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Review

Catalytic site forms and controls in ATP synthase catalysis

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Abstract

A suggested minimal scheme for substrate binding by and interconversion of three forms of the catalytic sites of the ATP synthase is presented. Each binding change, that drives simultaneous interchange of the three catalytic site forms, requires a 120° rotation of the γ with respect to the β subunits. The binding of substrate(s) at two catalytic sites is regarded as sufficing for near maximal catalytic rates to be attained. Although three sites do not need to be filled for rapid catalysis, during rapid bisite catalysis some enzyme may be transiently present with three sites filled. Forms with preferential binding for ADP and P_i or for ATP are considered to arise from the transition state and participate in other steps of the catalysis. Intermediate forms and steps that may be involved are evaluated. Experimental evidence for energy-dependent steps and for control of coupling to proton translocation and transition state forms are reviewed. Impact of relevant past data on present understanding of catalytic events is considered. In synthesis a key step is suggested in which proton translocation begins to deform an open site so as to increase the affinity for ADP and P_i, that then bind and pass through the transition state, and yield tightly bound ATP in one binding change. ADP binding appears to be a key parameter controlling rotation during synthesis. In hydrolysis ATP binding to a loose site likely precedes any proton translocation, with proton movement occurring as the tight site form develops. Aspects needing further study are noted. Characteristics of the related MgADP inhibition of the F_1 ATPases that have undermined many observations are summarized, and relations of three-site filling to catalysis are assessed.

Keywords: ATP synthase; Binding change; Rotational catalysis; Phosphorylation

1. Introduction

Researches of the past few decades have revealed some main features of the catalysis by ATP synthase, particularly the structure-function relations in the F_1 portion. The stage is now set for development of a better understanding of the binding and control parameters. The main purpose of this paper is to review present information about some important steps in the catalysis, forms of the enzyme that may participate, how the forms may arise, and controls that appear operative.

For this discussion some assumptions that seem adequately justified are made. It is assumed that all three catalytic sites pass sequentially through the same conformations as catalysis proceeds. It is also assumed, although it is not as convincingly proven, that covalent bond formation and cleavage occur only when substrates are bound at a site in the tight-binding conformation. A minimal reaction pattern is suggested in which near maximal reaction velocity of ATP synthesis and hydrolysis is attained

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when only two catalytic sites are filled. This serves as a base for consideration of other forms and interrelationships that may be operative as ATP synthesis or hydrolysis occurs.

2. A minimal reaction sequence

The scheme presented in Fig. 1 shows interconversion of the three catalytic sites present in different conformations, designated as open (O), loose (L) and tight (T) [1]. The forms are considered to be closely related to the subunits designated as β_E, β_{TP}, and β_{DP} in the structure of Abrahams et al. [2]. The T site has Mg^{2+} and either ATP or ADP bound, but does not have the inhibitory MgADP as in the Walker structure, and is regarded as the structure where covalent catalysis occurs. The conformation assumed by the T site during catalysis depends on whether ATP or ADP is bound as in active net hydrolysis or synthesis. The transformation that each site undergoes during ATP synthesis is designated as step 1, 2, or 3 for convenience. It is important to recognize that binding changes, coupled to proton translocation in F_0, drive rotation of the γ subunit so that *all three* catalytic sites undergo change *simultaneously* as one 120° rotation occurs. For each catalytic site to return to its original conformation a complete 360° rotation is needed.

An important feature that may not be evident in the depiction of Fig. 1 is that interactions between a rotating γ subunit and β subunits repeat themselves after 1/3 of a complete rotation. After each 120° rotation the β subunits have undergone conformational changes so that the γ and β subunits again have the same spatial relationships as before the rotation, although with different β subunits. Catalytic behavior of the β subunits would be expected to be identical unless the differences in nucleotide binding to the α subunits, and the presence of a stator to prevent rotation of the αβ subunits, impart some heterogeneity to the catalytic steps. But so far this has not been detected and, in the elegant design of the machine, may not be present.

Although rotation measurements reveal 120° steps [3–6], during rapid net catalysis proton translocation may continue at a near uniform rate, with resultant continued conformational changes. Movement without large irregular transitions finds support in mechanochemical models for the enzyme [6]. Even though conformational changes may be continual, one 'binding change', in the sense of the binding change mechanism, is considered to result from a 120° rotation driving the simultaneous conformational changes of three subunits between forms indicated in Fig. 1.

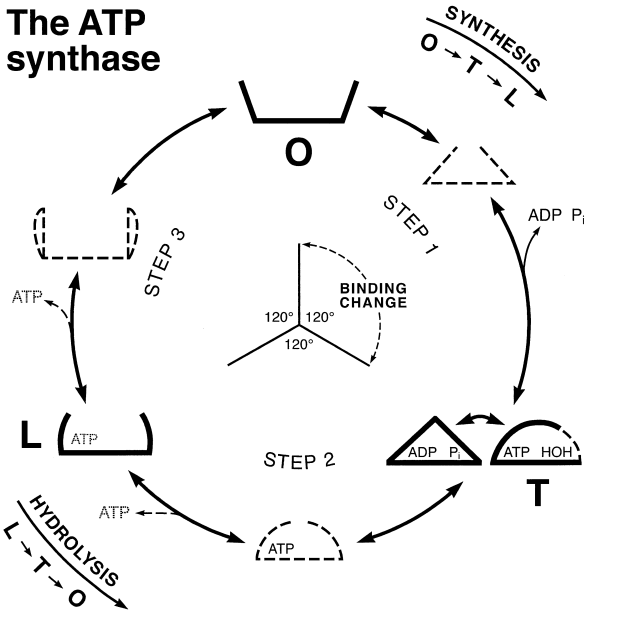

Fig. 1. The ATP synthase. Forms assumed by one catalytic site of the ATP synthase during synthesis or hydrolysis of ATP. The figure depicts the following. (a) The forms of one catalytic β subunit that appear during one complete rotation of the γ subunit in 120° steps. (b) The sites are designated as O (open), T (tight) or L (loose), and are related to forms designated as β_E, β_D, or β_T in the Walker structure. Tightness of ATP binding is indicated by the degree of openness of the depicted sites. (c) Intermediate forms during a 120° binding change are indicated with lighter dashed lines. (d) Forms with some curved lines preferentially bind ATP and those with all straight lines preferentially bind ADP and P_i. (e) The sequence of forms that appear as ATP synthesis or hydrolysis occurs is shown. (f) Appropriate arrows indicate reversible substrate binding or release for rapid bisite catalysis. (g) Dashed arrows and dotted ATP indicate that whether ATP is released primarily from the L or the O form during rapid synthesis is not known. (h) A dashed portion of the T site indicates that HOH formed when ATP is made interchanges readily with the medium water.

A number of features of Fig. 1 warrant additional comment. The scheme presents what is felt to be a likely sequence of catalytic events. Some alternate steps that may participate are considered later. Intermediate states with important properties must devel-

op as rotation proceeds. A prominent aspect of Fig. 1 is that most catalytic sites are regarded as being in conformations that preferentially bind either ATP or ADP and P_i. Other features are indicated in the legend to the figure and, together with alternate possibilities, in the following text.

3. The ATP synthesis sequence

3.1. The binding of ADP and P_i

Only one step is indicated in Fig. 1 for the binding of both P_i and ADP. This simplification is justified because there is good evidence that the binding is random [7,8], and which one binds first does not appear to have catalytic significance. There will obviously be two pathways operative and their relative participation will depend on reaction conditions. In one pathway P_i binds first, in the other ADP.

For photophosphorylation evidence has been presented that MgADP and free P_i are the forms that combine for net ATP synthesis [9], and it seems likely that similar considerations hold for the synthase from other sources. This means that the level of free Mg^{2+}, and thus the level of MgATP and free ATP could be important parameters influencing reaction rates under physiological conditions. More information is needed. In this paper the binding of ADP and ATP will usually be mentioned although it is recognized that it is likely they are bound as their complexes with Mg^{2+} (evidence for MgATP binding is mentioned later in Section 5.1).

Consideration of the Walker structure [2] and the direction of observed rotation [10] means that, during net ATP synthesis, ADP and P_i must bind to the O form, which is then converted to the T form. The O form as in the Walker structure has a low binding affinity for AMP-PNP and presumably for ATP. The enzyme was crystallized in the presence of 5 μM ADP and 250 μM AMPNP so the respective K_d values must be above these concentrations. Evidence for the affinity of ADP or ATP for the open site comes from competition with the binding of trinitrophenyl-ATP at this site in MF_1 [11]. The K_d for ATP was about 2 mM and that for ADP, 150 μM, more than 10-fold lower. This greater affinity for ADP harmonizes with the probability that ADP binds initially and preferentially to the O form to initiate catalysis.

P_i binds weakly to a catalytic site of F_1 and the K_d for ADP mentioned above is well above its K_m values for synthesis. The binding of both ADP and P_i must be increased for net ATP synthesis to occur. Two ways in which such binding may be promoted merit consideration. One way, considered less likely, is that during rapid net synthesis the O form as in the crystalline enzyme never completely arises as dynamics of rotation continue, and that a form that is transiently present has a good binding potential for ADP and P_i. Fig. 1 depicts a second, and perhaps more likely, way. This is that some rotational movement driven by migration of say one or two protons is necessary for the site to assume a conformation favorable for ADP and P_i binding. That presence of protonmotive force is necessary for competent P_i binding was recognized long ago in ^{18}O studies [12] and substantiated by observations in Sigman's [13] and Senior's [14] laboratories of the relatively high concentrations of P_i necessary to form tightly bound ATP from bound ADP. That availability of energy promotes ADP binding is suggested by various observations. These include a marked increase in the rate of ADP binding when photophosphorylation is initiated [15]; a lowering of the K_m of ADP for photophosphorylation (summarized in [16]) and oxidative phosphorylation [8] as the protonmotive force is increased; and an increase in the observed K_m for ADP in the presence of an uncoupler [17]. The promotion of ADP binding by energy input has been substantiated by direct measurement of the binding by chloroplast thylakoids in the dark or light [18].

The binding of ADP and P_i is relevant to an important control feature. It is evident that protonmotive translocation should not occur if ADP and P_i are not ready to form ATP; otherwise energy would be wasted. The extent of the control is indicated by the observation that when photophosphorylation is limited by low ADP, the tightly bound ADP, P_i and ATP interconvert about 50 times before ATP is released [19]. Tight control is also nicely shown by the observation that, with a reconstituted synthase in presence of protonmotive force, no movement of a tagged β subunit was observed unless ADP and P_i were added [20].

3.2. Passing through the transition state

An important feature, as presented in another recent publication [21], is that a form with tightly bound ADP and P_i, waiting to be converted to tightly bound ATP, is not considered to be an intermediate in a single binding change step when rapid net ATP synthesis is occurring. As the 120° rotation of one step is completed, the bound reactants are suggested to have passed through the transition state, and tightly bound ATP is ready to be released as the rotational movement continues and drives the sites through their next conformational changes. The nature of the transition state, although a topic of considerable interest, is not a primary concern for the features covered in this review. The suggestions of Abrahams et al. for ATP hydrolysis [2] mean that in synthesis a COO^- of βGlu188 of MF_1 could accept a proton from a water molecule adjacent to bound P_i and the guanidine αArg273 could accept a proton from the β phosphate of ADP to increase the propensity of nucleophilic attack of an oxygen of the β phosphate on the P_i. In this regard, reports of studies under way (J.E. Walker, private communication) with aluminum fluoride complexes with nucleotides that may mimic transition states are awaited with interest.

As mentioned earlier, as reactants pass through the transition state, the conformation of the catalytic site is suggested to change from one that binds ADP and P_i better than ATP to one that preferentially binds ATP[1]. Shifts in the position of the ε subunits occur when ATP replaces ADP at a catalytic site, as detected by cryoelectron microscopy of ECF_1 labeled with gold particles [23]. The capacity for a very tight binding of ATP, inherent in the enzyme structure, contributes critically to the overall process of net ATP synthesis. Changes in enzyme conformation accompanying covalent bond changes are indicated by EPR studies of the bound MnATP. Significant rearrangement is detected under conditions favoring unisite hydrolysis [24]. Enzyme residues critical for rapid rotation and likely to be involved in key conformational changes may be revealed by mutational studies, such as those from Futai's laboratory [25]. A suggested participation of an intermediate form in which all three β subunits have similar conformations [26] lacks sufficient experimental support to warrant serious consideration.

Additional enzyme forms may be identified as required for transition state formation. For catalysis of synthesis or hydrolysis it is probable that two groups or at least one group at the active site undergo protonation and deprotonation. This would be analogous to the fumarase catalysis, where cycling between protonated and deprotonated forms have been found to be relatively slow processes dependent upon accompanying conformational changes. These forms differing in protonation are what Rose considers as isoforms of fumarase[1]. Future studies will likely identify such isoforms in the ATP synthase catalysis.

3.3. The release of ATP

A salient requirement of the ATP synthase catalysis is that, when a T site is opened during rapid net ATP synthesis, essentially only ATP and not ADP is released. As has been well demonstrated, in the isolated F_1 ATPase the equilibrium between bound ATP and ADP is not far from unity. Somehow when synthesis is coupled to protonmotive force, the site must have essentially only ATP present when it is opened. Two ways that this might be accomplished are suggested. One is that in the presence of adequate protonmotive force the equilibrium is shifted substantially toward bound ATP. Then the proton translocation allowed when ADP and P_i bind could also drive the simultaneous step of release of tightly bound ATP from a T site. Another way of assuring that ATP would be released would be to have a control function such that the site opening cannot occur unless ATP is present.

There is important experimental evidence that the level of tightly bound ATP is not far from one per

[1] In enzyme catalysis a substrate undergoes a guided tour through a transition state to form a product. Likely in the process an enzyme conformation that preferably binds substrate is converted to one that preferentially binds product. An example is the recent demonstration by Rose that when fumarase catalyzes the reversible hydration of fumarate to malate a change in conformation preferentially binding fumarate to one preferentially binding malate occurs [22]. Rose also detects different protonation states of the malate and fumarate forms that participate in catalytic steps, and points out that conformational changes accompanying interconversions of such forms likely are general features of enzyme catalyses.

enzyme when high protonmotive force and adequate ADP are present. When net ATP synthesis rates are low, measurement of the amount of recently formed [γ-^{32}P]ATP in the reaction mixture that is inaccessible to hexokinase allows reasonable approximation of the level of tightly bound ATP. Such measurements show that about one ATP per enzyme is present when low P_i concentration limits the photophosphorylation rate [18]. The T-site appears to be ready for opening and ATP release. In contrast, when low medium ADP limits the photophosphorylation rate, only roughly one-half ATP per enzyme is present [18]. The presence of bound ADP at one site allows or promotes the formation of ATP at a T-site. When an uncoupler is added to the system, the amount of tightly bound ATP rapidly drops by over one-half [27], indicative that it is protonmotive force – pressure from the push toward rotation – that has driven the equilibrium toward ATP.

The above explanation harmonizes with evidence that opening of the tight site by protonmotive force can occur when inhibitory MgADP is tightly bound in the site [28]. This inhibitory MgADP is in a different chemical-conformational state than the MgADP and P_i that arrive at the site during rapid net synthesis. The important point is that presence of ATP does not seem to be necessary. But an alternate possibility is that the conformation assumed by the enzyme in the inhibited state with tightly bound MgADP actually resembles that during rapid net ATP synthesis when ATP is present at the tight site. As noted later (see Section 5.2) the formation of the MgADP-inhibited state depends on slow changes following binding of medium Mg^{2+}. This could reflect the need for conformational transitions that yield a form resembling that when ATP is present. There is another possibility that seems unlikely to me. This is that with the inhibitory MgADP present the rotation reverses, akin to the occasional reverses seen with the visual demonstration of rotation [3,5]. Again, more information is needed.

During rapid net ATP synthesis it seems uncertain whether the ATP would be released primarily from the L or the O forms or from both (see Fig. 1). The K_d of ATP for the loose site is not known, although the K_m values for ATP hydrolysis of 100–200 μM suggest that release from the site could be relatively rapid. The competitive inhibition of net ATP synthesis from ADP and P_i by ATP indicates K_d values for ATP in the mM range [8,29]. Such a high apparent K_d could result because the binding of either P_i or ADP could block ATP binding for hydrolysis [8]. But if rapid bisite catalysis occurs, with ADP and P_i binding at the transforming O site for synthesis, and ATP binding at the L site for hydrolysis, the competition of ATP for this O site during ATP synthesis would be indicative of the actual dissociation constant. As mentioned in Section 3.1, there is other evidence for a high K_d in the mM range for ATP binding to the O form. Such a low affinity for ATP would insure its rapid departure should some still remain as this state of rotational change is approached.

3.4. Energy requirements for various steps

Because of the close contacts between α and β subunits and their contacts with the γ subunit, conformational changes induced principally by interactions of the γ subunit with one or more β subunits will induce conformational changes in all three β subunits. Thus during synthesis O will become T, T will become L, and L will become O, irrespective of whether the transition of the form L to form O might require less energy than the transition of T to L. Also one cannot tell whether the requirement of energy input for competent binding of ADP and P_i in step 1 of Fig. 1 should be regarded as an addition to energy input required for ATP release in step 2. For example, it could even be that after a tighter binding site for ADP is created, the occupancy of this site by ADP could be exergonic. Compensatory changes in protein conformation could tend to decrease energy requirements for needed transitions. Nature may have achieved a design so that the energy barrier for rotational movement does not differ much as a different extent of rotation is achieved. This is consistent with direct observations of rotation that indicate a nearly constant torque is present.

Even though the manner in which energy input may be distributed among the catalytic site forms as their interchange is promoted, the net effect of energization must be able to promote competent binding of P_i and ADP, release of tightly bound ATP, and perhaps shift of the quasi-equilibrium at a T site toward ATP. There is particularly good in-

formation that energy must bring about release of tightly bound ATP. This includes the early ^{18}O studies (see [30]) and unisite catalysis studies [31] showing the reversible cleavage of tightly bound ATP, ADP, and P_i occurs in absence of protonmotive force; the very tight binding of ATP by the isolated F_1 ATPase [32]; the slow formation of tightly bound ATP from medium P_i by isolated F_1 ATPase [13]; and the affinity changes for ATP demonstrable with submitochondrial particles [33]. Thus, a recent suggestion that energy input serves primarily to drive the formation of ATP from ADP and P_i [34] does not seem apt.

4. The reversibility of reaction steps

A striking characteristic of the ATP synthase reaction is its ready reversibility. This is demonstrated by the rapid exchange between P_i and ATP of the reaction medium and the oxygen exchanges that accompany photo- or oxidative phosphorylation. Water oxygens appear in the ATP made even with a hexokinase trap to remove the ATP, meaning that reversals of ATP formation have occurred before ATP was released from the synthase. In addition, net ATP synthesis is accompanied by appreciable exchange of medium P_i oxygens with water oxygens, meaning that P_i has bound, been converted reversibly to bound ATP, and again released. These oxygen exchanges accompanying net ATP synthesis are more rapid than those found during ATP hydrolysis by the isolated F_1 ATPase (see [30]). The best explanation of these findings is that the rotational movement driving ATP synthesis is being rapidly reversed, and is accompanied by reversal of the proton translocation so that the reversibility does not waste energy. This is as indicated in Fig. 1. Such results and explanation add to the probability that synthesis and hydrolysis follow the same reaction pathways. This view gains support from a recent thermodynamic analysis of the catalysis [29]. Observations such as azide inhibiting hydrolysis but not synthesis [35] can result because the MgADP inhibition stabilized by azide (see Section 5.2) is readily displaced to allow synthesis to occur if ADP, P_i and protonmotive force are present.

5. The ATP hydrolysis sequence

5.1. The binding of ATP

The high affinity of the ATP synthase for ATP means that energy-requiring changes, 'reverse' proton transport to increase protonmotive force and enzyme conformational changes, can be driven by the binding step. This is as indicated in Fig. 1 by the 120° rotational change accompanying the change of an L site to a T site form. The major problem faced by Nature in designing the ATP synthase was not how to achieve rapid covalent bond formation and cleavage at a catalytic site, but how to couple energy reversibly to the release and binding of ATP. How the problem was solved, as crudely indicated in Fig. 1, now seems somewhat obvious.

Present information favors the view that combination with MgATP gives an active complex, although some uncertainty remains. Recently suggestions have been made that free ATP may be a preferred substrate for the F_1 ATPase, based on effects of ATP and Mg^{2+} concentrations on hydrolysis rates [36–38]. In one instance [37], an assumption of a rapid equilibrium binding for ATP seems untenable. Also, with the reports there is inadequate assurance that interference by the MgADP inhibition did not occur, particularly at higher added Mg^{2+} concentrations. The fact that in all the studies excess Mg^{2+} is considered to be an inhibitor suggests that the MgADP inhibition was overlooked. Important data of Murataliev give evidence that up to 5 mM Mg^{2+} is not an inhibitor of the MF_1 ATPase if the initial velocity, free of MgADP inhibition, is measured [39]. Also Murataliev's data are hard to reconcile with free ATP as a preferred substrate. In the presence of 100 μM added ATP an increase in added Mg^{2+} up to 5 mM, that would reduce the free ATP concentration to a very low value, did not decrease the initial velocity [39]. It remains possible that a competent complex can form when free ATP adds to an enzyme with bound Mg^{2+} already present. There is ample evidence for tight binding of Mg^{2+} or Mn^{2+} at or near catalytic sites in the absence of bound nucleotide (see [24,40]). Binding sites for Mg^{2+} separate from nucleotide binding sites could exist. Some results indicate that free Mg^{2+} might be an essential activator, and the

inhibition of ATPase activity by excess ATP could result from Mg^{2+} depletion [41].

5.2. ADP and P_i formation and release

These steps for the intact synthase are the reverse of those for synthesis. When ATP is added, and protonmotive force is low and catalytic sites are unoccupied, the binding of ATP to the L site would rapidly drive step 2 of Fig. 1 in the hydrolysis direction, accompanied by proton pumping. The ATP would rapidly form tightly bound ADP and P_i. With binding of the next ATP, the ADP and P_i would be released. In the intact synthase, some intermediate oxygen exchange accompanies ATP hydrolysis even with relatively high uncoupler concentrations present [42], indicative that some intermediate state or conformation present prior to proton translocation is sufficiently rate limiting to allow reversal of ATP cleavage. This merits further investigation.

With the isolated F_1 ATPase hydrolyzing excess medium ATP only one water oxygen appears in the P_i released, meaning that no intermediate oxygen exchange occurs. But with both the intact synthase and the F_1 ATPase, as the ATP concentration is lowered the extent of intermediate exchange per ATP cleaved rises markedly (see [30]). The rate of exchange is considerably less than the rate of ATP cleavage with excess ATP present. The release of the product ADP and P_i depends on the binding of ATP to another catalytic site.

5.3. The MgADP inhibition

The interpretation of many kinetic studies is clouded by the complex patterns of MgADP inhibition. In nearly all steady-state measurements of the rate of ATP hydrolysis, a fair fraction, and often most, of the enzyme is in the MgADP-inhibited form, which is being slowly reactivated and reinhibited. It is surprisingly difficult to escape some or considerable such inhibition with the F_1 ATPase from various sources. That initial velocity of the MF_1 ATPase reaction readily decreased in the presence of Mg^{2+} was clearly recognized in early studies of Moyle and Mitchell [43]. This was an expression of what is discussed here as the MgADP inhibition, a clearly distinct phenomenon from the combination of MgADP as a substrate for net synthesis. Molecular details of the phenomenon still need to be clarified, but all who measure F_1 ATPase rates should be aware of the following characteristics of the MgADP inhibition (see [44] and references therein).

(1) The inhibition arises when medium Mg^{2+} combines with enzyme containing ADP bound at a single catalytic site without bound P_i present. The onset of the inhibition is relatively slow and under most assay conditions reaching equilibrium between inhibited and active stages requires seconds to minutes of time.

(2) The inhibition can be slowly and partially reversed by hydrolysis of ATP, and such recovery is aided by anions such as bicarbonate and sulfite.

(3) The binding of ATP at noncatalytic sites is essential for, or promotes, the recovery of activity during ATP hydrolysis.

(4) The inhibitory ADP can arise from the medium ADP or from ATP hydrolyzed at the site. Thus although medium ADP can promote the inhibition it is not essential, and under some conditions, ATP hydrolysis can favor the onset of the inhibition.

(5) The initial inhibitory MgADP-enzyme detected can slowly isomerize to a form with tighter Mg^{2+} binding.

(6) The presence of azide considerably stabilizes the inhibitory complex, and azide sensitivity serves as a diagnostic probe for the presence of the inhibition.

(7) All F_1 ATPases are likely susceptible to the inhibition, but differences exist. For example, the CF_1 and ECF_1 are more readily inhibited than the MF_1.

(8) Most or all observations of Mg^{2+} inhibition are likely due to its promotion of the MgADP inhibited state.

(9) The inhibition of ATP hydrolysis also occurs with the intact ATP synthase when protonmotive force is low or absent, but no inhibition of net ATP synthesis occurs because the inhibitory MgADP is removed by adequate protonmotive force.

As a result of the above characteristics, when a steady-state rate of ATP hydrolysis is achieved under most assay conditions, even with activating anions present, a considerable portion of the enzyme is in a MgADP-inhibited form, being slowly interconverted with the active form.

6. F_1-F_0 interactions

Coupled solute transport models often need to include a step for reorientation of a binding site across a membrane after a solute molecule has been transported. For the coupling of proton translocation to rotation of the γ subunit of the ATP synthase, such reorientation does not seem necessary, although it has been invoked in some models (e.g. [45]). Translocation of a proton can give rise to some type of circular displacement in F_0 so that another protonatable group or channel opening is now available without further reorientation. The direction of circular displacement would obviously change depending upon which direction the proton moves through the membrane.

During synthesis the most important control of relative γ-β movement appears to depend on ADP binding. Another possible mode of control would be that the rotational movement could not continue unless ATP was present at the tight site. This seems unlikely because of an old observation. When photophosphorylation is induced by an acid-base transition, a tightly bound ADP on the enzyme was not converted to ATP, but the first ATP made came from ADP of the reaction medium [28]. However, the tightly bound ADP was released to the medium in the initial turnover. Subsequent observations have made it clear that the initial tightly bound ADP was very likely present at a catalytic site with Mg in the form that blocked ATP hydrolysis. When ADP, P_i and energy for proton translocation became available, the T site with only ADP and not P_i present, was rapidly converted to the L and then to the O form etc.

When net ATP hydrolysis drives proton translocation in the intact synthase, unlike with ADP, the initiation of ATP binding can likely precede any proton translocation. The completion of a binding step would only occur as a 120° binding change occurs accompanied by proton translocation. If protonmotive force is sufficiently high no rotation would be expected, or if the rotation and proton translocation occurred, they would be rapidly reversed. No controls on ATP cleavage other than tight coupling to proton translocation appear necessary to stop ATP cleavage under fully energized conditions. Other important controls may operate that will not be considered here, such as the control of ATPase action by the mitochondrial inhibitor protein extensively studied by Harris [46]; the activation of the chloroplast enzyme by disulfide bond reduction (see [16]); and the largely unknown controls suggested by the large number of subunits in the mitochondrial F_0 component [47].

7. Site filling and catalytic rates

7.1. Rapid bisite catalysis

Measurements of the dependence of the rate of ATP synthesis on the ADP concentration support the view that rapid net synthesis is attained when a second catalytic site fills. As mentioned above, rate measurements of net synthesis are not complicated by the strong and unusual inhibition by MgADP. A number of laboratories have reported only single apparent K_m values in the range of about 20–100 μM for ADP during photophosphorylation. An apparent K_m below μM with a low V_{max} appears to represent unisite synthesis [19]. Careful studies of oxidative phosphorylation in a bacterial system by Perez and Ferguson [8] also showed a low K_m attributable to unisite catalysis and a single higher K_m for rapid ATP synthesis, as if second site filling gave rapid catalysis. Similarly, Matsuno-Yagi and Hatefi [48] found a low and a higher K_m value with heart submitochondrial particles. They also detected very tight binding of an ADP at submicromolar concentrations under nonphosphorylating conditions. This likely represents the tight binding of an inhibitory MgADP at a catalytic site, and not an additional site that must be filled for rapid net synthesis. In a recent report for the *Escherichia coli* F_1-F_0 reconstituted into liposomes only one K_m was observed above the micromolar range [49].

The limitations of substrate-velocity measurements make desirable assessments of bisite or trisite catalysis by other methods. For photophosphorylation direct measurements of nucleotide binding by a filtration technique show that the K_m below μM mentioned above results when only one catalytic site is filled, and that the onset of rapid catalysis occurs with the filling of a second catalytic site [18]. The extent of such studies is limited and additional evaluations could be helpful.

The relation between site filling and catalysis during ATP hydrolysis remains controversial. Kinetic evaluations can be misleading. A common assay for ATPase (coupled assay) depends on the steady-state rate of NADH disappearance in the presence of lactate dehydrogenase and pyruvate kinase. The observed rates will depend upon the fraction of enzyme not in the MgADP-inhibited form and this fraction can change with a change in added ATP concentrations. For example, the steady-state level of medium ADP will drop as ATP concentration is lowered and pyruvate kinase rate decreases. This may result in less MgADP inhibition, and suggest a non-existent K_m near micromolar concentrations of added ATP. With MF_1, measurements of initial velocity by extrapolation to zero time, starting with enzyme depleted of inhibitory MgADP at the tight site, can give valid kinetic assessments. The coupled assay is not suitable for this with concentrations of added ATP below about 5 μM because small amounts of ADP and/or ATP impurities in the NADH, phosphoenolpyruvate, lactate dehydrogenase, pyruvate kinase or other reagents used can suggest a low K_m that does not exist. Use of radioactive ATP is satisfactory at low substrate concentrations where a readily measurable fraction of the total radioactivity appears in the product. Obtaining of identical rates in the regions around 5 μM where assays overlap can validate the results. Measurements made with MF_1 in this fashion in my laboratory show the presence of only one K_m value for the increase in velocity from less than μM to mM concentrations of ATP [50]. Hence kinetic measurements are regarded as supporting the occurrence of rapid bisite catalysis.

Convincing support for occurrence of rapid bisite catalysis during ATP hydrolysis has come from a somewhat unexpected source. This is from the visual demonstrations of rotation by attachment of an actin filament to an appropriately modified and mounted TF_1. Single rotational steps of 120° occurred when ATP was added at nM concentrations, with velocity of the step as with excess ATP [3,5]. If rapid catalysis were attained only when three catalytic sites are filled, this would require simultaneous filling of a second and a third catalytic site. If the sites had K_m values in the μM range, as suggested in the fluorescence quenching studies mentioned above, such filling seems unlikely and perhaps impossible. The observed stepwise rotation at low ATP concentrations very likely results when a second catalytic site is filled.

7.2. Advantages of bisite catalysis

Additional reasons for favoring rapid bisite catalysis come from consideration of the catalytic advantages from such behavior. During synthesis the key binding change is that of an O form (see Fig. 1) with ADP and P_i present to the T form with tightly bound reactants. To also require ADP and P_i to be present at the L site introduces another control that seems unnecessary. In the next binding change an L site with bound ADP and P_i would next become an O site with bound ADP and P_i – not an essential catalytic change. Also, rapid bisite catalysis would allow synthesis to continue whether or not the L site had ATP or ADP present or was empty. This could help when synthesis needs to occur with ATP concentrations greater than ADP concentrations.

Similar considerations hold for rapid bisite ATP hydrolysis. The crucial catalytic step is getting the substrate into the tight site. The essential conversion of an L site with ATP bound to a T site does not also require a conversion of ATP at an O site to ATP at an L site. During both synthesis and hydrolysis there could be some effects of whether the third site not undergoing essential change is empty or occupied with ATP or ADP. Such information will come from future studies.

The demonstration of rotational catalysis by the F_1 ATPase has led to interesting biophysical models for how the catalysis may occur with high efficiency. Kinosita and Yasuda present a model derived from analogy with electric motors in which bisite catalysis occurs with changing affinities for binding of ADP, P_i and ATP [5]. Wang and Oster present a sophisticated analysis on how elastic strain accompanying rotation may operate [6]. Their model can accommodate either rapid bisite or trisite catalysis.

8. Note added in proof

Results from the laboratories of Senior [51,52] and

of Allison [53] have given evidence that three catalytic sites are filled when sufficient ATP is present for rapid catalysis by ECF_1 or TF_1. A recent paper shows that this occurs with ECF_1 even when the ε subunit that slows catalysis is removed [54]. Catalytic site filling in these studies was assessed from fluorescence quenching measurements with mutants containing tryptophan replacements at catalytic sites. The data were interpreted to mean that rapid catalysis requires binding of ATP to the O site after the other two catalytic sites are already filled with nucleotide.

However, from present information alternative explanations appear preferable. A prominent one of these is as follows. The fluorescence measurements show that ADP binds with a K_d of about 20 μM to both the O and L sites [54,55]. This relatively tight binding is consistent with the well-recognized departure of ATP as a slow step in the catalysis and the facile inhibition of hydrolysis by ADP. During steady-state catalysis bound ADP will appear at the O and L sites. Only when the L site becomes vacant and ATP binds to this site will rapid catalysis continue. This is bisite catalysis, not dependent on whether the O site contains ATP or ADP or is vacant.

If the off constant for ADP dissociation from the O site is less than that from the L site, nearly three sites may be filled during rapid hydrolysis with excess ATP present. This is not because of binding of ATP to an O site that then converts to an L and then a T site. It is because sticky ADP tends to remain at both the O and L sites, and catalysis continues only when the L site becomes vacated and ATP can bind to the site.

In addition, in measurements of ATP binding with the relatively high enzyme concentrations required, ADP will be rapidly formed before or during the fluorescence measurements. Such ADP, as well as ATP, could bind to any empty O sites and tend to keep nearly three catalytic sites filled as bisite catalysis continues. That bisite catalysis prevails is also in harmony with the observed K_m of only about 20–30 μM [54]. Other studies have indicated a K_d in the mM range for ATP binding to the O site of MF_1 [11], and in the crystalline state ECF_1 like MF_1 has the O site empty in the presence of 250 μM AMP-PNP [56].

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