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Inhibition of mitochondrial F_1-ATPase activity by binding of (2-azido-)ADP to a slowly exchangeable non-catalytic nucleotide binding site

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F_1-ATPase was treated so that it contained three tightly bound nucleotides per molecule. One of these was bound at a catalytic site and was rapidly exchangeable, the two remaining nucleotides were nonexchangeable. Incubation of this preparation with ADP in the presence of Mg^{2+} results in 40–45% inhibition of the ATPase activity. With 2-azido-ADP instead of ADP, the ligand was covalently bound to F_1 by illumination, in the presence or absence of turnover of the enzyme, and the site of binding was determined. In this way, one site could be identified, which induces the inhibition. The attachment of the covalently bound 2-nitreno-ADP is at Tyr-368 of a β-subunit, characterized in the literature as a non-catalytic site. A second, non-catalytic site also binds 2-azido-ADP, but this binding is partially reversed by the addition of ATP and does not cause further inhibition of the ATPase activity. It is concluded that the slowly exchangeable non-catalytic site is the site of inhibition by ADP.

Introduction

The mitochondrial F_1F_0-ATPsynthase performs the last step of the oxidative phosphorylation, the synthesis of ATP. The energy needed for this phosphorylation process is provided by a proton gradient built up over the mitochondrial inner membrane by the respiratory chain [1], or by direct energy transduction from a respiratory chain complex to the ATP synthase [2]. The catalytic moiety of the enzyme, F_1, can be relatively easily dissociated from the membranous F_0 part. In the isolated form, F_1 shows only ATP hydrolysis activity. The mechanism of this process has been the subject of study for many years, but a clear understanding has still not yet been achieved.

The F_1-ATPase is composed of five different subunits, with a stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$ [3,4]. To determine the number, function and location of nucleotide binding sites on F_1, a large number of different ADP and ATP analogues have been used (see, for examples Ref. 5). The enzyme contains six nucleotide binding sites, located on the α and β subunits [6–8], and these are divided into catalytic and noncatalytic sites. The F_1 preparations, as isolated in most laboratories, contain three tightly bound nucleotides [9,10]. Although it has been assumed by some authors [11,12] that these sites are all non-catalytic, it has become evident that only two of these bound nucleotides are not exchangeable and the other one is exchangeable and catalytic [8,13]. Additionally, after saturation of the enzyme, only three nucleotides are easily exchangeable [14]. So one of the three sites that does not contain a tightly bound nucleotide, binds a nucleotide (ADP) in such a way that it is not readily exchanged. This site, then, has to be non-catalytic.

Although there is no agreement on the total number of catalytic and non-catalytic sites (but see Ref. 15), it has been shown that the catalytic sites are located on the β-subunits. Photo-affinity labeling studies with 8-azido-ATP and 8-azido-ADP [16] have, furthermore, indicated that, in addition to two catalytic sites on β-subunits, two non-catalytic sites, located at the interface between α- and β-subunits, can be labeled with these analogues.

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Abbreviations: DEAE, diethylaminoethyl; NADH, nicotinamide adenine dinucleotide; NAP_3-($2N_3$)ADP, 3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}(2-azido)adenosine-5′-diphosphate; PEP, phospho*enol*pyruvate; SDS, sodium dodecyl sulphate; TDAB, tetradecyltrimethylammonium bromide; Tea, triethylamine.

The total number and the functions of the non-catalytic sites of F_1 are not clear. The two non-exchangeable, tightly bound nucleotides are clearly not catalytic, but their location (at two non-catalytic α/β-sites or at both one potentially catalytic β-site and one non-catalytic α/β-site) is not known. Recently we have given arguments for the last possibility [15,17]. A regulatory function for at least one non-catalytic site has been shown by several groups [18,19]. Harris et al. [20] showed that incubation of bovine heart F_1 with ADP results in a partial (about 50%) inhibition of the ATPase activity when this activity was measured in the absence of ADP. Hysteretic inhibition after incubation with ADP or ADP analogues was demonstrated with pig heart F_1-ATPase by the group of Gautheron [21,22]. ADP inhibition of nucleotide-depleted F_1-ATPase was shown by Drobinskaya et al. [23] and has been explained by tight binding of ADP to a catalytic site [23,24]. Experiments in which ADP was replaced by 2-azido-ADP [25] confirmed this conclusion.

Kironde and Cross [8,13] described some properties of a non-catalytic site that could be easily saturated with ADP in the presence of Mg^{2+}. The bound ADP or 2-azido-ADP was not removed from the site upon subsequent addition of ATP and start of hydrolysis. Although it was assumed that occupation of this site with ADP had no effect on the ATPase activity of F_1, the high affinity of ADP for this site makes it a candidate for an inhibitory site.

On the basis of the work with 2-azido-adenine nucleotides from Garin et al. [26], Cross et al. [27] and Lunardi et al. [28], a distinction can be made between catalytic and non-catalytic sites. Covalent binding of this nucleotide analogue by ultraviolet irradiation takes place mainly on the β-subunit, at two distinct sites. One site is at, or near, Tyr-345, which is supposed to be part of a catalytic site. Under conditions of labeling of the non-catalytic sites, label was found at or near Tyr-368 [27]. However, in other experiments, labeling of the β-region Gly-72 to Asp-83 was found, which is also supposed to belong to a non-catalytic site [28].

Our aim in this paper is to determine where the ADP binds that induces inhibition of ATPase activity, as described by Harris et al. [20]. To this end, ADP was replaced by 2-azido-ADP and illumination was performed with and without turnover. The results point to covalent binding of the inhibiting ligand, under turnover conditions, to Tyr-368, a non-catalytic site. The data give additional evidence for a two-site mechanism of catalysis of F_1.

Materials and Methods

F_1 preparation

F_1-ATPase, isolated from bovine hearts according to the method of Knowles and Penefsky [29], was stored in liquid nitrogen in a medium consisting of 10 mM Tris-HCl (pH 7.5), 250 mM sucrose, 4 mM ATP and 4 mM EDTA. Before each experiment, the stored F_1 was precipitated twice by adding an equal volume of saturated ammonium sulphate (pH 7.0–7.5). After 10 min on ice the precipitate was spun down in an Eppendorf table centrifuge for 2 min at $10000 \times g$. The pellet was dissolved in 50 mM Tris-HCl (pH 7.5), 150 mM sucrose and 4 mM EDTA (TSE 4). After the second precipitation residual salt and loosely bound nucleotides were removed by filtering the sample twice by column centrifugation, as described by Penefsky [30]. The first column was equilibrated in TSE 4, the second column, 10 min later, in TSE 0.2 (0.2 mM instead of 4 mM EDTA). Colums of 1×5 cm, containing Sephadex G-50 coarse, equilibrated in the desired buffer, were centrifuged for 1 min at 2000 rpm in a Homef LC-30 table centrifuge. After the centrifugation steps the F_1 preparations were diluted to 2 mg protein ml^{-1} with TSE 0.2 and Mg^{2+} was added from a 1 M $MgCl_2$ solution up to a concentration of 4 mM. The specific activities of these preparations were 140–160 μmol ATP min^{-1} mg^{-1} protein.

Synthesis of 2-azido-[α-^{32}P]ADP

The non-radioactive compounds 2-azido-adenosine, 2-azido-AMP and 2-azido-ADP were synthesized as published earlier [31]. The method described by Boulay et al. [32] was used for the ^{32}P-labeled compounds, with some minor changes. For the 2-azido-[α-^{32}P]AMP synthesis according to Symons [33] we used siliconised glassware, which improved the yield. The coupling of pyrophosphate to the 2-azido-[α-^{32}P]AMP was performed according to the procedure of Hoard and Ott [34]. For purification Sephadex DEAE A-25 columns were used and eluted with a Tea-HCO_3 gradient. The 2-azido-[α-^{32}P]ADP was prepared from 2-azido-[α-^{32}P]ATP with hexokinase in the presence of glucose and Mg^{2+}. All preparations were used as Tea-salt solution in H_2O, with a specific activity between 500 and 750 dpm per pmol.

Incubation and illumination with 2-azido-[α-^{32}P]ADP

Incubation with ADP or ADP analogues took place in the dark. After 1 h in the dark with 100 μM 2-azido-[α-^{32}P]ADP, F_1 was illuminated in the absence or presence of turnover. Turnover conditions were created by adding an equal volume of 50 mM Tris-HCl (pH 7.5), 150 mM sucrose, 4 mM Mg^{2+}, 1 mM ATP, 20 mM PEP and 5 U/ml pyruvate kinase. After 30 s of turnover a 20 s illumination period with a Penray ultraviolet lamp followed. The turnover conditions were sufficient for at least 1 min. When no turnover was wanted the same medium was added, which lacked ATP, PEP and pyruvate kinase.

ATPase assay and inhibition

ATPase activity was measured with an ATP regenerating system consisting of 33 mM Tris-HCl (pH 8.0), 83 mM sucrose, 6 mM $MgCl_2$, 10 mM $KHCO_3$, 5 mM ATP, 0.5 mM PEP, 5 U/ml pruvate kinase, 5 U/ml lactate dehydrogenase and 250 μM NADH. The oxidation of NADH was followed at 340 nm with a Zeiss M4Q III spectrophotometer at 30°C. All calculated inhibition percentages were determined against specific activities of control F_1 preparations, with the same protein concentration and treatment, to which no ADP or ADP analogue was added.

Determination of covalently bound 2-azido-[α-^{32}P]ADP

Samples for determination of covalently bound 2-nitreno-ADP were incubated first for 10 min in 1 mM ADP. Protein was precipitated by adding perchloric acid up to 4% (w/v). After 3 min on ice the samples were centrifuged in an Eppendorf centrifuge, for 5 min at $10000 \times g$. The pellet was washed twice with 4% perchloric acid and dissolved in 200 mM NaOH, 0.1% SDS. Radioactivity (^{32}P) was measured in glass vials with a Packard Scintillator 299 scintillation cocktail with a LKB 1214 Rackbeta liquid scintillation counter.

Gel electrophoresis, protein and nucleotide determination

TDAB gel electrophoresis was performed according to Penin et al. [35], with the adjustments described by Fellous et al. [22]. After drying the gels, ^{32}P-labeling was visualized by autoradiography on a Agfa X-ray film. Protein concentrations were determined with the Bio-Rad protein assay, a reagent based on the method of Bradford [36], with bovine serum albumin as standard. Bound nucleotides (ATP and ADP) were determined luminometrically, as described by Van Dongen and Berden [37].

Analysis of 2-nitreno-[α-^{32}P]ADP modified peptides

Tryptic digests of F_1, labeled with 2-nitreno-[α-^{32}P]ADP, were prepared as described in Ref. 38, and peptides were separated according to the method described by Wise et al. [39]. The peptides were brought onto a Whatman Partisil PX S25/SAX10 ion exchange column, eluted with a linear gradient (see Results) of 0.01 M sodium phosphate (pH 4.0) (eluent A) and 0.4 M sodium phosphate (pH 3.0) (eluent B), both mixed with acetonitrile in a ratio of 71:29, with a flow rate of 1.25 ml/min. The column was operated with LKB 2150 HPLC pumps with a gradient mixer, operated by a LKB 2151 controller. Protein was detected at 215 nm with a Pharmacia/LKB VWM 2141 detector. Radioactivity in the collected fractions was determined by Cerenkov radiation. Fractions containing protein and radioactivity were further analysed on a Vydac C_4 reverse-phase column, eluted with a linear gradient of 0.1% trifluoroacetic acid (eluent C) and 0.1% trifluoroacetic acid, 90% acetonitril (eluent D). The column was operated with the same setup as mentioned before with a flow of 1.1 ml/min. Collected fractions with radioactivity and protein were lyophilised and sequence analysis was performed by automated Edman degradation at the S.O.N. gas-phase sequenator facility in Leiden.

TABLE I

Nucleotides bound on F_1-ATPase

A, stored F_1 and excess ATP removed; B, standard F_1 preparation after treatment, as described in Materials and Methods, and addition of 4 mM Mg^{2+}; C, after incubation of standard F_1 with 100 μM 2-azido-ADP in the presence of Mg^{2+} in the dark and a column centrifugation step; D, control for C, no 2-azido-ADP added.

	Total	ATP	ADP
A	4.75	4.45	0.30
B	3.00	1.30	1.70
C [a]	1.95	0.80	1.15
D	3.00	0.80	2.20

[a] The contribution of 2-azido-ADP and 2-azido-ATP to the signal of ATP in the used luciferine-luciferase assay was negligible.

Materials

Tris, ATP, ADP, PEP, pyruvate kinase, lactate dehydrogenase and NADH were obtained from Boehringer, luciferine/luciferase was from ITL. Sucrose and $MgCl_2$ from BDH, acetonitril, trifluoroacetic acid, perchloric acid, EDTA and NaH_2PO_4 from Merck. All reagents used were of analytical grade.

Results

Nucleotide content of isolated F_1

When F_1-ATPase, isolated from bovine heart mitochondria according to the method of Knowles and Penefsky [29] and stored in the presence of ATP and EDTA, is separated from excess nucleotides in the medium by column centrifugation, the preparation contains about 5 mol adenine nucleotides per mol F_1, mainly ATP (Table IA). This nucleotide content can be lowered by ammonium sulphate precipitation. When the enzyme is precipitated twice, followed by two column centrifugation steps in the continuous presence of EDTA, the nucleotide content equals 3 mol per mol F_1. The bound nucleotides are in a ratio of around one ADP and two ATP, and this is our standard F_1 preparation. A further lowering of the nucleotide content requires several precipitation- and column-centrifugation steps. Therefore, the three residual nucleotides are considered to be tightly bound nucleotides.

The addition of Mg^{2+} to this preparation induces the conversion of one ATP to ADP. In Mg^{2+} the three tightly bound nucleotides are divided in a ratio of about two ADP and one ATP (Table IB and D).

TABLE II

Inhibition of ATPase activity after incubation of F_1 with ADP or ADP analogues in a Mg^{2+}-containing medium in the dark

8-Azido-ADP was synthesized as described in Ref. 16. NAP_γ-ADP and NAP_γ-$2N_\gamma$-ADP as described in Ref. 47.

	Inhibition (%)
100 μM ADP	42
100 μM $2N_3$ADP	26
2.5 mM $8N_3$ADP	3
100 μM NAP_γ-ADP	35
100 μM NAP_γ-$2N_\gamma$ADP	45

Incubation with 100 μM 2-azido-ADP in the dark induces dissociation of one mol ADP per mol F_1, compared with a control experiment (Table IC and D). Thus, one tightly bound nucleotide, an ADP, exchanges with 2-azido-ADP during this incubation.

Inhibition of ATPase activity by ADP

F_1 preparations, containing three tightly bound nucleotides, were incubated with ADP and some ADP analogues in the presence of Mg^{2+} in the dark, and ATPase inhibition was measured (Table II). For ADP a maximal inhibition was observed of 40–45%. Aryl-azido analogues induce the same inhibition as ADP, while 2-azido-ADP induces a slightly lower level of inhibition. The inhibitions shown in Table II were measured after incubation with 100 μM ADP or ADP analogues. Maximal inhibition was reached with concentrations between 50 and 75 μM ADP or ADP analogues. Incubation with 8-azido-ADP, an analogue in the *syn*-conformation instead of the *anti*-configuration, had no influence on the ATPase activity. Even up to a concentration of 2.5 mM no inhibition was measured after dilution in the assay medium.

TABLE III

Activity of and bound label to F_1-ATPase after incubation with 100 μM 2-azido-[α-^{32}P]ADP in Mg^{2+}

Inhibition and bound label after incubation in the dark (1), after illumination (2), and after illumination during turnover (3), in the presence of excess label (A), or excess label removed by column centrifugation (B). After illumination, (2 and 3), the label was covalently bound. After dark incubation and removal of excess label, (B1), the label was not covalently bound. The results are average of three experiments ± S.D.

Conditions	A		B	
	Inhibition (%)	Mol label/ mol F_1	Inhibition (%)	Mol label/ mol F_1
1. Dark	28.5 ± 1.7	–	17.1 + 0.85	(3.4 ± 0.3)
2. Illumination	95.2 ± 1.4	3.85 ± 0.15	90.7 ± 3.6	2.15 ± 0.05
3. Illumination + Turnover	41.3 + 1.7	1.5 ± 0.1	42.7 + 3.9	0.85 ± 0.05

Inhibition of ATPase by covalently bound 2-nitreno-ADP

To determine the number and location of sites which induce the ATPase inhibition, F_1 was incubated with 2-azido-[α-^{32}P]ADP. After illumination with and without turnover the level of inhibition and the amount of covalently bound 2-nitreno-[α-^{32}P]ADP were measured. The results are represented in Table IIIA. Incubation with 100 μM 2-azido-[α-^{32}P]ADP inhibits the ATPase activity by about 30%. Illumination in the absence of turnover with ATP resulted in nearly complete inhibition of ATPase activity, accompanied by covalent binding of nearly 4 mol of 2-nitreno-ADP per mol of F_1. Under these conditions all four sites not occupied with non-exchangeable nucleotides are covalently labeled, including the catalytic sites (see Ref. 31).

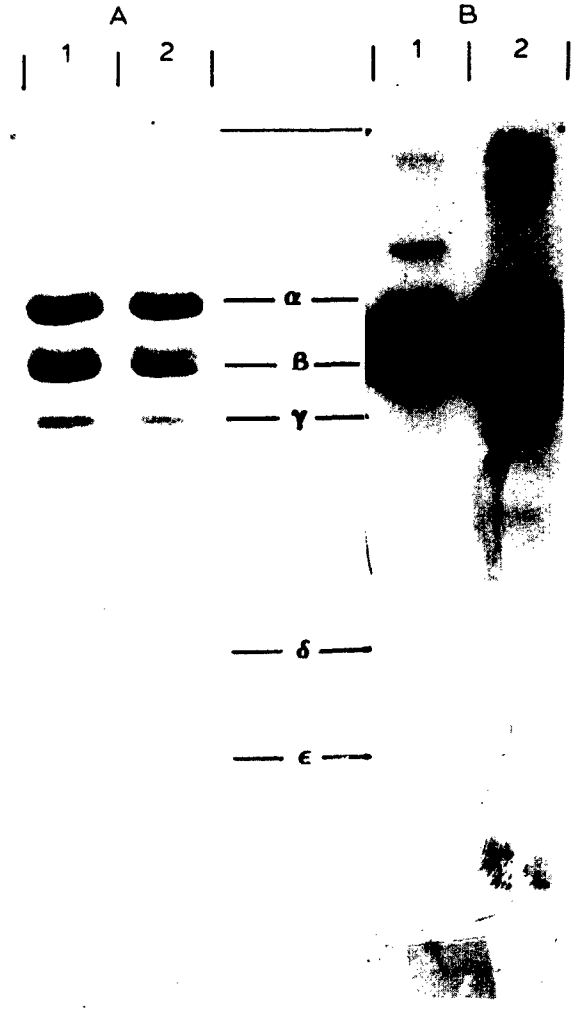

Fig. 1. (A) TDAB gel electrophoresis of 2-nitreno-[α-^{32}P]ADP-labeled F_1-ATPase in Mg^{2+}-containing buffer with turnover (1), and without turnover (2), protein stained. (B) Autoradiogram of the gel.

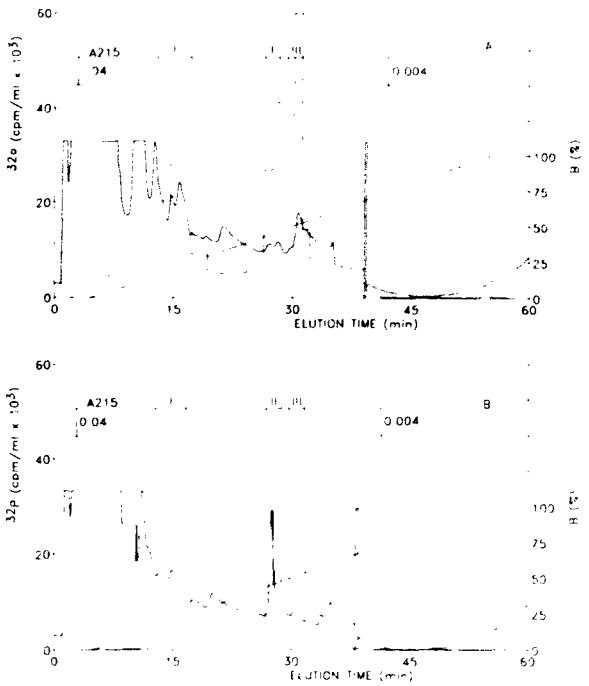

Fig. 2. Separation on an ion-exchange column of tryptic digests of F_1-ATPase, labeled with 2-nitreno-[α-^{32}P]ADP. (A) without turnover and (B) with turnover. Protein absorption at 215 nm, radioactivity in collected fractions (left) and gradient of eluent B (right) against the elution time. For technical details see Materials and Methods.

Under turnover conditions at least the catalytic sites, but also exchangeable non-catalytic sites, are occupied with ATP or ADP, and only non-catalytic sites, at which 2-azido-ADP remains bound in the presence of excess ATP, become labeled. The inhibition, after illumination, was 42%, slightly more than without illumination and the same as obtained after incubation with ADP. 1.5 mol of 2-nitreno-ADP per mol F_1 were covalently bound under these conditions (Table IIIA). Apparently, more than one non-catalytic site is available for binding 2-azido-ADP, but it could well be that only one of these two sites is responsible for the inhibition.

To find out whether one site is responsible for the observed inhibition or two sites are involved, the free and loosely bound 2-azido-ADP were removed from the incubation mixture by passing the sample over a Sephadex column (using column centrifugation). 3.4 Mol of labeled 2-azido-ADP remained bound per mol F_1 (Table IIIB). After illumination the inhibition was again nearly complete and 2.15 mol of 2-nitreno-ADP were covalently bound per mol of F_1. When illumination was started after the start of turnover with ATP and Mg^{2+}, the final inhibition was again 42%, just as without the intermediate column centrifugation step, but now only 0.85 mol of 2-nitreno-ADP per mol F_1 were covalently bound.

We conclude that after the column step only about one mol of 2-azido-ADP remains bound at a site from which it is not removed by ATP during turnover. The binding of 2-azido-ADP at this one non-catalytic site is responsible for the inhibition of the ATPase activity. We are dealing here with a situation in which the character of the binding is not relevant for the effect: covalently bound 2-nitreno-ADP has the same effect on ATPase activity as non-covalently bound ADP. Non-covalently bound 2-azido-ADP has slightly less effect on the ATPase activity. The non-catalytic character of the binding site involved could not be demonstrated more clearly.

Localisation of the inhibitory 2-nitreno-ADP

Electrophoresis on TDAB-gels, followed by autoradiography, was used to detect which subunits were labeled with 2-nitreno-ADP after illumination in the presence or absence of ATP-induced turnover, as described in Table IIIA. In both cases the large majority of the label was connected with the β-subunit (Fig. 1). The same results were obtained when excess label was removed before illumination (situation as in Table IIIB, results not shown). These findings agree with the data in the literature [26–28].

To analyse where the β-subunit was labeled, tryptic digests were made of F_1 preparations, illuminated either in the presence or absence of turnover, as described in Table IIIA. The tryptic digests were separated on HPLC in two steps: first on an ion-exchange column, followed by a reverse phase column (see Materials and Methods).

From the ion-exchange column (see Fig. 2) three radioactive fractions were obtained, respectively, fraction I at 23% eluent B, fraction II at 45% eluent B and fraction III at 50% of eluent B. Fraction I was not present when the illumination was performed in the presence of turnover (Fig. 2B). So this fraction contains a peptide fragment to which the photoaffinity label is linked when a catalytic site is occupied.

Further analysis of all three fractions from the first column on a reverse phase column (Fig. 3) also revealed three peak fractions, respectively, at 18%, 23% and 30% of eluent D. Fraction I contained a labeled peptide eluting at 30% D. Fraction II contained two labeled peptides, eluting respectively at 30% and 23% D. In fraction III, also, two labeled peptides were detected: they eluted at 23% D and 18% D.

The results of the sequence analysis of the three collected peptides from the reverse phase column are shown in Fig. 4 and were compared with the sequence of the β-subunit, as determined by Runswick and Walker [40]. The peptide at 30% D showed a sequence which corresponds with Ala-338 till Arg-356 of the

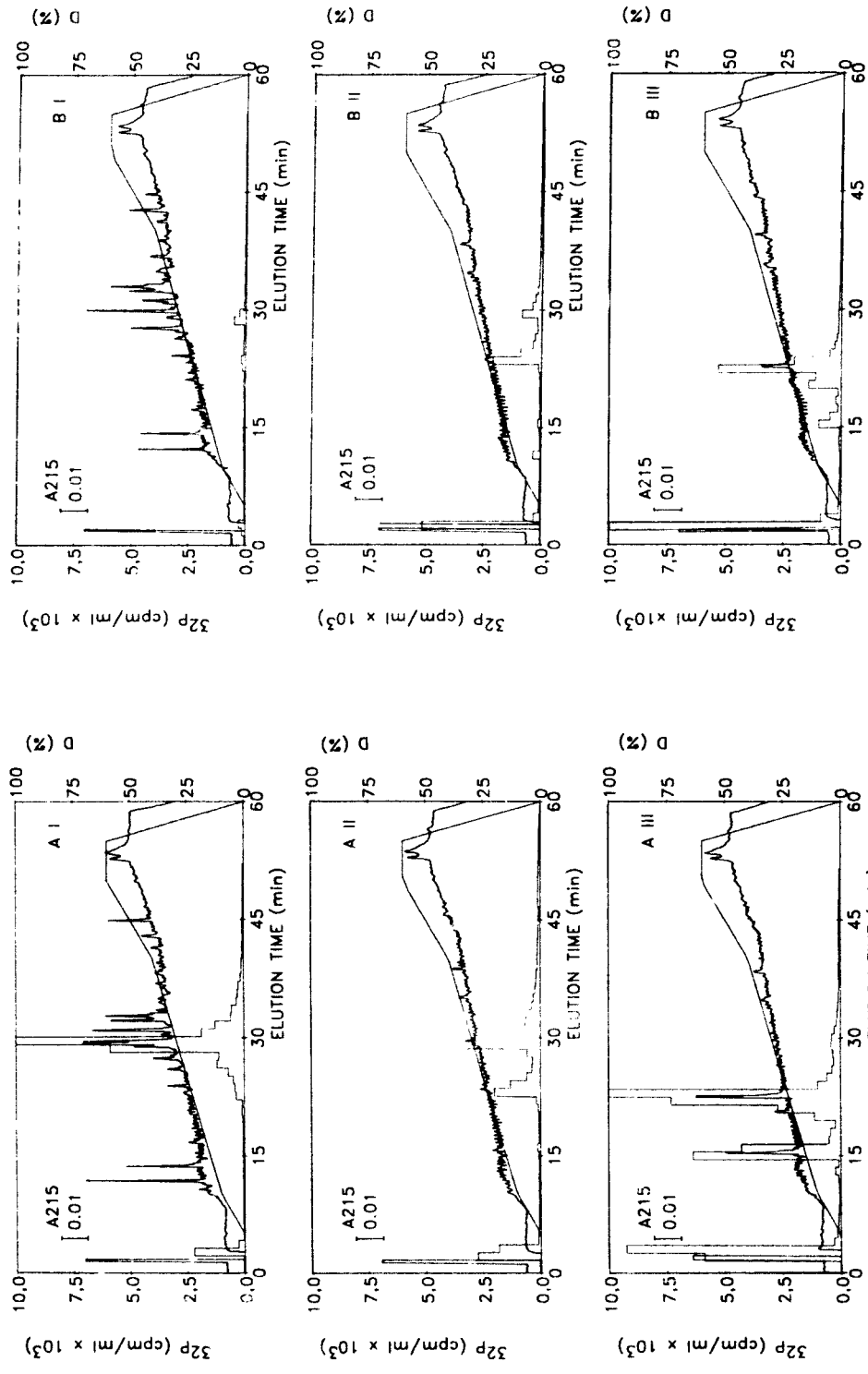

Fig. 3. Separation on a reversed-phase column of labeled fractions I, II and III, collected from the ion-exchange column (see Fig. 2), labeled without turnover (A) and with turnover (B). Protein absorption at 215 nm, radioactivity in collected fractions (left) and gradient of eluent D (right) against the elution time. For technical details see Materials and Methods.

β-subunit. In the eighth cycle of the sequence analysis no amino acid was detected at a level at which the other amino acids of this peptide were detected. This place corresponds to Tyr-345, the site of modification by 2-nitreno-ADP when the ligand is bound at a catalytic site [26,27]. The fraction eluting at 30% D also contained another peptide fragment (see A_{215} of Fig. 3A I) at a 2-fold lower concentration, that could be identified as Ser-191 till Arg-202 of the γ-subunit, on the basis of the sequence of Walker et al. [41]. Due to its negative charge, it eluted from the ion-exchange column together with the 2-nitreno-ADP-containing β-fragment.

The peptides in the fractions eluting at 18% and 23% D contained overlapping sequences and spanned the sequence of Ile-357 till Arg-372 of the β-subunit. No clear amino acid was detected at the positions corresponding with His-367 and Tyr-368, the site of modification of the non-catalytic site by 2-nitreno-ADP. A secondary finding was that at position 359 of the β-subunit no asparagine was found [40], but, rather an aspartate, in agreement with the report of Cross et al. [27].

We may conclude, then, that inhibition of the ATPase activity induced by 2-azido-ADP is the result of binding of the ligand to a non-catalytic site, from which the bound ligand is not removed under turnover conditions.

Discussion

The ADP-induced inhibition of ATPase activity, measured under V_{max} conditions, is supposed to be the result of binding of ADP to a non-catalytic site. Since the effect is measured at a high ATP concentration in the absence of free ADP, this site exhibits a high affinity for ADP, which does not exchange with medium nucleotides on a time scale of several minutes. Using illumination of F_1 in the presence of 2-azido-ADP under turnover conditions we could occupy this site covalently with 2-nitreno-ADP, resulting in a similar inhibition of the ATPase activity, as with non-covalently bound ADP. Analysis of the covalent modification showed that, indeed, a non-catalytic site was modified (modification of β Tyr-368). Whether the decreased rate of ATP hydrolysis is, indeed, the result of binding of ADP or 2-azido-ADP to this site, or of the prevention of ATP binding to this site during turnover in the presence of a regenerating system, cannot be decided. This regulatory effect of ADP could well be physiologically significant under conditions of high phosphate potential (low ADP concentrations) because of the high affinity of this site for ADP. The slow exchangeability of ADP on this site prevents, on the other hand, a fast response on changing physiological conditions. Modification of ATPase activity by ATP-ADP binding to this site, combined with factors such as a membrane potential and binding of the inhibitor protein or hormones to F_1 (e.g., see Refs. 42 and 43), can result in an efficient regulation of the F_1-F_0 ATP-synthase complex under physiological circumstances.

Milgrom and Murateliev [24] reported another type of ADP inhibition when nucleotide-depleted F_1 was used. The ADP-induced inhibition in their experiments is assumed to be the result of binding of ADP to a catalytic site. Binding of 2-nitreno-ADP to Tyr-345 in their experiments [25], confirmed this assumption. Comparable results were reported by Zhou et al. [44] for chloroplast ATP synthase. This kind of inhibition is quite different from the inhibition described in this paper, since it results in complete inhibition of ATPase activity; however, as a result of the slow dissociation of ADP from this catalytic site, the activity increases again during ATP incubation under assay conditions. It is important to note that nucleotide-depleted F_1 is quite different from freshly isolated F_1. All three tightly-bound nucleotides are largely removed and the site with highest affinity in nucleotide-depleted F_1 is the first catalytic site [28]. The affinity of the two originally non-exchangeable sites is not very high in glycerol-treated F_1.

After labeling nucleotide-depleted F_1 with 2-azido-ADP, Lunardi et al. [28] found, in addition to the labeling of Tyr-345, the catalytic site, no labeling at Tyr-368, the non-catalytic site, although four sites were labeled. Instead, another fragment of the β-subunit, Gly-72 till Asp-83, was labeled. This discrepancy can be explained by differences in starting material (use of nucleotide-depleted F_1) and the use of cyanogen bromide for peptide digestion. From the preparations

30 % D: A I A E L G I - P A V D P L D S T S R → Ala338-Arg356 of β-subunit

S' Y D D I D A D V L R → Ser191-Arg202 of γ-subunit

23 % D: I M D P N I V G S E - - D V A
18 % D: I V G S E - - - V A R } Ile357-Arg372 of β-subunit

Fig. 4. Sequence analysis of labeled peptides collected at 30%, 23% and 18% of eluent D from the reversed-phase column (see Fig. 3)

used in our own experiments, in which labeling of Tyr-368 was obtained, we also made cyanogen bromide digests and analyzed them on SDS polyacrylamide gels. With this method no labeled peptide was seen besides the CB9 fragment (containing Tyr-345), although the Tyr-368 (located on the CB10 fragment) was labeled. The band pattern on the gel and the autoradiogram were the same as those reported by Lunardi et al. The possibility exists that after cyanogen bromide digestion, which includes a long incubation in acid, the label on Tyr-368 is lost. Direct tryptic digestion of the 2-nitreno-ADP labeled preparation of Lunardi et al. should be used as a test for this possibility.

Yet another type of inhibition of F_1-ATPase activity by ADP has been reported by Di Pietro et al. [21] and Fellous et al. [22] for the pig heart enzyme. These authors described a phenomenon that was called hysteretic inhibition. After incubation of F_1 with ADP or NAP_3-ADP the ATPase activity became increasingly inhibited during an ATPase assay, from no inhibition at the start of the assay to 75–80% inhibition after about 1 min. With NAP_3-ADP a non-catalytic site on the β subunit was identified as the inhibitory site [22]. In our assays, inhibition was maximally 45% and already present at the start. The activity during the assay was constant. (Inhibition was measured against a control F_1 preparation to which no ADP (or analogue) was added.) The hysteretic inhibition is, therefore, not identical with the inhibition described in this report, although in both cases a non-catalytic site is involved in the inhibition phenomenon. We have recently proposed [15] that hysteretic inhibition is due to binding of ADP at one of the tight nucleotide binding sites. Possibly by the use of a different isolation method, the pig heart F_1 preparations contain two bound nucleotides, mainly ADP. The inhibitory site described in this paper seems to be occupied by ADP in the pig-heart F_1 preparations: comparison of the specific activities shows that the activity of the F_1 preparations used by Fellous et al. [22] is about twice as low as that of our preparations (but similar to that of our preparations after incubation with ADP). At the same time, we propose that the site on the pig-heart enzyme that is responsible for the hysteretic inhibition is occupied with ATP in our enzyme preparations [15].

The 2-azido-adenine nucleotide analogue exist in two forms, the photolysable azido form and the non-photolysable tetrazolium form, which are in slow equilibrium with each other [45]. Both forms are supposed to be able to bind to F_1 [32]. However, we found that in a short period of illumination (20 s) nearly all four available sites could be covalently modified. We have to conclude from this that either only the azido form binds to the enzyme, or the equilibrium between the azido and tetrazolium form is very rapid when the molecule is bound to F_1.

The sequence analysis showed, when compared with the β-sequence determined by Runswick and Walker [40], that amino acid 359 was an aspartate instead of an asparagine, which is in agreement with the report of Cross et al. [27]. The difference can be explained by a point mutation, changing an A into a G or the reverse, of the first nucleotide of the triplet which codes for this amino acid. This mutation, although it has a great influence on the charge, has no influence on the enzyme activity, possibly because it is located in the neighbourhood of the non-catalytic sites.

On the ion-exchange column the retention of the 2-nitreno-ADP-modified peptides is determined mainly by the nucleotide moiety. Running free 2-azido nucleotides on this column, 2-azido-AMP eluted at 25% B, 2-azido-ADP at about 48% B and 2-azido-ATP at about 70% B. The 2-nitreno-adenosine-modified peptides eluted at two positions, in the neighbourhood of 2-azido-ADP, but also at 23% B in the neighbourhood of 2-azido-AMP. (A tryptic digest of 2-nitreno-ATP-modified F_1 eluted, indeed, partly in the region of 2-azido-ATP.) Possibly, F_1 is capable of hydrolysing 2-azido-ADP to 2-azido-AMP, when bound at a catalytic site. A 2-nitreno-AMP-modification, after incubation with 2-azido-ADP, was found earlier for chloroplast ATPase by Melese et al. [46], and was explained by assumption of adenylate kinase activity of the F_1-ATPase. We would like to propose that the hydrolysis to 2-nitreno-AMP occurs after covalent binding to F_1, because 2-azido-AMP (or any other adenosine monophosphate) does not bind to F_1.

Of the three tightly-bound nucleotides one ADP exchanges upon incubation with 2-azido-ADP and is clearly bound at a catalytic site. (If it had been bound at a non-catalytic site no inhibition of the catalytic activity would have been measured, since exchange of ADP for 2-azido-ADP has no effect on activity.) This one catalytic site (Fig. 5, site 3) and the three remaining sites (Fig. 5, sites 4–6) can be modified with 2-

1 AXP	3 $2N_3AMP$	4 $2N_3AMP$
2 AXP	5 $2N_3ADP$	6 $2N_3ADP$

Fig. 5. Schematic representation of the nucleotide binding sites on F_1-ATPase. The main ligand at each site after labeling with 2-azido-ADP is indicated. Upper row, β-sites; lower row, interface sites. (1) and (2) represent the non-catalytic non-exchangeable sites; (3) to (6) the exchangeable sites, with, (3) and (4) being catalytic sites and (5) and (6) non-catalytic sites. (5) is the high-affinity site which induces the measured inhibition, while (6) has a low affinity. (X represents T or D.)

nitreno-ADP, and, in that case, the enzyme is fully inhibited because of modification of catalytic sites (Fig. 5, sites 3 and 4). Two sites, indeed, exchange rapidly and when illumination is started immediately after the initiation of ATP hydrolysis by addition of ATP, these two sites are not modified any more and the induced inhibition of enzyme activity is only partial. In fact, more than one site can be modified under these conditions (see Table IIIA), but the inhibition is the same when only about one site (site 5 of Fig. 5) is modified or two sites (sites 5 and 6 of Fig. 5). We wish to conclude from these experiments that two non-catalytic exchangeable sites bind 2-azido-ADP (Fig. 5, sites 5 and 6). One is the slowly exchangeable site, which induces the partial inhibition (site 5 of Fig. 5). The other is a non-catalytic exchangeable site with low affinity (Fig 5, site 6). Covalent binding of 2-nitreno-ADP to this latter site has no influence on the ATPase activity under V_{max} conditions. Such a non-catalytic site was found earlier in labeling experiments with 8-azido-ATP [16]. In our experiments this site was about half-occupied with 2-nitreno-ADP (Table IIIA). It cannot be a catalytic site because this would have resulted in an increased inhibition and attachment of label to Tyr-345; neither of the two was found. The possibility of aspecific binding can also be ruled out: first, because only the β-subunit was labeled (not to be expected for aspecific binding), and second since illumination was started after a turnover period of 30 s with 500 μM added ATP, eliminating aspecific binding.

Our conclusion from these results is that F_1-ATPase contains two exchangeable non-catalytic sites and two catalytic sites (see Fig. 5). The difference with a three-site catalytic mechanism lies in the non-catalytic exchangeable site with low affinity. A nucleotide at this site exchanges under turnover conditions (partially) with ATP and may look like a catalytic site. Further analysis of the properties of this site will be reported in a forthcoming paper.

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