Minireview

Catalytic site occupancy during ATP synthase catalysis

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Abstract An early proposal was that for rapid ATP synthesis by the rotational ATP synthase, a specific second site must bind ADP and P_i, and for rapid ATP hydrolysis a different second site must bind ATP. Such bi-site activation was considered to occur whether or not an ADP or ATP was at a third site. In contrast, a more recent proposal is that rapid ATP hydrolysis requires that all three sites have bound ADP or ATP present. However, discovery that one second site binds ADP better than ATP, together with other data and considerations support the earlier proposal. The retention or rebinding of ADP can explain why three sites fill during hydrolysis as ATP concentration is increased although bi-site activation still prevails. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: ATP synthase; F_1-ATPase; Bi-site activation; ATP and ADP binding

1. Introduction

From contributions of many investigators the following information about the ATP synthase is generally accepted: the intact synthase is comprised of a membrane-imbedded portion, F_0, with a ring of small subunits that functions in proton translocation, and an attached F_1 portion with three large α and three large β subunits, circularly arranged around a smaller γ subunit. The γ subunit and another associated smaller subunit interact with the ring of small subunits in F_0. The separated F_1, that catalyzes ATP hydrolysis, has three catalytic sites at α–β subunit interfaces, largely on the β subunits. In the intact synthase other subunits connect the outer portions of the F_0 and F_1. When proton translocation occurs, rotation of the ring of subunits in the F_0 and the γ subunit results in sequential conformational changes of the catalytic sites. One catalytic site on the intact synthase or separated F_1-ATPase can very tightly bind ADP or ATP. All three sites pass through identical conformations but at any one time all are in different conformations. The sequential conformational changes facilitate the binding, the chemical change, and the release of reactants.

An early proposal is that slowly reversible formation or hydrolysis of ATP is catalyzed at the tight binding site and that binding of ADP and P_i or ATP at other sites results in rapid enzyme turnover by a bi-site activation mechanism. Such activation meant that rapid ATP synthesis, in the presence of adequate protonmotive force, requires that ADP and P_i occupy a selected second site. Rapid ATP hydrolysis, when protonmotive force is low or absent, requires that ATP occupies a different second site. These rapid rates were considered to require only the occupancy of a specific second site, and thus that nucleotide occupancy at a third site gave little or no further activation. Also, it was postulated that the site to which ADP must bind for net synthesis had a greater affinity for ADP than ATP [1–3].

More recently a contrasting proposal has been developed, namely that rapid net ATP hydrolysis by the F_1-ATPase occurs only after both a second and a third site have initially bound ATP. The view is that ADP or ATP must occupy all three catalytic sites for rapid catalysis to occur [4–10]. The important consideration should be, however, not the number of catalytic sites that may be occupied, but what sites must be occupied for rapid enzyme turnover to occur. As noted in this paper, pertinent earlier data that have been overlooked and other recent data considerably strengthen the earlier suggestion of bi-site activation for rapid catalysis.

Knowing whether bi-site or tri-site activation prevails is of considerable interest because of the important differences in catalytic steps for operation by the two mechanisms.

2. The proposal for tri-site activation

Weber and Senior and associates, by use of an innovative fluorometric assay, reported that when sufficient ATP is present for rapid ATP hydrolysis by *Escherichia coli* F_1-ATPase, nearly three catalytic sites have nucleotide present. On this basis they postulated that rapid ATP hydrolysis required that a second and third site had initially bound ATP and that all three catalytic sites have ATP or ADP present [4–10]. At present, three leading research groups have suggested reaction pathways for ATP hydrolysis by F_1-ATPase that meet this requirement [4–16]. In two of the suggested mechanisms for continued rapid hydrolysis ATP binds to an enzyme that already has ATP bound at two catalytic sites [11,16], in the other the binding is to an enzyme with ADP and ATP bound [5,10]. In all three suggested mechanisms a rapid ATP hydrolysis step can occur only if all three sites have bound nucleotide present. Also ATP hydrolysis rather than binding is considered to play a more prominent role in driving rotation. These aspects are clearly incompatible with the earlier hypothesis [1–3].

Other difficulties arise with the proposed mechanisms. The same open form is proposed to serve for ATP binding during

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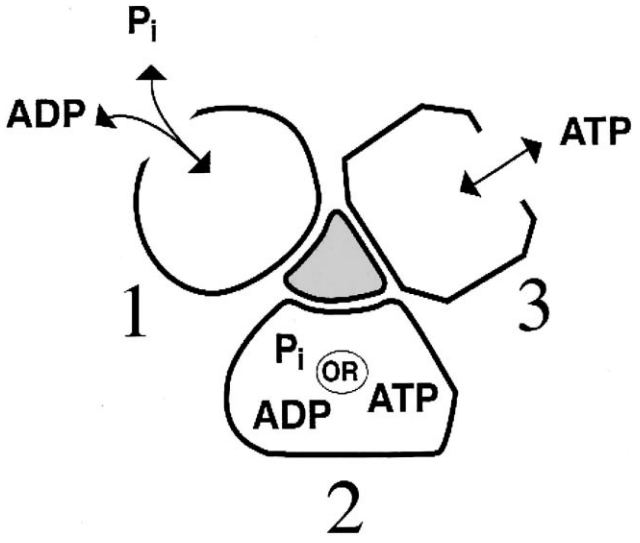

Fig. 1. A depiction of the three major conformations of catalytic sites for bi-site activation of ATP synthesis or hydrolysis by the ATP synthase. Three catalytic sites in different conformations are shown with asymmetric interactions to the shaded γ subunit. During catalysis sites are converted sequentially into three different states accompanying rotation of the γ subunit. The sequence for synthesis is $1\rightarrow2\rightarrow3$, for hydrolysis it is $3\rightarrow2\rightarrow1$. Site 1 binds ADP better than ATP and is the site at which ADP and P_i must be present for rapid synthesis to occur. Site 2 has the ability to catalyze chemical transformation and to be present as a form with ADP and P_i present or ATP present. ATP can be released from site 3 during synthesis and must be present at this site for rapid hydrolysis. Figure adapted from [29].

net hydrolysis or for ADP binding during net synthesis. This obviates the advantage that the bi-site activation mechanism provides for ADP participation in synthesis. Entry of ADP by binding to a site with low affinity for ATP offers an attractive explanation for how rapid ATP synthesis can proceed with relatively high ATP concentrations present, as is commonly observed. Also, different pathways for synthesis or hydrolysis are suggested [8,11], whereas in bi-site activation, Fig. 1, formation or hydrolysis of ATP occur via the same reaction steps.

In a recent paper, Weber and Senior concluded that a 'bi-site catalysis' has little or no role in rapid ATP hydrolysis at higher ATP concentrations [10]. But they regard such bi-site catalysis as that which occurs when only two sites are filled. All three catalytic sites readily bind ADP or ATP, thus at higher ATP concentrations more than two catalytic sites will have bound nucleotide present. Assume that rapid bi-site activation occurs at low ATP concentration with only two-site filling. Then, in order to require that such bi-site activation would not continue when ATP concentration is raised, it would be necessary to postulate that the presence of ATP or ADP at a third site would stop the bi-site activation. Otherwise, bi-site activation would have a predominant role at higher ATP concentrations. The analysis of Weber and Senior [10] actually gives no information about whether bi-site activation is operative at higher ATP concentrations.

3. Bi-site activation during ATP synthesis

In their evaluations Senior and associates [4–10] give no consideration to data supporting bi-site activation during ATP synthesis. Direct measurements of ADP binding during photophosphorylation at lower than K_m concentrations of ADP give evidence that the onset of rapid ATP synthesis occurs as a second catalytic site fills with ADP [2]. Bi-site activation during ATP synthesis supports the probability of bi-site activation during ATP hydrolysis.

4. A site with preferential affinity for ADP

Support for the postulate that one site binds ADP better than ATP [1–3] came from studies of the hydrolysis of low concentrations of trinitrophenyl ATP (TNP-ATP) by the mitochondrial F_1-ATPase [17]. ADP inhibited the hydrolysis of TNP-ATP with a K_d of about 0.15 mM compared to a K_d for inhibition by ATP of about 2 mM. The results are consistent with one very tight binding site for ATP, a second site where ATP binding allows rapid hydrolysis, and a third site that does not bind ATP until mM concentrations are reached. This suggests that explanations other than ATP binding must be sought for why three-site filling is observed with much less than mM concentrations of ATP in studies of Senior and associates [4–10].

In view of these results, it was gratifying when Menz et al. [16] recently reported a crystalline structure of mitochondrial F_1-ATPase with all three catalytic sites filled with nucleotide, and in which one site bound ADP in preference to ATP. This correlates well with the suggestions for bi-site activation. Indeed, the publication of their paper was a considerable stimulus for the preparation of this present contribution.

5. How bi-site activation may occur

The three catalytic sites of the ATP synthase are considered to be in different conformations, as depicted in Fig. 1. For net ATP synthesis site 1 binds ADP and P_i, site 2 catalyzes reversible ATP formation, and site 3 releases ATP. For net hydrolysis, the reverse sequence occurs. Sites 1 and 3 if empty will change conformations when substrates bind, and intermediate forms will arise as sites are interconverted in each 120° rotational step. Rapid ATP synthesis can occur when site 2 is already filled and site 1 binds ADP and P_i, whether or not site 3 has nucleotide present. Rapid hydrolysis can occur when site 2 is already filled and site 3 binds ATP, whether or not site 1 has nucleotide present.

An important property of the site 2 where chemical transformation occurs needs additional explanation; namely that a form with only ADP and P_i present may be favored during net ATP hydrolysis and that a form with only ATP present may be favored during synthesis. The different shapes of the sides of site 2 in Fig. 1 indicate this duality. For net ATP synthesis it is essential that when site 2 is converted to site 3, only ATP will be present so that only ATP will be released. The properties of site 2 suffice to result in the presence of about equal amounts of bound ADP and ATP. Our data indicate that during net ATP synthesis with ample protonmotive force, the quasi-equilibrium may be shifted toward ATP [2]. Such a shift, and/or a conformational signal that allows opening only when ATP is present, is essential. Analogously, during hydrolysis the quasi-equilibrium may be shifted toward bound ADP so that essentially only ADP will be released. How this could be achieved is not known. It may be that the capacity for chemical transformation is maintained as

the site changes properties to favor the presence of ADP and P_i or ATP, with the relative position of the γ and β subunits during a rotation step determining whether hydrolysis or water elimination is favored.

6. Bi-site activation and K_m evaluations

Reaction velocity measurements can serve primarily to eliminate rather than prove proposed reaction schemes. The literature is replete with measurements that could be interpreted as reflecting more than one K_m value above μM concentrations. These could rise from velocity changes when second or third sites bind ATP or ADP, or, for ATP hydrolysis, from interconversion of active and MgADP-induced inactive forms. With the mitochondrial F_1-ATPase, when adequate measurements of initial velocity are made, only a single K_m value consistent with bi-site activation is noted. For example, Cunningham and Cross found a linear increase in rate with nM to 10 μM added ATP, a 10 000-fold concentration range [18]. This is as expected if throughout the concentration range the enzyme was turning over by the same mechanism and had a K_m well above 10 μM, and if in the low nM concentration range a rapid turnover of an individual enzyme results when ATP adds to a second catalytic site. Extension of the measurements to higher ATP concentrations by Milgrom et al. showed a single K_m of about 130 μM [19].

Examples of careful measurements for ATP synthesis are those of Perez and Ferguson for oxidative phosphorylation with a bacterial enzyme [20] and of Richard and Gräber for photophosphorylation with a constant protonmotive force [21]. Both reported only a single K_m for ADP above μM concentrations.

These examples are consistent with the view that bi-site activation accounts for the very large increase in rate over the uni-site catalysis rate. Present velocity data do not, however, rule out minor effects on rates from third site filling. That any such effects are not large is indicated by the similar ^{18}O exchange patterns accompanying hydrolysis of 1 μM ATP with or without addition of 100 μM ADP [22]. It is also pertinent that present data do not eliminate the possibility of tri-site activation.

7. Rotational experiments favor a bi-site mechanism

Kinosita, Yoshida and colleagues have demonstrated a rotation of actin filaments or gold beads attached to immobilized F_1-ATPase preparations [23,24]. Although the frequency of rotation is decreased as ATP concentration is lowered, the rotational steps when they occur are as rapid at low as at higher ATP concentrations. They interpret these results to mean that a single mechanism for driving rotation prevails from 20 nM to 2 mM or higher ATP concentrations, and that the rotation is driven by ATP binding to a second catalytic site. These advances supply a novel support for the occurrence of bi-site activation.

Yasuda et al. [24] were able to separate the 120° rotational step into a 90° and 30° substep. It may be that the 90° step is associated with the large conformational change accompanying ATP binding, and the 30° step with the shift in the hydrolysis quasi-equilibrium toward ADP (for net hydrolysis) or ATP (for net synthesis).

8. Third site filling by ADP during ATP hydrolysis with bi-site activation

Measurements of ADP binding by quenching of fluorescence of a mutant F_1-ATPase show that ADP can bind quite tightly to all three catalytic sites. The estimated K_d for the first ADP bound is much less than μM and for the second and third is in the range of 20–30 μM [4,6,7,9]. When attempts are made to measure ATP binding by the fluorescence quenching, the relatively high concentrations of enzyme required result in considerable ADP being formed as measurements are made. Thus at higher ATP concentrations it is to be expected that ADP formed will readily rebind to any empty catalytic sites of the enzyme. Also the high affinity for ADP suggests that release of ADP may be a slow step in the catalysis. That such slow release and/or rebinding of ADP occurs is corroborated by measurements of the presence of bound ATP or ADP at catalytic sites [5]. During hydrolysis at higher ATP concentrations more than one ADP is reported to be present [5,7,9].

9. Relation of participating forms to X-ray structures

The crystal structures of the F_1-ATPase as observed with inhibited static enzymes are likely closely related to, but not identical with, principal and intermediate forms that appear in solution during active catalysis. That differences exist, for example, seems evident from data mentioned earlier that demonstrate three catalytic sites readily bind ADP at low μM concentrations. This means that a form such as the β_E of Abrahams et al. [25], that does not bind AMPPNP even at 5 mM [26], is likely not present in the assay solution. A form approaching the β_E could be a transient intermediate in the conversion of site 1 to site 3. Evidence favoring its appearance at least as a transient intermediate has been presented by Ren and Allison [14]. By the time the conversion of site 1 to site 3 is essentially complete, the site could still be quite open but now be more able to combine with ATP. Having the β_E as a transient intermediate could help assure the departure of ATP during net ATP synthesis, or of ADP during hydrolysis. Alternatively, a β_E form might not appear, and a site 1 with ADP bound could be converted to a site 3 with ADP still bound.

Site 1 with ADP and P_i bound could be closely represented by the ADP binding form with sulfate present as recently described by Menz et al. [16]. A form of site 2 with ADP and P_i present may be related to the β_{DP} form [25]. Site 3 when empty may not be represented by any reported X-ray structure, except that it may be readily derived from the β_E form if this form appears in catalysis. When ATP adds to site 3, the site may approach a configuration akin to the β_{TP} form observed in the 1994 studies [25]. The β_E might arise from site 1 being almost converted to site 3, the β_{DP} from site 2 being pushed (or pulled?) toward site 1 but blocked, and the β_{TP} from site 3 being nearly converted to site 2. In the active enzyme the appearance of intermediate forms might result in structures resembling β_{DP} and β_{TP} both being transiently present. This could account for the results of Tsundoa et al. [27]; their cross-linking studies with cysteine-inserted mutants indicated that closed forms resembling β_{DP} and β_{TP} might both be present during catalysis. Other forms observed in X-ray studies [16,28] may offer insights to intermediate or

transition states. Obviously, much interesting uncertainty and intriguing experimental challenges remain.

A more extended discussion of forms that may participate in enzyme catalysis in general as well as in ATP synthase catalysis has been presented elsewhere [29].

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