Tightly bound adenosine diphosphate, which inhibits the activity of mitochondrial F_1-ATPase, is located at the catalytic site of the enzyme

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The binding of one ADP molecule at the catalytic site of the nucleotide depleted F_1-ATPase results in a decrease in the initial rate of ATP hydrolysis. The addition of an equimolar amount of ATP to the nucleotide depleted F_1-ATPase leads to the same effect, but, in this case, inhibition is time dependent. The half-time of this process is about 30 s, and the inhibition is correlated with P_i dissociation from the F_1-ATPase catalytic site (uni-site catalysis). The F_1-ATPase–ADP complex formed under uni-site catalysis conditions can be reactivated in two ways: (i) slow ATP-dependent ADP release from the catalytic site ($\tau_{1/2}$ 20 s) or (ii) binding of P_i in addition to MgADP and the formation of the triple F_1-ATPase–MgADP–P_i complex. GTP and GDP are also capable of binding to the catalytic site, however, without changes in the kinetic properties of the F_1-ATPase. It is proposed that ATP-dependent dissociation of the F_1-ATPase–GDP complex occurs more rapidly, than that of the F_1-ATPase–ADP complex.

Mitochondrial F_1-ATPase *Nucleotide binding* *ADP inhibition* *Phosphate binding*

1. INTRODUCTION

The mitochondrial F_1-ATPase has several types of nucleotide-binding sites with different specificity and affinity to nucleotides (review [1,2]). By their affinity to nucleotides these sites can be divided into two groups: the sites of the reversible and practically irreversible (tight) binding of nucleotides [1,2]. To the latter belong the sites which preserve the bound nucleotides during gel filtration, activated charcoal treatment, or re-precipitation by ammonium sulphate [3–5]. The native mitochondrial F_1-ATPase contains 3 molecules of tightly bound nucleotides [4,6–9].

The F_1-ATPase nucleotide-binding sites can also be divided functionally into two classes. The sites capable of exchanging bound nucleotides in the course of ATP hydrolysis are believed to be catalytic ones [9]. The other 3 sites do not exchange bound nucleotides and are therefore noncatalytic sites [9]. The function of the noncatalytic sites in the mechanism of ATP hydrolysis is unknown, but they are believed to act as a regulator.

Numerous data testify to the fact that the catalytic activity of F_1-ATPase is controlled by the tight binding of ADP to the enzyme [10–16]. However, it was not shown where this ADP molecule is located: at catalytic or noncatalytic F_1-ATPase sites.

Recently, Vinogradov and co-workers [15,16] showed that ADP can be tightly bound to F_1-ATPase, resulting in the formation of an inactive complex. This complex may be formed both as a result of ADP binding to F_1-ATPase and in the course of ATP hydrolysis without ADP release from the enzyme [15]. The dissociation of the inactive F_1-ATPase–ADP complex is considerably accelerated in the presence of ATP [16].

Here we studied the binding of the nucleoside diphosphates, ADP and GDP, to mitochondrial F_1-ATPase. From the data obtained it follows that

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both the nucleoside diphosphates bind to the catalytic sites of F_1-ATPase, forming tightly bound complexes in the absence of ATP or GTP. The inhibition of F_1-ATPase occurs when ADP (and not GDP) is bound to one of the catalytic sites of the enzyme in the absence of phosphate. The F_1-ATPase–ADP–phosphate and F_1-ATPase–GDP complexes, stable in the absence of ATP and GTP, are rapidly dissociated in the presence of nucleoside triphosphates.

2. EXPERIMENTAL

ATP, ADP, GTP, GDP, NADH, phospho*enol*-pyruvate, pyruvate kinase and lactate dehydrogenase were from Reanal; Tris, crystalline bovine serum albumin (Vth fraction) from Sigma; Sephadex G-50, fine, from Pharmacia; [U-^{14}C]ATP, [U-^{14}C]GTP, [U-^{14}C]ADP and [U-^{14}C]GDP from UVVVR; [γ-^{32}P]ATP from Amersham. The sucrose was recrystallised from ethanol (non-recrystallized preparations of sucrose inactivated nucleotide-depleted F_1-ATPase).

The mitochondrial F_1-ATPase was isolated and stored according to Knowles and Penefsky [17]. Before use the enzyme was desalted by the centrifuge column procedure using Sephadex G-50 equilibrated with a solution containing 0.25 M sucrose, 20 mM Tris-H_2SO_4, 0.2 mM EDTA, pH 8.0 [18].

Nucleotide-depleted F_1-ATPase was prepared as described [4]. After gel filtration, the protein fractions with an A_{280}/A_{260} ratio of more than 1.90 were collected.

The experiments on the binding of equimolar quantities of nucleotides to nucleotide-depleted F_1-ATPase were carried out in a medium containing 250 mM sucrose, 20 mM Tris-HCl, 1.2 mM $MgSO_4$, 0.2 mM EDTA, ^{14}C-labelled nucleotides, pH 8.0. Following 2 min preincubation nucleotides were removed by the centrifuge column procedure [18]. The columns with the Sephadex G-50 were equilibrated with the same buffer, but excluding nucleotides.

The activity of F_1-ATPase was determined spectrophotometrically in the ATP-regenerating system as described [19]. The reaction was started by adding the F_1-ATPase preparation (1–10 μg).

Protein concentration was determined according to Lowry et al. [20], using crystalline bovine serum albumin as a standard. The M_r of the F_1-ATPase was taken to be equal to 360000 [21].

3. RESULTS AND DISCUSSION

Fig.1 shows the time course of the ATPase reaction for the native and nucleotide-depleted F_1-ATPase. It can be seen from fig.1a that the preincubation of the native F_1-ATPase with Mg^{2+} leads to the inhibition of the initial rate of ATP hydrolysis (cf. curves 1 and 2). Removal of the nucleotides tightly bound to F_1-ATPase abolishes this inhibition (fig.1b). Fitin et al. [15,16] interpreted the sharp decrease in the initial ATPase reaction rate after the addition of Mg^{2+} to the native F_1-ATPase as the result of the formation of the slowly dissociating inactive F_1-ATPase–MgADP complex, where ADP is one of the tightly bound nucleotides. The same complex is formed slowly in the course of ATP hydrolysis [16]. The steady-state rates of ATP hydrolysis which is the same in the case of the native and Mg^{2+} preincubated F_1-ATPase (fig.1a) reflect the functioning of the 'active' enzyme fraction. This fraction results from the equivalence of the formation rate of the inactive F_1-ATPase–MgADP complex and the rate of its ATP-dependent reactivation [16].

A similar picture to that observed in fig.1b was obtained by Fitin et al. [22] who studied the ATP

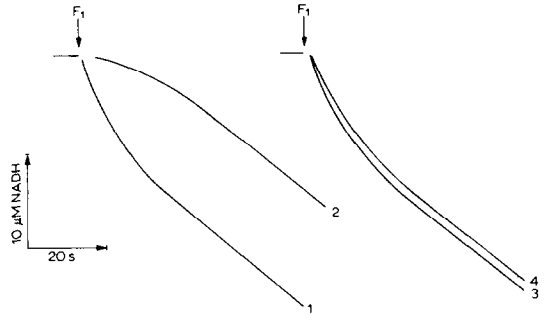

Fig.1. The kinetics of ATP hydrolysis of native (1,2) and nucleotide-depleted F_1-ATPase (3,4) before (1,3) and after (2,4) preincubation with $MgSO_4$. The enzyme was diluted to a concentration of 1 mg/ml in 20 mM Tris-HCl, pH 8.0, containing 0.2 mM EDTA and 0.25 M sucrose. 1 mM excess of $MgSO_4$ over EDTA was added and after 2 min aliquots of the enzyme were withdrawn to determine ATPase activity.

hydrolysis with the preparation of nucleotide-depleted F_1-ATPase, prepared according to Leimgruber and Senior [5]. This enzyme preparation is not, however, capable of rebinding the ADP added [5]. The advantage of the nucleotide-depleted F_1-ATPase preparation used in our work, lies in the fact that F_1-ATPase obtained by the method of Garrett and Penefsky [4] is capable of reconstructing a native enzyme with added nucleotides. This property makes possible the functional localization of the ADP-binding site.

Fig.2 shows the change in the time course of the ATPase reaction caused by preincubation of nucleotide-depleted F_1-ATPase with equimolar quantities of MgADP (fig.2, curve 6) and MgATP (fig.2, curves 3–5). From the results shown in fig.2 the conclusion can be drawn that the binding of one molecule of ADP to a molecule of nucleotide-depleted F_1-ATPase leads to the inhibition of the ATPase reaction. MgATP binding also results in the inhibition of the initial rate of ATP hydrolysis with nucleotide-depleted F_1-ATPase, but, in this case, the inhibition develops gradually (fig.2, curves 3–5). Grubmeyer et al. [23] have shown that MgATP added to the native F_1-ATPase in equimolar quantities binds to the catalytic site of the enzyme with K_s equal to 10^{-12} M. The dynamic equilibrium ATP + $H_2O \rightleftharpoons$ ADP + P_i is soon established with an equilibrium constant close to 1, followed by the slow dissociation of P_i. It seems logical to suggest that the inhibition of nucleotide-dependent F_1-ATPase which develops gradually in the course of preincubation with MgATP (fig.2, curves 3–5) has resulted from ATP hydrolysis with the subsequent release of P_i and the formation of the inactive F_1-ATPase–MgADP complex. The same complex is formed upon direct incubation of the nucleotide-depleted F_1-ATPase with MgADP (cf. curves 5 and 6 in fig.2). From fig.2 it follows that the inhibition develops by half in 0.5 min. Direct measurements of P_i release from the complex of nucleotide-depleted F_1-ATPase with stoichiometric quantities of [γ-^{32}P]ATP (uni-site catalysis) carried out in our group [19] showed that the half-time of P_i exit is indeed equal to 0.5 min. Thus, the kinetics of both the processes of ATPase inhibition and P_i release, coincide. This result supports the conclusion that ATPase inhibition (fig.2) is caused by P_i release from the catalytic site. It is interesting to note that P_i release in the course of the native F_1-ATPase uni-site catalysis occurs more slowly, in approx. 10 min [23].

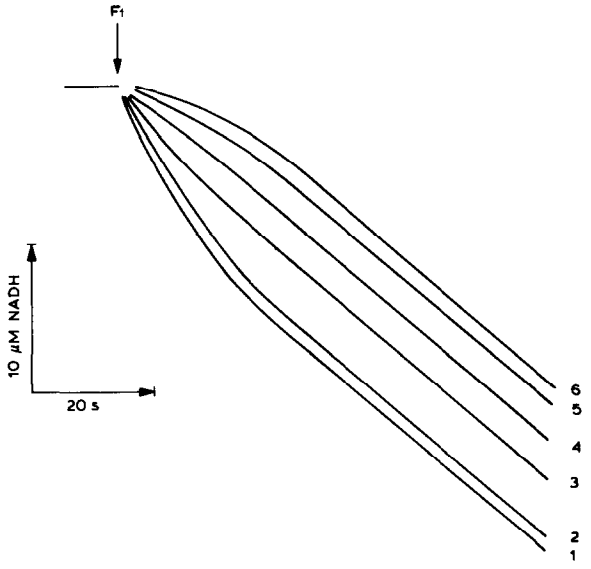

Fig.2. The kinetics of ATP hydrolysis catalysed with nucleotide-depleted F_1-ATPase preincubated in the presence of equimolar quantities of MgATP and MgADP. (Curve 1) The enzyme was diluted to a concentration of 1 mg/ml (2.8 μM) as described in the legend to fig.1. (Curves 2–6) The enzyme was diluted to 2.8 μM in the same buffer containing $MgSO_4$ and incubated for 5 min; ATP or ADP were added up to a final concentration of 2.8 μM and preincubated for the given time: 0 s (nucleotides not added), curve 2; 40 s after ATP addition, curve 3; 90 s after ATP addition, curve 4; 300 s after ATP addition, curve 5; 40 s after ADP addition, curve 6.

Fig.3 shows the change in the kinetics of ATP hydrolysis with nucleotide-depleted F_1-ATPase consecutively preincubated with stoichiometric quantities of MgADP and phosphate. As can be seen from the result obtained, P_i abolishes the effect of MgADP (fig.3, curves 2–5). It has also been established that P_i averts the inhibition induced by the addition of an equimolar quantity of MgATP (not shown). Therefore, the data in fig.3 are also in good agreement with the above conclusion that ATPase inhibition is connected with P_i release from the catalytic site.

The mitochondrial F_1-ATPase contains one tight binding site for P_i located on the β-subunit [18,24]. Thus, it may be suggested that the reactivation of the enzyme is conditional on the binding of P_i to

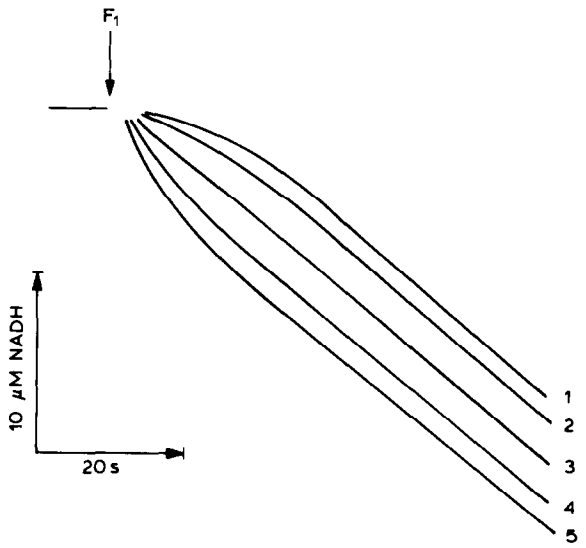

Fig.3. The phosphate-dependent reactivation of nucleotide-depleted F_1-ATPase preincubated with an equimolar quantity of ADP in the presence of Mg^{2+}. (Curve 1) The time course of the accumulation of the products of the ATPase reaction catalysed by the enzyme preincubated with an equimolar quantity of MgADP, as described in the caption to fig.2. (Curves 2–5) After preincubation with 2.8 μM MgADP the enzyme was incubated with 5 mM H_2PO_4 for 40 s (curve 2), 90 s (curve 3), 180 s (curve 4) and 300 s (curve 5).

the catalytic site of F_1-ATPase. Independent experiments (see table 1) showed that the P_i binding to F_1-ATPase and abolishing ATPase inhibition (fig.3) does not lead to dissociation of MgADP from the catalytic site. It seems probable that the incubation of the inactive MgADP–F_1-ATPase complex with P_i results in the formation of the triple F_1-ATPase–P_i–MgADP complex which is the same as an intermediate complex of the enzyme

Table 1

Binding of stoichiometric quantities of ^{14}C-labelled nucleoside di- and triphosphates to nucleotide-depleted F_1-ATPase

Additions to F_1-ATPase	ATP hydrolysis rate 5 s after the reaction starts	Steady-state rate of ATP hydrolysis	Quantity of bound ^{14}C-labelled nucleotide (mol/mol/F_1)	
			Before MgATP addition	After MgATP addition
Mg^{2+}	49.0	37.0	–	–
Mg^{2+} [^{14}C]ADP	8.0	38.0	0.92 ± 0.06	0.24 ± 0.03
Mg^{2+} [^{14}C]ATP	9.5	36.0	0.98 ± 0.07	0.20 ± 0.06
Mg^{2+} [^{14}C]GDP	44.0	36.0	0.82 ± 0.05	< 0.1
Mg^{2+} [^{14}C]GTP	47.0	37.0	0.86 ± 0.06	< 0.1
Mg^{2+} [γ-^{32}P]ATP (0.5 min)	21.0	36.0	0.44 ± 0.05	–
Mg^{2+} [γ-^{32}P]ATP	10.0	37.0	0.21 ± 0.03	–
Mg^{2+} [^{14}C]ADP + P_i (1 min)	23.0	36.0	0.91 ± 0.05	–
Mg^{2+} [^{14}C]ADP + P_i (5 min)	32.0	37.0	0.91 ± 0.06	–

2.8 μM nucleotide-depleted F_1-ATPase was preincubated with 1 mM free Mg^{2+} and 2.8 μM nucleotides for 5 min (except where indicated), as indicated in the caption to fig.2. Where indicated P_i was added to the enzyme preincubated with ADP to a final concentration of 0.5 mM. Aliquots of the enzyme were taken to determine the ATPase activity. After the incubation of the enzyme with ^{14}C-labelled nucleotides and the separation of the free label by centrifuge column procedure (see section 2), the enzyme was incubated (where indicated) with 1 mM unlabelled ATP and 1 mM $MgSO_4$ and then, after gel filtration the quantity of radioactive label bound to F_1-ATPase was determined. The specific radioactivity of [^{14}C]ATP, [^{14}C]ADP, [^{14}C]GTP and [^{14}C]GDP was equal to 4.3×10^3, 4.6×10^3, 4.7×10^3 and 4.6×10^3 dpm/nmol, respectively. The specific radioactivity of [γ-^{32}P]ATP was equal to 7.2×10^4 dpm/nmol. The error in measuring the steady-state rates of ATP hydrolysis was no more than 5% and in the rates measured 5 s after the beginning of the reaction no more than 10%

with the ATPase reaction products formed in the course of ATP hydrolysis. The rapid ATP-dependent dissociation of P_i and MgADP from this triple complex coincides with one of the steps in the ATPase reaction.

Table 1 summarizes the results of the experiments in which the binding of equimolar quantities of MgADP, MgATP, MgGTP and MgGDP with F_1-ATPase was studied. It can be seen that the preincubation of nucleotide-depleted F_1-ATPase with MgADP and MgATP leads to the binding of 1 mol ^{14}C-labelled nucleotide to 1 mol F_1-ATPase and to inhibition of the initial ATP hydrolysis rate. Preincubation with Mg^{2+}, ADP (in the absence of Mg^{2+}) or MgGDP does not cause a decrease in the initial ATP hydrolysis rate. From the data of table 1 it follows that the binding of 1 mol ADP (in the absence of Mg^{2+}) or MgGDP (MgGTP) to F_1-ATPase does not result in the formation of the inactive complex. The fact that all the ^{14}C-labelled nucleotides bound to F_1-ATPase in the experiments shown in table 1 can be rapidly replaced by an excess of MgATP indicates that they are located in the catalytic site of F_1-ATPase (table 1).

^{32}P release from F_1-ATPase in the experiments with [γ-^{32}P]ATP also provides evidence pointing to the location of nucleotides in the active site of F_1-ATPase (table 1).

As mentioned above, the addition of P_i to the F_1-ATPase–MgADP complex which results in the reactivation of the enzyme (fig.2) does not cause MgADP release (table 1).

The fact that MgGDP does not have any effect on the ATPase activity of the enzyme evidently means that the ATP-dependent dissociation of the F_1-ATPase–MgGDP complex occurs more swiftly than that of the similar complex with MgADP. This ATP-dependent MgGDP dissociation probably occurs in less than 2 s, and therefore the inhibition of the initial rate cannot be observed without using a rapid technique. In this connection, it is interesting to note that when measurements were made of the ATPase reaction catalysed by native F_1-ATPase on a millisecond time scale some authors [25,26] managed to discover a prestationary phase with $\tau_{1/2}$ equal to 100 ms, during which the reaction rate increased. The effect was not observed in the case of nucleotide-depleted F_1-ATPase [26]. It may be suggested that the preincubation with Mg^{2+} simply slows down the ATP-dependent dissociation of ADP from the complex with the enzyme by 2 or 3 orders.

The data in this and our previous papers [19] are evidence pointing unambiguously to the location of ADP inhibiting F_1-ATPase activity in the catalytic site. The results are in good agreement with the co-operative model of F_1-ATPase functioning proposed by Boyer and co-workers [27] and contradictory to Vinogradov's conclusion [28] that this ADP molecule is bound at the 'ATP-synthase', and not at the 'ATP-hydrolyase' site of the enzyme.

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