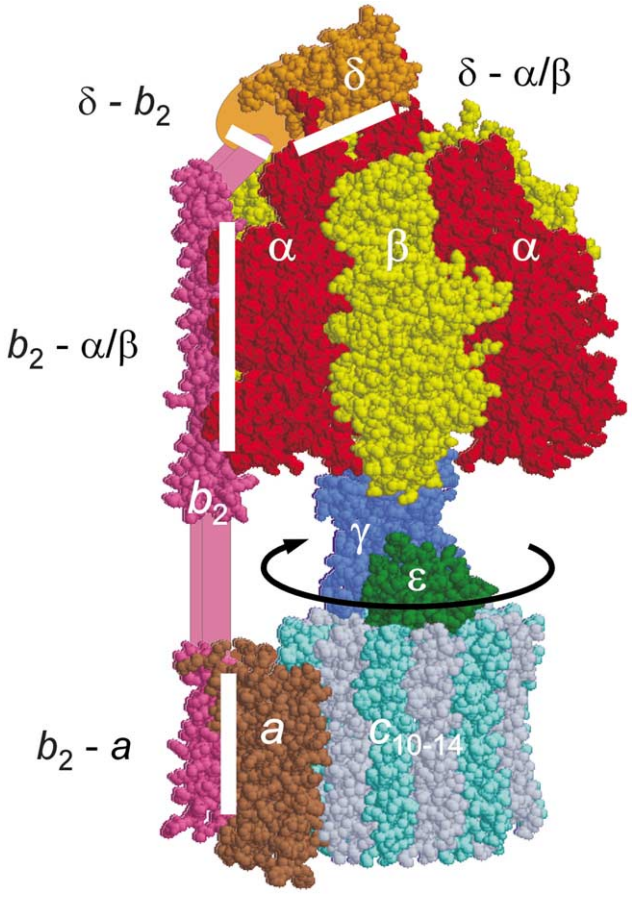

Fig. 2. Structure of *E. coli* ATP synthase showing stator interactions. White rectangles show stator interactions, established and probable, designed to counteract rotor torque.

of binding, structure, and other characteristics appear to be very similar to those of δ [51,52].

The b_2/δ interface is formed between the C-terminal domains of *b* and δ [53], for which high-resolution structure is lacking. There is good evidence for formation of a $b_2\delta$ complex, although the measured affinity (K_d ~ 5 μM) seems unexpectedly weak. Crosslinking studies have shown proximity of the very C-terminal residues of *b* to δMet158 [54], and mutations at conserved δGly150 are among the rare point mutations in δ to impair function [55]. Interactions between b_2 and $\alpha_3\beta_3$ shown in Fig. 2 are inferred from crosslinking experiments [56,57]. Binding of b_2 to *a*-subunit, implicit in the oligomeric stability of F_0, has been supported by crosslinking [56] and suppressor mutagenesis [58]. The N-terminal 22 residues of the two *b*-subunits provide anchoring transmembrane helices which lie close to each other [19]. Interaction sites between *b* and *a* within the membrane have not yet been identified.

Initially, binding affinity measurements in *E. coli* F_1 indicated that the stator resistance was finely balanced with rotor torque [49]. Further experiments in our laboratory have indicated however that the stator may be 'overengineered' to provide excess resistance. In the case of mitochondrial F_1F_0, where OSCP replaces δ and there are several other subunits likely involved in stator structure [52] this appears also to be the case [51].

[3] *E. coli* residue numbering used throughout.

3. How might protons drive rotation of the *c*-ring?

NMR structural studies by Fillingame, Girvin and colleagues revealed that isolated *E. coli* *c*-subunit forms a hairpin consisting of two antiparallel helices connected by a polar loop [18,22]. The two helices traverse the membrane, and the polar loop extends out of the membrane to interact with γ- and ε-subunits (Fig. 3). NMR and cryoelectronmicroscopy studies of *P. modestum* *c*-subunit agree with this structure [41,59]. Models for rings of *c*-subunits have been proposed and supported by crosslinking studies [60]. X-ray studies of F_1F_0 [39] and cryoelectronmicroscopy studies of *c*-rings [41] corroborate these models.

Charged residues lying in the center of the membrane are key players in the ion transport (H^+ or Na^+) mechanism (Fig. 3). A body of work, comprising chemical modification with dicyclohexylcarbodiimide (DCCD), mutagenesis studies, ion specificity studies, and other approaches, has established that residues *c*Asp61 and *a*Arg210, and polar residues in their environment, are directly involved [45,60–65]. Access channels are needed to allow the ions to move from the membrane surfaces to these residues, and the structures of such pathways, involving primarily residues in subunit *a*, have been proposed. Impeding progress is the current lack of high-resolution structure of subunit *a* and the *a*/*c* interface.

A conceptual breakthrough came from the demonstration that upon protonation of *c*Asp61, *c*-subunit undergoes a large rotation of its C-terminal-helix [22]. It was proposed that this helical rotation couples the protonation/deprotonation reaction to angular displacement of *c* versus *a*, resulting in net proton-driven *c*-ring rotation. Structural studies of mutant *c*-subunit [60], and extensive crosslinking of *c* with *a* [61] allowed development of this concept, such that swivelling of

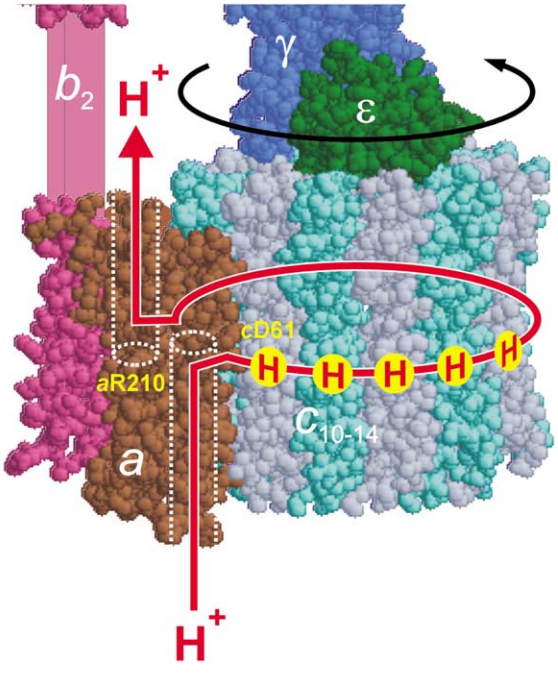

Fig. 3. Structure of ATP synthase showing proposed proton transport path. Residues *c*Asp61 and *a*Arg210 lie in the center of the bilayer, at the *a*/*c* interface. Their concerted interaction is required for proton movement. Putative access channels for ingress/egress of protons are shown. The *c*-ring carries protons around on protonated *c*Asp61 as it rotates.

helices of *c* and *a* in relation to each other is now considered integral to generation of *c*-ring rotation. Studies on Na^+-linked rotation have emphasized the important role of the electrical potential in generating torque [66], revealed details of the mode of interplay between residues *a*Arg210 and *c*Asp61 [64,67], and delineated specific determinants of ion selectivity for Na^+ vs H^+ [68].

There is general agreement that each subunit in the *c*-ring carries a proton or Na^+ ion as it rotates, and that deprotonated or 'unloaded' *c*-subunits occur only at the *a*/*c* interface [18,62]. Thus the effective pK_a (pNa) of *c*Asp61 must be unusually high to prevent dissociation of ion in the relatively long time needed for one full rotation (~30 ms at a typical ATP hydrolysis/synthesis rate of 100/s). *c*Asp61 has a pK_a of 8 [69] in detergent-dispersed F_1F_0 as measured by DCCD reactivity, but the reaction in membranes proved to be pH-insensitive, probably 'because the reacting *c*Asp61 residues are shielded from the bulk aqueous solvent when in the membrane'. This explains why detergent-dispersed preparations of F_1F_0 are 'uncoupled' – they lose the coupling ion during rotation. When *c*-ring rotation was first shown in detergent-dispersed preparations, questions were asked as to whether it was 'physiological' or not [70]. The above considerations show that it is feasible to have ATP-driven rotation without proton pumping. Indeed, a mutant *c*D61N ATP synthase which is unable to pump protons showed normal ATP-driven rotation [71].

4. X-ray structures of F_1

Table 1 lists high-resolution X-ray structures of F_1 now in the literature. The first (1994) structure (Table 1, line 1) and essentially similar subsequent structures from the Walker group (Table 1, lines 2–7) correspond to the enzyme state that has just released product and is about to bind substrate. They all have one catalytic site empty, called βE. The AlF_4^--inhibited structure (Table 1, line 8) is suggested to correspond to an immediate post-ATP hydrolysis state [16], it has all three sites occupied. The site in position of βE now binds ADP and SO_4^{2-} (mimicking Pi) and was named βADP+Pi. Table 1 makes evident other points. First, the strong asymmetry seen in the first structure (Table 1, line 1) has not been universally reported in subsequent studies. Structures with more symmetry have now been seen in rat and spinach chloroplast F_1, even with γ-subunit present. Reasons for this are not clear. Various factors including presence/absence of Mg^{2+} ion during crystallization, use of ammonium sulfate vs PEG as precipitant, or nucleotide occupancy of catalytic sites, have been discussed. Second, there is as yet no structure of a catalytic site with MgATP bound. This is important because MgAMPPNP (5′-adenylyl-β,γ-imidodiphosphate) binds with much lower affinity than MgATP [82] and so a site with MgAMPPNP bound will not faithfully mimic one with MgATP. Third, the catalytic sites can bind a range of nucleotides – the βDP and βTP sites are not limited to, nor determined by, ADP and AMPPNP binding, a misapprehension that seems widespread. Also, the conundrum remains that even though bovine, *E. coli*, chloroplast, and *Bacillus* PS3 enzymes have all been shown to bind three MgAMPPNP and other Mg-nucleotides at catalytic sites under saturation conditions [82–86] it has proven impossible so far to get F_1 to crystallize with three MgAMPPNP or other Mg-nucleotides

Table 1
High-resolution structures of F_1

Source	Description	Reference	Asymmetric/symmetric[a]	Nucleotides in catalytic sites[b]		
				βDP	βTP	βE
Bovine	'Native' F_1	[11,72]	Asymmetric	ADP	AMPPNP	Empty
Bovine	Aurovertin-inhibited F_1	[73]	Asymmetric	ADP	AMPPNP	Empty
Bovine	Efrapeptin-inhibited F_1	[74]	Asymmetric	ADP	AMPPNP	Empty
Bovine	NBD-Cl-inhibited F_1	[75]	Asymmetric	ADP	AMPPNP	Empty
Bovine	DCCD-inhibited F_1	[15]	Asymmetric	ADP	ADP	Empty
Bovine	AlF_3-inhibited F_1	[72]	Asymmetric	ADP·AlF_3	AMPPNP	Empty
Bovine	High [AMPPNP] F_1	[76]	Asymmetric	ADP	AMPPNP	Empty
Bovine	AlF_4^--inhibited F_1	[16]	Asymmetric	ADP·AlF_4^-	ADP·AlF_4^-	ADP+SO_4^{2-}[c]
E. coli	'Native' F_1	[77]	Asymmetric	Unknown[d]	Unknown	Unknown
Bacillus PS3	$\alpha_3\beta_3$ subcomplex	[78]	Symmetric	All three sites empty, βE-like		
Rat	'Native' F_1	[79]	Symmetric	ADP+Pi; ADP+Pi; ADP; all βDP-like		
Spinach	'Native' CF_1	[80]	Symmetric	All three sites empty, βDP-like		
Spinach	Tentoxin-inhibited CF_1	[81]	Symmetric	All three sites empty, βDP-like		

[a]Asymmetric and symmetric are relative terms in this context. Among the bovine structures those in lines 1–7 are essentially identical and all are strongly asymmetric. The AlF_4^--inhibited F_1 (line 8) is less asymmetric because its half-closed βADP+Pi subunit (see footnote c) differs less from βDP and βTP. Spinach chloroplast and rat liver F_1 show more symmetric arrangement of $\alpha_3\beta_3$ but still contain the (necessarily) asymmetric γ-subunit. The *Bacillus* PS3 $\alpha_3\beta_3$ subcomplex lacks γ, contains no nucleotide in catalytic sites, and is truly symmetric.
[b]Except in the rat F_1, all nucleotides were present as Mg complexes. We use the designations βDP, βTP and βE from [11] for the three catalytic sites in the strongly asymmetric bovine F_1 structures. βDP and βTP involve β-subunit in 'closed' conformation whereas β-subunit in βE is 'open'. The difference is that the C-terminal domain is hinged up in βTP and βDP, down in βE. In the bovine AlF_4^--inhibited F_1, βE is replaced by (βADP+Pi) in which the β-subunit is half-closed. Note that the actual catalytic sites are different again, because α-subunit contributes to the catalytic interface. Thus the βDP and βTP catalytic sites are substantially different from each other, despite similarity of β-subunit structures βDP and βTP.
[c]In this structure the catalytic site corresponding in position to βE is in a half-closed conformation called βADP+Pi. SO_4^{2-} is suggested to mimic Pi [16].
[d]Resolution of this structure was at 4.4 Å.

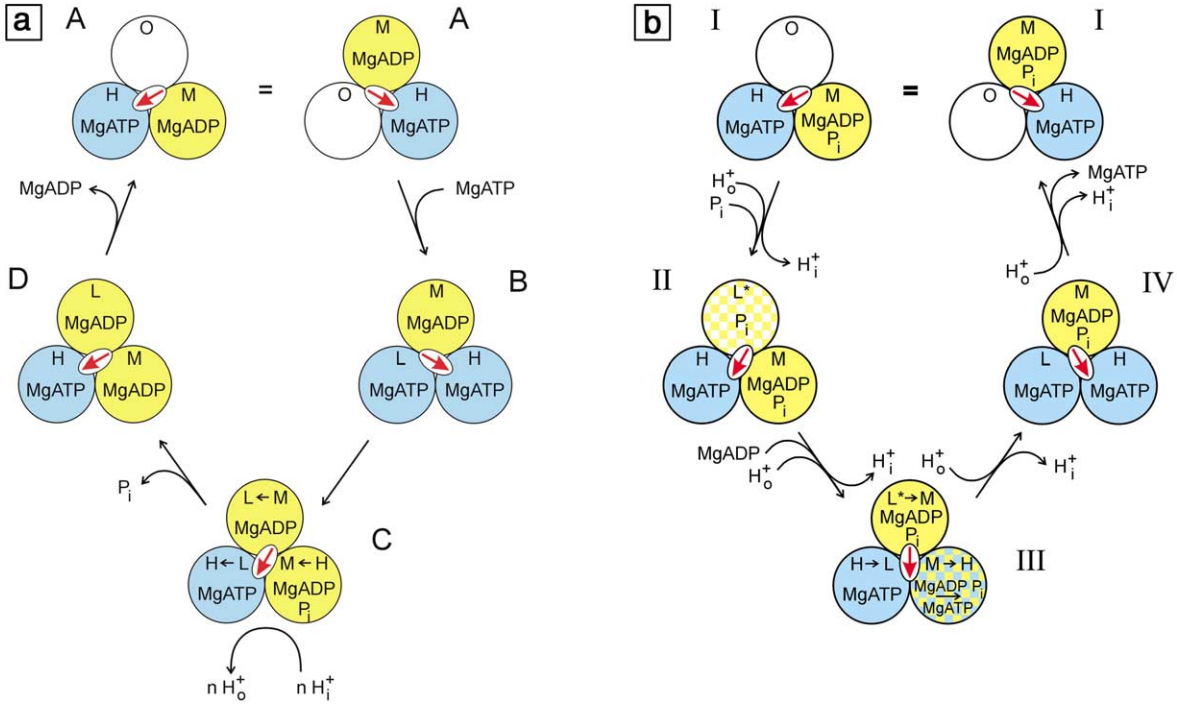

Fig. 4. Enzymatic mechanisms of ATP hydrolysis and synthesis. Catalytic site conformations are: O, open (unoccupied); H, highest affinity for nucleotide; M, medium affinity; L, lowest affinity; L*, site with Pi binding pocket present. The central arrow denotes γ-subunit rotation. a: ATP hydrolysis. Binding of ATP to the empty site O (A→B) brings about hydrolysis in the H site by catalytic site cooperativity (B→C). Combined binding and hydrolysis of ATP occur with 90° rotation of γ, which is also associated with switch in conformations of the catalytic sites ('binding change'). Pi is released (C→D) associated with 30° rotation of γ, followed by ADP release (D→A) to regenerate the starting ground state. b: ATP synthesis. Proton-driven γ rotation generates L* from O (I→II) so that Pi binds. This allows discrimination so that ADP binds (II→III) despite an unfavorable [ATP]/[ADP] ratio in the cell of ≥10/1. Next the binding change occurs (II→III→IV) and ADP+Pi condense chemically at the (new) H site. Release of ATP involves transformation of an H site via L to O site (III→IV→I).

bound in catalytic sites except by using the very tight binding MgADP·fluoroaluminate complexes. Clearly there are selective forces at work in the crystallization conditions that we do not as yet understand.

Beginnings have been made in integrating X-ray structure information with mechanics of rotation. The structures in Table 1, lines 1–7, are essentially identical in terms of spatial relationship of γ to $\alpha_3\beta_3$, but in the AlF_4^--inhibited structure (line 8) a rotation of γ by ~20° has occurred. From a detailed comparative analysis of native and AlF_4^--inhibited enzymes, Menz et al. [16] argue that this γ rotation is linked to partial closure of the hinge in the β-subunit, resultant upon binding and hydrolysis of ATP. Substantial movement of the C-terminal domain of β occurs during this hinge motion. The importance of interactions between the C-terminal domain of β and the γ-subunit for rotation had been anticipated by mutagenesis of γ [87].

Molecular dynamics simulations of γ rotation using bovine native F_1 X-ray structure as template have been reported [88,89]. Forced rotation of γ through a 120° step in ATP synthesis direction was deduced to result in specific conformational changes in β-subunits that were proposed to be linked

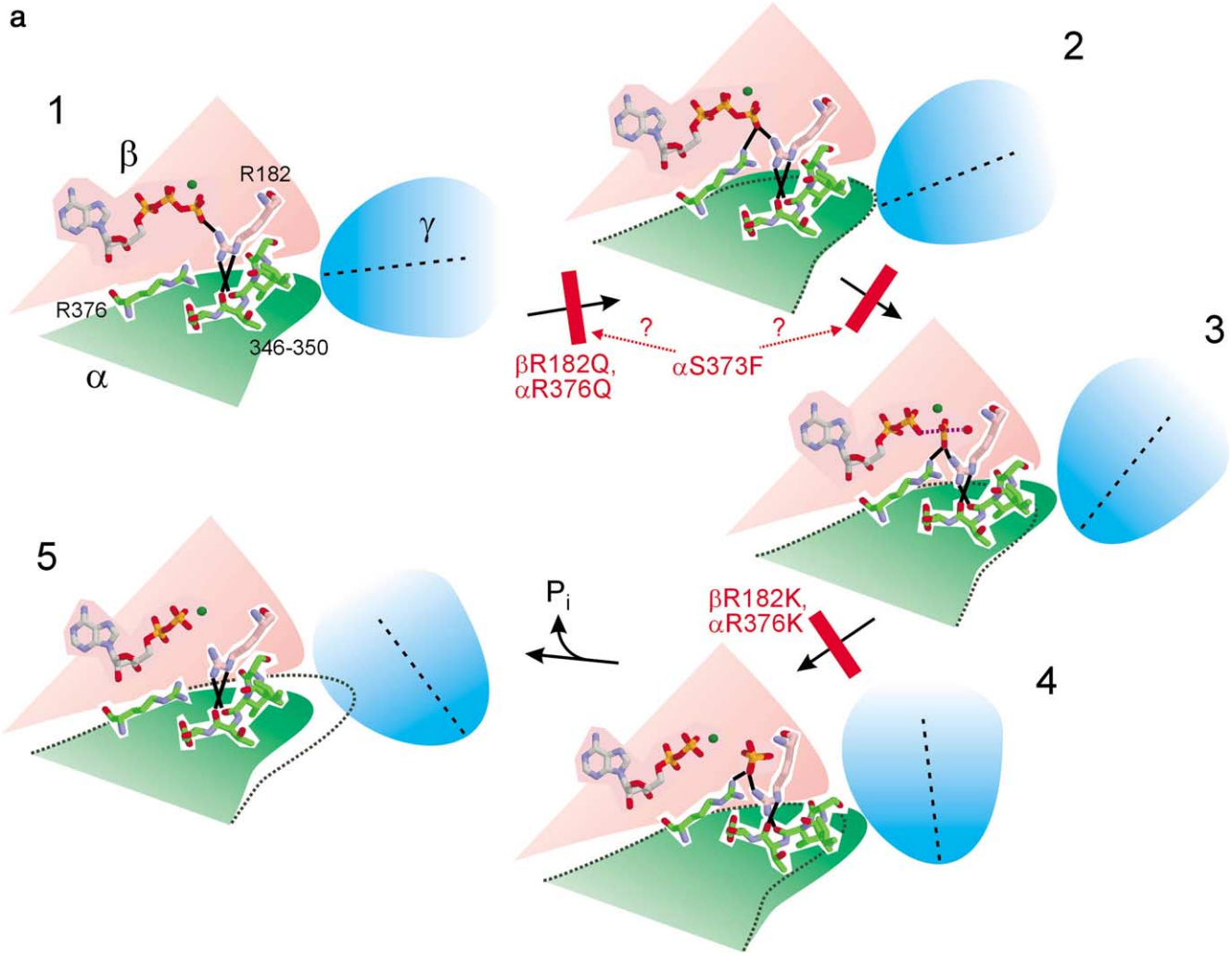

Fig. 5. Mechanoenzymatic mechanism of ATP hydrolysis. a: Proposed pathway of ATP hydrolysis. β-Subunit in pink, α in green, γ in blue, Mg^{2+} cation as green sphere. State 1, MgATP binds to an empty catalytic site at the α/β interface, eliciting partial rotation of γ as the site closes by β-hinging motion. This causes insertion of the 'arginine finger' (αArg376) into the site (State 2), triggering formation of the catalytic transition state (State 3). Formation and collapse (State 4) of the transition state involves lengthening of the ADP-O to P bond, then electrostatic repulsion moves Pi away from ADP. Residue αArg376 moves with the Pi. Residue βArg182, which initially ligates to ATP (State 1) likewise moves with Pi. Both Arg residues are linked by H-bonding to residues α346–350, hence dislocation of the α/β interface occurs as the hydrolytic event proceeds. Rotation of γ also occurs in these steps, and as State 4 is reached γ has rotated 90°. The final 30° rotation occurs as Pi is released (State 4→5), leaving both αArg376 and βArg182 disengaged. Spontaneous release of ADP from State 5 will yield an empty site to which ATP can bind to initiate another round of hydrolysis. Effects of mutations (αS373F, αR376Q, αR376K, βR182Q, βR182K) to block ATP hydrolysis at indicated points are referenced and discussed in [35]; see also [1,16] for further information. b: The catalytic transition state. Upper: the natural catalytic transition state occurring in State 3 of panel a (above) as originally proposed from mutagenesis studies in our laboratory [1]. Lower: X-ray structure of the transition state analog MgADP·AlF_4^- in the βDP catalytic site [16] (F atoms, brown; Al, gray; proposed attacking water, blue; P-loop, yellow ribbon). c: The Pi binding pocket. Details of the Pi binding pocket occurring in the βADP+Pi catalytic site in the X-ray structure [16], with Pi replacing the observed SO_4^{2-}. This is the conformation proposed to occur in State 4 of panel a (above), and also in the L* catalytic site in ATP synthesis (Fig. 4b). The optical probe βR323W, which senses bound Pi and nucleotide γ-P [36], is shown in pink, water molecules in blue (not all water molecules H-bonded to the Pi are shown, for clarity).

to substrate binding and product release. In [89] an 'ionic track' composed of charged residues on γ was suggested to guide motions of β (although as noted the residues involved are not highly conserved). In addition, unwinding of the γ coiled-coil region was predicted to occur during the rotation step [89]. Hausrath et al. [77] noted that when comparing their crystals of *E. coli* F_1 to the bovine enzyme, unwinding of the coiled-coil region of γ was evident. Unwinding of γ may well represent a physiological state related to elastic energy transmission.

A displacement of ε in *E. coli* F_1 vs bovine F_1 was seen by [77], with the structure of ε conforming to isolated *E. coli* γε complex [90]. A relatively large-scale motion in the C-terminal region of ε is predicted from comparison of various X-ray structures, which is consistent with crosslinking and immunological studies [91,92]. On this basis ε-subunit has been proposed to regulate ATP synthesis vs hydrolysis by acting as a ratchet to control directionality of rotation [93].

5. Mechanism at the level of F_1: ATP hydrolysis and synthesis

5.1. Enzymatic mechanism

We define the term 'enzymatic mechanism' in the usual sense, as a series of enzyme states starting before substrate binding and ending after product release, encompassing all significant conformational and chemical intermediate states in between. A diagram consisting of a series of enzyme states, starting and finishing at the same state, is the accepted presentation. In F_1, rotation of γ also comes into play – for each enzyme state along the pathway there will be a corresponding

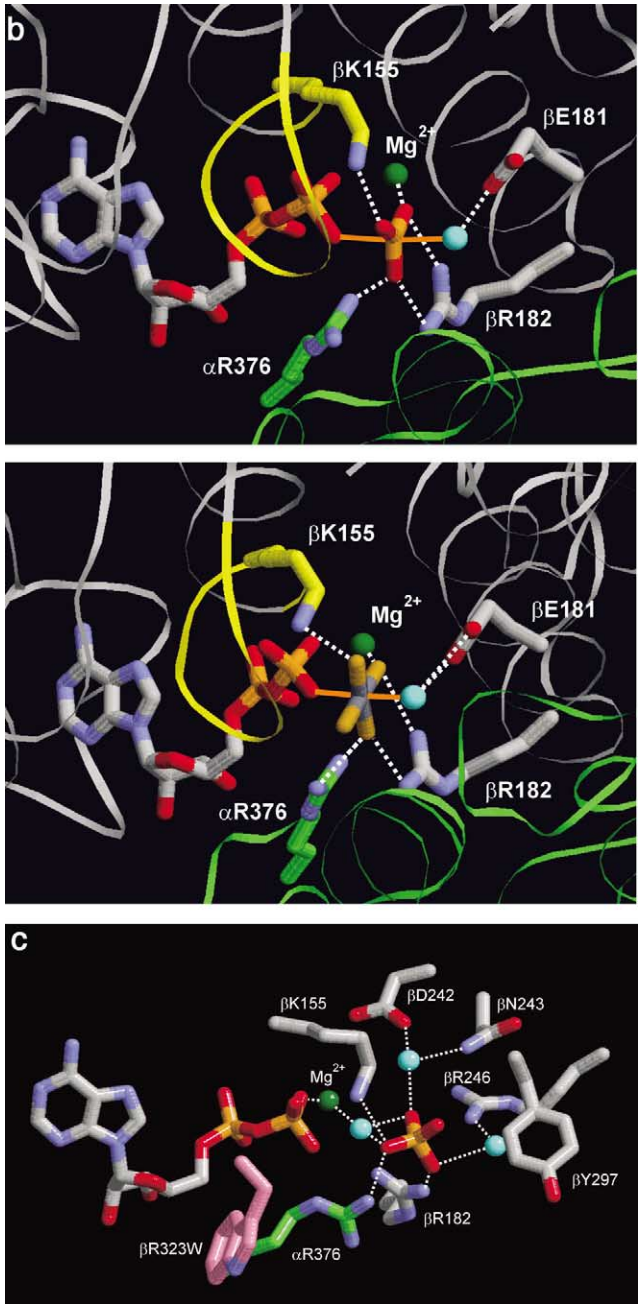

Fig. 5 (*Continued*).

position of γ within its arc. We call the resultant diagram a 'mechanoenzymatic mechanism'. Because there are three catalytic sites in F_1, there is the possibility of three different types of catalysis, namely unisite, bisite and trisite. Following the first usage of these terms [94] we define unisite catalysis as that occurring in an F_1 with only one site occupied by substrate, bisite as that which occurs when only two sites fill leaving one empty, and trisite as that which occurs when all three sites fill with substrate. By this definition, bisite catalysis can be dismissed if an F_1 with only two sites filled shows insignificant rate, which is in fact the case [33].

Principles developed by Boyer provide the guide for development of mechanisms in this area. Boyer proposed that the three catalytic sites pass sequentially through three different conformations ('binding changes') linked to subunit rotation, such that at any moment in time different steps of the enzymatic mechanism are occurring at each of the three sites. In ATP synthesis this was envisaged to entail binding and sequestration of substrates MgADP and Pi, chemical synthesis of ATP, and release of ATP. Binding of substrates and release of product were considered to be 'energy-linked' steps (we can now say 'rotation-linked') whereas the chemical step was thought to occur without free energy change.

Several attempts to write an enzymatic mechanism incorporating Boyer's principles together with newly available structure and nucleotide binding information have been made [2–4,16,27,79,95,96]. It is apparent from the diversity of opinion that no uniform enzymatic mechanism has yet emerged. Therefore, until a written enzymatic mechanism becomes generally accepted, it is preferable to refer to Boyer's 'binding change principles' rather than to a 'binding change mechanism'. Our suggested enzymatic mechanisms for ATP hydrolysis and synthesis are shown and discussed in Fig. 4a,b. We propose a mechanoenzymatic mechanism later.

Evidence now convincingly supports trisite mechanisms as in Fig. 4a,b, because molecules with two sites filled show negligible activity [82,86,97] and writing a bisite mechanism is problematic [33,98]. A corollary is that only a molecule with all three sites filled can rotate [1,33]. This emphasizes a major point, that the F_1 motor functions obligatorily as a three-cylinder engine. Two sites are not enough, it is the filling of the third catalytic site that brings about the conformational changes and catalytic cooperativity that are critical for operation of the mechanoenzymatic mechanism. In interesting contrast, other transport ATPases also come as two-cylinder or one-cylinder models (ABC transporters, P-type).

5.2. Catalytic site conformations

Originally, three different catalytic site conformations were proposed [13,99], namely 'tight', 'loose' and 'open'. In effect this was a semantic device to conveniently designate three disparate conformations, since actual equilibrium binding data were not yet available. Recent data have shown that these designations are insufficient and actually confusing, thus they should now be abandoned.

There is general agreement that each of the three catalytic sites can bind nucleoside tri- and diphosphate, with widely different affinities [82]. In *E. coli* F_1, K_d values for MgATP binding are around 1 nM, 1 μM, and 30 μM, respectively. We designate these binding conformations as H (highest affinity), M (medium affinity) and L (lowest affinity). An open (O) site conformation is different again – by definition an O site is unoccupied. In *E. coli* F_1 it has K_d for MgATP > 10 mM, precluding binding of nucleotide under effectively all circumstances. Our scheme for ATP hydrolysis in Fig. 4a requires these four different catalytic site conformations. In ATP synthesis, a fifth, distinct catalytic site conformation that can specifically bind Pi is generated by energy-linked γ rotation [1]. We designate this as L* in Fig. 4b. Formation of the Pi binding pocket in the L* conformation is important in synthesis direction to prevent unwanted binding of ATP from the medium instead of Pi+ADP. The model of Menz et al. [16], based on X-ray structures, is generally similar to our models, although using different designations (T, L, L′, L″, O) and with differences in detail.

Correlations with X-ray structures are as follows. The O site corresponds to βE; the L* site corresponds to βADP+Pi in the AlF_4^--inhibited structure; L likely corresponds to a site that is similarly half-closed, but with ATP bound, which has not yet been seen crystallographically [16]. M and H will correspond to βTP and βDP, likely in that order (this is the scenario favored in [6,16] but we note that only MgAMPPNP has so far been seen in the βTP site; a structure with MgATP in either βTP or βDP could yet alter our views as to which is the highest affinity site).

5.3. Catalytic site cooperativity

Promotion of ATP hydrolysis at the highest affinity site upon binding of ATP to the other catalytic sites ('positive catalytic cooperativity') is an integral mechanistic feature, demonstrated in [94]. It is evident also from the nucleotide binding affinities that there exists apparent negative binding cooperativity. The alternating arrangement of α and β in $\alpha_3\beta_3$ hexagon provides a possible communication route between catalytic sites via β-α-β interactions. This was anticipated by studies of defective catalysis in α mutants [100]. However the central location of γ potentially provides a different kind of cooperativity, in which each face of γ enforces a separate nucleotide binding and catalytic behavior on each catalytic site. In this scenario, each site is independent, but three different behaviors can be encompassed. Analysis of nucleotide binding characteristics showed that either type of cooperativity was consistent with the data [33] but crosslinking studies supported the idea that the position of γ determines the nucleotide binding affinity of each site [101]. Mutational studies continue to support β-α-β signal transmission for positive catalytic cooperativity [4,86]. Menz et al. [16] incorporate both β-α-β and γ-imposed cooperativity between catalytic sites into their X-ray structure-derived mechanism of catalysis.

5.4. A mechanoenzymatic mechanism of ATP hydrolysis

Combined use of optical probes, mutagenesis of catalytic site residues, and X-ray structural information, allows us to formulate a mechanoenzymatic mechanism for binding and hydrolysis of ATP and release of products, shown and discussed in Fig. 5a. The chemical transition state is shown in Fig. 5b, and the pocket within which product Pi is contained transiently after hydrolysis is shown in Fig. 5c. In the mechanism presented in Fig. 5a, the hydrolysis step is linked to γ rotation. Menz et al. [16] concur, but Boyer [8,13] favors a mechanism in which the hydrolysis step is not energy-linked.

In ATP synthesis direction the Pi binding pocket (Fig. 5c) is formed by proton-driven γ rotation, with Pi binding preceding ADP binding (Fig. 4b). Bound Pi must then be moved close to

bound ADP to form ATP, essentially by reversal of States 4→1 of Fig. 5a, utilizing the same transition state as in hydrolysis (Fig. 5b). An interesting question is whether the substeps of γ rotation occurring during ATP synthesis will be the same as seen during hydrolysis, or whether more, incremental, steps are needed. Once formed, ATP must be released from the high affinity site. This is believed to occur as the β-subunit C-terminal domain is forced to hinge downward to open the catalytic site, and the mechanism by which rotation of γ-subunit drives opening of the β-subunit has been extensively discussed [16,38,46,90]. It is essential that the affinity of the catalytic site for ATP is drastically reduced, to maintain high cellular ATP concentration; as noted earlier K_d(MgATP) upon opening is >10 mM. Other enzymes that face the same challenge are creatine kinase, pyruvate kinase (PK) and phosphoglycerate kinase (PGK). Interestingly, PGK is known to operate using a 'hinging of domains' mechanism [102], and the structure of PK shows that this enzyme might also do so [103].

5.5. Two potential challenges to current dogma

Whether the chemical step occurs reversibly without free energy change during steady-state ATP synthesis is controversial. We have argued [1] that evidence usually proffered, namely data derived from unisite catalysis and ^{18}O-exchange measurements, is insufficient to establish the point. For example, at high rates of net ATP synthesis the average number of reversals of the chemical reaction per ATP released fell to ~2 [104], which can easily be accounted for by a minority of enzyme molecules undergoing rapid reversals but not engaged in steady-state synthesis. Possibly the reversible synthesis/hydrolysis reaction that is measured by unisite catalysis and by the ^{18}O-exchange technique is an idling mode, manifested at low occupancy of catalytic sites or in absence of a proton gradient, where γ rotation is not possible, to conserve the proton gradient or ATP when the enzyme is not engaged in productive work.

An interesting situation has arisen regarding how many catalytic sites are actively engaged in chemical catalysis at any one time. Complementary biochemical [105] and structural [16] evidence has shown that two sites in F_1 can form a transition state-like structure simultaneously with bound MgADP·fluoroaluminate (AlF_4^-). In other enzyme systems (e.g. myosin, G-proteins) MgADP·fluoroaluminate is accepted as a transition state analog, and indeed its use contributed substantially to understanding the mechanism of G-protein activation. Had there been no previous history, we might now be discussing seriously mechanisms utilizing two chemically active sites, since at the very least the new data show that such a situation is not structurally precluded in F_1. This could be an area of development in the future.

6. Conclusions

How do protons drive rotation and how is rotation linked to ATP synthesis? How does ATP hydrolysis drive rotation and how is rotation linked to uphill transport of protons? In trying to answer these questions the ATP synthase field has expanded into 'mechanoenzymology', encompassing not only enzymology and membrane transport, but also engineering and nanotechnology. It represents virgin territory for physicists, biophysicists, biochemical engineers, membrane transport researchers, and enzymologists, to collaborate in understanding two of biology's fundamental processes.

Acknowledgements: Supported by NIH Grant GM25349. The authors thank G. Groth, R.A. Capaldi, A.G.W. Leslie, J.E. Walker, L.M. Amzel, and P.L. Pedersen for advice concerning X-ray structures.

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