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Review

ATP synthase: what we know about ATP hydrolysis and what we do not know about ATP synthesis

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Abstract

In ATP synthase, X-ray structures, demonstration of ATP-driven γ-subunit rotation, and tryptophan fluorescence techniques to determine catalytic site occupancy and nucleotide binding affinities have resulted in pronounced progress in understanding ATP hydrolysis, for which a mechanism is presented here. In contrast, ATP synthesis remains enigmatic. The molecular mechanism by which ADP is bound in presence of a high ATP/ADP concentration ratio is a fundamental unknown; similarly P_i binding is not understood. Techniques to measure catalytic site occupancy and ligand binding affinity changes during net ATP synthesis are much needed. Relation of these parameters to γ-rotation is a further goal. A speculative model for ATP synthesis is offered.

Keywords: ATP synthase; Proton pumping; Oxidative phosphorylation

1. Introduction

The 3 years since we wrote a comprehensive review of ATP synthase [1] have been characterized by attention-catching developments. Scientifically, the big advance was the demonstration of ATP-driven rotation of the γ subunit [2,3], meaning that the enzyme is truly a 'molecular motor', as P.D. Boyer and G.B. Cox had hypothesized. Another headline event was the award of the 1997 Nobel Prize in Chemistry to P.D. Boyer and J.E. Walker for their contributions to the field [4,5]. Now that the excitement generated by these events has subsided, we must not lose sight of the fact that there are still many aspects of the mechanism which are only partially or not at all understood. Although the mechanism of ATP hydrolysis and proton pumping is becoming clearer, our understanding of ATP synthesis remains rudimentary in molecular terms. There are large portions of the enzyme for which high-resolution structure is not yet available, i.e., F_0 and the stalk(s) connecting F_1 and F_0. In this short review we describe recent advances in the understanding of the catalytic mechanism of F_1, and address aspects which are controversial or await resolution.

2. Models

The majority of current models for ATP synthesis and ATP hydrolysis have the following features in common.

1. There are three catalytic sites, each directly involved, which are in principle identical, but have different affinities for substrate at any given moment in time during catalysis, i.e., they show asymmetry. The catalytic sites are termed 'high'-,

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'medium'- or 'low'-affinity sites, or sites 'one', 'two', and 'three', respectively.
2. The three sites switch their affinities at a fixed step in the catalytic sequence.
3. Only one catalytic site, the one with high affinity (site one), is able to perform catalysis at any given time.
4. Substrate binding to sites two and/or three is necessary for product release from site one to occur at a rate consistent with physiological catalysis. This 'site to site' positive cooperativity is responsible for a significant fraction of catalytic rate acceleration.
5. In ATP synthesis, energy contained in the proton gradient is used to reduce affinity for product MgATP, also to enhance affinity for substrate P_i.

Additionally it may be noted that most authors, in drawing mechanistic schemes, use bidirectional reaction arrows, implying that ATP synthesis and ATP hydrolysis are reversible by the same intermediate steps. Contrarily, a key obstacle to ATP synthesis, namely the binding of ADP by ATP synthase in the face of a seemingly prohibitive ATP to ADP concentration ratio inside bacteria or mitochondria, has been addressed only rarely.

In the following, we discuss experimental evidence supporting these features, and the findings which favor certain models over others. First, we examine the models from the perspective of ATP hydrolysis. This is the reaction for which most experimental data has been obtained. Then we examine ATP synthesis.

3. ATP hydrolysis

3.1. Features of ATP hydrolysis

Clear evidence for presence of a single site of high affinity for substrate MgATP on F_1, and for promotion of release of products MgADP and P_i from this site by binding of substrate MgATP to additional site(s), was presented by Penefsky and colleagues (reviewed in [6]). These experiments were carried out however under conditions far from physiological in terms of MgATP concentration, and measured only a single turnover of MgATP hydrolysis (termed 'unisite catalysis'). The first (and so far only) demonstration that all three catalytic sites are asymmetric in their behavior toward substrate MgATP came from direct measurement of the binding affinities of all three catalytic sites for substrate MgATP published by us 6 years ago [7], using as a probe the fluorescence of a genetically-engineered Trp, β-Trp-331,[1] inserted into the adenine-binding subdomain of the catalytic site of *Escherichia coli* F_1. These experiments established that all three sites are filled at cell MgATP concentrations. In accordance with data from the Penefsky laboratory, site one was found to be of high affinity ($K_d \leq$ nM); the other two sites had K_d of the order of 1 μM and 100 μM, respectively. The recently-demonstrated rotation of the central γ subunit upon hydrolysis of MgATP [2,3] provides a compelling argument for a synchronized switch of affinities of the three catalytic sites during physiological catalysis.

It has not proven an easy task to detect or monitor the high-affinity site during steady-state MgATP hydrolysis turnover, nor to establish that only this site is catalytically competent. Results from two studies are relevant in this context. First, using F_1 from two Trp mutants which responded specifically to nucleotide binding at the high-affinity site, αF291W and βY297W, it was shown that one of the three catalytic sites exists in high-affinity conformation during steady-state hydrolysis [8]. Secondly, use of fluoroaluminate has been helpful [9]. MgADP•AlF_x complex is an analog for the catalytic transition state of ATPases and GTPases, and transition state analogs bind tightly to enzymes by capturing a fraction of the binding energy for the true transition state species [10]. Using β-Trp-331 fluorescence as signal, we determined that fluoroaluminate increases binding affinity for MgADP at site one by several orders of magnitude; binding of MgADP at site two was moderately increased, and site three was not affected [9]. The results demonstrate convincingly that site one can assume transition-state conformation, while site three cannot. Taking the results of earlier studies [11–13] into consideration, we concluded that site two does bind MgADP•AlF_x, but with only partial transition state-like structure [9]. Overall these results demonstrate that in the catalytic transition state, F_1

[1] *E. coli* numbering is used throughout.

contains three total bound nucleotides in catalytic sites, and that site one can perform catalysis whereas site three cannot. Whether site two can actually perform ATP hydrolysis, or a partial reaction thereof, remains to be clarified.

3.2. Site occupancy during rapid steady-state ATP hydrolysis

Earlier 'unisite' work [6,14,15] had shown that net hydrolysis of enzyme-bound MgATP to MgADP+P_i on the high-affinity site was accelerated by $\sim 10^5$-fold when 'chase' MgATP was added. Acceleration is due both to enhanced product release and enhanced rate of the catalytic step. Using the fluorescence signal of β-Trp-331, comparison of MgATP binding stoichiometry with hydrolysis data under conditions of rapid steady-state hydrolysis showed that K_{d3} (K_d for binding of MgATP to the low-affinity site) corresponds to K_m(MgATP), and that all three catalytic sites must be filled to obtain V_{max} ATPase activity. This was the case for isolated F_1 [1,7], for detergent-solubilized F_1F_0 [16], for ε-depleted F_1 [17], and for $\alpha_3\beta_3\gamma$ complex [13]. The data unambiguously establish that an F_1 molecule with just two catalytic sites filled by MgATP, while it may show residual 'bi-site' activity equivalent to a few percent of V_{max}, cannot attain catalysis rates necessary to sustain cell viability.

This work eliminates all models which require that one site remains unfilled or 'open' throughout the catalytic cycle, such as the one shown in Fig. 1. In Fig. 1, ATP hydrolysis proceeds from right to left; this model involves only enzyme species with maximally two sites filled. For reasons stated above, prevailing cellular MgATP concentration would dictate filling of all three sites, and the mechanism as described could not reach physiological catalytic rates. A further problem with this model is that at the substrate binding step, MgATP binds to the low-affinity site three while the medium-affinity site two remains empty. This binding pattern could occur only infrequently. A corollary of the conclusion that models which incorporate a constantly 'open' catalytic site are non-viable is that the concept that substrate binding and product release steps occur simultaneously must now be discarded. Rather, the binding and release events occur sequentially, as in other enzymes.

Subsequent to development of the fluorescent probe β-Trp-331 to measure nucleotide occupancy of the catalytic sites, we generated a probe that was able to differentiate between bound MgADP and MgATP, by introducing a Trp in the vicinity of the γ-phosphate-binding subdomain at residue β-Phe-148. Using the fluorescence response of β-Trp-148, we determined that during rapid MgATP hydrolysis (at V_{max}), in time-average, the bulk of enzyme molecules contain two catalytic sites filled with MgADP, and one with MgATP [18]. Thus, hydrolysis models in which all participating enzyme species have either two or three sites filled with MgATP also cannot be correct [19,20].

A mechanism for ATP hydrolysis encompassing results described above is shown in Fig. 2. Starting at the end of the cycle after release of product MgADP from site three (step C→D), site one is filled with MgATP, site two with MgADP, and site three is empty (state D). This is the state of the enzyme captured in the first X-ray structure [5]. Under V_{max} conditions and at physiological concentration, substrate MgATP binds rapidly to site three

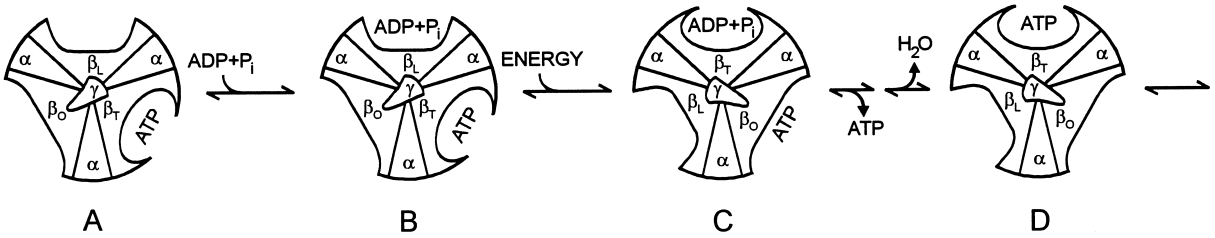

Fig. 1. Earlier mechanism of ATP hydrolysis. This model is from the Nobel Prize in Chemistry (1997) (poster), Royal Swedish Academy of Sciences, with permission. ATP hydrolysis proceeds from right to left (C→B→A, note that A and D are the same). The three catalytic sites are labelled open (β_O), loose (β_L) and tight (β_T). In the text we discuss reasons why this mechanism is incorrect.

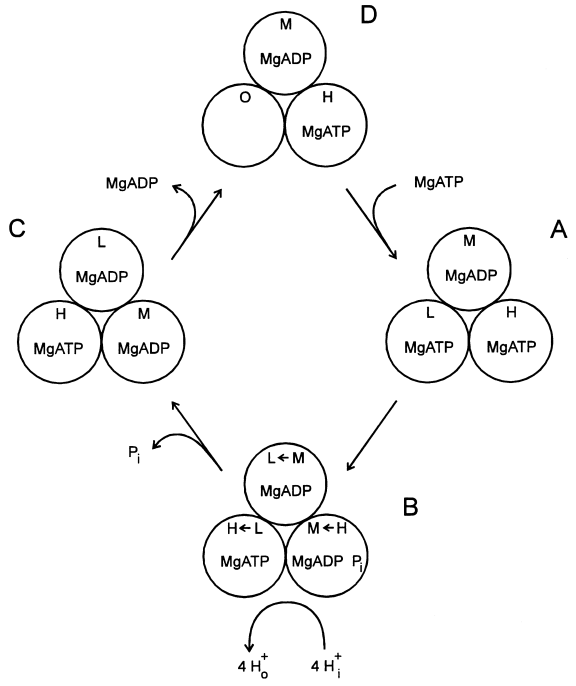

Fig. 2. New mechanism of ATP hydrolysis. This mechanism was developed in our laboratory [1,18,23]. The enzyme passes through four states, A→D, in one complete cycle of catalysis. The three catalytic sites are labelled H (high-affinity site one), M (medium-affinity site two) and L (low-affinity site three). In each of these forms the β subunits are in 'closed' conformation, with bound nucleotide. In state D, an 'open' conformation (O) of the catalytic site occurs transiently, after product release leaves the site temporarily empty. See the text for further details.

yielding state A [21]. The MgATP binding event promotes MgATP hydrolysis at site one, with the catalytic transition state occurring as an intermediate between states A and B. MgATP hydrolysis at site one in turn triggers a concerted affinity change of the three catalytic sites (indicated by the arrows in state B). The affinity change coincides with a 120° rotation of the γ subunit from one β subunit to the next [22], which is coupled to proton translocation through F_0. (We suggest that the hydrolysis event itself generates the initial mechanical forces to ultimately drive γ-rotation, see later). The next steps are release of P_i from (now) site two (B→C) and then release of MgADP from site three (C→D).

The experimental determination that in time average the majority of enzyme molecules contain two bound MgADP and one bound MgATP (state C) led us originally to propose that MgADP release is the rate-determining step of steady-state hydrolysis [18,23]. However, as noted [18], because of the low affinity of the enzyme for P_i (see below), it was not possible to determine the β-Trp-148 fluorescence spectrum for a catalytic site containing both MgADP and P_i. Thus, we cannot formally exclude the possibility that sites containing bound MgADP might also contain P_i. In that case, any step occurring after the actual hydrolysis reaction, i.e., nucleotide binding affinity change, P_i release (as long as it precedes MgADP release), or MgADP release, could be rate-limiting. Some authors [24,25] favor the affinity-change step as rate-limiting.

The mechanism in Fig. 2 is the first to be based on direct measurements of catalytic sites nucleotide occupancy made during steady-state hydrolysis, which is its major attractive feature. So far, no such measurements have been made during ATP synthesis, hence the arrows are unidirectional.

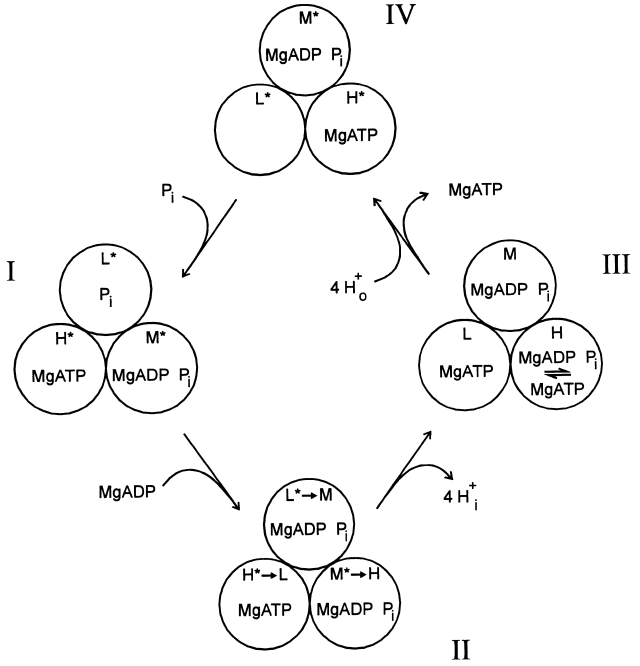

Fig. 3. Speculative model of ATP synthesis. H, M, L refer to high-affinity site one, medium-affinity site two, and low-affinity site three, respectively. An asterisk denotes that the relative affinity of that site for P_i or MgATP is modulated from its normal value by the proton gradient. See text for further discussion.

3.3. Conformation of the low-affinity site

In the first X-ray structure [5] the β subunit carrying the empty catalytic site (βE) adopted an 'open' conformation, quite different from the 'closed' conformations of the two occupied β subunits (βTP and βDP). In our model the open conformation occurs in state D (Fig. 2). An interesting question is, what is the conformation of βE after it fills with MgATP in steady-state catalysis? Several groups propose that the empty site closes, wholly or partly, upon binding of nucleotide [20,21,25,26]. Such a conformational change appears logical. The difference in affinity between sites two and three is relatively small, between zero and two orders of magnitude depending upon the nucleotide, corresponding to only zero to <2.7 kcal/mol difference in binding energy [1]. Thus, the structures of sites two and three, when filled with Mg-nucleotide, are expected to be similar. A recent X-ray structure of mitochondrial F_1 [26] shows that even in absence of Mg^{2+}, where the nucleotide-binding affinity of all three catalytic sites is low (K_d for ATP and ADP about 100 μM [1]), all the sites adopt a closed type of conformation.

A rough estimate for the lower limit for K_d of a catalytic site in open (βE) conformation can be obtained from experiments with NBD-modified F_1. The X-ray structure of NBD-modified mitochondrial F_1 suggested that the NBD moiety on β-Tyr-297 in the βE subunit does not affect nucleotide accessibility of the catalytic site but prevents it from closing [27]. With NBD-modified *E. coli* F_1, we did not see any MgATP binding to site three using concentrations up to 1 mM and we estimated K_{d3}(MgATP) to be at least 10 mM [28]. K_d(MgATP) of the site transiently opened during steady-state ATP hydrolysis (Fig. 2, state D, site O) is expected to be in the same range. Our conclusion is that after product release one site exists in open (βE) conformation of exceedingly low affinity, whereas F_1 with three Mg-nucleotides bound exists with three catalytic sites each in a 'closed' or 'partly-closed' conformation, with high, medium and low affinity.

3.4. What determines the different MgATP binding affinities of the three catalytic sites?

Mg^{2+} ions are of crucial importance for conferral of high affinity to catalytic site one and medium affinity to site two, because in absence of Mg^{2+} the affinity of all three sites for ATP or ADP is the same and low [1]. Three catalytic site residues are involved in coordination of the Mg^{2+} ion of the Mg-nucleotide, either directly (β-Thr-156) or via an intervening water molecule (β-Glu-185 and β-Asp-242). Elimination of the relevant side-chain group of any one of these results in nearly complete loss of activity and loss of catalytic sites binding asymmetry. Thus, in βT156A, βE185Q, βD242N mutant enzymes, MgATP is bound to all three sites with the same, low affinity, similar to that for free ATP [29]. There is no significant effect of the mutations on Mg-nucleotide affinity at site three, and in wild-type F_1, Mg^{2+} has only a small influence on nucleotide binding affinity at site three, demonstrating that poor Mg^{2+} coordination is a major reason for the low affinity of this site. At site two, Mg^{2+} coordination is somewhat better; however, only at site one is an optimal octahedral geometry seen [29]. Thus, correct Mg^{2+} coordination is a prerequisite for catalysis. It is interesting to note that the contribution of the three functional groups to the overall binding energy is not additive, but cooperative, in that elimination of any one group abolishes the effects of the other two.

Besides the Mg^{2+}-coordinating residues, two other residues have been shown to specifically contribute to binding of substrate MgATP, namely β-Lys-155 [30] and β-Arg-182 [31,32]. The contribution of these residues to the overall binding energy is highest at site one, intermediate at site two, and small at site three.

Summarizing, there is no single residue responsible for the affinity differences between catalytic sites one and two or two and three; instead, the overall structure of the catalytic sites shows a continuum of subtle differences, from a highly compact conformation with optimized interactions between ligand and protein at site one, to a looser conformation with less favorable ligand–protein interactions at site three. In absence of Mg^{2+}, the latter conformation is assumed by all three sites.

An affinity-change mechanism involving γ-rotation implies that position of γ subunit vis-a-vis the three β subunits ultimately determines location of the high-, medium- and low-affinity sites. In effect, γ presents three different faces, one to each β subunit. When all three catalytic sites are empty, they all adopt an open

conformation [33],[2] and MgATP can in principle bind to all three. However, only binding to a specific one will result in the conformation change necessary to form the high-affinity site. In one position, γ 'sets' the catalytic site so that all the requisite protein-to-MgATP ligands will properly engage, and high-affinity binding is achieved. How the position of γ determines the potential high-affinity site is a very intriguing question, since no residue of γ is less than 15 Å away from the nucleotide binding site, or about 23 Å from the Mg^{2+} ion [5].

3.5. The molecular mechanism of ATP hydrolysis

Residues responsible for binding of substrate MgATP to the high-affinity site, discussed above, are obviously important for hydrolysis, and of at least equal importance are residues which stabilize the catalytic transition state. As described in Section 3.1, we have recently used high-affinity binding of the analog MgADP•AlF_x as indicator of a catalytic transition state [9].

The structure of the catalytic site [5] suggests three positively-charged residues as candidates for hydrogen-bonding to the three equatorial oxygen atoms around the γ-phosphorus in a pentacoordinate transition state: β-Lys-155, β-Arg-182, and α-Arg-376. We mutated each of these residues to Gln and found that coincident with a very large impairment of catalysis, the ability for high-affinity binding of MgADP•AlF_x complex at site one was abolished, demonstrating that all three residues are involved in stabilization of the transition state ([9,32]; S. Nadanaciva, J.W., A.E.S., submitted). As expected, Mg^{2+} was essential for tight binding of ADP•AlF_x complex [9] and therefore also essential for stabilization of the transition state.

We found in addition that the carboxyl group of residue β-Glu-181 is essential for transition state stabilization [9]. Based on the X-ray structure [5] it had been suggested that this residue might function as a general base to activate a defined neighboring water molecule by abstracting a proton (debated in [1]). Subsequent studies [29] showed that residue β-Glu-181 is not involved in substrate MgATP or product MgADP binding. While unisite experiments had already indicated a possible role for this residue in formation of the catalytic transition state [34], this was impressively confirmed by the failure of the (inactive) βE181Q mutant to bind MgADP•AlF_x [9]. An obvious mechanism by which residue β-Glu-181 contributes to transition state stabilization is by hydrogen-bonding the water molecule whose oxygen atom is one of the axial ligands of the γ-phosphorus in the transition state. Thus, the function of β-Glu-181 is to correctly align the substrate water molecule. Our results indicate that the hydrolysis reaction is to a large degree associative, i.e., there is significant bond formation between γ-phosphorus and both entering and leaving oxygens [35].

Little is known yet as to how MgATP hydrolysis drives rotation of γ. Areas of interaction between β and γ are apparent in the X-ray structure [5] and functionally important β/γ interface residues were identified by mutational analysis [25]. We currently favor the view that the hydrolysis event itself, occurring at the α/β subunit interface of the catalytic site (see Fig. 9 in [1]) initiates the mechanical movements that lead ultimately to γ-rotation. We conceive that separation of MgADP from P_i drags liganded residues (e.g., α-Arg-376 [5]) across the catalytic site, distorting the α/β interface, and thereby triggering more global α/β, α/γ and β/γ movements. This speculative idea derives from older work on mutants that abolish positive cooperativity between catalytic sites and which were subsequently found in the X-ray structure to cluster at the α/β interface (discussed in [1]). Recent work on the βR182K mutant [32] affirms its plausibility. In this mutant, MgADP•AlF_x binding is maintained, but catalysis is strongly-impaired. Scrutiny of the X-ray structure shows the guanidinium group of β-Arg-182 makes hydrogen bonds with two main-chain carbonyl oxygens of α subunit residues across the α/β catalytic site interface. The Lys mutant supports transition state stabilization, but presumably interrupts correct interaction across the α/β interface.

Obviously much more work is needed for an adequate description of catalysis at the molecular level.

[2] One might argue that the open conformation of the three empty β subunits in the $\alpha_3\beta_3$ complex in [33] is due to the lack of γ subunit. However, fluorescence quenching experiments (J.W. and A.E.S., unpublished results) indicate that in *E. coli*, F_1 containing a full complement of subunits all three catalytic sites are open when unoccupied.

It is, however, exactly at this juncture that the need to combine the experimental approaches of enzymology with those of protein mechanics becomes self-evident, adding novelty and spice to future research.

4. ATP synthesis

4.1. Features of ATP synthesis

ATP synthesis is much more difficult to study experimentally than hydrolysis due to requirement for a proton gradient and necessity to work with membrane protein. Some quite basic problems remain unsolved. Demonstration of rotation of γ and its direction during ATP synthesis would be satisfying. Even more basic is the need for measurement of catalytic site nucleotide occupancy, and effects of the proton gradient on nucleotide binding affinities at the three catalytic sites during steady-state ATP synthesis, which has not yet been achieved. Here we review available information about binding affinities for substrates MgADP and P_i and product MgATP in the presence of a proton gradient, and discuss why ATP synthesis cannot be described adequately by simply reversing the reaction arrows in the ATP hydrolysis scheme of Fig. 2.

4.2. P_i binding

Data on direct binding of P_i to isolated F_1 in absence of a proton gradient are few, and the results controversial. Penefsky [36] reported a single site with K_d of 80 μM for mitochondrial F_1. Using [^{32}P]P_i from the same source, a similar result was obtained with *E. coli* F_1 [37]. However, in the latter report it was shown that further purification of the [^{32}P]P_i eliminated binding of radioactivity, suggesting that it was due to an impurity in the commercially available product, likely PP_i or tripolyphosphate (PPP_i), which is not unexpected. The fact that ADP was as potent as ATP in inhibiting P_i binding might indicate that a similar problem occurred in [36], as both ADP and ATP would be expected to compete with PP_i or PPP_i binding, whereas P_i binding would not necessarily be prevented by ADP. Furthermore we showed that PP_i [38] and PPP_i (J.W. and A.E.S., unpublished results) do bind tightly to *E. coli* F_1 (K_d = 20 and 50 μM, respectively), although the binding is to noncatalytic and not to catalytic sites. We failed to detect any P_i binding to F_1 or F_1F_0 using a fluorescence competition assay with MgAMPPNP or ATP [7,16], and concluded that $K_d(P_i)$ in *E. coli* F_1 is at least 10 mM at catalytic or noncatalytic sites [1].

Unisite experiments have allowed calculation of $K_d(P_i)$ at site one. Up to pH 7.5, K_d values of about 1 M were found [39], which is consistent with lack of direct P_i binding noted above. At higher pH, $K_d(P_i)$ increased markedly, suggesting that $H_2PO_4^-$ is the species bound [39]. Since these values apply to the high-affinity site, $K_d(P_i)$ at sites two and three might be even higher. Evaluation of unisite P_i binding and release data for a series of mutant and wild-type enzymes led to the conclusion that enhancement of P_i binding affinity is one major energy-requiring step in ATP synthesis and a major function of the proton gradient [34,39].

In the presence of a proton gradient, affinity for P_i can be assessed from $K_m(P_i)$ for ATP synthesis. With *E. coli* ATP synthase, a K_m of 0.7 mM [40] or 3.5 mM [25] was found. Although it is not sure whether K_m values describe P_i binding to high- or low-affinity sites (and indeed whether the site with highest affinity for nucleotides has also highest affinity for P_i) nevertheless it is obvious that the proton gradient increases affinity for P_i by a factor of at least 300, probably by much more. The molecular basis for this P_i affinity increase is totally unknown. What happens in effect is that the proton gradient causes a phosphate-binding pocket to form in the catalytic site, in a place where one did not previously exist. From its location in the catalytic site, a candidate for P_i-binding is β-Glu-181 [5,26]. According to $K_d(P_i)$ calculations from unisite experiments [34], β-Glu-181 is not involved in P_i binding in absence of a proton gradient.

4.3. MgADP binding

Affinities of the three catalytic sites for MgADP in absence of a proton gradient are well-established; for *E. coli* F_1, using β-Trp-331 fluorescence, K_{d1} for (MgADP) was found to be 0.1 μM, K_{d2} and K_{d3} about 20 μM [1,7] and very similar values were seen in F_1F_0 [16]. Direct MgADP binding measurements in the presence of a proton gradient have not

yet been reported, so, as for P_i, we must resort to K_m(MgADP) values in ATP synthesis for indication of affinity. For *E. coli* ATP synthase, most K_m(MgADP) values fall between 20 and 40 μM [25,40,41], and are of similar order of magnitude in mitochondrial enzyme [42]. Therefore the proton gradient does not affect affinity for MgADP.

4.4. MgATP release

The only experiments in which MgATP binding affinity has been directly measured in the presence of a proton gradient are those of Penefsky and colleagues [43], which showed that the affinity of MgATP bound stoichiometrically to site one in mitochondrial ATP synthase was reduced by more than six orders of magnitude upon generation of a proton gradient. K_d(MgATP) increased from 10^{-12} M to 3 μM in absence of added nucleotide, and to 60 μM in the presence of 0.1 mM ADP [43]. The authors concluded that under optimal conditions it should be possible to reach a mM K_d(MgATP) and physiological rate of MgATP dissociation. It is important to note, however, that these measurements were not made under conditions of net ATP synthesis; rather the conditions were non-physiological. The bulk of the affinity decrease was observed even when the two other catalytic sites were empty, in contradiction of widely held assumptions regarding catalytic site cooperativity. Under conditions of steady-state ATP synthesis, a K_i(MgATP) of 5 mM was seen [25] which is also indicative of a mM K_d value expected for an open conformation of a catalytic site. Thus, while proton-gradient-induced release of product MgATP is supported by the available data, the need for a technique allowing direct, equilibrium measurement of MgATP (and MgADP) binding under steady-state ATP synthesis conditions is clear.

4.5. Energized release of MgATP is good but not sufficient

In light of the high ATP to ADP concentration ratios in mitochondria and bacteria (3 mM ATP, 0.4 mM ADP in *E. coli* [44]), the truly fundamental problem of ATP synthesis is, how, in those moments immediately after release of MgATP, does the empty catalytic site bind MgADP, and how is MgATP rebinding prevented? In absence of a proton gradient, binding affinities of site three for MgATP and MgADP are similar [1,7]. In the presence of a proton gradient, MgADP binding must be favored.

One possibility, suggested by us in [1], is that proton-gradient-enhanced P_i binding is a critical step. Once P_i is bound to the catalytic site, only MgADP, not MgATP, can bind. Another possibility would be a mechanism in which, in the presence of a proton gradient, the conformational change which closes the catalytic site can occur only when MgADP binds, not with MgATP as ligand, perhaps controlled by a residue reaching into the binding space of the γ-phosphate.

4.6. The mechanism of ATP synthesis

Mere reversal of the reaction arrows in Fig. 2 does not take proton gradient-induced binding site affinity modulations into account. Also, since ATP-induced rotation of γ subunit likely is initiated by the reaction step of hydrolysis, whereas in synthesis direction rotation is F_0-induced and drives affinity changes, with the reaction step thought to be largely spontaneous, we believe there are now strong arguments for writing separate schemes for ATP synthesis vs. hydrolysis. Without detailed knowledge of the affinities for substrates and product at each of the three catalytic binding sites, any model for steady-state ATP synthesis is necessarily speculative. Fig. 3 presents a model.

In this hypothesis, presence of a proton gradient modulates the affinities of the catalytic sites at certain steps, indicated by asterisks. Starting with state IV, the low-affinity site three has just released MgATP product (state III → IV) and has passed through the open conformation as it did so. It now assumes a conformation L* in which P_i and MgADP binding are strongly favored over MgATP. L* occurs only in the presence of a proton gradient. Binding of P_i and MgADP now occur (state IV → state I → state II). The order of P_i and MgADP binding might be reversed or random. Once state II is reached, rotation of γ brings about the 'binding change' and the three sites change affinities (arrows in state II). State III has the sites in their normal, non-proton-gradient-modulated conformations and affinities. MgATP is

now in site three (L) and is released by spontaneous opening of the site.

It is generally assumed that at the high-affinity site one, bound MgATP and MgADP+P_i are in an equilibrium with $K_{eq} \sim 1$ [6]. This would mean that the 'binding change' step would be just as likely to put MgADP as MgATP into site L in state III, leading to futile release of MgADP. The problem has been recognised, and suggestions are that the binding change occurs only when the high-affinity site contains MgATP [26], or that presence of the proton gradient shifts the equilibrium towards MgATP [45]. In our model, H* indicates a high-affinity site which has the property of only allowing ultimate release of MgATP, for whatever reason. Again we wish to emphasize that Fig. 3 is speculation.

5. Conclusion

The availability of high-resolution structural information, the demonstration of ATP-driven rotation, and the application of fluorescence techniques to determine catalytic site occupancy and ligand binding affinities has resulted in pronounced progress in our knowledge of the mechanism of ATP hydrolysis by the ATP synthase. In contrast, information on the mechanism of ATP synthesis is still scarce, and a tremendous amount of work remains to be done.

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