THE ATP- AND ADP-BINDING SITES IN MITOCHONDRIAL COUPLING FACTOR F_1 AND THEIR POSSIBLE ROLE IN OXIDATIVE PHOSPHORYLATION

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Received 19 April 1979

1. Introduction

Different lines of evidence point to the presence of several ATP- and ADP-binding sites in mitochondrial coupling factor F_1.

(1) Its ATPase activity and direct involvement in the phosphorylation of ADP in oxidative phosphorylation [1] show that it contains at least one ATP- and ADP-binding site.

(2) ADP is initially a weak competitive inhibitor of the ATPase activity [2]. However, the degree of inhibition increases with time [2,3].

(3) Radioactively-labelled ADP and ATP are bound to F_1 [4].

(4) Both membrane-bound and isolated F_1 contain 2 molecules of firmly bound ATP and 1 of firmly bound ADP [5,6]. The firmly bound ATP is not accessible to the ATPase catalytic site. Only part of the nucleotides exchange with added nucleotide [5].

(5) Hydrolysis of ATP by F_1 in the presence of Mg^{2+} proceeds with an appreciable lag time [2,7] the duration of which is dependent on the concentration of ATP [2].

(6) In the presence of Mg^{2+}, 8-azido-ATP is a substrate for the ATPase, competitive with ATP [8]. Irradiation of F_1 with 8-azido-ATP in the absence of Mg^{2+} causes inactivation of the ATPase activity, the inactivation being proportional to the amount of 8-nitreno-ATP (the photolysis product of 8-azido-ATP) that is covalently bound to the F_1, complete inactivation being obtained when 2 molecules 8-nitreno-ATP are bound per molecule of F_1 [9]. ATP and ADP (but not AMP) protect against the inactivation. The nitreno-ATP is bound to the β-subunit [9]. The ATPase is also completely inactivated when 2 molecules of 8-nitreno-ADP are covalently bound by irradiation of F_1 with 8-azido-ADP, either in the presence or absence of Mg^{2+}. ATP and ADP (but not AMP) protect against the inactivation. In the presence of Mg^{2+}, the 8-nitreno-ADP is covalently bound mainly to the α-subunit; in the absence of Mg^{2+}, it is bound about equally to both the α- and β-subunits. The amount of firmly bound ATP and ADP is not appreciably altered by irradiation in the presence of azido-ATP [8]. When F_1 is irradiated in the presence of azido-ATP in the absence of Mg^{2+}, followed by irradiation in the presence of both azido-ADP and Mg^{2+}, a total of 3.1 mol photoaffinity label was covalently bound to the F_1 without appreciably affecting the amount of firmly (but not covalently) bound ATP and ADP (R.J.W., unpublished observations). After the first irradiation, the label was practically entirely in the β-subunit; the extra label after the second irradiation was predominantly in the α-subunit. Thus, the binding site for ATP in the absence of Mg^{2+} and that for ADP in its presence appear to be independent and to be located on the β- and α-subunits, respectively.

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(7) ATP binds to a regulatory site [10], which also binds certain anions [11]. Binding of ATP or SO_4^{2-} to this site induces low-affinity binding of ATP to the catalytic site [11]. Binding of HSO_3^- [11] and probably HCO_3^- [10] to the regulatory site stabilizes a conformation of the catalytic site with a high affinity for ATP.

2. Types of ATP- and ADP-binding sites

An attempt will now be made to rationalize the observations summarized in section 1 in terms of a minimum number of types of ATP- and ADP-binding sites in mitochondrial F_1 (see table 1).

I. Two very strong ATP-binding sites
The binding of ATP to these sites in isolated F_1 is weakened when isolated F_1 is partly dissociated into subunits by cold treatment [12] or modified by low pH [13], and in membrane-bound F_1 when the membrane is energized by electron flow through the electron-transfer chain [14,15] or by ATP hydrolysis [15].

II. One strong ADP-binding site
Binding to this site is weakened by the same treatments that weaken binding to the two strong ATP-binding sites.

III. Two catalytic ATP-binding sites
One on each β-subunit of the enzyme.

IV. Two allosteric anion-binding sites
One on each α-subunit of the enzyme.

2.1. *Type-I sites*

The dissociation constants of these sites have been estimated to be $< 10^{-11}$ M [6]. At the time of their discovery, it was postulated that ATP binding to these sites is an intermediate step in the mechanism of oxidative phosphorylation [5,16]. This postulate was given strong support by the finding that ATP dissociates from these sites in membrane-bound F_1 when coupled electron transfer takes place in the membrane [14,15]. However, it has been seriously weakened by the recent finding that, during a single flash of chromatophore membranes, dADP, IDP and GDP are phosphorylated without dissociation of the firmly bound ATP [17]. Although one could argue that this experiment should not be extrapolated to other energy-transducing membranes, since chromatophores can phosphorylate a wider range of nucleoside diphosphates (e.g., GDP and IDP) than heart F_1, it is clearly unlikely that a basically different mechanism operates. Replacement of ATP bound to type-1 sites by incubation at low pH with ADP or the closely related 2′-deoxy or 2-hydroxy analogues or by formycin diphosphate (in which the C-8 and N-9 atoms of adenine are interchanged) results in inhibition of the ATPase activity [13]. Thus, it appears that type-I sites act as regulatory or allosteric sites for the ATPase. Replacement of these sites by ADP is responsible for inhibition of the ATPase activity by preincubation of F_1 with ADP. A dissociation constant for ADP has been calculated at most 25 μM, but it is probably much less [13].

Table 1
ADP- and ATP-binding sites in isolated heart mitochondrial F_1

Type	No.	Description	Function in ATPase reaction	Subunit	K_d (ATP) (μM)		K_d (ADP) (μM)	
					+Mg	−Mg	+Mg	−Mg
I	2	Strong ATP	Positive effector		$\leqslant 10^{-5}$	$\leqslant 10^{-5}$		$\ll 25$
II	1	Strong ADP	Negative effector			$\geqslant 0.3$		0.3
III	2	Catalytic ATP	Catalysis	β		40	140	140
IV	2	Anion Sites	Positive and negative effector	α	(40)[a]	> 140	< 140	140

[a] Yeast

2.2. *Type-II site*

The dissociation constant of ADP bound to this site has been determined by ultrafiltration in the absence of Mg^{2+} to be $\sim 3 \times 10^{-7}$ M [18], which is considerably higher than that estimated for the type-I sites. Indeed, ADP bound to this site can probably exchange with added nucleotides (0.6–0.75 mol firmly bound nucleotide is rapidly exchanged [5,13]). Since, in the absence of Mg^{2+}, ADP is not replaced by ATP, the dissociation constant of the latter must be $\gg 0.3$ μM. When ADP is stripped from this site by treatment with glycerol, added ATP is hydrolysed within the first 200 ms at a rate much greater than that in the steady state or with normal F_1 [2]. It is possible, then, that ADP bound to this site acts as a negative effector and it has been proposed [2] that the lag in the hydrolysis of ATP [2,7] is due to replacement of this ADP by ATP in the presence of Mg^{2+}. ADP bound to this site is not the initial phosphate acceptor in oxidative phosphorylation [17,19].

2.3. *Type-III and type-IV sites*

Added ADP binds first to the type-II site and then to weak sites. If it is assumed that there are 4 weak sites with the same intrinsic dissociation constant, the latter is ~140 μM [18]. This is the same as the dissociation constant of ADP bound to the catalytic site, in the presence of Mg^{2+}, calculated from the K_i for competitive inhibition by ADP measured immediately after adding ADP [2]. Since azido-ADP binds equally well to both α- and β-subunits in the absence of Mg^{2+}, the dissociation constant of ADP bound to the α-subunit is probably also ~140 μM. Since ADP and ATP, under these conditions, are approximately equally effective in protecting against azido-ATP [20], we may conclude that the dissociation constant to the β-subunit under these conditions is also ~140 μM. Since ATP binds almost conclusively to the β-subunit, the dissociation constant of ATP bound to the α-subunit must be substantially > 140 μM. The finding that in the absence of Mg^{2+}, ATP is about one-half as effective as ADP in protecting against azido-ADP is roughly in agreement with these calculations.

Since in the presence of Mg^{2+}, azido-ADP binds almost exclusively to the α-subunit, the binding constant of azido-ADP under these conditions is presumably appreciably < 140 μM. Mg^{2+} has little effect on the degree of inactivation, indicating that covalent binding to either the α- or the β-subunit is sufficient for inactivation. Since, however, the K_i for competitive inhibition by ADP in the presence of Mg^{2+} is 140 μM, the ADP-binding site on the α-subunit cannot be the source of the competitive inhibition. This gives support to our designation of the β-subunit as the site of catalysis, which is in agreement with the work of others (e.g. [21]).

The important dissociation constant of ATP bound to the catalytic site in the presence of Mg^{2+} is not known. From the steady-state kinetics a dissociation constant of ~230 μM in the presence of Mg^{2+} can be calculated [2], but this refers to the first kinetically competent complex formed and does not take account of possible subsequent isomerizations (protein.conformation changes) leading to stronger binding of ATP at this site (cf. myosin [22]). The strong inhibition by incubation with non-hydrolyzable analogues [13] suggests that this might be the case.

It has been shown [13] that the catalytic site is relatively non-specific for ATP. It can be replaced by a number of related compounds, including 2′-dATP, iso-GTP, ITP, GTP, ε-ATP, *N*′-ATP, *rro*-ATP (ATP in which the ribose ring has been opened between C-2′ and C-3′) [13] as well as 8-azido-ATP [8].

Type-IV sites may well be identical with the anion-binding site [10] the occupation of which by ATP (in the presence of Mg^{2+}) causes a large decrease of the affinity of the catalytic site for ATP [11]. A dissociation constant of 40 μM has been calculated [11] for ATP bound to the yeast enzyme.

3. Discussion

Whatever the mechanism of oxidative phosphorylation, relatively strong ADP-binding sites and relatively weak ATP-binding sites must be involved, since oxidative phosphorylation has a high affinity for ADP and is not inhibited by ATP. However, since it has been demonstrated clearly that extensive conformational changes in membrane-bound F_1 take place during coupled electron-transfer reactions in the membrane, the binding constants of some or all of the 4 types of binding sites may be quite different under conditions of oxidative phosphorylation. In particular, the tightly bound nucleotides become exchangeable

under these conditions [14,15]. This makes it difficult at present to assign a specific rôle in oxidative phosphorylation to any of the 7 sites enlisted in table 1. However, some tentative conclusions can be drawn from the study of the ATP-driven reduction of NAD^+ by succinate (the so-called reversal of the respiratory chain), which proceeds according to eq. (1):

$$ATP + NAD^+ + \text{succinate} \rightleftharpoons ADP + P_i + NADH + \text{fumarate} \quad (1)$$

Since the reverse reaction represents site-1 oxidative phosphorylation, one might expect that the same ADP- and ATP-binding sites are involved in the coupled hydrolysis of ATP (eq. (1) from left to right) and oxidative phosphorylation (eq. (1) from right to left). Indeed, covalent binding of 8-nitreno-ATP to the β-subunits of membrane-bound F_1 causes inactivation of the ATP-driven reversal of the respiratory chain, indicating that type-III sites are involved in oxidative phosphorylation. Moreover, the degree of inactivation of this reaction brought about by incomplete binding to the β-subunits is greater than the inactivation of the ATPase and the difference agrees quantitatively with a model in which the two β-subunits interact in the coupled but not in the uncoupled reaction [8], thus giving support to the two-site, flip–flop type of mechanism proposed [23].

However, the relative non-specificity for nucleotides of type-III sites compared with that for the reaction indicated in eq. (1) suggests that these are not the only sites involved. Indeed, the close correlation between the specificity of binding to type-I sites and the specificity of nucleotides for oxidative phosphorylation and the ATP-driven reversal suggests that these sites are involved in some way [13], although it seems likely that they are not where ATP synthesis takes place [17]. It seems more likely that these sites are allosteric, and that the higher specificity of oxidative phosphorylation than of the uncoupled ATPase is a property of this allosteric site. Since, under conditions of oxidative phosphorylation or of coupled ATP hydrolysis type-I sites are readily exchangeable, it is probable that they are occupied by ATP during coupled ATP hydrolysis and by ADP during oxidative phosphorylation. It is not unlikely that the conformations induced by ADP and ATP are different.

The type-II site may be also allosteric. Under conditions of oxidative phosphorylation, ADP will be bound to this site, resulting in inhibition of the ATPase activity. Under conditions of the ATP-driven reversal, the type-II site will be occupied by ATP, yielding a conformation suitable for ATP hydrolysis. Thus, the condition of the enzyme system driving eq. (1) from right to left is quite different from that when running from left to right. It is conceivable that the conformation of the type-III site is so altered when ADP is bound to type-II sites (and perhaps to type-I) as to favour ADP binding to the former. However, it is equally possible that the translocation of protons at high electrochemical potential induces conformational changes leading to increased ADP binding [19], decreased ATP binding [16,19] and/or promotes the esterification reaction [24].

Type-IV sites are also allosteric, and may be replaced be certain anions.

The picture that emerges is that the two type-III sites on the β-subunit are the only sites at which hydrolysis of ATP takes place, the other sites having a structural or allosteric rôle. The question might well be asked: what is the biological sense of an enzyme system that involves up to 5 allosteric sites for substrate and product as well as 2 catalytic sites. To state that the allosteric sites have a control function without stating what is controlled or why, does not help our understanding. Since we cannot give a satisfactory answer to this question, we must conclude that there is still a lot of chemistry in the proton-translocating ATPase or protonmotive-driven ATP synthase to be unravelled.

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