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Interaction of nucleotide-depleted F_1-ATPase with ADP. Characterization of a high-affinity binding site responsible for ADP-induced inhibition

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The ADP-induced inhibition of nucleotide-depleted F_1-ATPase (ndF_1-ATPase) from bovine heart mitochondria has been shown to be the result of ADP binding at one site with a dissociation constant of 4–5 nM. ADP binding at this site is at least biphasic: the initially formed inactive complex ($ADP \cdot ndF_1$) dissociating with the rate of about 0.01 s^{-1} is transformed with a half-time of 2–3 min to another state ($ADP \cdot ndF_1^*$) with the ADP-release rate of about $4 \cdot 10^{-4}$ s^{-1}. ATP and ADP were found to accelerate the dissociation of ADP from the inhibitory site; half-maximal effects were exerted by 170 μM ATP and 120 μM ADP. The ATP-dependent reactivation of the inactive complex of ndF_1-ATPase and ADP is suggested to result from the ATP-induced shift of the equilibrium between the $ADP \cdot ndF_1$ and $ADP \cdot ndF_1^*$ complexes to the former one. The nature of the inhibitory ADP-binding site is finally discussed, and it is concluded that this ADP-binding site is clearly distinct from the catalytic sites participating in the normal turnover of the enzyme.

Introduction

F_1-ATPase is a catalytic component of H^+-ATP synthase which catalyzes the final step of oxidative phosphorylation in mitochondria. The properties and the mechanism of functioning of this enzyme were investigated by many workers (see for reviews Refs. 1–6). One of the interesting and not fully understood properties of the enzyme are the transitions between the active and inactive states. So preincubation of the native F_1-ATPase in the presence of Mg^{2+} induces an inhibited state of the enzyme which is reactivated by EDTA or during ATP hydrolysis [7]. This phenomenon may be related to the slow changes of the enzyme activity during ATP hydrolysis and to the modulations of the F_1-ATPase by various anions [8–18].

In the case of F_1-ATPase form bovine heart mitochondria, the depletion of tightly bound nucleotides prevents the ability of Mg^{2+} to inhibit the enzyme [19]. But this ability is restored after a reconstitution of the nucleotide-depleted F_1-ATPase (ndF_1-ATPase) with 1 mol of ADP or ATP (which under experimental conditions is hydrolyzed and forms ADP) per mol of the enzyme [19]. These results suggest the conclusion that ndF_1-ATPase has an ADP-binding site responsible for the slow interconversions between the active and inactive forms of the enzyme.

The aim of the present work was to investigate the properties of this ADP-binding site of the ndF_1-ATPase. We found the binding of ADP in this site to be at least biphasic. Initially ADP binds to this site with a dissociation constant of 4–5 nM, and then the $ADP \cdot ndF_1$-ATPase complex isomerizes into another state. We also investigated the influence of ATP and ADP on the dissociation of this bound ADP and discussed the nature of the site with such a high affinity for ADP.

Materials and Methods

Materials. Mops, Tris and ATP were from Sigma (St. Louis, MO, U.S.A.) BSA from Serva (Heidelberg, F.R.G.), Sephadex G-50 fine from Pharmacia (Uppsala, Sweden), ADP from Calbiochem-Behring (La Jolla, CA,

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Enzymes and abbreviations: F_1-ATPase, a catalytic component of the proton-translocating ATPase complex from bovine heart mitochondria (EC 3.6.1.34); pyruvate kinase (EC 2.7.1.40); lactate dehydrogenase (EC 1.1.1.27); ndF_1-ATPase, F_1-ATPase, depleted from tightly bound nucleotides; BSA, bovine serum albumin; P_i, inorganic phosphate.

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U.S.A.), phosphoenolpyruvate, NADH, pyruvate kinase and lactate dehydrogenase from Reanal (Budapest, Hungary), [U-^{14}C]ADP (440 mCi/mmol) from UV-VVR (Prague, Czechoslovakia) and [α-^{32}P]ADP (more than 1000 Ci/mmol) from Isotop (Moscow, U.S.S.R.). The purity of radiochemicals was about 90%.

F_1-ATPase from bovine heart mitochondria was isolated according to Knowles and Penefsky [20]. Nucleotide-depleted F_1-ATPase (ndF_1-ATPase) was obtained according to Garrett and Penefsky [21]. Enzyme fractions with $A_{280}/A_{260} > 1.95$ were used. They contained less than 0.2 mol nucleotide/mol ndF_1-ATPase.

Binding of [α-^{32}P]ADP and [^{14}C]ADP. The binding of labelled ADP to ndF_1-ATPase was measured in a medium containing 50 mM sucrose, 20 mM Mops-Tris (pH 8.0), 0.2 mM EDTA, 2.5 mM $MgCl_2$ and 1.1 mg/ml BSA (buffer A). The concentrations of nucleotide and enzyme are specified in the figure legends. The separation of the bound ligand was carried out by the centrifuge-column method [22] on columns filled with 1 ml (for 50-μl samples) or 2 ml (for 100- and 200-μl samples) of swollen Sephadex G-50 fine. Sephadex was preequilibrated with buffer A and the columns were pre-centrifugated at $700 \times g$ for 30 s. The separation of the bound ligand was then performed by centrifugation of the samples through Sephadex in the same regime. The yield of the enzyme in the eluate was 90%. To calculate the stoichiometry of ADP binding to F_1-ATPase, the radioactivity of the eluate obtained in the absence of the enzyme at each ADP concentration was subtracted from the value obtained in the presence of the enzyme. This correction never exceeded 10% of the measured value.

Release of the bound [α-^{32}P]ADP and [^{14}C]ADP. The preliminary prepared complex of ndF_1-ATPase with radioactive ADP was diluted by buffer A to the concentrations specified in the figure legends, and the free ADP was separated by the centrifuge-column method as described above. The rate constants for release of the bound ligand were determined graphically from the plots of $\log(L_{b,t} - L_c)$ versus time t, where $L_{b,t}$ is the radioactivity of the bound ligand (ADP) at the time t and L_c is the radioactivity of the bound ligand after 2 min chaise in the presence of 1 mM ATP. The value of L_c never exceeded 10% of $L_{b,0}$.

Other methods. ATPase activity was measured spectrophotometrically [8] in buffer A lacking BSA but containing additionally 50 mM KCl, 0.15 mg/ml pyruvate kinase, 0.04 mg/ml lactate dehydrogenase and 0.1 mM ATP. The protein was assayed according to Lowry et al. [23] using BSA as standard. The molecular mass of F_1-ATPase was taken to be 360 kDa. The radioactivity of ^{14}C was counted in a dioxan scintillator [24] and the radioactivity of ^{32}P was measured in water by Cherenkov radiation.

Results

In the Mg^{2+}-containing medium, incubation of ndF_1-ATPase with stoichiometric amounts of ADP or ATP leads to the formation of an inhibited state of the enzyme as a result of ADP binding [19]. From this inhibited stoichiometric (1 : 1) complex of ndF_1-ATPase and ADP, the release of the bound nucleotide occurs very slowly but may be accelerated upon loading some other nucleotide-binding sites during the incubation of this complex in the presence of ATP [25] (such an incubation also results in a restoration of the ATPase activity of the enzyme [18,19]). In other words, the properties of the site responsible for ADP-induced inhibition may depend on the degree of the filling of the other nucleotide-binding sites. Therefore, at first we investigated the properties of this ADP-binding site under conditions ensuring that the other nucleotide-binding sites of ndF_1-ATPase are empty. These conditions were provided by using very low concentrations of the ligand (carrier-free [α-^{32}P]ADP) and a molar excess of the ndF_1-ATPase over the ADP.

As there were indications that the affinity for ADP of the site responsible for the ADP-induced inhibition could lie in a nanomolar range [25], in some of the experiments we had to use ndF_1-ATPase at very low concentrations. In the absence of the nucleotides, however, ndF_1-ATPase in highly diluted state was found to be unstable and to lose its activity. The enzyme could be stabilized by an inclusion of glycerol or sucrose into solutions. But at concentration fully protecting the ndF_1-ATPase, these stabilizers increased the time required for the separation of the free ligands by the centrifuge-column method [22] and diminished the extent of the separation (see Ref. 25). Therefore as a stabilizer we used sucrose at a concentration of 50 mM, since this sucrose concentration had no significant effect on the degree of separation but protected markedly the enzyme. So, after an incubation in buffer A (containing 50 mM sucrose) at room temperature for 2 min, 4 nM ndF_1-ATPase retained about 50% of both the ATPase activity and the ability to bind the carrier-free [α-^{32}P]ADP. Meanwhile, the binding of only 1 mol of ADP per mol of the ndF_1-ATPase completely prevented the irreversible inactivation of the enzyme (ADP-induced reversible inhibition of the enzyme and the irreversible inactivation due to high dilution could be discriminated by measuring the enzyme activity in the presence of sulfite which induced rapid reactivation of the ndF_1-ATPase preincubated with the stoichiometric ADP [18]).

Incubation of the ndF_1-ATPase in the presence of the carrier-free [α-^{32}P]ADP resulted in nucleotide binding in a concentration- and time-dependent fashion. As seen in Fig. 1, in a wide range of enzyme concentrations

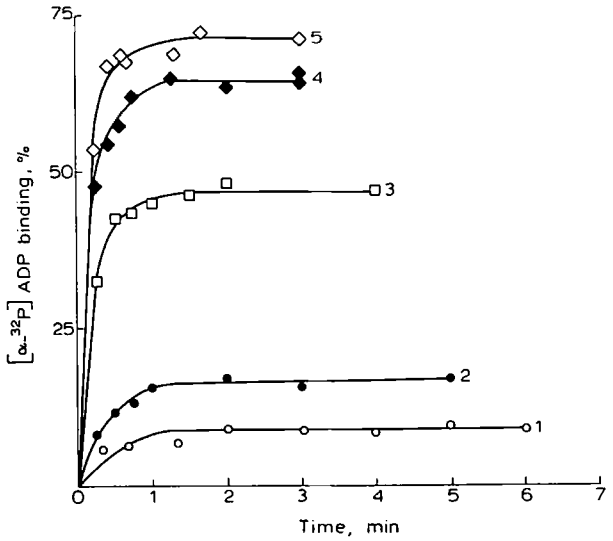

Fig. 1. Kinetics of the carrier-free [α-^{32}P]ADP binding by ndF$_1$-ATPase. ndF$_1$-ATPase, at concentration of 0.5 nM (curve 1), 1 nM (curve 2), 4 nM (curve 3), 8 nM (curve 4) and 12 nM (curve 5), was incubated in buffer A in the presence of carrier-free [α-^{32}P]ADP ($75 \cdot 10^3$ cpm/ml, ADP concentration was less than 0.075 nM), and 0.2 ml samples were processed as described in Materials and Methods. The radioactivity of the eluate is expressed in % of the radioactivity of added [α-^{32}P]ADP ($15 \cdot 10^3$ cpm) and is not corrected for the enzyme recovery in the eluate.

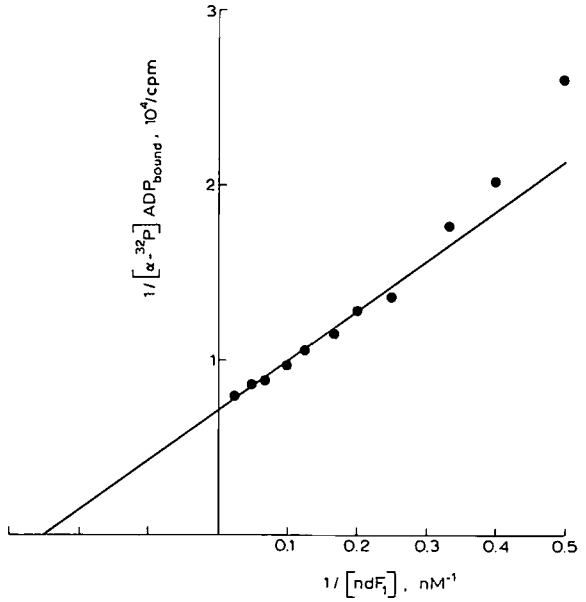

Fig. 2. Double reciprocal plot of the carrier-free [α-^{32}P]ADP binding by ndF$_1$-ATPase. ndF$_1$-ATPase was incubated in buffer A in the presence of carrier-free [α-^{32}P]ADP ($155 \cdot 10^3$ cpm/ml, ADP concentration less than 0.15 nM) for 2 min and 0.1 ml samples were processed as described in Materials and Methods. The results were not corrected for enzyme recovery in the eluate.

(0.5–12 nM), the process of nucleotide binding was completed within 2 min. The double-reciprocal plot of the radioactivity of the carirer-free [α-^{32}P]ADP bound to the ndF$_1$-ATPase during 2 min incubation vs. the enzyme concentration is shown in Fig. 2. This plot indicates that the ndF$_1$-ATPase possesses a binding site with the affinity for ADP of about 4 nM. Some deviations from the linearity at low enzyme concentrations in Fig. 2 are obviously due to the ndF$_1$-ATPase inactivation upon the dilution described above.

The existence of a site with the unusually high affinity for ADP can be also deduced from the Scatchard plot of [^{14}C]ADP binding to the ndF$_1$-ATPase (Fig. 3). This plot is curvilinear and, in spite of some data point scattering, can be approximated by two straight lines corresponding to the binding of 1.1 mol of ADP with K_d of about 5 nM and 1.2 mol of ADP with K_d of about 200 nM. We will designate the sites of ndF$_1$-ATPase with the K_d values of 5 and 200 nM as sites 1 and 2, respectively. Since the binding of only 1 mol of ADP per mol of ndF$_1$-ATPase is sufficient for the reversible inhibition of the enzyme [19], we can conclude that this inhibition results from the filling of site 1 with ADP.

The kinetics of ADP release from site 1 of ndF$_1$-ATPase was investigated in experiments shown in Fig. 4. In these experiments, 40 nM ndF$_1$-ATPase was allowed to bind the carrier-free [α-^{32}P]ADP at site 1, and then, after various time intervals, the incubation mixture was diluted 50-fold to obtain an enzyme concentration far below the K_d value. The radioactivity of the bound [α-^{32}P]ADP was then measured by the centrifuge-column method. When the results of these experiments were presented in a semi-logarithmic plot (Fig. 4), the process of [α-^{32}P]ADP release was found to be clearly biphasic. The rapid phase is characterized with the half-time, τ, of about 1 min, and the slow phase has the τ of about 30 min. It is interesting that the proportions of the slowly and the rapidly dissociable nucleotides depend on the time of preincubation before the dilution of the [α-^{32}P]ADP-enzyme complex. Initially,

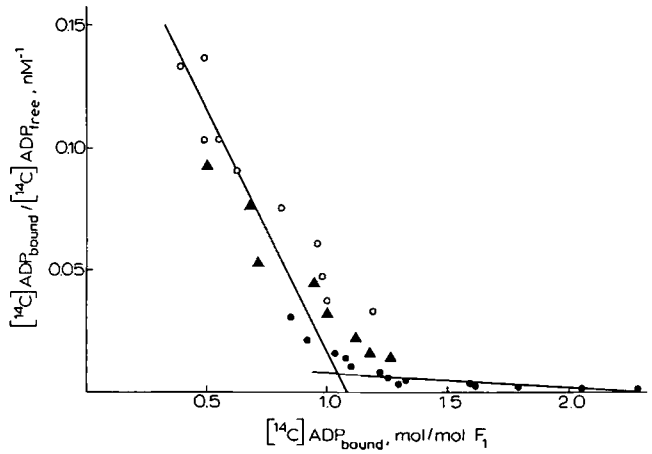

Fig. 3. Scatchard plot of [^{14}C]ADP binding by ndF$_1$-ATPase. ndF$_1$-ATPase, at concentration of 5 nM (○), 10 nM (▲) and 40 nM (●), was incubated in buffer A in the presence of [^{14}C]ADP for 2 min, and 0.2 ml (○, ▲) or 50 µl (●) samples were processed as described in Materials and Methods.

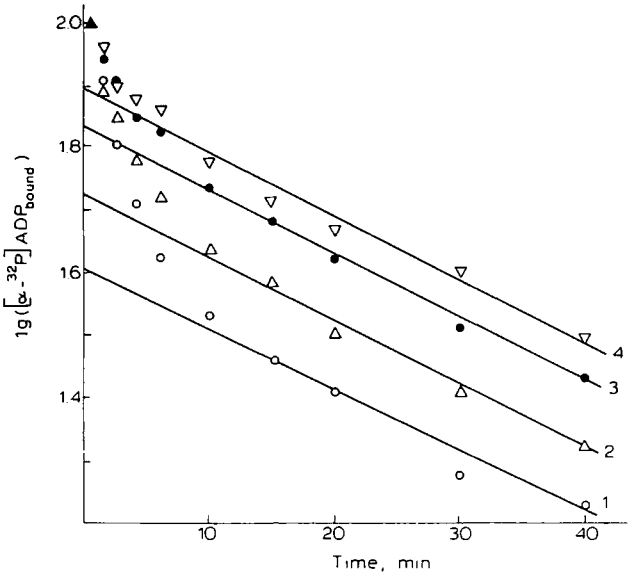

Fig 4. Release of [α-^{32}P]ADP from the complex with ndF$_1$-ATPase. 40 nM ndF$_1$-ATPase was incubated in 60 μl of buffer A in the presence of $4 \cdot 10^5$ cpm of carrier free [α-^{32}P]ADP (ADP concentration was less than 7 nM) for 10 s (○, curve 1), 3 min (△, curve 2), 6 min (●, curve 3) and 12 min (▽, curve 4), and the reaction mixture was diluted with 3 ml of buffer A. This point was taken as zero time, and 0.2 ml samples were processed as described in Materials and Methods. The radioactivity of bound [α-^{32}P]ADP at 20 s after dilution was taken as 100%. The data are presented in a semilogarithmic plot.

for the shortest time of preincubation (10 s), more than 60% of the bound [α-^{32}P]ADP dissociated rapidly (curve 1, Fig. 4). But the increasing of preincubation time reduced the proportion of the rapidly dissociable nucleotide and increased the proportion of the slowly releasable nucleotide (Fig. 4). After a preincubation for 12 min, more than 80% of the bound [α-^{32}P]ADP was released with τ of 30 min (curve 4, Fig. 4).

Two possible alternatives may be suggested to explain the ADP-release pattern shown in Fig. 4. According to the first one, the biphasic pattern of the bound ADP release reflects the possible heterogeneity of the high-affinity binding site, and the time-dependent changes in this pattern reflect a redistribution of the bound ADP including the dissociation and rebinding steps. According to the second alternative, the process of ADP binding at site 1 is multiphasic and, in the simplest case, can be approximated by the biphasic scheme

$$\mathrm{ADP} + \mathrm{ndF_1} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} \mathrm{ADP \cdot ndF_1} \underset{k'_{-1}}{\overset{k'_{+1}}{\rightleftarrows}} \mathrm{ADP \cdot ndF_1^*} \qquad (1)$$

The fact that the dissociation of the stoichiometric equimolar complex of [^{14}C]ADP and ndF$_1$-ATPase was also biphasic in a mode similar to that shown in Fig. 4 for the release of the carrier-free [α-^{32}P]ADP from the complex obtained under the conditions of enzyme molar excess argues in favor of the second alternative.

According to Eqn. 1, the results in Fig. 4 can be explained as follows. Initially, ADP binds at site 1 of ndF$_1$-ATPase, forming the ADP-ndF$_1$ complex (Eqn. 1) which subsequently slowly isomerizes to produce the ADP-ndF$_1$* complex. The rapid phase of bound ADP release ($\tau \approx 1$ min) corresponds to the dissociation of the first ADP-ndF$_1$ complex with $k_{-1} \approx 0.01$ s^{-1}, and the slow phase ($\tau \approx 30$ min) reflects the reverse isomerization of the second ADP-ndF$_1$* complex to the ADP-ndF$_1$ complex. The rate constant k'_{-1} for the reverse isomerization is therefore equal to $3.8 \cdot 10^{-4}$ s^{-1}. Since the transformation of the rapidly dissociable nucleotide to the slowly releasable one proceeds at τ of 2–3 min (Fig. 4), the rate constant for the isomerization of the first complex to the second one (Eqn. 1), k'_{+1}, can be estimated to be about $5 \cdot 10^{-3}$ s^{-1}.

The second-order rate constant, k_{+1}, for ADP binding at site 1 was derived from the results shown in Fig. 5. In these experiments, the ndF$_1$-ATPase was incubated at increasing concentrations of [α-^{32}P]ADP for 5 s; then cold ADP was added to a final concentration of 0.5 μM to reduce the specific radioactivity of [α-^{32}P]ADP, and, after this quenching, the bound ligad was separated by the centrifuge-column method. At the concentration used for quenching, the cold ADP did not yet induce a significant release of the bound [α-^{32}P]ADP (see below Fig. 7), and the contribution of the nucleotide binding after the quenching (which could be measured by adding the ndF$_1$-ATPase to the [α-^{32}P]ADP solution already containing cold ADP) did not exceed 10–15% of the values obtained. From the data in Fig. 5, the k_{+1} value can be obtained by dividing the slope of the initial linear part by the time of the [α-^{32}P]ADP binding, 5 s.

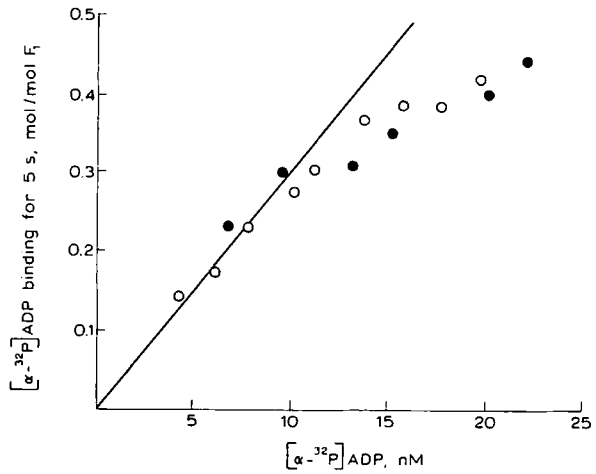

Fig. 5. The determination of the second-order rate constant for ADP binding by ndF$_1$-ATPase. ndF$_1$-ATPase, at concentration of 2 nM (○) or 4 nM (●), was incubated for 5 s in buffer A in the presence of [α-^{32}P]ADP ($8.7 \cdot 10^3$ cpm/pmol), and the [α-^{32}P]ADP binding was blocked by the addition of the cold ADP solution (50 μM) to the final concentration of 0.5 μM. After 25 s, the 0.1 ml samples were processed as described in Materials and Methods.

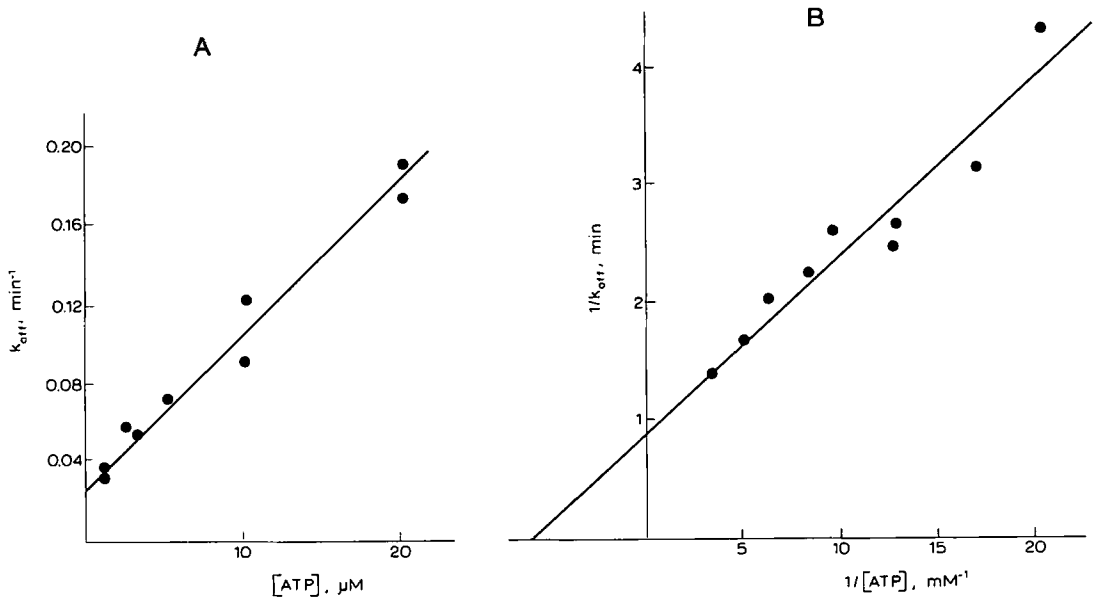

Fig. 6. The dependence of rate constant k_{off} for release of [^{14}C]ADP from the complex with ndF$_1$-ATPase on ATP concentration in the medium. 2 μM ndF$_1$-ATPase was incubated with 1.8 μM [^{14}C]ADP for 3 min in a medium, containing 10% glycerol, 20 mM Mops/Tris (pH 8.0), 0.6 mM EDTA and 3 mM $MgCl_2$. Then the reaction mixture was diluted 40-fold with buffer A, containing additionally 50 mM KCl and 1 mM phospho*enol*pyruvate. After 8 min pyruvate kinase (final concentration, 0.2 mg/ml) and ATP were added, and k_{off} values were determined as described in Materials and Methods. (A) Low ATP concentration. (B) ATP-dependence of k_{off} in double-reciprocal plots.

The k_{+1} value thus calculated is equal to $6 \cdot 10^6$ $M^{-1} \cdot s^{-1}$.

According to Eqn. 1, the K_d value for the ADP-ndF$_1$ complex may be calculated as a k_{-1}/k_{+1} ratio and is equal to about 2 nM. This value rather fairly agrees with the experimentally determined K_d for site 1 (Figs. 2 and 3). Eqn. 1 also predicts that, due to isomerization to the ADP-ndF$_1$* complex during incubation, the affinity of site 1 for ADP must be increased by the factor numerically equal to the k'_{+1}/k'_{-1} ratio. This ratio is equal to about 15 and, consequently, due to formation of the ADP-ndF$_1$* complex, K_d must be lowered to 0.1–0.2 nM. We attempted to test this prediction but failed, since our experiments required a long-term incubation of ndF$_1$-ATPase in the subnanomolar range. At these conditions, the irreversible inactivation of the ndF$_1$-ATPase (see above) was more rapid than ADP binding, which could stabilize the enzyme.

TABLE I

The binding of [^{14}C]ADP in the sites 1 and 2 of ndF$_1$-ATPase

5 μM ndF$_1$-ATPase was preincubated in buffer A for 15 min in the absence of any additions (Expt. 1) or in the presence of either 6 μM ADP (Expt. 2) or 5 μM [^{14}C]ADP (Expts. 3 and 4). Then the enzyme was diluted to the final concentration of 10 nM (a) or 40 nM (b) by buffer A, containing additionally 0.1 μM (a) or 1.5 μM (b) [^{14}C]ADP for Expts. 1 and 2, and 0.1 μM (a) or 1.5 μM (b) cold ADP for Expt. 4. After the incubation for 2 min, the amount of bound [^{14}C]ADP in 200 μl (a) or 50 μl (b) samples was determined by centrifuge-column procedure as described in Materials and Methods.

Addition in the course of preincubation		Addition after dilution	[^{14}C]ADP binding mol per mol of F$_1$
1. –			
	(a)	0.1 μM [^{14}C]ADP	1.07
	(b)	1.5 μM [^{14}C]ADP	1.77
2. 6 μM ADP			
	(a)	0.1 μM [^{14}C]ADP	0.35
	(b)	1.5 μM [^{14}C]ADP	1.15
3. 5 μM [^{14}C]ADP			
	(a)	–	0.82
	(b)	–	0.90
4. 5 μM [^{14}C]ADP			
	(a)	0.1 μM ADP	0.70
	(b)	1.5 μM ADP	0.60

In further experiments we investigated the possible effect of site 2 and other nucleotide-binding sites on the ADP bound at site 1. The data presented in Table I show that the formation of the ADP-ndF$_1$* complex after ADP binding at site 1 did not significantly affect the binding of the second ADP molecule at site 2 (cf. Expts. 1 and 2). On the other hand, ADP at concentration of 1.5 μM, which nearly fully saturated site 2 (Expt. 1), affected the amount of ADP bound to site 1 slightly (cf. Expts. 3 and 4). In the latter case, the loss of ADP from site 1 was comparable to the spontaneous release of ADP after dilution (see Fig. 4). Therefore, we can conclude that the binding of ADP at site 2 does not significantly accelerate the release of ADP from site 1.

Since ATP was shown to induce reactivation of the inactive stoichiometric (1 : 1) complex of ndF$_1$-ATPase and ADP [18,19], we examined the effect of ATP on the dissociation of this complex. ATP was found to accelerate the release of ADP from ADP · ndF$_1$* (Fig. 6). At

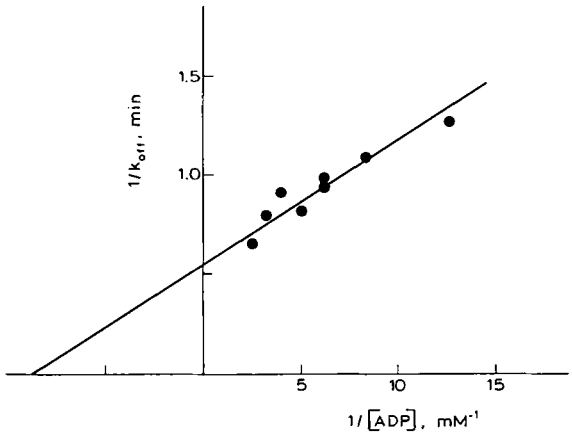

Fig. 7. Double-reciprocal plots of the dependence of the rate constant k_{off} for [^{14}C]ADP release from the complex with ndF$_1$-ATPase on ADP concentration in the medium. 2 μM ndF$_1$-ATPase was incubated with 1.8 μM [^{14}C]ADP for 3 min in a medium, containing 10% glycerol, 20 mM Mops/Tris (pH 8.0), 0.6 mM EDTA and 3 mM $MgCl_2$. Then the reaction mixture was diluted 40-fold with buffer A. After 8 min ADP was added and k_{off} values were determined as described in Materials and Methods.

low ATP, k_{off} for ADP release was linearly dependent on the ATP concentration, and the slope was $1.3 \cdot 10^2$ $M^{-1} \cdot s^{-1}$ (Fig. 6A). The k_{off} value extrapolated to the zero ATP concentration is equal to $4.3 \cdot 10^{-4}$ s^{-1} and concurs well enough with the rate constant for the non-promoted ADP release (k'_{-1}, $3.8 \cdot 10^{-4}$ s^{-1}, Fig. 4). At higher ATP concentrations, the k_{off} values reached a plateau in a hyperbolic fashion. From the double-reciprocal plot (Fig. 6B), the ATP concentration inducing a half-maximal value of k_{off} (K_{ATP}) was found to be 170 μM. From an intercept on the abscissa, the limiting k_{off}^{ATP} value was found to be $1.8 \cdot 10^{-2}$ s^{-1}. The k_{off}^{ATP}/K_{ATP} ratio (a pseudo-bimolecular rate constant) was equal to $1.1 \cdot 10^2$ $M^{-1} \cdot s^{-1}$ and agreed with the slope at low ATP concentrations.

ADP also accelerated the dissociation of the preformed ADP-ndF$_1$* complex in a mode similar to ATP (Fig. 7). The parameters K_{ADP} and k_{off}^{ADP} were calculated from the data shown in Fig. 7 to be 120 μM and $3.2 \cdot 10^{-2}$ s^{-1}, respectively.

Discussion

The nucleotide (including ADP-) binding properties of ndF$_1$-ATPase were investigated in various laboratories [15,20,21,26–29]. In total, six binding sites were found in ndF$_1$-ATPase [30–32], and the models, involving both interactive and non-interactive sites, were proposed to explain the ADP-binding curves [28,31,32]. The most essential result of the present work is that an ADP-binding site with unusually high affinity for the nucleotide (site 1, K_d 4–5 nM, Figs. 2 and 3) was detected. While the existence of ADP-binding sites with K_d values in the 50–500 nM range was shown previously [28,32], site 1 with such a high affinity escaped detection. We believe that the most probable reason for this omission in the previous investigations could be the using of ndF$_1$-ATPase at concentrations to high for detecting the binding site with K_d in a nanomolar range.

Site 2 (Fig. 3) with K_d of about 200 nM seems to belong to the class of high-affinity ADP-binding sites previously described [28,32]. At present it is hard to tell whether sites 1 and 2 are a priori different or both site 1 and 2 are initially indistinguishable, but ADP binding at one of them changes the affinity of the second site for the nucleotide. From the data in Table I it may be only pointed out that sites 1 and 2 can be clearly differentiated from each other in the complex of the ndF$_1$-ATPase with two molecules of the bound ADP, i.e., when both site 1 and 2 are loaded with ADP, they do not interconvert rapidly at any rate.

A photoactivable ADP derivative, 2-azido-ADP, added at a concentration sufficient to load only one nucleotide-binding site both in native F$_1$-ATPase and ndF$_1$-ATPase was shown by Lunardi et al. [32] to photolabel mainly the fragment of the β subunit containing Tyr-345. This Tyr-345 residue was shown to react with 5′-*p*-fluorosulfonylbenzoyladenosine, an active site directed analog [33], and to be photolabeled by the 2-azidonucleotides bound in the catalytic sites [34]. From the binding characteristics, the first high-affinity binding site reported by Lunardi et al. [32] for the ndF$_1$-ATPase (K_d equals 0.1 μM and 0.3 μM for ADP and 2-azido-ADP, respectively) seems to correspond to site 2 (K_d for ADP of 200 nM) in a greater extent than to the site 1 (K_d for ADP of 4–5 nM). Therefore, site 2 may be catalytic.

ADP binding to site 1 is responsible for the ADP-induced inhibition of the ndF$_1$-ATPase. This phenomenon seems to be closely related to the Mg^{2+}-dependent inhibition of the native mitochondrial F$_1$-ATPase [7,11,12] and the chloroplast CF$_1$-ATPase [35,36]. ADP binding at site 1 of ndF$_1$-ATPase is multiphasic, as evident from the time-dependent changes in the kinetics of the bound nucleotide release (Fig. 4). Recently, the multiphasic nature was also noted for the high-affinity binding of the fluorescent ADP derivative, 3′-*O*-(1-naphthoyl)adenosine-5′-diphosphate [37].

The ability of ATP to accelerate the dissociation of ADP from site 1 (Fig. 6) seems to be responsible for the process of ATP-dependent reactivation of the ADP-inhibited form of the enzyme. This process was investigated by Vinogradov and co-workers [38] and was indeed explained as a simple acceleration of the inhibitory ADP dissociation [39]. Our data (Fig. 6) shed some light on the mechanism of this process. k_{off}^{ATP} ($1.8 \cdot 10^{-2}$ s^{-1}) is found to be only slightly higher than k_{-1} (approx. $1 \cdot 10^{-2}$ s^{-1}) for the non-promoted dissociation of ADP · ndF$_1$ (see Eqn. 1). Thus, it may be proposed that the

effect of ATP lies primarily in the shift of the equilibrium between the ADP-ndF$_1$ and ADP-ndF$_1$* complexes (Eqn. 1) to the first one as a result of the k'_{-1} increase.

In the case of chloroplast CF$_1$-ATPase, strong evidence was recently presented for catalytic site location of the inhibitory ADP [40,41]. The same location was suggested by Drobinskaya et al. [19] for the case of mitochondrial F$_1$-ATPase. The conclusion of Drobinskaya et al. [19] was derived from the facts of ndF$_1$-ATPase inhibition following stoichiometric ATP hydrolysis under single-site conditions and the ability of P$_i$ to reverse this inhibition. It was proposed that, during single-site ATP hydrolysis by ndF$_1$-ATPase, the release of P$_i$, which was believed to be faster than that of ADP, resulted in an abortive ADP-enzyme complex, which might be also formed directly after ADP addition [19]. The P$_i$-induced reactivation of this abortive complex was suggested to be the result of the formation of a triple P$_i$-ADP-enzyme complex which was assumed to be identical with the normal intermediate complex of the enzyme and products [19]. The lower rate constant k_{-1} (Eqn. 1) for non-promoted ADP release from the inhibitory site (approx. 0.01 s^{-1}) is yet comparable to the rate of ADP release during single-site ATP hydrolysis by ndF$_1$-ATPase (0.05–0.1 s^{-1}) [42–44]. However, as we have found, there is no inhibition during steady-state uni-site ATP hydrolysis by the ndF$_1$-ATPase (Milgrom, Ya.M. and Murtaliev, M.B., unpublished results). Inhibition of the enzyme was observed only under single turnover conditions [19,25]. These facts mean that the inactive and slowly reactivating complex ADP · enzyme is not formed during ATP hydrolysis. To form this inactive complex ADP, which appears in the catalytic site during ATP hydrolysis, should be first released from the catalytic site and only then can be bound at some site to induce inhibition. Naturally, this inhibitory site may be catalytic or noncatalytic, but it clearly differs from the normal catalytic site.

Relevant to this may be the data obtained for the TF$_1$-ATPase from thermophilic bacterium PS3 which in isolated state does not contain endogenous nucleotides. A lag-phase was reported to occur before attainment of the steady-state rate of ATP hydrolysis by TF$_1$-ATPase [45,46]. This lag-phase increased greatly for the 1 : 1 ADP-TF$_1$ complex and, as in the case of bovine heart F$_1$-ATPase, the rate of the enzyme activation was dependent on the ATP concentration during assay [45]. It was reported recently that this lag-phase was not detected with the complex reassembled from the wild-type β and γ subunits of TF$_1$-ATPase and the α subunits with Asp-261 replaced by Asn by means of site-directed mutagenesis [47]. In addition, the mutant noncatalytic α subunit was shown to bind ADP weaker than the wild-type α subunit [47]. These results indicate that, at least in the case of the TF$_1$-ATPase, the noncatalytic sits may be responsible for the ADP-dependent modulation of activity. In this respect it is interesting that ADP formed during ATP hydrolysis by the TF$_1$-ATPase under the single-site catalysis conditions was shown to be released from the catalytic site and rebound to another site on the noncatalytic α subunit [46].

Returning to the mitochondrial F$_1$-ATPase it may be noted that the ternary complex P$_i$ · ADP · enzyme, which appears during uni-site ATP hydrolysis by ndF$_1$-ATPase, differs from that formed upon P$_i$ binding to the ADP-inhibited enzyme. During uni-site ATP hydrolysis, the rate of P$_i$ release was found to be about 0.1 s^{-1} [42–44,48,49]. The rate constant for the dissociation of the P$_i$-enzyme complex formed as a result of P$_i$ binding was shown to be more than 10-fold lower [17,25]. P$_i$, originating in the catalytic site of F$_1$-ATPase due to the hydrolysis of the prebound ATP, was found also to differ in the susceptibility to the promotion of the dissociation by the nucleotides from the phosphate prebound to the enzyme as P$_i$ [50].

It is surprising that the rate of non-promoted release of ADP from the ADP-ndF$_1$* complex ($3.8 \cdot 10^{-4}$ s^{-1}) is strikingly close to the value of $3.6 \cdot 10^{-4}$ s^{-1} reported by Grubmeyer et al. [51] for the release of ADP formed during ATP hydrolysis by the native F$_1$-ATPase under the single-site conditions. It should be noted here that the parameters of the uni-site ATP hydrolysis measured by Grubmeyer et al. [51] differ significantly from those measured in our group [42–44] and by Bullough et al. [48]. Although some differences in the enzyme preparations can be responsible for the variations between laboratories, we have to agree with Penefsky [52] that our experiments and the experiments made in his laboratory "are not measuring the same phenomenon". Under single-turnover conditions, ATP hydrolysis by F$_1$-ATPase is biphasic [42,48,51,53]. The first rapid phase is characterized by the high rate of the product release but during the second slow phase the products are released very slowly. The rapid phase dominates in the preparations of ndF$_1$-ATPase [42,53]. In the preparations of the native F$_1$-ATPase, the relative proportions of the two phases seem to be variable, depending on the composition of incubation medium and the number of tightly bound nucleotides in the enzyme [48,51,52]. Contrary to Penefsky [52], we believe that it is the first phase of ATP hydrolysis with the subsequent rapid release of the products that is a normal functioning of the normal catalytic site of F$_1$-ATPase. The first phase of ATP hydrolysis, unlike the second one, was completely prevented by omitting Mg^{2+} from the medium [53]. If the slowly hydrolysable ATP was bound at the normal catalytic site of the active F$_1$-ATPase, it would be released during the first enzyme turnover. However, we have shown that the F$_1$-ATPase preloaded with [γ-^{32}P]ATP and subsequently preincubated in the presence of 20 μM cold ATP during 20 s (that is sufficient

for hundreds of enzyme turnovers) retains the slowly dissociating ^{32}P label [52]. This result contradicts also with the assumption that the slow phase of ATP hydrolysis is a normal functioning of the catalytic site participating in the alternating-site cooperativity. Retorting against our conclusions, Penefsky [52] has shown that the slowly hydrolysable [γ-^{32}P]ATP bound in the high-affinity site is splitted at a rate of about 300 s^{-1} upon addition of 5 μM ATP as a cold chase. However, the acceleration of the product release step was not verified. To accelerate the hydrolysis of [γ-^{32}P]ATP in the experiments of Penefsky [52], cold ATP should be first bound to the enzyme at the minimal apparent second-order rate constant of $6 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ ($300\ s^{-1}/5 \cdot 10^{-6}$ M). However, the apparent second-order rate constant for the binding of ATP to a second catalytic site on F_1-ATPase was found to be in the range of $6.3 \cdot 10^6$–$7.6\ 10^6$ $M^{-1} \cdot s^{-1}$ [48,54,55] that is considerably lower than the value calculated from the data of Penefsky [52]. Also, we have not found in the literature a value of the pseudo-bimolecular rate constant (V_{max}/K_m) for F_1-ATPase higher than $1 \cdot 10^7$ $M^{-1} \cdot s^{-1}$. For these reasons also, the slow phase of ATP hydrolysis cannot be treated as a normal functioning of a normal catalytic site.

In conclusion, the properties of the ADP bound at site 1 of ndF_1-ATPase differ distinctly from those of ADP originating due to ATP hydrolysis in the normal catalytic site of the enzyme. The question, whether ADP-binding site 1 belongs to the class of the catalytic sites described by Penefsky and coworkers [51,52] or this site is purely noncatalytic, requires further study.

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