

The nucleotide binding site of F_1 -ATPase which carries out uni-site catalysis is one of the alternating active sites of the enzyme

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Nucleotide-depleted mitochondrial F_1 -ATPase binds 3'-(2')-O-(2-nitro-4-azidobenzoyl)-derivatives of ATP (NAB-ATP) and GTP (NAB-GTP) when these nucleotide analogues are added to the enzyme in equimolar quantities in the presence of Mg^{2+} (uni-site catalysis conditions). The binding of NAB-ATP is accompanied by its hydrolysis and inorganic phosphate dissociation from the enzyme; NAB-ADP remains bound to F_1 -ATPase. The F_1 -ATPase·NAB-ADP complex has no ATPase activity and its reactivation in the presence of an excess of ATP is accompanied by NAB-ADP release. The illumination of the F_1 -ATPase complexes with NAB-ADP or NAB-GDP leads to the covalent binding of one nucleotide analogue molecule to the enzyme and to the irreversible inactivation of F_1 -ATPase. It follows from the results obtained that the modification of just one of the F_1 -ATPase catalytic sites is sufficient to complete the inhibition of ATPase activity.

F_1 -ATPase Uni-site catalysis Photoactive nucleotide analog

1. INTRODUCTION

F_1 -ATPase is the catalytic part of the mitochondrial H^+ -ATPase complex. The enzyme consists of 5 types of subunits which are in stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$ and contains 6 nucleotide binding sites (for review see [1,2]). It has been shown [3] that these sites can be divided into 2 groups. Three sites are not capable of exchanging bound nucleotides in the course of ATP hydrolysis and therefore are noncatalytic. The other sites exchange bound nucleotides and are believed to be catalytic [3].

Grubmeyer et al. [4] showed that ATP added to F_1 -ATPase in equimolar quantities can be bound to the enzyme with a dissociation constant of

10^{-12} M. This binding is accompanied by ATP hydrolysis followed by the slow release of inorganic phosphate (uni-site catalysis). The binding of a second ATP molecule results in the manifold acceleration of ADP and P_i release from the first site [5]. These results have been confirmed in a number of studies [6,7]. They convincingly support Boyer's conception on alternating catalytic sites of F_1 -ATPase [8,9].

However, interpretation of the above cited data depended on the question of whether the site performing uni-site catalysis is indeed one of the catalytic sites of F_1 -ATPase. This question is justified since ADP and P_i release in uni-site catalysis conditions occurs very slowly and it cannot thus be excluded that this ATP hydrolysis is some kind of superficial activity on the part of one of the non-catalytic sites.

In this work we investigated this problem, using new photoactivated ATP and GTP analogues. A number of activated substrate analogues were used

Abbreviations: NAB-AD(T)P, 3'-(2')-O-(2-nitro-4-azidobenzoyl)adenosine-5'-di(tri)phosphate; NAB-GD(T)P, 3'-(2')-O-(2-nitro-4-azidobenzoyl)guanosine-5'-di(tri)phosphate; Mops, 4-morpholinepropane-sulfonic acid

previously to study the location and properties of nucleotide binding sites of mitochondrial F_1 -ATPase [10–20]. However, contrary to the previous studies we labelled F_1 -ATPase in uni-site catalysis conditions i.e. all the nucleotide analogue added is bound to the enzyme.

From the results obtained it follows that blocking the F_1 -ATPase site, which carries out uni-site catalysis, leads to the complete inactivation of the enzyme. It can be concluded that uni-site catalysis is carried out by one of the catalytic sites of F_1 -ATPase and that all 3 active sites have to operate for F_1 -ATPase to demonstrate ATPase activity.

2. MATERIALS AND METHODS

ATP from Reanal, Tris, Mops, bovine serum albumin from Sigma, triethanolamine from Serva, [U - ^{14}C]ATP, [U - ^{14}C]GTP from UVVVR and [γ - ^{33}P]ATP from Isotop (USSR) were used.

^{14}C and ^{33}P labelled NAB-ATP and [^{14}C]NAB-GTP (50 mCi/mmol) were synthesized as described in [21].

F_1 -ATPase was isolated according to Knowles and Penefsky [22]. Nucleotide-depleted F_1 -ATPase was obtained according to Garrett and Penefsky [23]. For that purpose, F_1 -ATPase was gel-filtrated on a Sephadex G-50 column equilibrated with 100 mM Tris- H_2SO_4 , pH 8.0, containing 4 mM EDTA and 50% glycerol (buffer A). Protein fractions with $A_{280}/A_{260} > 1.90$ were collected.

For NAB-ATP or NAB-GTP binding with nucleotide-depleted F_1 -ATPase 30 μ M solution of the enzyme in the buffer A was added to buffer B (40 mM Mops-NaOH, pH 8.0, 0.25 M sucrose, 2.5 mM $MgSO_4$ and 0.2 mM EDTA) up to a final concentration of F_1 -ATPase 3.4 μ M. Quantities of nucleotide analogs stoichiometric or substoichiometric to F_1 -ATPase were added to this solution, and the mixture was incubated for 1 min at room temperature. Free nucleotides and P_i were separated by centrifuge column procedure [24] using a Sephadex G-50 equilibrated with a buffer B.

When the exchange of bound nucleotide analogues was studied, 1 mM ATP or GTP was added to the complex of F_1 -ATPase with nucleotide analogues and after 2 min preincubation free nucleotides were separated by the centrifuge column method [24].

The illumination of the F_1 -ATPase·NAB-A(G)DP complex was carried out at a wavelength of 300–380 nm for 10 min. To determine the degree of F_1 -ATPase modification with [^{14}C]NAB-A(G)DP, aliquots of the protein (10 μ l) were added to the solution containing 5 mM ATP and the albumin (0.5 mg/ml). Then trichloroacetic acid up to a final concentration of 8% was added and the mixture was incubated for 10 min at 4°C. The protein pellet was collected after centrifugation (3 min, 9000 $\times g$) and dissolved in a 0.4 ml buffer, containing 30 mM Mops-NaOH, pH 9.5, and 1% SDS.

The protein was determined according to Lowry et al. [25].

ATPase activity was determined spectrophotometrically in the presence of an ATP regenerating system as described in [6].

3. RESULTS

As can be seen from table 1, tight (but non-covalent) binding of the ^{14}C label to the enzyme is observed when stoichiometric quantities of [^{14}C]NAB-ATP are added to the nucleotide depleted F_1 -ATPase in the dark. The ^{14}C label is largely removed from the enzyme as a result of the subsequent incubation with non-labelled ATP.

Table 1
NAB-ATP or NAB-GTP binding to nucleotide-depleted F_1 -ATPase in the uni-site catalysis conditions

Nucleotide added (0.75 mol/mol F_1)	Nucleotide bound (mol/mol F_1)	
	–	After chase by 1 mM ATP
[^{14}C]NAB-ATP	0.69	0.19
[^{14}C]NAB-ATP ^a	0.69	ND ^b
[γ - ^{33}P]NAB-ATP	0.36	0.15
[^{14}C]NAB-GTP	0.65	0.1
[^{14}C]NAB-GTP	0.65	0.1 ^c

^a The unbound nucleotide was separated from the enzyme, an eluate was incubated in buffer B for 30 min and repeatedly centrifuged through Sephadex G-50 column as described in section 2

^b Not determined

^c 1 mM GTP instead of 1 mM ATP was used in the chase experiment

The same results were obtained when [^{14}C]NAB-GTP was substituted for [^{14}C]NAB-ATP and the bound nucleotide was chased with ATP or GTP. When [$\gamma\text{-}^{33}\text{P}$]NAB-ATP is added to the $\text{F}_1\text{-ATPase}$, the quantity of the radioactive label bound to the enzyme after 1 min incubation is considerably less than in the case of [^{14}C]NAB-ATP (table 1). A time course of ^{33}P release from the $\text{F}_1\text{-ATPase} \cdot [\gamma\text{-}^{33}\text{P}]\text{NAB-ATP}$ complex is shown in fig.1. Since all the radioactive label remained bound to the enzyme when [^{14}C]NAB-ATP instead of [$\gamma\text{-}^{33}\text{P}$]NAB-ATP was used (table 1), the results obtained (fig.1) indicate that ^{33}P is released from the complex as the inorganic phosphate.

From the data in fig.1 and table 1 it follows that F_1ATPase can bind and hydrolyse NAB-ATP at a single catalytic site (uni-site catalysis). The P_i formed as a result of NAB-ATP hydrolysis dissociates slowly and the NAB-ADP (like ADP in the case of uni-site hydrolysis of ATP [6]) remains bound to the enzyme.

As can be seen from fig.2, the preincubation of $\text{F}_1\text{-ATPase}$ with stoichiometric quantities of NAB-ATP causes a decrease in the initial ATP hydrolysis rate. This inhibition of the enzyme develops in time and correlates with the dissociation of the P_i formed as a result of uni-site

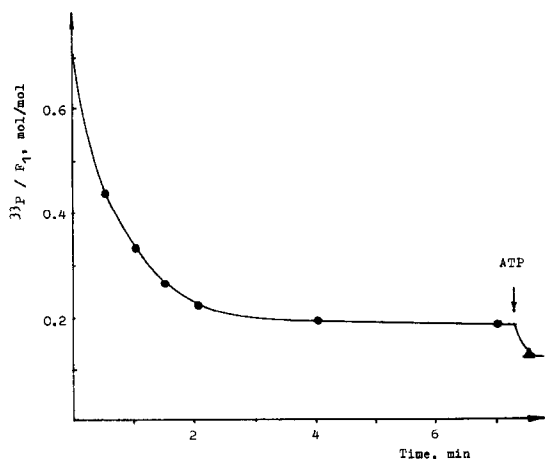


Fig.1. $^{33}\text{P}_i$ release from the complex of $\text{F}_1\text{-ATPase}$ with [$\gamma\text{-}^{33}\text{P}$]NAB-ATP. To $3\text{ }\mu\text{M}$ solution of nucleotide-depleted $\text{F}_1\text{-ATPase}$ in buffer B $2.1\text{ }\mu\text{M}$ [$\gamma\text{-}^{33}\text{P}$]NAB-ATP was added. After the time intervals indicated free nucleotides and P_i were separated and radioactivity which remained bound to the enzyme was determined (see section 2).

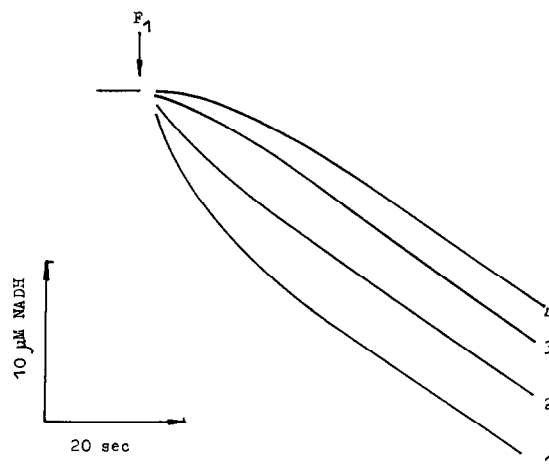


Fig.2. The kinetics of ATP hydrolysis with nucleotide-depleted $\text{F}_1\text{-ATPase}$ preincubated with equimolar quantities of NAB-ATP. The enzyme ($3\text{ }\mu\text{M}$) in buffer B was preincubated with $3\text{ }\mu\text{M}$ NAB-ATP for 10 s (curve 1), 30 s (curve 2), 60 s (curve 3) and 120 s (curve 4) and then its ATPase activity was measured.

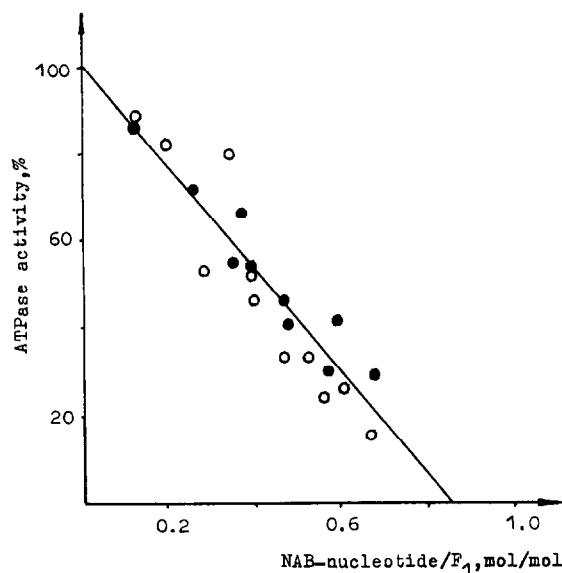


Fig.3. The dependence of the $\text{F}_1\text{-ATPase}$ modification with [^{14}C]NAB-ATP on the illumination time in uni-site catalysis conditions. $2\text{ }\mu\text{M}$ [^{14}C]NAB-ATP was added to nucleotide-depleted $\text{F}_1\text{-ATPase}$ ($4\text{ }\mu\text{M}$) in buffer B. The mixture was subjected to the centrifuge column procedure, protein fractions were illuminated for the different periods of time and the radioactivity covalently bound to $\text{F}_1\text{-ATPase}$ was determined as described in section 2.

hydrolysis of [γ - ^{33}P]NAB-ATP (fig.1). Thus, uni-site hydrolysis of NAB-ATP occurs in the same way as uni-site hydrolysis of ATP [6], resulting in the formation of the inactive $\text{F}_1\text{-ATPase}\cdot\text{NAB-ADP}$ complex. The dissociation of this inactive complex leads to the reactivation of $\text{F}_1\text{-ATPase}$ and the steady state ATPase activity does not depend on the preincubation of the enzyme with NAB-ATP (fig.2).

The illumination of $\text{F}_1\text{-ATPase}$ preincubated with [^{14}C]NAB-ATP in uni-site catalysis conditions causes the covalent binding of the ^{14}C label to the protein. The kinetics of photo-induced

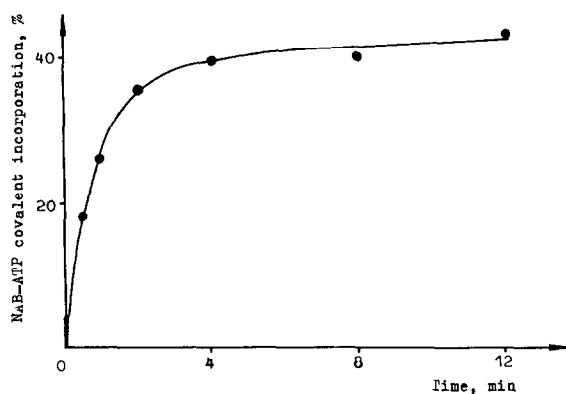


Fig.4. The irreversible inhibition of $\text{F}_1\text{-ATPase}$ activity as a result of enzyme modification with [^{14}C]NAB-ATP (●) or [^{14}C]NAB-GTP (○) in uni-site catalysis conditions. Nucleotide-depleted $\text{F}_1\text{-ATPase}$ ($3\text{--}4\ \mu\text{M}$) in buffer B was incubated for 1 min with $1\text{--}4\ \mu\text{M}$ [^{14}C]NAB-ATP or [^{14}C]NAB-GTP (NAB-nucleotide/ $\text{F}_1\text{-ATPase}$ ratio never exceeded one). The mixture was illuminated for 10 min and the protein aliquots were taken to determine ATPase activity and the quantity of covalently bound radioactive label (see section 2). To increase the degree of $\text{F}_1\text{-ATPase}$ modification with [^{14}C]NAB-nucleotides in some experiments the procedure of $\text{F}_1\text{-ATPase}$ labelling was repeated several times (up to three). In all cases the total concentrations of [^{14}C]NAB-ATP or [^{14}C]NAB-GTP (free and covalently bound to the enzyme) during the incubation with $\text{F}_1\text{-ATPase}$ never exceeded the concentration of $\text{F}_1\text{-ATPase}$ itself. After each case of illumination all the nucleotide analogue which remained non-covalently bound to $\text{F}_1\text{-ATPase}$ was removed by the incubation with 4 mM EDTA and centrifugation through Sephadex G-50 column equilibrated with 20% glycerol, 1 mM EDTA, 40 mM Mops, pH 8.0. 2 mM MgSO_4 was added to the eluate before the new addition of the nucleotide analogue.

covalent labelling of $\text{F}_1\text{-ATPase}$ with [^{14}C]NAB-ATP is shown in fig.3. The maximal yields of covalent $\text{F}_1\text{-ATPase}$ modification are 40–45% bound NAB-ATP (fig.3) and NAB-GTP (not shown).

The illumination of the $\text{F}_1\text{-ATPase}$ complexes with nucleotide analogues formed in conditions of uni-site hydrolysis causes the ATPase activity of the enzyme to decrease irreversibly. Fig.4 shows the dependence of the $\text{F}_1\text{-ATPase}$ activity on the degree of the modification of the enzyme as a result of the illumination. Since only 40–45% of the nucleotide is covalently bound to the enzyme as a result of the illumination of the $\text{F}_1\text{-ATPase}\cdot\text{NAB-nucleotide}$ complexes, several cycles of the illumination were used to increase the extent of covalent photoinduced modification of the enzyme (fig.4).

From fig.4 it can be seen that the activity of $\text{F}_1\text{-ATPase}$ monotonously decreases as the degree of the modification of $\text{F}_1\text{-ATPase}$ increases, while the covalent binding of 1 mol of both the nucleotide analogues per mol of $\text{F}_1\text{-ATPase}$ corresponds to complete inactivation.

4. DISCUSSION

In our previous paper we showed that $\text{F}_1\text{-ATPase}$ hydrolyses NAB-ATP with $K_m = 0.85\ \text{mM}$ (for ATP K_m in similar conditions is equal to 0.3 mM) and V_{\max} 16-times less than for ATP [21].

From the results cited in this paper it follows that in uni-site catalysis conditions NAB-ATP also behaves as a substrate for $\text{F}_1\text{-ATPase}$. Like the ATP [6], the NAB-ATP added in quantities stoichiometric to the nucleotide-depleted $\text{F}_1\text{-ATPase}$, is bound and hydrolysed by the enzyme; P_i formed dissociates, and NAB-ADP remains at the catalytic site (table 1 and fig.1). The $\text{F}_1\text{-ATPase}\cdot\text{NAB-ADP}$ complex formed as a result of P_i release, like the $\text{F}_1\text{-ATPase}\cdot\text{ADP}$ complex formed in uni-site catalysis conditions [6,26], is catalytically inactive, until the dissociation of NAB-ADP occurs (fig.2).

From the results obtained it follows that the illumination of the NAB-ADP· $\text{F}_1\text{-ATPase}$ complex leads to the covalent binding of the molecule of nucleotide analogue to the enzyme (figs 3 and 4).

When photo-inactivation of $\text{F}_1\text{-ATPase}$ with

8-azido-ATP [15] or 3'-O-(5-azidonaphthoyl)-ADP [20] was carried out, the covalent binding of 2 mol of the nucleotide analogue per mol of the enzyme corresponds to complete inhibition of the ATPase activity. On the basis of these data, Lübben et al. [20] came to the conclusion that, when one of the catalytic sites is modified the two remaining catalytic sites of F_1 -ATPase are effective for ATP hydrolysis.

In accordance with the data obtained (fig.4), the blocking of just one catalytic site causes the complete inhibition of the ATPase activity of the enzyme. The discrepancy between our data and those of Wagenvoort et al. [15] and Lübben et al. [20] may ensue from the fact that in our experiments the non-specific binding of the nucleotide analogue is minimized since the F_1 -ATPase·NAB-ADP complex (as well as F_1 -ATPase·NAB-GDP complex) is illuminated in conditions when there is hardly any free nucleotide analogue in the solution. It should also be noted that the data obtained by us cannot be explained by the presence in our preparations of F_1 -ATPase of a considerable quantity of the inactive (not able to bind nucleotides to the catalytic site) enzyme. The following facts contradict such a possibility: (i) the preincubation with equimolar quantities of NAB-ATP (fig.2) or ATP and ADP (not shown) results in a complete decrease in the initial ATP hydrolysis rate; (ii) reconstruction of our nucleotide depleted preparations of F_1 -ATPase with [14 C]adenine nucleotides according to Cross and Nalin [3] leads to the binding of approx. 5 mol of the adenine nucleotides per mol of the enzyme.

Thus, from the results obtained in this paper it follows that co-operative interaction of all 3 catalytic sites is necessary for the functioning of F_1 -ATPase in steady state conditions and that the site performing uni-site catalysis is indeed one of the alternating catalytic sites of the enzyme.

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