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Identification of an exchangeable non-catalytic site on mitochondrial F_1-ATPase which is involved in the negative cooperativity of ATP hydrolysis

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Labeling of mitochondrial F_1-ATPase with 8-azido-ATP or 8-azido-ADP under turnover conditions with Mg^{2+}-ATP resulted in the identification of one exchangeable non-catalytic site whose occupation with a ligand does not influence the ATPase activity of F_1 when measured at V_{max}. With 8-azido-ADP two exchangeable non-catalytic sites could be labeled, but at one of them the bound ligand exchanges, at least partly, during the illumination under turnover conditions. After labeling an exchangeable non-catalytic site under turnover conditions with 8-azido-ATP or with 8-azido-ADP, F_1-ATPase kept the ability to bind NAP_3-$2N_3$ADP at the slowly exchangeable noncatalytic site, thereby inhibiting the ATPase activity by 45%, as recently described (Edel et al. (1992) Biochim. Biophys. Acta 1101, 329–338). Covalent modification of the low-affinity non-catalytic site with 8-nitreno-AT(D)P increased the K_m of ATP and abolished the negative cooperativity of ATP hydrolysis. This site can therefore be marked as a regulatory site, whose occupation with a nucleotide decreases the affinity of the catalytic sites for ATP.

Introduction

F_1-ATPase is the water-soluble part of the F_1F_0-ATPsynthase, the complex that catalyses the last step of the process of oxidative phosphorylation, the synthesis of ATP from ADP and P_i. In the isolated form, detached from F_0, F_1 catalyses only the hydrolysis of ATP. It is composed of five different subunits, the large α and β subunits, and the small γ, δ and ϵ subunits. These subunits are present in the stoicheiometry of α_3, β_3, γ_1, δ_1 and ϵ_1 [1]. F_1-ATPase contains six nucleotide binding sites, located on the α and β subunits, which can be divided in catalytic and non-catalytic sites [2,3].

Incubation of F_1 with the adenine nucleotide analogue 8-azido-ATP at high concentrations (≥ 300 μM) results after ultraviolet-irradiation in covalent binding of 8-nitreno-ATP to F_1, accompanied by inhibition of the ATPase activity. The inhibition is linearly related to the amount of covalently bound ligand. For complete inhibition the binding of two moles 8-nitreno-ATP per mol F_1 is necessary [4,5]. The label is bound to the β and α subunits in a typical 3:1 ratio [5]. At low concentrations of 8-azido-ATP (20 μM) the inhibition (after ultraviolet-illumination) is also linearly related to the amount of covalently bound label, but extrapolation shows that under these conditions for complete inhibition the binding of 1 mol 8-nitreno-ATP per mol F_1 is sufficient. Under these circumstances only the β subunit is labeled [5]. Van Dongen and Berden [5] concluded from these results that at low 8-azido-ATP concentrations only one catalytic site, located on a β-subunit, is labeled. At high concentrations an additional mol 8-nitreno-ATP per mol F_1 is bound to a non-catalytic site, located at the interface between an α- and a β-subunit. Because of the linear relation between amount of bound 8-nitreno-ATP and inhibition of ATPase activity, modification of this latter site is supposed to have no influence on the enzyme activity, when measured under V_{max} conditions.

8-Nitreno-ATP binds covalently at two regions of the β subunit, at the N-terminus and at or near Tyr-311 [6]. The proposal that this last region is part of the catalytic site [6] is supported by the finding that the affinity label Nbf-Cl modifies initially also Tyr-311. This inhibitor is supposed to bind only to one catalytic site of F_1 [7,8].

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Abbreviations: FSBA, 5′-*p*-fluorosulfonylbenzoyl-5′-adenosine; NAP_3-$2N_3$ADP, 3′-*O*-(3-(*N*-(4-azido-2-nitrophenyl)amino)propionyl)-2-azido-adenosine-5′-diphosphate; Nbf-Cl, 4-chloro-7-nitrobenzofurazan; TDAB, tetradecyltrimethyl-ammonium bromide.

After labeling of F_1 with 8-azido-ATP (2 mol of label bound per mol F_1), further labeling with 8-azido-ADP in the presence of Mg^{2+} results in the additional covalent binding of 2 mol 8-nitreno-ADP per mol F_1, but the β/α labeling ratio of 3:1 is not affected [5]. After these two labeling steps still 2 mol adenine nucleotides per mol F_1 are tightly bound [2,5]. From these results it was concluded (see Ref. 5) that F_1 contains two catalytic sites – each located on a β subunit –, two exchangeable non-catalytic sites – each located on an α/β interface –, and two non-exchangeable sites – one located on a β subunit and one on an α/β interface. In contrast with the conclusions described above and further analysed in Ref. 9, a catalytic mechanism for ATP hydrolysis based on the involvement of three catalytic sites is still generally accepted (e.g., see Ref. 10).

Independent of a two- or three-site mechanism, the catalytic sites on F_1-ATPase are functioning in a cooperative manner; binding of ATP at a catalytic site accelerates the dissociation of bound ADP from another catalytic site [11–13]. Additional cooperativity between catalytic sites may also be responsible for the non-linear kinetics (negative cooperativity) of ATP hydrolysis at substrate concentrations between 10 μM and 1 mM [13–15]. This negative cooperativity is influenced by anions and nucleotide analogues [15,16]. In a three-site-mechanism the increase of K_m (and V_{max}) at concentrations higher than about 50 μM is ascribed to binding of the substrate at these concentrations to the third catalytic site [13]. The kinetic data can also be explained, however, as due to the presence of a regulatory non-catalytic site [17–19].

In the labeling experiments with 8-azido-ATP and 8-azido-ADP performed thus far, the effect of covalent modification of non-catalytic sites was not visible because of the concomitant modification of catalytic site(s). The present paper describes our attempts to label specifically the exchangeable non-catalytic sites with 8-azido-ATP or 8-azido-ADP and to measure the effect of this modification on the kinetics of F_1-catalysed ATP hydrolysis.

Materials and Methods

F_1 preparation

F_1-ATPase was isolated from bovine hearts according to the method described by Knowles and Penefsky [20], and stored in liquid nitrogen in a medium consisting of 10 mM Tris-HCl (pH 7.5), 250 mM sucrose, 4 mM EDTA and 4 mM ATP. Before an experiment the stored F_1 was precipitated two times with ammonium sulphate, followed by filtration twice over a centrifugation column [21]. The precipitated F_1 was dissolved in and the column material (Sephadex G-50 coarse) equilibrated with 50 mM Tris-HCl (pH 7.5), 150 mM sucrose and 4 mM EDTA (EDTA-medium). Column filtration (1 × 5 cm columns) was performed as described by Penefsky [22]. After the last centrifugation step the F_1 samples were diluted to 4 or 2 mg protein/ml in the above mentioned buffer. The specific activity of these preparations was 140–150 μmol ATP hydrolysed/min per mg protein.

Synthesis of 8-azido-ATP and 8-azido-ADP

The tritium-labeled forms of 8-azido-ATP and 8-azido-ADP were synthesised from [2-^{3}H]ATP (Amersham) as described by Schäfer et al. [23]. 8-Azido[2-^{3}H]ATP and 8-azido[2-^{3}H]ADP were stored in 50% (v/v) ethanol-water at −20°C, The concentration and specific activity of 8-azido[2-^{3}H]ATP were respectively 6.5 mM and $95 \cdot 10^3$ dpm/nmol and those of 8-azido[2-^{3}H]ADP 9.75 mM and $109 \cdot 10^3$ dpm/nmol.

Photolabeling of F_1-ATPase with 8-azido[2-^{3}H]AT(D)P

F_1 samples, prepared as described above, were incubated with 100–2000 μM 8-azido[2-^{3}H]AT(D)P for 30 min in the dark (see Results). Illumination was performed with a Penray UV-lamp as described in Ref. 21. When illumination was performed under turnover conditions, an equal volume of 50 mM Tris-HCl (pH 7.5), 150 mM sucrose, 20 mM $MgCl_2$, 2 mM ATP, 20 mM PEP and 5 U/ml pyruvate kinase was added, 30 s before illumination was started. When no turnover was desired the same medium, but without ATP, PEP and pyruvate kinase, was added before starting the illumination. After illumination the samples were filtrated by column centrifugation, using gel material equilibrated in the original EDTA-medium. When indicated in Results, this incubation and illumination procedure was repeated.

In experiments in which the catalytic sites were protected, 5 mM pyrophosphate or 5 mM ITP was added to the F_1 preparations (in EDTA-medium) 10 min before the addition of 100 μM 8-azido[2-^{3}H]AT(D)P. After 30 min in the dark the samples where illuminated for another 30 min with a CAMAG lamp at 350 nm. The procedure of label addition, incubation and illumination was repeated once. After the second illumination the samples were filtrated by column centrifugation, the gel material being equilibrated with the original EDTA-medium. When indicated the procedure was repeated, starting with the addition of ITP or PP_i. All incubations and illuminations were performed at room temprature.

ATPase activity was measured with an ATP-regenerating system as described in Ref. 21. Inhibition was determined relative to the activity of control samples not incubated with 8-azido-AT(D)P, but further treated in the same way as samples to which label was added. Protein concentrations were determined with the

method described by Lowry et al. [24] with bovine serum albumin as standard.

Determination of covalently bound 8-nitreno[2-^{3}H]-AT(D)P

Covalently bound 8-nitreno[2-^{3}H]AT(D)P was determined via acid precipitation, as described in Ref. 21. ^{3}H was measured in glass vials with an LKB 1214 Rackbeta liquid scintillation counter, the samples being dissolved in Packard Scintillator 299 scintillation cocktail. The subunits of F_1 preparations, modified with 8-nitreno[2-^{3}H]AT(D)P, were separated by TDAB gel electrophoresis as described by Penin et al. [25], with the adjustments of Fellous et al. [26]. The gels were stained with Coomassie Brilliant Blue G and scanned on a Bio-Rad model 1650 scanning densitometer. The gels were sliced by cutting out the stained bands and protein was extracted by incubation with 2 ml 90% Protosol (New England Nuclear Dupont) in glass vials, for 36 h at room temperature. After this solubilization procedure 200 μl acetic acid and 4 ml scintillation cocktail were added. After mixing, ^{3}H was measured in the liquid scintillation counter as described above.

Results

On the basis of their labeling experiments with 8-azido-ATP, Van Dongen and Berden [5] postulated the presence on F_1-ATPase of an exchangeable non-catalytic site. The covalent modification with 8-nitreno-ATP of this site is expected to have no influence on the enzyme activity (when determined under V_{max} conditions, i.e., 5 mM ATP in an ATP regenerating system) and to show a distribution in a 1:1 ratio of bound 8-nitreno-ATP over the β and α subunits. In order to label only the exchangeable non-catalytic site(s) with 8-nitreno-ATP or 8-nitreno-ADP, without concomitant labeling of catalytic sites, we performed the ultraviolet illumination either under turnover conditions or in the presence of compounds that are supposed to protect the catalytic sites.

Covalent modification of F_1 with 8-nitreno[2-^{3}H]-AT(D)P in the presence or absence of turnover with ATP

Mitochondrial F_1, prepared as described in Materials and Methods, contains three tightly bound nucleotides, of which one is exchanged easily after addition of (NAP_3-) $2N_3ADP$ in Mg-medium. This nucleotide has been shown to be bound at a catalytic site (Ref. 21, unpublished results). Such F_1-ATPase was incubated with tritiated 8-azido-AT(D)P and 30 s before ultraviolet-illumination was started Mg^{2+}-ATP was added in order to remove the labeled analog from the catalytic sites before the start of the illumination. A short illumination period (20 s) in combination with the reconversion of formed ADP into ATP (by using an ATP-regenerating system), was chosen to minimize dissociation of the labeled analog from exchangeable non-catalytic sites and/or exchange with AT(D)P. This method we had used successfully in experiments with (NAP_3-)2-azido-ADP (Ref. 21 and unpublished data).

Labeling with 8-azido[2-^{3}H]ATP. F_1-ATPase was incubated with 100 to 1600 μM 8-azido[2-^{3}H]ATP in the dark. ATPase activity was measured both after dark incubation and after subsequent illumination, with or without turnover. As control for 100% activity a sample was used to which no 8-azido-analog was added. Incubation in the dark with 8-azido[2-^{3}H]ATP had no influence on the ATPase activity (Fig. 1A). After illumination without turnover the ATPase activity was inhibited depending on ligand concentration added. The inhibition increased at higher label concentrations and was maximally 80% (Fig. 1A). The relation between the amount of covalently bound 8-nitreno[2-^{3}H]ATP and the measured inhibition is shown in Fig. 1B. After illumination in the absence of turnover this relation is linear and complete inhibition is reached when 2 mol 8-nitreno[2-^{3}H]ATP per mol F_1 are bound. Illumination under turnover conditions does not result in any significant inhibition of the ATPase activity (5–10%), whatever the concentration of 8-azido[2-^{3}H]ATP (Fig. 1A). The amount of covalently bound label, however, increases with increasing concentrations of 8-azido[2-^{3}H]ATP. Maximally 0.7 mol 8-nitreno[2-^{3}H]ATP per mol F_1 is covalently bound (Fig. 1B).

The partition of covalently bound 8-nitreno[2-^{3}H]ATP over the α and β subunits was detemined by TDAB gel electroforesis. Fig. 2A shows the staining pattern of a gel and the measured amount of ^{3}H in the bands of the α, β and γ subunits, obtained after incubation of F_1 with 400 μM 8-azido[2-^{3}H]ATP, followed by illumination in the absence or presence of turnover. In this experiment the distribution of label over β- and α-subunits was 2.9:1 when the illumination was performed in the absence of turnover, a ratio of 1.2:1 was found after illumination in the presence of turnover. Fig. 2B shows that the β/α labeling ratio does not significantly vary with the concentration 8-azido[2-^{3}H]ATP. The mean ratio after illumination without turnover is 2.75:1, with turnover this ratio is 1.05:1.

The finding that the inhibition of ATPase activity is linearly related with the amount of covalently bound 8-nitreno[2-^{3}H]ATP, complete inhibition being obtained by the binding of 2 mol 8-nitreno-ATP per mol F_1, and that the bound label is distributed over the β- and α-subunits in a ratio of 2.75:1, confirms the results obtained by Van Dongen and Berden [5]. The further results, obtained under conditions of turnover, show that these authors reached the right conclusions about the presence of an exchangeable non-catalytic

site whose ligation has no influence on the ATPase activity and which is located at the interface between an α- and a β-subunit.

Labeling with 8-azido[2-^{3}H]ADP. With 8-azido[2-^{3}H]ADP comparable experiments were performed as described for 8-azido[2-^{3}H]ATP. Incubation of F_1 with concentrations between 100 and 2000 μM 8-azido[2-^{3}H]ADP in the dark had no influence on the ATPase activity (Fig. 3A). Subsequent illumination (without turnover) resulted in inhibition of the ATPase activity, depending on ligand concentration. The inhibition shows saturation at a ligand concentration of 400 μM, the maximal inhibition being 40–45%. Repeated incubation with 8-azido[2-^{3}H]ADP, followed by illumination, induced a further inhibition of the ATPase activity. A 3-fold incubation and illumination with 100 μM ligand induced about the same inhibition as a 2-fold incubation and illumination with 800 or 2000 μM 8-azido[2-^{3}H]ADP. Maximal inhibition in our experiments was about 75%. After illumination under conditions of turnover, the ATPase activity was only marginally inhibited, about 5%.

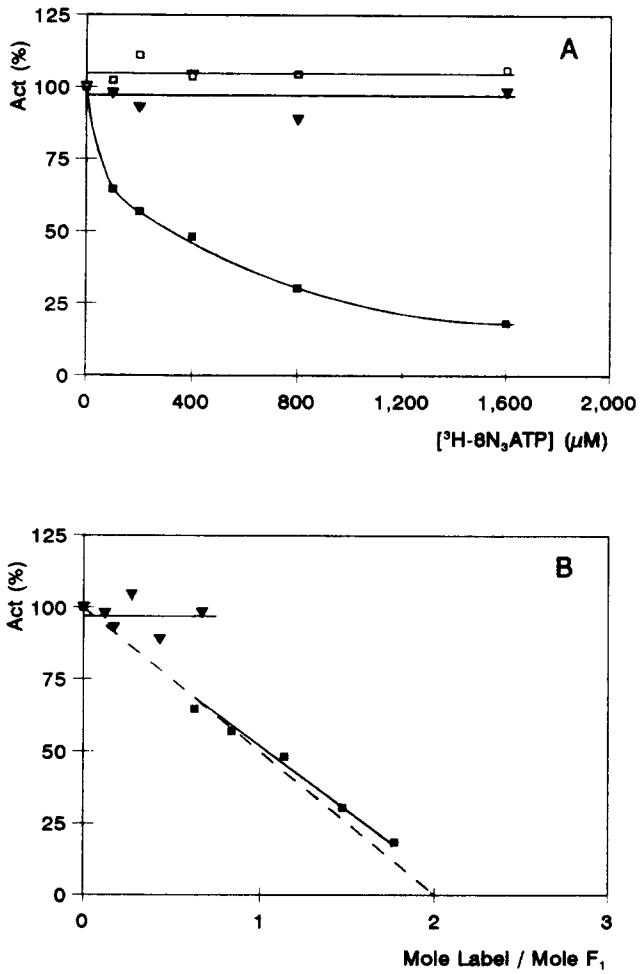

Fig. 1. Inhibition of F_1-ATPase activity by labeling with 8-azido-ATP. F_1 was incubated and then illuminated in the presence of variable concentrations of 8-azido[2-^{3}H]ATP, in the absence or presence of MgATP-induced turnover. The residual ATPase activity was measured and plotted as a function of the concentration of label (A) or as a function of covalently-bound label (B). (□), incubation in the dark, without ATP and Mg^{2+}; (■), illuminated in the absence of turnover; (▼), illuminated in the presence of turnover. The experimental procedures are described in Materials and Methods. The dotted line represents the theoretical curve on the basis of labeling of one catalytic and one non-catalytic site, assuming that modification of the non-catalytic site does not influence the V_{max} (see Ref. 5).

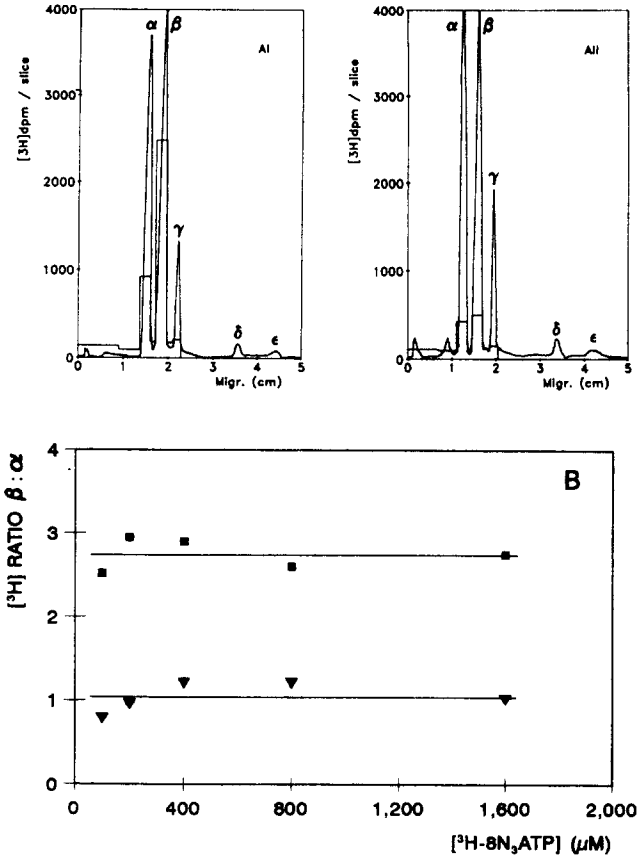

Fig. 2. Distribution of bound radioactivity over the α- and β-subunits of F_1. (A) After illumination in the presence of 400 μM 8-azido[2-^{3}H]ATP without turnover (AI) or with turnover (AII), F_1-ATPase (30 μg) was subjected to TDAB polyacrylamide gel electrophoresis. The gel scans after staining with Coomassie brilliant blue and the radioactivity extracted from the protein bands are shown. (B) The distribution of ^{3}H over the α- and β-subunits after illumination in the presence of 100–1600 μM 8-azido[2-^{3}H]ATP is shown as the β/α ratio. (▼), illumination in the presence of turnover; (■), illumination in the absence of turnover.

The relation between the amount of covalently bound 8-nitreno[2-^{3}H]ADP after illumination and the level of inhibition is shown in Fig. 3B. In the absence of turnover this relation is linear. Extrapolation of the line to complete inhibition shows that under these conditions 3 mol of 8-nitreno-ADP per mol F_1 are required for complete inhibition. The relation between inhibition and amount of bound 8-nitreno[2-^{3}H]ADP is independent of the number of incubations and illuminations. Illumination in the presence of turnover re-

sults, dependent on ligand concentration and times of incubation and illumination, in covalent attachment of different amounts of 8-nitreno[2-^{3}H]ADP. Maximally

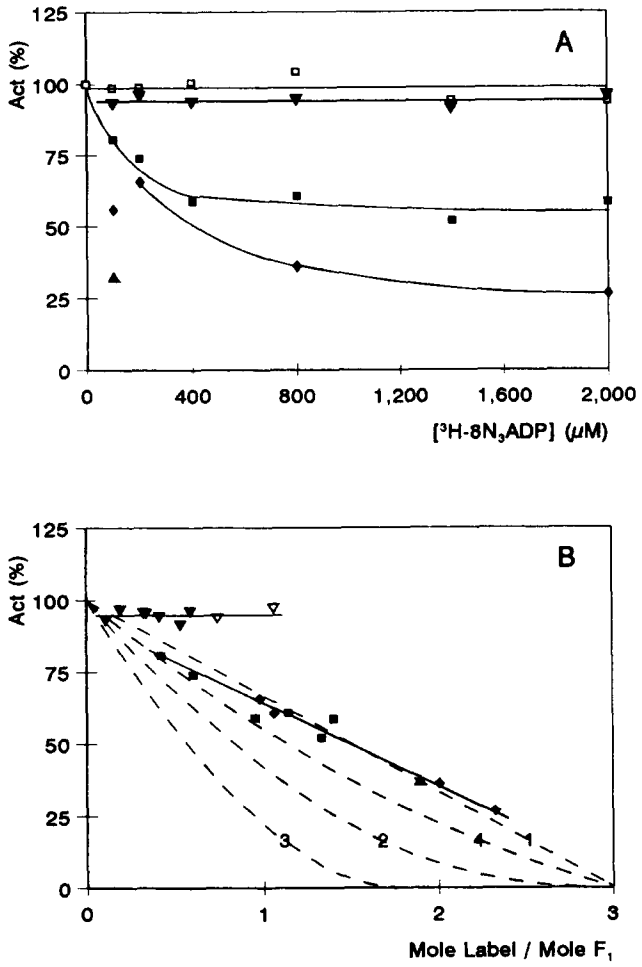

Fig. 3. Inhibition of F_1-ATPase activity by labeling with 8-azido-ADP. F_1 was incubated and then illuminated in the presence of variable concentrations of 8-azido[2-^{3}H]ADP, in the absence or presence of MgATP-induced turnover. The residual ATPase activity was measured and plotted as a function of the concentration of label (A) or as a function of covalently-bound label (B). (□), incubation in the dark, without ATP and Mg^{2+}; (■ and ♦), illuminated in the absence of turnover; (▼ and ▽), illuminated in the presence of turnover. At the indicated concentrations of 8-azido[2-^{3}H]ADP the incubation and illumination procedures were repeated (♦ and △), and at 100 μM 8-azido[2-^{3}H]ADP the illumination without turnover was performed once (■), twice (♦), and four times (▲). The experimental procedures are described in Materials and Methods. Dotted lines: these curves represent the theoretically expected relation between inhibition and amount of covalently-bound 8-nitreno[2-^{3}H]ADP when the ligand binds at three sites on the F_1-ATPase. These sites may consist of one catalytic and two non-catalytic sites (1 and 4), or two catalytic and one non-catalytic site (2) or three catalytic sites (3). Binding at a catalytic site is supposed to inhibit the activity by 100%, while binding at a non-catalytic site may have no effect on the activity (curves 1 and 2). Curve 4 assumes 45% inhibition upon binding at one non-catalytic site and no inhibition upon binding to the other one. In all cases equal labeling of all involved sites is assumed.

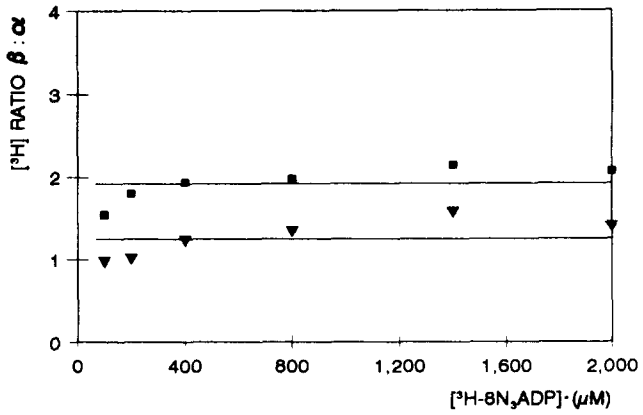

Fig. 4. The distribution of [^{3}H] over the α- and β-subunits after illumination in the presence of 100–2000 μM 8-azido[2-^{3}H]ADP is shown as the β/α ratio. (▼), illumination in the presence of turnover; (■), illumination in the absence of turnover.

1.05 mol 8-nitreno[2-^{3}H]ADP per mol F_1 was bound, accompanied by less than 5% inhibition.

The distribution of label over β- and α-subunits under the two conditions is shown in Fig. 4. The distribution of 8-nitreno[2-^{3}H]ADP covalently bound after illumination in the absence of added Mg-ATP, over β- and α-subunits is on average 1.9:1. After illumination under turnover conditions this average is 1.25:1.

Compared with the results obtained with 8-azido-ATP, 8-azido-ADP is capable of modifing one additional site in the absence of turnover. This extra modification results in a lowering of the label-distribution ratio over β- and α-subunits from about 3 to about 2. Since modification of two or three catalytic sites would result in a non-linear inhibition curve, the linear relationship between amount of covalently bound 8-nitreno[2-^{3}H]ADP and inhibition indicates that only one catalytic site is modified, together with two non-catalytic sites (see Fig. 3B dotted lines). The shift in label distribution also shows that the additional site cannot be a catalytic site (labeling of only β-subunit), but has to be a site that is located at the interface between β- and α-subunits (about equal labeling of both subunits). So two non-catalytic and one catalytic site seem to be modified with 8-nitreno-ADP in the absence of ATP + Mg^{2+}.

Effect of protection of catalytic sites on photo-affinity labeling of F_1 with 8-azido[2-^{3}H]AT(D)P

Another approach for labeling the exchangeable non-catalytic site(s) specifically with 8-azido-AT(D)P, is protection of the catalytic sites during the incubation and illumination period. ITP and pyrophosphate (PP_i) are compounds of which it has been described/suggested in the literature that they have only interaction with catalytic sites. ITP has been reported to be a substrate which only binds to (part of) the catalytic

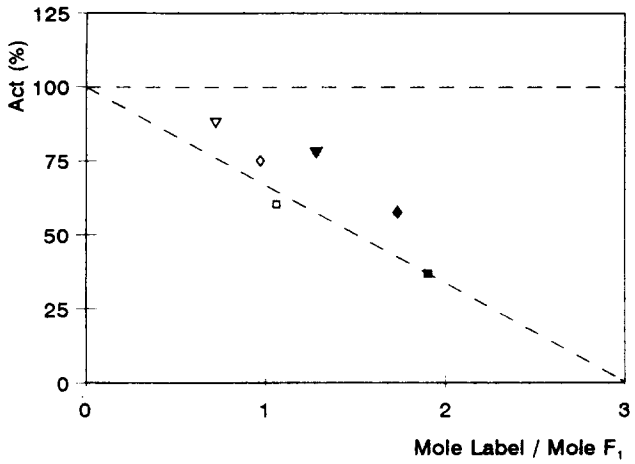

Fig. 5. Inhibition of ATPase activity by covalently-bound 8-nitreno-ADP. F_1 was incubated and illuminated two times (open symbols) or four times (closed symbols) with 100 μM 8-azido[2-^{3}H]ADP, in EDTA-medium without addition (□, ■), with 5 mM ITP (◇, ◆) or with 5 mM PP_i (▽, ▼) added. The residual ATPase activity is plotted as a function of the amount of covalently bound 8-nitreno[2-^{3}H]ADP. The experiments were performed as described in Materials and Methods. The dotted curve, intersecting the abscissa at 3 mol/mol F_1, represents equal labeling of the one catalytic and the two non-catalytic sites (see Fig. 3B). The horizontal dotted curve represents labeling of non-catalytic sites only (see Fig. 3B).

sites of F_1 [16]. PP_i is capable to bind to and to displace adenine nucleotides from catalytic sites [27]. Reported are interactions with two [27] or three sites [28]. In our experiments first 5 mM ITP or PP_i were added to F_1 preparations before they were incubated with a low concentration, 100 μM, of 8-azido[2-^{3}H]AT(D)P. This low label concentration should avoid competition between ITP/PP_i and 8-azido-AT(D)P for the catalytic sites, but is high enough to obtain labeling of exchangeable non-catalytic site(s).

In the control experiment (no added ITP or PP_i) the amount of covalently bound 8-nitreno-ADP in relation to the observed inhibition of ATPase activity fits with the linear relationship reported in Fig. 3B, 3 mol covalently bound label per mol F_1 being required for complete inhibition (Fig. 5). The measured β/α distribution of the ^{3}H label was 1.8:1, a ratio comparable with the ratio reported above. In the presence of ITP the inhibition after illumination was lower and a smaller amount of covalently bound 8-nitreno[2-^{3}H]ADP was detected. The β/α label distribution ratio was also lowered, but significant inhibition was still observed (see Fig. 5). In the presence of PP_i also a smaller inhibition and a lower amount of covalently bound 8-nitreno-ADP was found, compared with the control experiment (i.e., no PP_i added). The relation between inhibition and bound label, however, is close to the relation measured in the control experiment. The measured β/α labeling ratio was 1.6:1, which lies in between the values obtained for turnover and non-turnover conditions. These results point towards labeling of a substantial part of the catalytic sites in the presence of PP_i. Similar experiments with 8-azido-ATP in stead of 8-azido-ADP show in the presence of ITP and PP_i also a lower level of inhibition of ATPase activity and a lower amount of covalently bound 8-nitreno-ATP, compared with the control, but the β/α label distribution ratio was in all cases the same, 2.9:1, suggesting that ITP and PP_i compete with 8-azido-ATP for both catalytic and non-catalytic sites (results not shown).

From these results we may conclude that ITP in combination with 8-azido-ADP (but not in combination with 8-azido-ATP) protects the catalytic site more than the non-catalytic sites. PP_i, however, is clearly not specific. It protects the catalytic and non-catalytic sites equally, both not very efficiently.

Discrimination between two exchangeable non-catalytic sites

Recently we investigated the effect of binding of ADP or a suitable analog in the anticonfiguration such as 2-azido-ADP [21] and NAP_3-2-azido-ADP (unpublished data) to a non-catalytic site of F_1, in the presence of Mg^{2+}. Both non-covalent and covalent binding were shown to induce a partial (40–45%) inhibition of ATPase activity. This inhibition was not found with 8-azido-ADP [21]. To investigate the relation beteen the site(s) modified with 8-azido-AT(D)P and the site modified with 2-azido-ADP or NAP_3-$2N_3$ADP, F_1 was illuminated in the presence of 8-azido[2-^{3}H]ATP or 8-azido[2-^{3}H]ADP under turnover conditions and afterwards incubated and illuminated (again under turnover conditions) in the presence of NAP_3-$2N_3$ADP (see Table I). After labeling with an 8-azido-analogue under turnover conditions, the F_1 preparations were slightly inhibited and a substantial amount of covalently bound 8-nitreno-analogue (0.6–0.8 mol/mol F_1) was measured. These preparations showed, upon incubation and illumination in the presence of NAP_3-$2N_3$ADP, a further inhibition of the ATPase activity up to about 50%. The amount of covalently bound

TABLE I

Percentage inhibition and amount of covalently bound ^{3}H after labeling of F_1-ATPase under turnover conditions

F_1 was labeled two times with 800 μM 8-azido[2-^{3}H]ATP or 8-azido[2-^{3}H]ADP (A), followed by a single labeling with 100 μM [^{3}H]NAP_3-$2N_3$ADP (B). For experimental procedures see Materials and Methods.

	A		B	
	inhibition (%)	mol label/ mol F_1	inhibition (%)	mol label/ mol F_1
Control			44.6	0.78
$8N_3$ATP	9.7	0.76	50.0	0.78
$8N_3$ADP	8.3	0.59	53.3	0.66

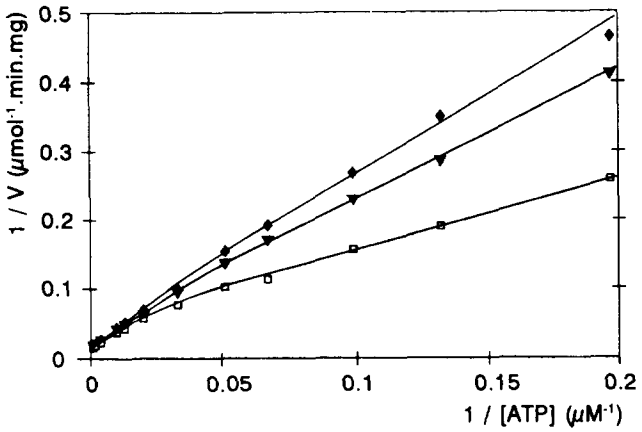

Fig. 6. Lineweaver-Burk plot of ATP hydrolysis by F_1, illuminated during turnover in the presence of 8-azido-ATP or 8-azido-ADP. (♦), 0.8 mol 8-nitreno-ATP bound per mol F_1; (▼), 0.6 mol 8-nitreno-ADP bound per mol F_1; (□), control F_1, illuminated in the absence of any 8-azido-adenine nucleotide. The activity measurements were performed at 30°C in an ATP regenerating system containing 33 mM Tris-HCl (pH 8.0), 83 mM sucrose, 6 mM $MgCl_2$, 20 mM KCl, 2 mM PEP, 250 μM NADH, 12.5 U/ml pyruvate kinase, 4 U/ml lactate dehydrogenase, with ATP concentrations between 5 μM and 1 mM. The activity was measured as NADH oxidation, followed at 340 nm.

NAP_3-$2N_3$ADP was comparable to the amount measured for a control preparation, not pretreated with an 8-azido-adenine nucleotide. Also the additional inhibition was similar to the inhibition measured for the control experiment. The latter value was comparable with the results obtained previously (Ref. 21, unpublished results).

By performing the illumination in the presence of ATP + Mg^{2+}, conditions are created that enable us to label with 8-azido-AT(D)P and NAP_3-$2N_3$ADP only exchangeable non-catalytic sites. The additional labeling of F_1 with NAP_3-$2N_3$ADP after a prelabeling with 8-azido-AT(D)P, as described here, shows that the two ligands (NAP_3-$2N_3$ADP vs. 8-azido-AT(D)P), modify different binding sites. From this we may conclude that mitochondrial F_1-ATPase contains two distinct exchangeable non-catalytic sites.

Kinetics of 8-nitreno-AT(D)P-modified F_1

Thus far the ATPase activity of F_1-ATPase after covalent binding of 8-nitreno-ADP or 8-nitreno-ATP to an exchangeable non-catalytic site was measured under V_{max} conditions (5 mM ATP). Fig. 6 shows the ATPase kinetics, for substrate concentrations between 5 μM and 1 mM, of F_1 preparations labeled with 8-nitreno-ATP, 8-nitreno-ADP or not labeled, the same preparations as used for Table I. The control sample shows the well-known negative cooperativity of catalysis, visible as a biphasic Lineweaver-Burk plot, as is usually measured for F_1-ATPase. After covalent labeling with 8-azido-ATP or 8-azido-ADP the kinetics tend to monophasicity, due to the disappearence of the high-affinity phase (at low ATP concentrations). The occupation of an exchangeable non-catalytic site with 8-nitreno-AT(D)P seems to induce the low affinity phase also at low concentrations of ATP. It can be seen that in the preparation with the highest level of ligation (the preparation with 0.8 mol 8-nitreno-ATP/mol F_1) the biphasic character has disappeared to the largest extent. We may conclude that binding of an 8-nitreno-adenine nucleotide to an exchangeable non-catalytic site lowers the affinity of the catalytic sites for the substrate ATP. Apparently the modified site is responsible for the negative cooperativity in the mechanism of multi-site catalysis. The binding of the substrate ATP to this regulatory site seems to induce a decreased affinity of the catalytic sites for ATP.

Discussion

The labeling of mitochondrial F_1-ATPase with 8-azido-ATP by ultraviolet illumination during turnover with ATP, led to the identification of an exchangeable non-catalytic site located at an α/β interface. Ligation of this site with 8-nitreno-ATP has no influence on the catalytic activity of the enzyme when the measurement is carried out under V_{max} conditions. These results confirm the interpretation of the results obtained earlier by Van Dongen and Berden [5].

The non-catalytic sites can be divided in non-exchangeable sites, occupied by tightly-bound nucleotides, and exchangeable sites. The tightly-bound nucleotides do not dissociate and/or are not exchanged upon ammonium sulphate precipitation, during column centrifugation, during ATP hydrolysis or during incubation with a nucleotide analogue. The experiments of Van Dongen and Berden [5] showed, after combined labeling with 8-azido-AT(D)P, still two tightly-bound nucleotides being present on F_1. The F_1 preparations used in our experiments contained at the start of an experiment three tightly-bound nucleotides. One of them exchanged upon incubation with (NAP_3-) 2-azido-ADP and appeared to be bound at a catalytic site (Ref. 21, unpublished data). After labeling with 2-azido-ADP these F_1 preparations bound maximally four mol 2-nitreno-ADP per mol F_1 and contained still two tightly bound adenine nucleotides [21]. In the experiments presented in this paper the amount of covalently bound label never exceeded 3 mol per mol F_1. From the combination of the data just summarized we may conclude that the two non-exchangeable sites are not affected and still contained adenine nucleotides after labeling with 8-nitreno-AT(D)P as described in this paper. So labeling took only place at the exchangeable sites, catalytic as well as non-catalytic.

Of the four exchangeable sites three sites exchange bound nucleotides upon ATP hydrolysis and are for this reason generally assumed to be catalytic [29]. The

remaining exchangeable site exchanges bound nucleotides very slowly: the (slowly) exchangeable non-catalytic site of bovine heart F_1 has been described by Kironde and Cross [27,30]. Earlier experiments with 8-azido-AT(D)P [5] and recently with 2-azido-ADP [21] pointed to the existence of two catalytic and two exchangeable non-catalytic sites. The labeling experiments with 8-azido-ATP and 8-azido-ADP confirm the existence of two distinct exchangeable non-catalytic sites. One is modified by both 8-azido-analogues, the second only by 8-azido-ADP. The arguments for this conclusion can be formulated as follows:

First: compared with 8-azido-ATP, 8-azido-ADP labels three instead of two sites after illumination in the absence of turnover. (In the presence of turnover the 8-azido-ADP at this additionally labeled site exchanges largely with ATP.) The β/α label distribution ratio and the linear relationship between inhibition and amount of covalently bound label, indicate that only one catalytic and two non-catalytic sites are labeled. If the site, additionally labeled with 8-azido-ADP and not with 8-azido-ATP, had been a catalytic site, the β/α label distribution ratio had to be 5:1 (i.e., two β-sites and one α-β site) instead of 2:1 (one β-site and two α-β sites), and the relationship between binding and inhibition should have been non-linear. The linear relation between inhibition and amount of covalently bound label can only be explained by one catalytic site which induces complete inhibition upon covalent binding and two non-catalytic sites with no influence on the enzyme activity upon covalent binding (see dotted lines in Fig. 3). When two catalytic sites are labeled, e.g., with 2-azido-AT(D)P, this relation is indeed not linear [31].

Second: after labeling of one exchangeable non-catalytic site with 8-nitreno-ATP or 8-nitreno-ADP, a labeling with NAP_3-$2N_3$ADP under turnover conditions gave identical results as obtained with a control preparation, not labeled with 8-nitreno-AT(D)P. We have shown previously that under these circumstances (NAP_3-)2-azido-ADP binds at a slowly-exchangeable non-catalytic site, inducing a 45% inhibition of the ATPase activity (Ref. 21, unpublished results). From this we may conclude that 8-nitreno-AT(D)P and (NAP_3-)2-azido-ADP are bound at two distinct exchangeable non-catalytic sites.

The dotted curves in Fig. 3 show the expected relation between binding and inhibition for various possibilities for the number of labeled catalytic sites. For the case that one catalytic site and two non-catalytic sites are modified, the possibility is included that modification of one of the non-catalytic sites causes 45% inhibition [21]. The points indicate not only that one catalytic and two non-catalytic sites are modified, but also that occupation of the two non-catalytic sites does not affect activity. We have to conclude, then, that occupation of the slowly exchangeable site with an adenine nucleotide in the syn-configuration (the 8-nitreno-analog) does not induce a partial inhibition of the ATPase activity, while occupation with a nucleotide in the anti-configuration does (Ref. 21 and Table I).

We are unsure as to the reason for our finding slightly lower ratios at low concentrations of ligand than at higher concentrations, especially under conditions of turnover. Although not evident from the inhibition, it is possible that at the higher concentrations of 8-azido-ADP the catalytic site is not fully exchanged with ATP and some 8-nitreno-ADP is bound at catalytic sites, causing an increased ratio of the label distribution. Another possibility is that the second non-catalytic site has a lower affinity for 8-azido-ADP than the first one and that the label distribution for covalent binding to this site is not 1:1, but slightly higher.

As shown by kinetic measurements, the negative cooperativity of ATP hydrolysis is largely lost after covalent binding of 0.8 mol 8-nitreno-AT(D)P to an exchangeable non-catalytic site. Covalent binding of the ligand apparently induces a high K_m value (i.e., low-affinity state) for the substrate also at low concentrations of ATP. With non-modified F_1 the K_m is low at low ATP concentrations and increases at higher ATP concentrations. The binding of an adenine nucleotide to this one non-catalytic site apparently decreases the affinity of the catalytic sites for ATP. Our data agree with the model for negative cooperativity as proposed previously by Recktenwald and Hess [17]. In experiments with F_1 from *Saccharomyces cerevisiae* the ions sulphate, phosphate and perchlorate [17] and the ADP chromium complex α,β-CrADP [32] induced the low-affinity state, while certain ions like sulphite and maleate [17,18], induced the high-affinity state. Recently a regulatory site located on the α subunit of mitochondrial F_1-ATPase from *Schizosaccharomyces pombe* was described [33,34]. A mutation located in the nucleotide binding site of the α subunit (i.e., α-Gln173 to Leu) induced a lower affinity for ADP, decreased the sensitivity for inactivation by FSBA, (a ligand which is supposed to bind specifically only to non-catalytic sites [35]) and affected the negative cooperativity of ATP hydrolysis. This mutant shows besides the existence of a regulatory site which controls the negative cooperativity, also that the α subunits form at least part of the domain of the non-catalytic sites.

Specific labeling of the exchangeable non-catalytic sites by protection of the catalytic sites was not very successful. Protection by ITP gave a more or less specific labeling of the non-catalytic sites when 8-azido-ADP was used. In all other cases protection of sites by ITP or PP_i was visible, (as lower amounts of covalently bound 8-nitreno-AT(D)P and lower inhibi-

tion levels compared with the control experiment) but this was not specific towards the catalytic sites. The difference between 8-azido-ADP and 8-azido-ATP in the presence of ITP can be the difference in number of modified sites; three instead of two. Competition between ITP/PP_i versus 8-azido-AT(D)P for binding to the catalytic sites can not be the explanation. In that case deviations from the label ratios would have been expected (i.e., shift to the α-subunit). ITP and PP_i seem capable to bind at exchangeable sites, the catalytic sites as well as the non-catalytic sites, ITP possibly one, PP_i possibly both exchangeable non-catalytic sites. The finding that PP_i binds to exchangeable non-catalytic sites is in agreement with the work of Kalashnikova et al. [36] with PP_i and the work of Michel et al. [37] with azido-nitrophenyl-PP_i. Recently Peinnequin et al. [38] postulated three PP_i binding sites, located in such a way that nucleotide binding at both catalytic and non-catalytic sites is affected by PP_i. The sum of bound nucleotides and bound PP_i never exceeded 6 mol per mol of F_1. These data agree with our finding that PP_i affects nucleotide binding on both catalytic and non-catalytic sites. An explanation less complicated than the postulation of PP_i binding sites would be that PP_i binds just to exchangeable nucleotide binding sites, catalytic- as well as exchangeable non-catalytic sites.

In conclusion: our data in combination with work published before [5,21] show that isolated F_1 contains two distinct exchangeable non-catalytic nucleotide binding sites. Both sites have a regulatory function, one affects the negative cooperativity, the other the V_{max} of ATP hydrolysis. Because of the presence of two non-exchangeable binding sites and a total of six nucleotide binding sites, the number of catalytic sites can not be higher than two. Two catalytic sites, however, are sufficient to explain the kinetics of ATP hydrolysis, since the observed negative cooperativity can be explained by the influence of one of the non-catalytic sites: binding of a nucleotide to this site lowers the affinity of the catalytic sites for ATP.

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