Biochimica et Biophysica Acta, 1057 (1991) 151–156

ADONIS 000527289100092W

BBABIO 43356

Review

Hydrolysis of ATP by F_1 can be described only on the basis of a dual-site mechanism

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(Received 19 July 1990)

Key words: ATP hydrolysis; ATPase, F_1F_0-; Catalytic site

Contents

I. Introduction

In recent books on bioenergetics [1] and also in textbooks [2] the description of the F_1F_0-ATPase is often accompanied with the model for the mechanism of ATP hydrolysis and synthesis that has been proposed by Cross and Boyer (e.g., Ref. 3). Apart from the well established cooperativity between catalytic sites [4], this model also includes the sequential involvement of three catalytic sites in the hydrolysis or synthesis reaction. As far as we know, this is the only example in biochemistry of a (postulated) cooperative three-site mechanism and it should be enzymologically very important to establish firmly the existence of such a mechanism. For many years controversy has existed between various research groups on the number of catalytic sites involved in multi-site catalysis. At present, the three site-model is favoured by most biochemists (and not only by those who are not themselves directly involved in the research on the ATP synthase), but alternatives are available [5,6]. A clear, indisputable experimental test for any of the possible models has not existed until now and many data on asymmetry between sequentially identical subunits can be fitted with each existing model.

At the moment, however, it seems to us that, as far as the number of catalytic sites involved in hydrolysis by F_1 is concerned, a definite choice between the various

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models can be made: recent experiments by the group of Yoshida (Ref. 7, see below) have provided a solid basis for such a conclusion. Having made this definite choice – for a two-site model – the functions and properties of the other four nucleotide binding sites can also be well described on the basis of accumulated knowledge about the properties of the various sites and the reported kinetics of the enzyme.

II. Why was a three-site model proposed?

All F_1 molecules, whether originating from bacteria, chloroplasts or mitochondria, contain three α- and three β-subunits, together with a γ-, δ- and ϵ-subunit [8–11]. Apart from the enzyme from thermophylic bacteria, all preparations contain tightly bound nucleotides, first discovered with the mitochondrial enzyme. Mitochondrial F_1, isolated in the absence of Mg^{2+}, contains three tightly bound nucleotides: in some laboratories 2 ADP and 1 ATP [12], in other laboratories 2 ATP and 1 ADP [13]. In total, six nucleotides can be bound to the enzyme, of which three become bound to β-subunits and three at the interfaces between α- and β-subunits. The catalytic sites are located on β-subunits [14] and the question was and is whether all three β-subunits are catalytic or not. Since the enzyme contains three tightly bound nucleotides upon isolation and also on three sites the bound nucleotides exchange very rapidly [15], it seemed attractive to assume that the rapidly exchangeable sites are catalytic and the tightly bound sites non-catalytic [16]. The kinetic analysis of ATP hydrolysis by F_1, as carried out very accurately by both Gresser et al. [17] and Roveri and Calcaterra [18], revealed three kinetic phases, and these data were considered as further evidence for a three-site mechanism. In the literature more sophisticated arguments can be found, but these arguments are too detailed for this contribution.

III. Problems with the three-site model

The groups of Boyer and Slater had concluded already in the early 1970's that synthesis and hydrolysis of ATP occur when the substrate is tightly bound at a catalytic site. The hydrolysis or synthesis reaction itself is not accompanied by a significant change in ΔG. This change occurs during the binding and dissociation reaction.

Thanks to the discovery by the group of Penefsky [19] of the very slow single-site catalysis it became clear that, under normal conditions, at least two catalytic sites cooperate: at low concentrations of ATP (stoicheiometric with the enzyme) one catalytic site binds ATP very strongly and hydrolysis occurs at a rate of 12 s^{-1}. The rate of dissociation of product ADP is still smaller, although faster than originally measured by Grubmeyer et al.: both Milgrom and Murateliev [20] and we (Berden, J.A., Roveri, O.A. and Calcaterra, N.B., unpublished results) have measured a rate of about 0.05–0.1 s^{-1} instead of $3 \cdot 10^{-4}$ s^{-1} in Ref. 19 (see below). When more ATP is present a second catalytic site also becomes occupied and, due to cooperative interaction, both the rate of hydrolysis (at both sites) and the rate of dissociation of product increase by several orders of magnitude (the data of Bullough et al. [21] and Gräber et al. [22] will be discussed later). From these results two conclusions can be drawn: first, one of the three tightly bound nucleotides is bound at a catalytic site, and second, to the three kinetic phases distinguished by Gresser et al. [17] and Roveri and Calcaterra [18] a fourth phase has to be added, since these authors did not measure the single-site catalysis. The reported kinetic data themselves as well as their interpretation in terms of the existence of three kinetic phases have never been contested by other authors.

Since one of the three tightly bound nucleotides has to be bound to a catalytic site (and is therefore rapidly exchangeable), at least one of the three sites without tightly bound nucleotide has to be non- or slowly exchangeable after binding a nucleotide, because only three out of the six nucleotide binding sites exchange rapidly [15]. A site with these properties has been described by Kironde and Cross [23]: one non-catalytic site binds ADP very strongly in the presence of Mg^{2+}, but much more weakly in the presence of EDTA. ADP bound at this site is, however, largely lost upon ammonium sulphate precipitation, one of the steps in the isolation procedure. If no Mg^{2+} is present in the further steps of the isolation, this site remains free. So this problem is solved: one of the tightly bound nucleotides is catalytic and exchangeable and one of the other three sites, a non-catalytic one, binds a nucleotide (MgADP) that exchanges only very slowly. The question can now be reformulated: are all the three rapidly exchangeable sites catalytic? The only possible answer seems to be no, since with just three catalytic sites no four kinetic phases can be explained. To explain four kinetic phases at least one rapidly exchangeable non-catalytic site is required which has interaction with the catalytic sites. The consequence of this argument is that only two of the rapidly exchangeable sites can be catalytic and that one of them has to be non-catalytic. A further consequence of this conclusion is that the two tightly bound, non-exchangeable nucleotides cannot both be bound to a (non-catalytic) α–β interface site, as is postulated by Kironde and Cross [24]; rather, one of them has to be bound to the catalytic domain of a β-subunit.

IV. Experimental data indicative of a two-site model

The reasoning given above leads to the conclusion that the six nucleotide binding sites on F_1 can be distinguished as follows: the two tightly bound non-ex-

changeable nucleotides are bound to different types of site, one to a β-site and one to an interface site. It is likely, then, that these two sites belong to the same α/β couple and, on basis of the observed asymmetry between the three α/β couples, Boekema et al. have proposed such an arrangement [25], in line with other data on asymmetry [26,27]. Two of the four remaining sites, then, are catalytic and during isolation, when nucleotides are removed, one of these two sites retains a tightly bound nucleotide. The residual two sites are non-catalytic and one of them is slowly exchangeable when ADP is bound [23] and one is rapidly exchangeable. Indeed, Van Dongen et al. [28] and Boulay et al. [29] have reported that three sites bind 2-azido adenine nucleotides rapidly, but only two of them are catalytic on the basis of the inhibition pattern [28]. A fourth site, non-catalytic, binds more slowly.

The data obtained with 8-azido-adenine nucleotides [30] are even more convincing: isolated F_1 can bind 2 mol 8-azido-ATP in the presence of EDTA and, after covalent attachment of these two nucleotides one of them appears to be bound to a catalytic site (on a β-subunit) and one to a non-catalytic site (at an $\alpha-\beta$ interface). At this stage, the enzyme still contains between two and three tightly bound adenine nucleotides. After subsequent addition of Mg^{2+} 2 mol of 8-azido-ADP can be covalently bound as well; again, one at a β-subunit and one at an interface site [30]. At this stage, two adenine nucleotides are still tightly bound, the two non-exchangeable ones, about one ATP and one ADP. According to simple logic, one of them has to be bound at a β-site and one at an $\alpha-\beta$ interface site (see scheme of Fig. 1).

More direct evidence for the presence of only two catalytic sites has been provided by reconstitution experiments with Nbf-modified F_1 [31]: Upon dissociation of at least the β-subunits with LiCl, followed by reconstitution according to a procedure developed by Wang [32], the inhibition of the enzymic activity by covalently bound Nbf was partly abolished, indicating that the Nbf modification (maximally one Nbf/F_1 was bound, causing full inhibition) in some molecules was no longer blocking ATPase activity. Statistical analysis showed that, assuming random incorporation of the modified and non-modified β-subunits during the reconstitution step, two positions of the modified subunit result in an inactive molecule while in one position of the β-subunit the modification does not affect activity. The possibility that the modified β-subunits reconstitute preferentially with other modified β-subunits, resulting in a preparation with more molecules without modified β-subunit than expected on the basis of random distribution, is excluded by the experiments of Wang, who showed that such preference does not exist [33].

V. Final evidence for a two-sites mechanism

For some unknown reason, the use of statistics was an argument for the defendants of a three-site mechanism not to accept the conclusions from these latter experiments. But recently, the group of Yoshida has shown with very elegant experiments that our conclusions were correct. Miwa et al. [7] could avoid the above-mentioned problem of a possible non-statistical distribution of modified and non-modified β-subunits over the reconstituted F_1 molecules: via genetic engineering they constructed β-subunits (from TF_1), modified in the catalytic site or not modified, which could be covalently attached to TS-6B beads via an additional cysteine. Such immobilized β-subunits were then reconstituted with all the required subunits of TF_1 into intact F_1 and in such a way F_1 preparations could be obtained containing only molecules of one type: molecules with only non-modified β-subunits; with one modified and two non-modified β-subunits; with two modified and one non-modified β-subunits; or with only modified β-subunits. The result was that F_1 containing two or three modified β-subunits, was always inactive (not taking into account single-site catalysis), thereby excluding the postulate of only one catalytic site as promoted by Wang [5], but F_1 containing one modified β-subunit was partially active and the kinetic pattern of the resid-

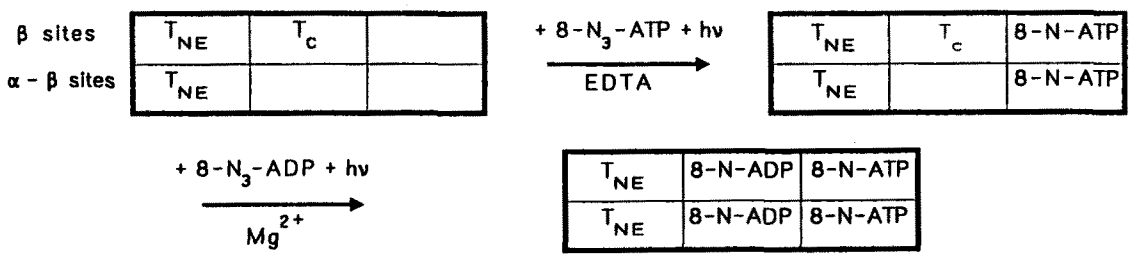

Fig. 1. Schematic representation of the covalent modification of F_1-ATPase with 8-azido-adenine nucleotides. T_{NE}, tightly bound non-exchangeable adenine nucleotide; T_C, adenine nucleotide tightly bound at a catalytic site; 8-N-AT(D)P, covalently bound 8-nitreno-AT(D)P; T_C, as T_C, but less than stoichiometric. In the final form (with 4 8-N-AT(D)P), T_{NE} can be replaced with 2-azido-adenine nucleotides, but not with 8-azido-adenine nucleotides.

ual ATPase activity was similar to that of the unmodified TF_1. This result excludes any model with three cooperative catalytic sites.

The experiments still contain one residual statistical problem. If two β-subunits of F_1 are catalytic and one is non-catalytic and therefore the whole molecule is indeed asymmetrical, the three positions of the β-subunits in the molecule have to be different. Based on random distribution of the modified and non-modified subunits over the possible positions in one molecule, the activity of the whole population of molecules with one modified and two non-modified β-subunits should be 33% of the activity of intact F_1. The authors do not discuss this point, but the reported activities vary between 8 and 45%, depending on the modification [7]. It is not unlikely that the type of modification of the catalytic site influences the preference of the modified subunit for a catalytic or a non-catalytic position in the molecule, but this point should be further analysed. The basic experimental result that F_1 containing one β-subunit without an active center can perform ATP hydrolysis with the same kinetics as unmodified F_1 can not be reconciled with a sequential three-site mechanism, but only with a dual-site mechanism. The negative cooperativity at high substrate concentrations, often considered as the result of binding of ATP to a third catalytic site [3], has to be explained by binding of substrate to a (rapidly exchangeable) regulatory site, as proposed by Recktenwald and Hess [34] and Stutterheim et al. [35].

A schematic representation of the resulting model is depicted in Fig. 2. One of the three α/β couples – the one exhibiting the strongest interaction with the small subunits [25] – contains the two tightly bound, non-exchangeable, structural nucleotides, while the two other couples contain a catalytic and a non-catalytic site each. The two catalytic sites interact with each other, but also the non-catalytic sites interact with the catalytic sites, their affinity for substrate and product being modified by binding of ATP to the non-catalytic sites as described previously [6,34,35]. Additionally, the data obtained by Lardy's group in the '70's, on the presence of regulatory sites [36,37], can be incorporated into this model.

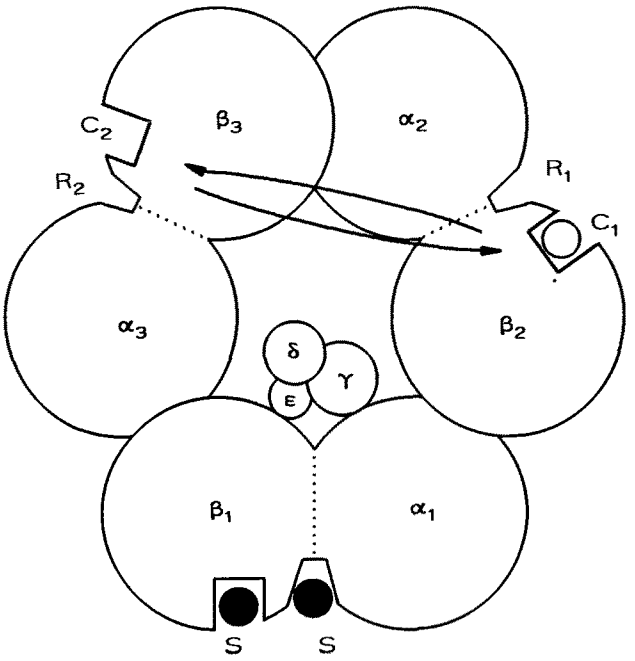

Fig. 2. Model for isolated F_1-ATPase. S, structural adenine nucleotide binding site; C, catalytic site; R, regulatory site; ●, tightly bound non-exchangeable nucleotide; ○, tightly bound exchangeable nucleotide. The regulatory sites bind ADP with a higher affinity than ATP, while the catalytic sites bind ATP with a higher affinity than ADP.

VI. Further test of the dual-site model

Since the model outlined above predicts that one of the tightly bound, non-exchangeable nucleotides in mitochondrial F_1 is bound at a 'β-site', a final additional control can be made: the analysis of the localisation of the two non-exchangeable nucleotides. Our approach at present is to dissociate the β-subunits with LiCl (resulting in loss of tightly bound nucleotides) in a way similar to the one used in earlier experiments [31,33], and to reconstitute F_1 in the presence of EDTA and 2-azido-ATP (we have established that 2-azido-ATP can fully replace ATP in the reconstitution procedure). After reconstitution, all exchangeable 2-azido-nucleotides are replaced by AT(D)P and the residual 2-azido-nucleotides (at non-exchangeable sites) are partly covalently bound by illumination. As expected, preliminary experiments have shown that this illumination does not cause significant inhibition of ATPase activity of the reconstituted molecules. Proteolytic digestion of such F_1, radiolabeled with 2-azido-[α-^{32}P]ATP, followed by peptide analysis and possibly sequence analysis will show where the label is attached. From the labelling studies in the literature it is known that 2-azido-ATP at the 'β-site' becomes covalently attached to predominantly Tyr-345 and 2-azido-ATP at the 'α/β-site becomes predominantly attached to Tyr-368 [38]. If indeed Tyr-345, or another amino acid that is known to be part of the catalytic site, becomes labelled under the conditions of our experiments, any reasonable doubt about the basic model may belong to the past.

VII. Single-site and multi-site catalysis

As concluded above, isolated F_1 (in EDTA) contains two nucleotides, tightly bound at one α/β pair (at a β- and an α–β site), which do not exchange and a third nucleotide bound at the second β-site, which is the first catalytic site. On this basis the reported kinetic data can be explained as follows.

To obtain single site catalysis of added ATP at a single site, the site has to be (partly) emptied by treat-

ment with phosphate [19] or pyrophosphate (Berden, J.A., Roveri, O.A. and Calcaterra, N.B., unpublished results). After hydrolysis of added ATP (in the presence of Mg^{2+}) the formed ADP dissociates from the catalytic site at a rate of 0.05–0.1 s^{-1} (see above), but this ADP binds rapidly to the α–β site in the same α/β pair, unless a regenerating system is present. It dissociates from this non-catalytic site at a rate of $3 \cdot 10^{-4}$ s^{-1}. This latter value is measured both by Penefsky's group (but interpreted as the rate of dissociation from the catalytic site, Ref. 19) and Kironde and Cross [23].

In the presence of a regenerating system, as used by Gresser et al. [17] and Roveri and Calcaterra [18] in their kinetic measurements, addition of ATP above stoichiometric concentrations results in the binding of ATP to this non-catalytic site. The binding of ATP at this site is much weaker than the binding of ADP. We suppose a K_d of 1–5 μM on the basis of the kinetic data – the value of this constant has never been measured – while the K_d of ADP equals 50 nM as measured by Kironde and Cross. The binding of ATP to this non-catalytic site results in a faster dissociation of ADP from the catalytic site and the measured rate of catalysis now equals the rate of ATP hydrolysis at the catalytic site, measured by Penefsky's group as 12 s^{-1} [19]. This value equals the V_m of the first kinetic phase determined by Gresser et al. [17] and Roveri and Calcaterra [18], i.e., 2 μmol/min per mg for the bovine heart enzyme.

Upon addition of more ATP the second catalytic site (the third β-site) becomes occupied, resulting in an increased rate of catalysis (300 s^{-1} at each site, Ref. 19), but now the rate of dissociation of ADP is again the rate-limiting step. The resulting K_m value equals 20–50 μM.

Upon addition of still more ATP the third α–β site (the second regulatory site) becomes occupied by ATP and the dissociation rate of ADP from the catalytic sites is increased again, so that the measured V_m equals the rate of hydrolysis of ATP at the catalytic sites. The resulting K_m value equals 200–500 μM. It should be emphasized that only binding of ATP or anions like sulphate to the regulatory site(s) influences the affinity of the catalytic sites for substrate and product. Binding of bicarbonate or sulphite (and possibly ADP) does not increase the measured K_m values (see Refs. 34 and 35).

VIII. Deviations by differences in the F_1 preparation

In the literature some data can be found which are not easily understood on the basis of the above description of the mechanism of hydrolysis of ATP by F_1. One type of data is related to the absence of a drastic increase in the rate of catalysis of ATP bound at a single catalytic site upon addition of more ATP [21,22], the other type to the so-called hysteretic inhibition of F_1 by ADP [39]. Both phenomena are probably related to the type of preparation of F_1, and we would like to propose the following explanation.

The preparations from the laboratories of Allison [21] and Gautheron [40] contain only slightly more than two tightly bound nucleotides, mainly ADP. This property is probably due to differences in the isolation procedure (presence of Mg^{2+}, together with ATP, during certain isolation steps). We suppose that in these preparations not only the non-exchangeable α–β site, but also the slowly-exchangeable non-catalytic site, described by Kironde and Cross [23,24], is occupied with ADP. On the other hand, the ATP bound at the 'non-exchangeable' β-site in the preparations obtained in the laboratories of Penefsky, Boyer and ourselves, is largely hydrolysed to ADP. But this ADP is not very tightly bound, except in non-activated chloroplast F_1, and is lost in the final preparation. When with this type of enzyme (no β-site fully occupied and two α–β sites occupied with ADP) single-site catalysis is carried out with substoichiometric amounts of ATP, this ATP is bound to the first β-site. This site has retained the capability of single-site hydrolysis (the rate is also 0.05–0.1 s^{-1}, as measured for CF_1, Ref. 22). But upon addition of more ATP the catalysis of ATP bound at this site is not largely enhanced, since this site does not take part in multi-site catalysis. Only ATP molecules at the second and third β-sites are involved in multi-site catalysis.

Hysteretic inhibition of mitochondrial F_1 by ADP is not well understood in relation to the occupation of the nucleotide binding sites. The phenomenon has been extensively described by the group of Gautheron [39,41,42], but also observed by Allison (personal communication). With hysteretic inhibition the following phenomenon is meant. If F_1 is preincubated with ADP, in the presence of Mg^{2+}, the enzyme starts to hydrolyze added ATP at a normal uninhibited rate, but with time ($t_{1/2} = 0.2$–0.3 min) the rate decreases till nearly full inhibition, up to 80%. Di Pietro et al. [41] have shown that the phosphate, released during the initial ATP hydrolysis, is essential for the inhibition. The hysteretic inhibition has never been observed in the laboratories of Boyer and Penefsky or by ourselves. If we preincubate the enzyme with ADP in the presence of Mg^{2+}, the enzyme starts ATP hydrolysis at a very low rate, but with time this rate increases. One may speculate, then, that for the observation of hysteretic inhibition added ADP has to become bound at the first β-site, just like ATP in the above-mentioned experiments on single-site catalysis. This suggestion is in agreement with the data on labelling and specificity of the hysteretic site [42]. Under these conditions, then, three sites are occupied with ADP, one β-site and two α–β sites. Additional binding of phosphate at a high-affinity phosphate-binding site induces a stable inhibited state of the enzyme.

We wish to stress, however, that the above interpretation of experimental data which we ourselves have never observed, is speculative. More data on the precise localisation of bound nucleotides under these special conditions are required. It may be evident, however, that the experiments of Bullough et al. [21] and Fromme and Gräber [22] on heterogeneity of catalytic sites in F_1 can be understood only when no more than two catalytic sites are involved in multi-site catalysis.

Acknowledgements

The authors thank Professor K. van Dam for stimulating discussions. This work was supported in part by grants from the Netherlands Organization for the Advancement of Scientific Research (N.W.O.) under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.).

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