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The ADP that binds tightly to nucleotide-depleted mitochondrial F_1-ATPase and inhibits catalysis is bound at a catalytic site

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Previous studies have shown that the initial complex formed when ADP binds to nucleotide-depleted F_1-ATPase is transformed with a half time of 2 to 3 min to a form with a much lower rate of ADP release. The ADP binding results in a strong inhibition of ATPase activity. The present paper reports appraisal of where the inhibitory ADP binds by use of the photoreactive ADP analog, 2-N_3-ADP. In presence of Mg^{2+} the 2-N_3-ADP like ADP induces reversible inhibition of nucleotide-depleted F_1 (ndF_1) with a K_d of about 10 nM. Photoirradiation of the inactive 2-N_3-[β-^{32}P]ADP-ndF_1 complex results in labeling of only the β-subunit. The major labeled peptide isolated from a trypic digest consists of residues from Ala-338 to Arg-356, with Tyr-345 as the site of labeling. This identifies the site of the inhibitory ADP binding as one of the catalytic sites of the enzyme.

Introduction

F_1-ATPases from various sources are strongly inhibited by the binding of ADP in presence of Mg^{2+} or upon the addition of Mg^{2+} if the requisite tightly bound ADP is already present [1–9]. Catalytic activity is slowly recovered upon addition of excess MgATP, correlated with release of the inhibitory ADP. Studies of labeling with 2-azido-ADP and 2-azido-ATP have established that with the enzyme from chloroplasts the inhibitory ADP is bound at a catalytic site [10]. Drobinskaya et al. [7] have shown that inhibition of nucleotide-depleted F_1 from beef-heart mitochondria may be induced by stoichiometric amounts of ATP and proposed that release of P_i during uni-site ATP hydrolysis by ndF_1 leads to the formation of inactive ADP · F_1 complex with ADP remaining bound at catalytic site, thus suggesting a catalytic site location of the inhibitory ADP on mitochondrial F_1. This possibility draws some support from the observations of Lundardi et al. [11], who demonstrated with ndF_1 that 2-N_3-ADP labeled a peptide fragment containing Tyr-345. Cross et al. [12] showed that labeling by 2-N_3-ADP bound at catalytic sites labeled Tyr-345 but that Tyr-368 was labeled when the 2-N_3-ADP was bound at a noncatalytic sites. Lunardi et al. [11] did not report Tyr-368 labeling or whether the 2-N_3-ADP binding they observed sufficed for the Mg^{2+} and ADP-induced inhibition. In addition, they reported an affinity for the initial 2-N_3-ADP binding to ndF_1 about 100-times less than we observe, as reported in this paper. These findings thus left a troublesome uncertainty as to whether the inhibitory ADP on ndF_1 was bound at catalytic or noncatalytic sites.

Some uncertainty also arose from some unusual aspects of the ADP inhibition of ndF_1. The bound ADP that induces the inhibition of ndF_1 initially has a relatively rapid dissociation rate but converts with a half-time of 2 to 3 min to a form with a relatively slow dissociation and a low K_d of less than 5 nM [13]. ADP release during uni-site hydrolysis by ndF_1 can be relatively rapid [14,15] and the ADP released could bind at a noncatalytic regulatory site [16,17]. Alternatively, it is even possible, as has been suggested [18], that a shift or dislocation of the ADP from a catalytic to an inhibitory site occurs.

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Abbreviations: F_1, the ATPase portion of ATP synthase from bovine heart mitochondria (EC 3.6.1.34); ndF_1, F_1 with tightly bound nucleotides removed.

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The experiments reported in this paper were conducted because of the uncertainties mentioned and were designed to provide a firm establishment of the location of the inhibitory ADP. Results reported on labeling by 2-N_3-ADP establish that the inhibitory ADP on ndF_1 is bound at a catalytic site.

Materials and Methods

Materials. Mops, phospho*enol*pyruvate, Sephadex G-50–80, pyruvate kinase, lactate dehydrogenase and TPCK-treated trypsin were from Sigma (St. Louis, MO, U.S.A.), Tris from Fisher Scientific (Fair Lawn, NJ, U.S.A.), bovine serum albumin from Calbiochem-Behring (La Jolla, CA, U.S.A.) and carrier-free [^{32}P]orthophosphoric acid from ICN Radiochemicals (Irvine, CA, U.S.A.).

2-Azido-[β-^{32}P]ADP was prepared by phosphorylating 2-azido-AMP using 3-phosphoglycerate kinase and adenylate kinase as described [19], except that 2-azido-AMP concentration was 2-fold higher.

F_1 from bovine heart mitochondria was isolated according to Knowles and Penefsky [20] and nucleotide depleted to obtain ndF_1 according to Garrett and Penefsky [21]. Enzyme fractions with $A_{280}/A_{260} > 1.95$ were used; they contained less than 0.2 mol of nucleotides per mol of F_1.

Binding of 2-N_3-[β-^{32}P]ADP. Binding of 2-N_3-[β-^{32}P]ADP to ndF_1 was measured in buffer A (50 mM sucrose, 20 mM Mops-Tris (pH 8.0), 0.2 mM EDTA and 2.5 mM $MgCl_2$) containing 1.1 mg/ml bovine serum albumin. The concentrations of nucleotide and enzyme are specified in the figure legends. Separation of the bound nucleotide was carried out by the centrifuge-column method [22] on columns filled with 2 ml (for 200-μl samples) or 5 ml (for 500-μl samples) of the Sephadex G-50-80 preequilibrated with buffer A containing 1.1 mg/ml bovine serum albumin.

Photoaffinity labeling and purification of ^{32}P-labeled peptides. In a typical experiment, 5 μM ndF_1 was preincubated with 4 μM 2-N_3-[β-^{32}P]ADP in buffer A for 10 min and then photolyzed for 40 min by using a Mineral-light lamp at 254 nm setting. In some experiments before photolysis the enzyme was separated from any unbound ligand by the centrifuge-column method. Typically, photolysis resulted in covalent incorporation of about 30% of bound ligand.

The labeled enzyme was precipitated and digested by TPCK-treated trypsin essentially as described in Ref. 12, except that succinylation was omitted. ^{32}P-labeled peptides were isolated by ion-exchange HPLC using Partisil PXS 10/25 SAX column (4.6 × 250 mm) and linear gradient from 100% solvent A (40% acetonitrile, 60% 0.01 M NaH_2PO_4, pH 3) to 100% solvent B (40% acetonitrile, 60% 0.35 M NaH_2PO_4, pH 4) and further purified by reversed-phase HPLC using Supelcosil LC-308 column (4.6 × 250 mm) and linear gradient from 100% solvent A (0.1% trifluoroacetic acid) to 100% solvent B (0.1% trifluoroacetic acid in 90% acetonitrile).

Other methods. Sequencing of purified peptides was performed by the automated Edman degradation method on an Applied Biosystems 475A protein sequencer. ATPase activity was measured spectrophotometrically [23] in buffer A-containing additionally 50 mM KCl, 0.15 mg/ml pyruvate kinase, 0.04 mg/ml lactate dehydrogenase, 1 mM phospho*enol*pyruvate, 0.3 mM NADH and 0.1 mM ATP. In some experiments 1 mM ATP in the presence of 2 mM Na_2SO_3 was used; the presence of the sulfite overcomes the inhibitory effect of bound ADP. The amount of F_1 was measured according to Lowry et al. [24] using bovine serum albumin as standard. Polyacrylamide gel electrophoresis in the presence of tetradecyltrimethylammonium bromide was performed according to Amory et al. [25] using solubilizing solution of Kironde and Cross [26]. The molecular mass of F_1 was taken to be 360 kDa. Radioactivity of ^{32}P was measured in water by Cherenkov radiation.

Results

To investigate whether 2-N_3-ADP can substitute for ADP in inducing the inhibited state of ndF_1 in Mg^{2+}-containing medium, ndF_1 was incubated with an equal molar amount of 2-N_3-ADP, then the initial rate of ATP hydrolysis was measured. As shown in Fig. 1, the incubation of ndF_1 with a stoichiometric amount of 2-N_3-ADP results in inhibition of initial rate of ATP

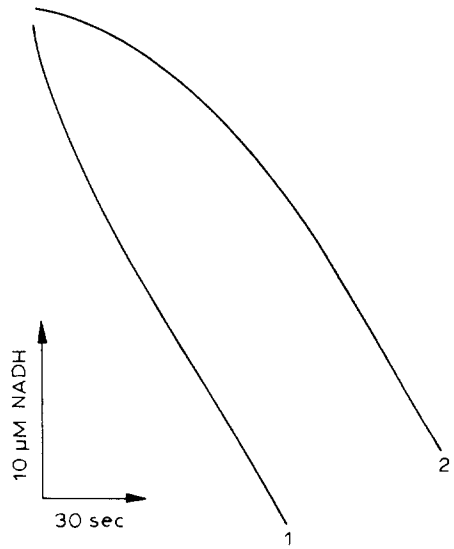

Fig. 1. Ability of 2-N_3-ADP to induce reversible inhibition of ndF_1. 3 μM ndF_1 was preincubated in buffer A for 5 min in the absence (trace 1) or in the presence of 2.7 μM 2-N_3-ADP (trace 2). Then the activity was assayed as described in Materials and Methods with 100 μM ATP as substrate. The ndF_1 concentration in the spectrophotometer cell was 15 nM.

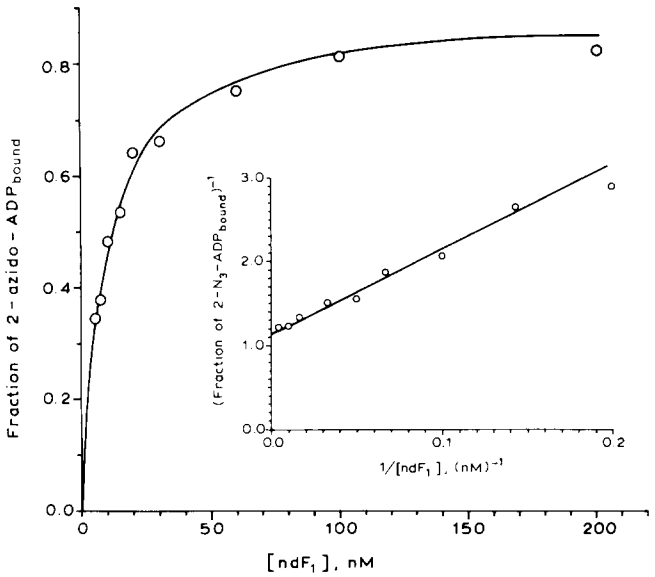

Fig. 2. Binding of 2-N_3-[β-^{32}P]ADP to ndF_1 under conditions of enzyme excess. ndF_1 was incubated in buffer A containing 1.1 mg/ml bovine serum albumin in the presence of 4.5 nM 2-N_3-[β-^{32}P]ADP for 10 min and unbound ligand was separated as described in Materials and Methods. The inset shows a double-reciprocal plot of the data.

splitting by the enzyme similar to that reported for ordinary ADP [7]. The 2-N_3-ADP thus can readily form the inactive 2-N_3-ADP · ndF_1 complex. Photolysis of the inactive 2-N_3-[β-^{32}P]ADP · ndF_1 complex for 40 min resulted in covalent incorporation of about 0.3 mol of label/mol F_1 with irreversible inhibition of the enzyme by about 30%. For this assay 1 mM ATP was used as substrate in the presence of 2 mM Na_2SO_3, i.e., the conditions were employed allowing rapid release of ADP-induced reversible inhibition [27].

The affinity of inhibitory site for 2-N_3-ADP can be evaluated from the data presented in Figs 2 and 3. Titration of 4.5 nM 2-N_3-[β-^{32}P]ADP with increasing concentrations of ndF_1 (Fig. 2) indicated an existence of a site (or sites) with K_d of about 8 nM. 2-N_3-[β-^{32}P]ADP binds to this site in exchangeable manner, since about 90% of bound label can be released from the enzyme after incubation with 2 mM MgATP for 3 min. The binding is thus nearly all at catalytic sites because ADP bound to noncatalytic sites in the presence of Mg^{2+} is not replaced under these conditions.

When binding is measured by titration of ndF_1 with increasing concentrations of 2-N_3-[β-^{32}P]ADP, an increasing fraction of label becomes bound nonexchangeably and is thus bound at noncatalytic sites. Under conditions employed, the size of this fraction is dependent on both 2-N_3-[β-^{32}P]ADP concentration and time of ndF_1 preincubation with 2-N_3-[β-^{32}P]ADP. The extent of exchangeable binding of 2-N_3-[β-^{32}P]ADP seems to be dependent only on the nucleotide concentration and does not change when incubation time increases from 2 to 10 min (compare Fig. 3A and Fig. 3B). It can be seen from Fig. 3 that half-saturation for the first molecule of 2-N_3-[β-^{32}P]ADP bound exchangeably to ndF_1 occurs with about 10 nM nucleotide, and the next molecule(s) of 2-N_3-[β-^{32}P]ADP binds exchangeably with $K_d > 0.5$ μM. The binding with K_d of about 10 nM is to the site revealed by 2-N_3-[β-^{32}P]ADP titration with an excess of the ndF_1 (Fig. 2) and is responsible for reversible inhibition of the enzyme by ADP [13] or 2-N_3-ADP (Fig. 1).

The data show that in Mg^{2+}-containing medium, both ADP and 2-N_3-ADP are able to induce reversible inhibition of ndF_1 when added at stoichiometric to the enzyme concentration and have similar binding characteristics. This allows using 2-N_3-ADP to locate the inhibitory site responsible for ADP-dependent inhibition.

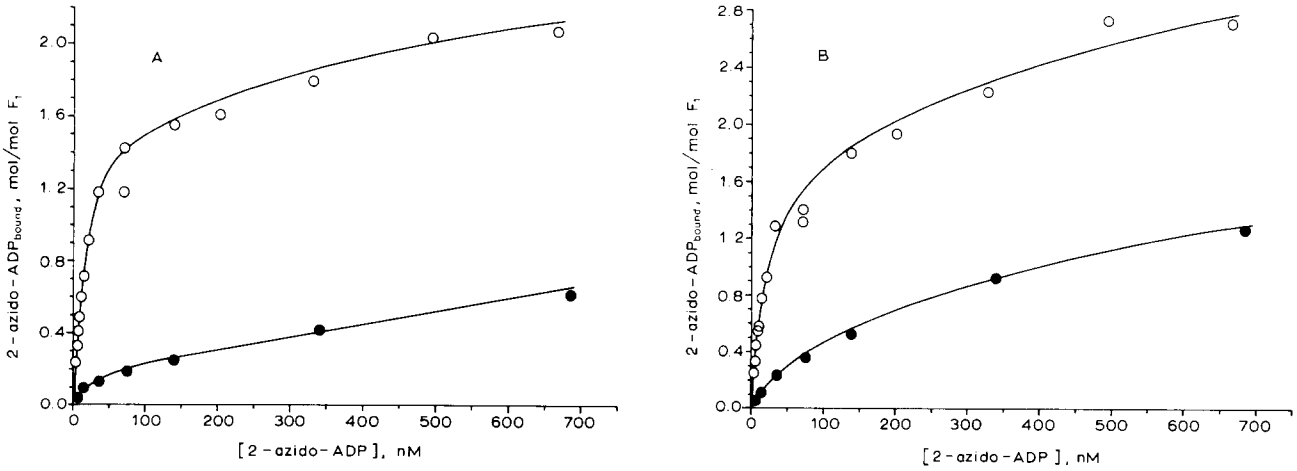

Fig. 3. Binding of 2-N_3-[β-^{32}P]ADP by ndF_1. ndF_1 was incubated with 2-N_3-[β-^{32}P]ADP in buffer A containing 1.1 mg/ml bovine serum albumin for 2 min (A) or 10 min (B), and unbound ligand was separated as described in Materials and Methods with (●) or without (○) additional incubation with 1 mM ATP for 3 min. ndF_1 concentration was 5 or 10 nM at 2-N_3-[β^{32}P]ADP concentration lower or higher than 100 nM, respectively.

When an inactive stoichiometric complex of 2-N_3-[β-^{32}P]ADP and ndF$_1$ was photolyzed and then subjected to acidic polyacrylamide gel electrophoresis in the presence of tetradecyltrimethylammonium bromide, all the covalently incorporated label was found attached to β subunit (not shown). To identify site(s) of labeling in β subunit, the photolyzed stoichiometric complex of 2-N_3-[β-^{32}P]ADP and ndF$_1$ was digested with trypsin, and labeled peptides were purified by means of ion-exchange and reversed-phase HPLC.

Fig. 4 shows an elution profile of ^{32}P radioactivity of the tryptic digest during ion-exchange HPLC. Typically, more than 60% of injected radioactivity was recovered, and major part of recovered radioactivity was eluted as a broad peak centered at 63% of solvent B. When the pooled fractions of this peak were subjected to reversed-phase HPLC, one main radioactive peak with ultraviolet absorbtion at 260 nm (as expected for presence of an adenine moiety) and 214 nm (for peptide bond absorption) was eluted at about 60% of solvent B. The peptide constituting this peak represented about 60% of recovered radioactivity. The results of amino acid sequence analysis of this peptide are shown in Table I. The sequence obtained belongs to the fragment of F$_1$ β subunit from Ala-338 to Arg-356. This fragment includes Tyr-345, which has been shown to be modified by 2-N_3-AT(D)P bound at catalytic sites of F$_1$ [12]. This result shows that the inhibitory high-affinity ADP-binding site of ndF$_1$ is one of the catalytic sites on the enzyme. As in the case of 2-N_3-AT(D)P-labeled catalytic site peptide [12], no phenylthiohydantoin derivative was obtained at cycle 8 during sequencing (Table I). This results because the inhibitory 2-N_3-ADP at catalytic site upon photolysis is cross-linked to Tyr-345.

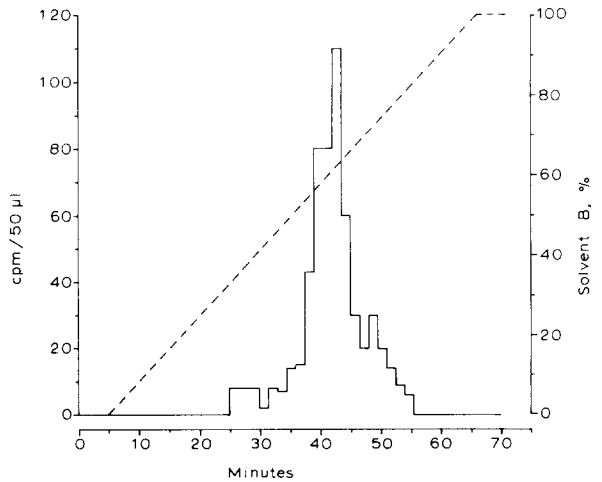

Fig. 4. Elution profile of ^{32}P radioactivity from ion-exchange HPLC of a tryptic digest of a photoirradiated stoichiometric 2-N_3-[β-^{32}P]ADP·ndF$_1$ complex. The complex of ndF$_1$ and 2-N_3-[β-^{32}P]ADP was prepared, photoirradiated, trypsin digested and subjected to ion-exchange HPLC as described in Materials and Methods. Fractions of 5 ml for first 30 min of the run and then of 1.5 ml were collected, and portions of 50 µl were counted for ^{32}P radioactivity. The dashed line shows percentage of solvent B. The bar shows the fractions pooled for subsequent purification.

TABLE I

Amino-acid sequence analysis of ^{32}P-labeled tryptic peptide purified after labeling ndF$_1$ with stoichiometric amount of 2-N_3-[β-^{32}P]ADP

The peptide was purified as described in Results. About 200 pmol were subjected to sequencing, and the yield of the phenylthiohydantoin amino acid (aa) residues is shown for each cycle. No phenylthiohydantoin derivative was detected at cycle 8. The β-subunit sequence was taken from Ref. 28.

Cycle	aa	pmol	β (338–356)
1	Ala	139	Ala
2	Ile	156	Ile
3	Ala	90	Ala
4	Glu	68	Glu
5	Leu	71	Leu
6	Gly	58	Gly
7	Ile	78	Ile
8	Xaa	–	Tyr
9	Pro	31	Pro
10	Ala	37	Ala
11	Val	28	Val
12	Asp	29	Asp
13	Pro	23	Pro
14	Leu	26	Leu
15	Asp	22	Asp
16	Ser	26	Ser
17	Thr	19	Thr
18	Ser	24	Ser
19	Arg	7	Arg

Noncatalytic site labeling was also observed under suitable conditions. ndF$_1$ was exposed overnight to 30–70 µM 2-N_3-ADP or 2-N_3-ATP in the presence of EDTA. This allows both exchangeable and nonexchangeable nucleotide binding [29]. After separation of unbound nucleotides and photolysis, both labeled catalytic (Ala-338–Arg-356) and noncatalytic (Ile-357–Arg-372) site peptides were obtained from the tryptic digests, in accord with the data of Cross et al. [12].

Discussion

The results show that, in Mg^{2+}-containing medium, 2-N_3-ADP can substitute for ordinary ADP in inducing the reversibly inhibited state of ndF$_1$ (Fig. 1) and, during photolysis of stoichiometric 2-N_3-ADP · ndF$_1$ complex, modifies Tyr-345 in a β subunit catalytic site. This result means that, as in the case of chloroplast CF$_1$ [10,30], with mitochondrial F$_1$ the ADP-binding site responsible for reversible inhibition of the enzyme is one of the catalytic sites. It seems likely, therefore, that catalytic site location of inhibitory ADP is a common feature of F$_1$-type ATPases.

The reversible, potent inhibition of mitochondrial F$_1$ in the presence of Mg^{2+} can be induced by the binding

in the catalytic site of a molecule of ADP [7,27], 2-N_3-ADP (present study) and 2′(3′)-*O*-2-nitro-4-azidobenzoyl-ADP (Milgrom and Murataliev, unpublished data) but not GDP [7]. Similarly, Mg^{2+} can induce potent reversible inhibition of chloroplast CF_1 only when ADP but not when GDP is bound in a catalytic site [31,32]. Therefore, it seems likely that interaction of an NH_2 group at C-6 of nucleoside diphosphate purine ring with some residue(s) in catalytic site is crucial for inducing the inhibited state of F_1.

The characteristics of 2-N_3-ADP binding to ndF_1 presented in this study are close to those observed by Milgrom and Murataliev [13] for ADP but differ significantly from the data reported by Lunardi et al. [11] for 2-N_3-ADP. Lunardi et al. [11] may not have noted the high-affinity binding of 2-N_3-ADP we observed (K_d of about 10 nM) because the high concentration of ndF_1 (about 1 μM) they used in the binding assay does not allow measurement of K_d values of about 100 nM or less.

In other studies, Cunningham and Cross [33] have shown that preparations of F_1 that have two or three noncatalytic sites filled bind ADP in the presence of P_i at one of exchangeable sites with K_d of about 1 nM. The pattern of both non-promoted and ATP-induced release of ADP from this site is different from those reported by Milgrom and Murataliev [13] for the inactive stoichiometric ADP · ndF_1 complex. This could reflect effects of nucleotides bound at noncatalytic sites or binding of P_i. P_i is known to reverse MgADP-induced inhibition of F_1 [8,34,35] and affects ADP and 2-N_3-ADP binding to ndF_1 (unpublished data). It is likely that the binding of exchangeable ADP reported by Cunningham and Cross [33] takes place at the same catalytic site where ADP [13] or 2-N_3-ADP (this work) binds to induce ndF_1 inhibition.

This paper focuses on and clarifies the ability of an ADP, when bound at a catalytic site and in the presence of medium Mg^{2+}, to strongly inhibit the initial rate of ATP hydrolysis. The interesting inhibitions of mitochondrial F_1-ATPase that have been noted by ADP binding under different conditions by Harris et al. [36] in the absence of Mg^{2+} and by Di Pietro et al. [37] the presence of Mg^{2+} appear to be caused by ADP that is not removed by extensive MgATP hydrolysis and thus is likely at noncatalytic sites. More than one type of inhibition by tightly bound ADP thus occurs. The relationship of nucleotides at noncatalytic sites to enzyme activity needs further clarification.

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