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Analysis of the inhibitory non-catalytic ADP binding site on mitochondrial F_1, using NAP_3-$2N_3$ADP as probe. Effects of the modification on ATPase and ITPase activity

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Abstract

The ADP analogue NAP_3-$2N_3$ADP is able to bind to one or two high-affinity sites on mitochondrial F_1-ATPase, depending on the nucleotide content of the F_1 preparation. In both cases studied (enzyme with three bound nucleotides and enzyme with four bound nucleotides), the binding is accompanied by the exchange of one tightly-bound adenine nucleotide and nearly complete inhibition of the ATPase activity upon UV illumination. In both cases the ADP-analogue binds at a high-affinity catalytic site, replacing a bound nucleotide. The apparent K_D value for the exchange equals 25–30 μM, but the newly bound ligand does not dissociate. With F_1 containing 3 bound nucleotides NAP_3-$2N_3$ADP is able to bind to a second high-affinity site as well. This binding induces already in the absence of illumination 45% inhibition of the ATPase activity. The additionally bound molecule does not exchange within a short period of turnover with Mg-ATP. Therefore it has to be bound at a slowly exchangeable non-catalytic site, with a regulatory influence on the activity of the enzyme. Binding of NAP_3-$2N_3$ADP to this non-catalytic site is influenced by the presence of Mg^{2+} or EDTA: tight binding requires Mg^{2+} and in the absence of Mg^{2+} and presence of EDTA the ligand is removed from this site relatively easily, just like ADP. The presence of EDTA instead of Mg^{2+} lowers the measured affinity of this site for NAP_3-$2N_3$ADP with a factor 5. Kinetic measurements after an incubation of F_1 with NAP_3-$2N_3$ADP show a decrease of the V_{max} with ATP as substrate, without effect on the two measured K_m values. With ITP as substrate, however, incubation of F_1 with NAP_3-$2N_3$ADP results in an increase of the K_m values, without effect on the V_{max}. Comparison of our data with the literature shows that this non-catalytic site is not the site responsible for hysteretic inhibition by ADP. We conclude that this latter form of inhibition is observed when ADP or a suitable analogue is bound at the first (potentially catalytic) β-site, in disagreement with the conclusions of Jault and Allison (J. Biol. Chem. 269 (1994) 319–325).

Keywords: F_1; ATPase; Photoaffinity labeling; NAP_3-2-azido-ADP; Tight binding; Cooperativity; Non-catalytic site; Hysteretic inhibition

1. Introduction

Mitochondrial F_1-ATPase, the soluble part of the F_1-F_o ATPsynthase complex, is composed of five different subunits: α, β, γ, δ and ϵ, present in a stoichiometry of 3:3:1:1:1, respectively [1,2]. The large subunits (α and β) are arranged in a hexagonal structure of alternating α and β subunits with the three small subunits (γ, δ and ϵ) in the centre, asymmetrically associated with one of the α/β pairs [3–5]. The six nucleotide binding sites on this enzyme [6] are located on the large subunits [7,8], three at each of the β-subunits and three at α/β interfaces. In the reaction catalysed by isolated F_1 -the hydrolysis of ATP- not all these six sites are equally involved. It is generally accepted that the catalytic sites are located on the β subunits [8,9], functioning in a cooperative manner [10,11]. Although many authors assume that all three β-sites are equally involved in catalysis, only two sites can be experimentally defined as involved in cooperative catalysis. We have previously shown that two α/β-sites with quite different properties can be identified as exchangeable non-catalytic sites [12,13]. This implies that of the two non-exchangeable nucleotides present in isolated F_1 [6,8], only one can be located at an α/β-site. The other one, therefore, has to be located at a β-site. In combination with structural data [4] we have concluded that both non-ex-

Abbreviations: NAP_3-$2N_3$ADP, 3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)-amino]-propionyl} 2-azidoadenosine-5′-diphosphate; TDAB, tetradecyltrimethylammonium bromide.

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changeable nucleotides are bound at the same α/β-pair. This pair is not directly involved in catalysis.

In the work of Kironde and Cross [14,15], a distinction between catalytic and non-catalytic sites has been made on the basis of exchangeability during ATP hydrolysis. Three sites exchange rapidly under ATP hydrolysis conditions and are called catalytic, the other three do not exchange under turnover conditions and are called non-catalytic. One of these latter sites appeared to be a slowly exchangeable site. This site releases bound nucleotides upon precipitation with ammonium sulfate or in the presence of EDTA, and becomes rapidly filled with adenine nucleotides during Mg-ATP hydrolysis or after addition of Mg-ADP. This site is identical with the site at which bound ADP or 2-azido-ADP cause partial inhibition, as described in [16]. Since two sites are non-exchangeable and one is slowly exchangeable, the model of Cross implies that no rapidly exchangeable α/β-site exists, in contrast with the data on binding of 8-azido-adenine nucleotides [12,13].

Regulation of enzyme activity has been proposed as a possible function for the non-catalytic sites. Examples of regulation are the hysteretic inhibition, induced by ADP or some ADP analogues [17,18], the ADP-induced partial inhibition of bovine-heart F_1-ATPase [16,19], the ATP-stimulated dissociation of ADP from a catalytic site [20,21] and the ATP-induced increase of steady-state activity [22,23]. In the two cases of inhibition by ADP mentioned, the inhibition is supposed to be the result of binding of ADP (or an ADP analogue) to a non-catalytic site, in distinction of the inhibition by ADP bound at a catalytic site [20,21]. By using 2-azido-ADP we have shown that the partial inhibition of bovine-heart F_1-ATPase activity induced by ADP is indeed the result of its binding to a (slowly exchangeable) non-catalytic site, an α/β-site [16]. For the site of ADP binding in the case of hysteretic inhibition, real evidence is still lacking and we have proposed previously that hysteretic inhibition is related to binding of ADP at a β-site [12]. The ADP analogue 3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl} 2-azidoadenosine-5′-diphosphate (abbreviated as NAP_3-$2N_3$ADP) induces an inhibition of the bovine heart F_1-ATPase comparable with that induced by ADP and 2-azido-ADP ([16], Table 2). The ATP form of this adenine nucleotide analogue has been reported to bind to nucleotide binding sites (both catalytic as well as non-catalytic) of F_1-ATPase from the thermophilic bacterium PS3 [24]. The use of NAP_3-$2N_3$ADP as photoaffinity analogue has the advantage that it shows the same binding characteristics as ADP and that, because of its two azido groups, the efficiency of covalent binding upon UV irradiation is very large (more than 80%). In the present study, the number and type of sites (catalytic or non-catalytic) of bovine-heart F_1-ATPase which are able to bind NAP_3-$2N_3$ADP are determined, in relation with the inhibition induced by this binding. Also some factors which influence this inhibition were investigated, such as the number of (tightly) bound nucleotides and the presence of Mg^{2+} or EDTA in the incubation medium. Additionally, the kinetics of F_1-ATPase were measured after dark incubation with this ADP analogue, using ATP as well as ITP as substrate. Finally, our results on binding and inhibition are used to interpret the reported data on hysteretic inhibition induced by ADP or suitable analogues. The conclusion is drawn that hysteretic inhibition requires binding of ADP at a β-site, in disagreement with the conclusion of Jault and Allison [25].

2. Materials and methods

2.1. Preparation of F_1

F_1-ATPase was isolated from bovine hearts according to the method of Knowles and Penefsky [26] and stored in liquid nitrogen in 10 mM Tris-HCl (pH 7.5), 250 mM sucrose, 4 mM ATP and 4 mM EDTA. From the isolated F_1-ATPase, stored in liquid nitrogen, preparations were made containing three or four bound adenine nucleotides. F_1 samples containing three tightly-bound nucleotides were prepared as described previously [17]. After the last step of this procedure (dilution of F_1 to 2 mg protein ml^{-1}), dependent on the experiment, Mg^{2+} or EDTA was added to a concentration of 4 mM from an 1 M $MgCl_2$ or a 500 mM EDTA (pH 7.5) solution, respectively. These preparations had a specific activity of 145–165 μmol ATP min^{-1} (mg protein)$^{-1}$.

Preparations with four bound nucleotides were obtained by adding Mg^{2+} to a concentration of 10 mM to the stored F_1. After 10 min, one ammonium sulfate precipitation was performed by adding an equal volume of saturated ammonium sulfate (pH 7.0–7.5). After 10 min on ice the precipitate was spun down for 2 min at $10\,000 \times g$. The pellet was dissolved in 50 mM Tris-HCl (pH 7.5), 150 mM sucrose and 4 mM Mg^{2+}. Residual salt and loosely bound nucleotides were removed by filtrating the sample once by column centrifugation as described by Penefsky [27]. Columns of 1×5 cm, containing Sephadex G-50 coarse equilibrated in the above mentioned buffer, were centrifuged for 1 min at 2000 rpm in a Homef LC-30 table centrifuge. These preparations were diluted to 2 mg protein ml^{-1}. The specific activity of these preparations was 100–120 μmol ATP min^{-1} mg^{-1}.

2.2. Synthesis of NAP_3-$2N_3$ADP

NAP_3-$2N_3$ADP was synthesized by esterification of *N*-(4-azido-2-nitrophenyl)-β-alanine with 2-azido-ADP as described by Jeng and Guillory [28]. 2-Azido-ADP was synthesized as described in [16]. In the synthesis of [^{3}H]NAP_3-$2N_3$ADP, β-[3-^{3}H]alanine (from NEN Dupont) was used for the synthesis of *N*-(4-azido-2-nitrophenyl)-β-alanine. The preparations used had a concentration of about 2 mM and were stored in DMSO at $-20°$ C. The

spectra of the preparations used were identical with reported spectra [28]. The specific activity of the [^{3}H]NAP_3-$2N_3$ADP preparations was 40000 dpm/nmol.

2.3. Incubation and labelling of F_1-ATPase with NAP_3-$2N_3$ ADP

F_1 was incubated with concentrations between 0 and 200 μM NAP_3-$2N_3$ADP for 1 h in the dark (see Results). After this incubation usually excess and loosely bound label was removed by column centrifugation as described above. The gel material of these columns was equilibrated with the same buffer as used during the dark incubation. Illumination was performed with a CAMAG UV lamp at 350 nm, for 2×20 min, separated by 20 min in the dark. The procedure for illumination under turnover has been described in [16].

ATPase activities, both after dark incubation and illumination, were measured with an ATP regenerating system as described in [16]. Inhibitions were calculated relative to the activity of control samples treated in the same way as the samples incubated with NAP_3-$2N_3$ADP except that no NAP_3-$2N_3$ADP was added.

2.4. Determination of bound [^{3}H]NAP_3-$2N_3$ ADP

In the binding studies bound label was determined after column centrifugation of samples incubated in the dark with different concentrations [^{3}H]NAP_3-$2N_3$ADP. Covalently bound label after illumination was determined as described in [16]. Radioactivity (^{3}H) was measured on samples in glass vials with Packard Scintillator 299 scintillation cocktail in an LKB 1214 Rackbeta liquid scintillation counter.

Tetradecyltrimethylammonium bromide (TDAB) gel electrophoresis of F_1 samples with covalently bound [^{3}H]NAP_3-$2N_3$ADP was performed as described by Penin et al. [29] with the adjustments of Fellous et al. [18]. After staining with Coomassie Brilliant Blue the gels where scanned with an Bio-Rad Model 1650 scanning densitometer. The Coomassie-stained gels were sliced by cutting out the stained bands and protein was extracted from these slices with 1 ml 50% Solvable (NEN Dupont), during 48 h at 50° C. After this period 100 μl acetic acid and 6 ml scintillation cocktail were added and after repeated mixing radioactivity was measured in the scintillation counter as mentioned before.

2.5. Protein and nucleotide determination

Protein concentrations were measured with the method of Lowry et al. [30], with bovine serum albumine as standard. Bound nucleotides (ATP and ADP) were determined luminometrically as described [31].

3. Results

3.1. Dissociation of bound nucleotides from F_1 upon incubation with NAP_3-$2N_3$ ADP

Our F_1 preparations contain after two ammonium sulfate precipitations and two column centrifugation steps, all in EDTA-containing medium, three bound nucleotides, about two ATP and one ADP (Table 1A, see also [16]), at variance with the preparations isolated by the groups of Allison and Gautheron, which contain only 2.2 mol of bound nucleotide (2 ADP and 0.2 ATP) per mol F_1 [18,32]. After subsequent addition of Mg^{2+} a time-dependent shift to one ATP and two ADP is induced (Table 1, compare A, B and D). An additional column centrifugation step has no influence on the amount of bound nucleotides (Table 1D), so these three nucleotides may be considered as tightly bound.

When the stored F_1 (4 mM ATP present) was precipitated with ammonium sulfate in Mg^{2+} containing medium, followed by a column centrifugation step, also in the presence of Mg^{2+}, four nucleotides are bound to F_1, one ATP and three ADP (Table 1E). The presence of Mg^{2+} instead of EDTA during the preparation period, possibly in combination with a lower number of ammonium sulfate precipitations and column centrifugation steps, results in retaining one extra bound nucleotide (ADP) per F_1.

Incubation of F_1 containing three tightly-bound nucleotides (F_1(3)) with 100 μM NAP_3-$2N_3$ADP in the presence of Mg^{2+}, resulted in dissociation of one nucleotide, an ADP (Table 1C). Incubation of F_1 containing four bound nucleotides (F_1(4)) with 100 μM NAP_3-$2N_3$ADP, resulted in dissociation of about 1.4 adenine nucleotides per F_1, also ADP (Table 1F). Under these latter conditions also the control experiment (Table 1G) showed

Table 1
Bound nucleotides in F_1 preparations (expressed as mol per mol F_1), as determined luminometrically

	Total	ATP	ADP
A	2.95 ± 0.07	2.21 ± 0.05	0.74 ± 0.11
B	2.98 ± 0.04	1.35 ± 0.13	1.63 ± 0.11
C	2.01 ± 0.08	0.86 ± 0.05	1.15 ± 0.12
D	2.92 ± 0.10	0.81 ± 0.01	2.11 ± 0.10
E	3.92 ± 0.14	0.96 ± 0.05	2.96 ± 0.17
F	2.50 ± 0.16	0.91 ± 0.14	1.59 ± 0.27
G	3.72 ± 0.17	0.84 ± 0.15	2.88 ± 0.27

Results are the average of three experiments ± S.D. A. Stored F_1 after two ammonium sulfate precipitations and two column centrifugation steps in EDTA buffer (0.2 mM EDTA in last column); B. A after addition of 4 mM Mg^{2+}; C. B after incubation with 100 μM NAP_3-$2N_3$ADP for 1 h in the dark, followed by a column centrifugation step; D. control experiment of C (no NAP_3-$2N_3$ADP added); E. stored F_1 after addition of 10 mM Mg^{2+} followed by one ammonium sulfate precipitation and one column centrifugation step in Mg^{2+}-medium; F. E after incubation with 100 μM NAP_3-$2N_3$ADP for 1 h in the dark, followed by a column centrifugation step; G. control experiment of F (no NAP_3-$2N_3$ADP added)

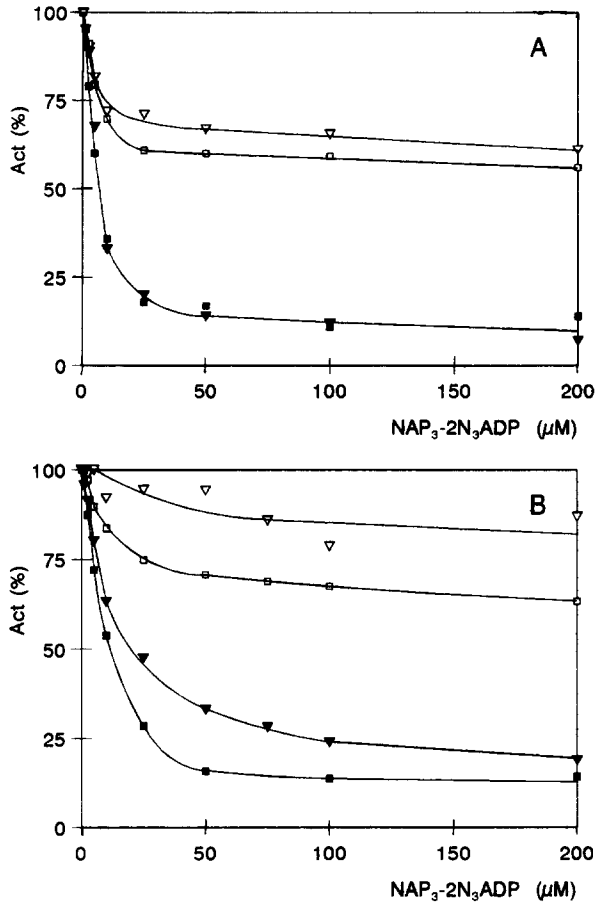

Fig. 1. The inhibition of ATPase activity after incubation of $F_1(3)$ with 0–200 μM NAP_3-$2N_3$ADP in Mg-medium (A) or EDTA-medium (B) (see Materials and methods). Inhibition was measured after one hour in the dark (□), followed by illumination (■); inhibition was also measured after dark incubation and removal of excess and loosely-bound label (▽), followed by illumination (▼). Illumination was performed with a CAMAG lamp as described in materials and methods.

some loss of bound ADP. Since an additional column centifugation step (after 1 h) removes some of the bound nucleotides in $F_1(4)$ preparations, the nucleotide additionally bound in these preparations seems to dissociate slowly. The binding of this nucleotide is therefore not as tight as that of the other three tightly-bound nucleotides.

3.2. *Influence of Mg^{2+} and EDTA on binding of NAP_3-$2N_3$ ADP*

F_1-ATPase with three tightly-bound nucleotides was incubated with NAP_3-$2N_3$ADP in concentrations between 0 and 200 μM, in a medium containing Mg^{2+} or EDTA. In Mg^{2+}-medium the ATPase activity of $F_1(3)$, measured after dark incubation with NAP_3-$2N_3$ADP, was inhibited for maximally 40–45% (Fig. 1A). Maximal inhibition was already reached at ligand concentrations of 25–50 μM. After removal of excess and loosely bound ligand by column centrifugation, the maximal inhibition was slightly less (max. 40%). This indicates that the site responsible for the measured inhibition is still nearly fully occupied with NAP_3-$2N_3$ADP. UV illumination gave in both cases (in the presence of excess ligand as well as after removal of the excess) an inhibition curve which was dependent on the (original) ligand concentration. Nearly complete inhibition of enzyme activity was reached at an (original) concentration of 100 μM.

In EDTA-medium (Fig. 1B) the maximal inhibition after dark incubation was slightly lower, around 35%. Maximal inhibition was only reached at higher concentrations of ligand (> 100 μM), compared with the experiments in Mg^{2+}-medium. When excess ligand was removed, maximal inhibition decreased to 15–20%, so part of the inhibitory site had lost the ligand as consequence of the column centrifugation. UV illumination resulted again in nearly complete inhibition. Compared with illumination in the presence of excess ligand, complete inhibition by illumination after removal of the excess was reached at slightly higher original concentrations. The inhibition curve in the first situation (illumination in the presence of excess ligand) is very similar to the analogous curve in Mg^{2+}-medium. The inhibition of ATPase activity after dark incubation, whether followed by removal of excess ligand or not, is clearly influenced by the used media: in EDTA-medium higher ligand concentrations are needed for inhibition and removal of excess ligand results in a decrease of the observed inhibition.

The amount of covalently bound ^{3}H-labelled ligand after illumination was determined. At first, the amount of covalently bound label was related to the measured inhibition when excess and loosely-bound [^{3}H]NAP_3-$2N_3$ADP were removed before illumination (Fig. 2). In Mg^{2+}-medium as well as in EDTA-medium at least 2 mol of covalently bound label per mol F_1 were needed for complete inhibition. In Mg^{2+}-medium the relation between amount of covalently bound label and inhibition is nearly linear. In EDTA-medium this relation is biphasic. The extrapolation of the first part shows that for complete inhibition the covalent binding of 1 mol label per mol F_1 would be sufficient, indicating that the site with the highest affinity results in complete inhibition after covalent attachment. The second site apparently binds with a lower affinity, which is not the case in the presence of Mg^{2+}. NAP_3-$2N_3$ADP seems to be able to bind at two high-affinity sites on F_1 preparations with three bound nucleotides, independent of the used incubation medium. One of the sites induces full inhibition after covalent attachment of the ligand, the other one causes partial inhibition already without covalent attachment. The binding of the ligand to this latter site is influenced by the presence of Mg^{2+}.

3.3. *Influence of bound nucleotides*

F_1-ATPase preparations with four bound nucleotides were incubated with [^{3}H]NAP_3-$2N_3$ADP in Mg^{2+}-medium and inhibition of ATPase activity was measured as men-

tioned before (Fig. 3A). After dark incubation the maximal inhibition measured was only about 5–10%, reached at a ligand concentration of 25 μM. This inhibition is not influenced by removal of excess and loosely-bound ligand. UV illumination after removal of excess ligand results in nearly complete inhibition of enzyme activity at an original ligand concentration of 200 μM. After illumination, a linear relation between covalently bound label and inhibition was found. Extrapolation shows that for complete inhibition of ATPase activity the covalent binding of 1 mol ligand per mol F_1 is required.

Compared with $F_1(3)$ preparations, $F_1(4)$ preparations are able to bind only 1 mol of NAP_3-$2N_3ADP$ per mol F_1 with a high affinity, instead of 2. Binding of ligand to $F_1(4)$ induces little inhibition of ATPase activity after dark incubation but gives complete inhibition after UV illumination. Therefore, it seems that under these circumstances the label is bound to a high-affinity catalytic site. Starting with $F_1(3)$, a second mole of label is bound with high affinity, which induces a partial inhibition of ATPase activity after dark incubation. This additional mole of ligand, therefore, does not exchange rapidly and is bound at a site where no turnover takes place, a non-catalytic site. The extra nucleotide that is present in $F_1(4)$ preparations and prevents the binding of a second NAP_3-$2N_3ADP$ molecule, must be bound at this same non-catalytic site, which is partially inhibitory when a diphosphate is bound.

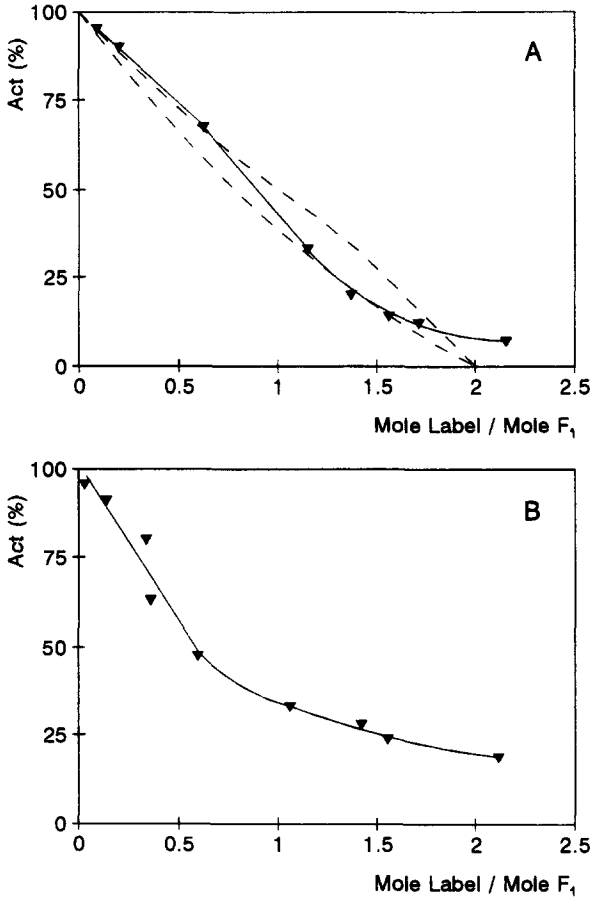

Fig. 2. Inhibition of ATPase activity related to amount of covalently bound [^{3}H]NAP_3-$2N_3ADP$ to $F_1(3)$ in mol ligand per mol F_1. Incubation of $F_1(3)$ in Mg-medium (A) or EDTA-medium (B) with variable concentrations of [^{3}H]NAP_3-$2N_3ADP$ was followed by removal of excess and loosely-bound label and illumination before the activity was determined. The dotted lines are explained in the Discussion section.

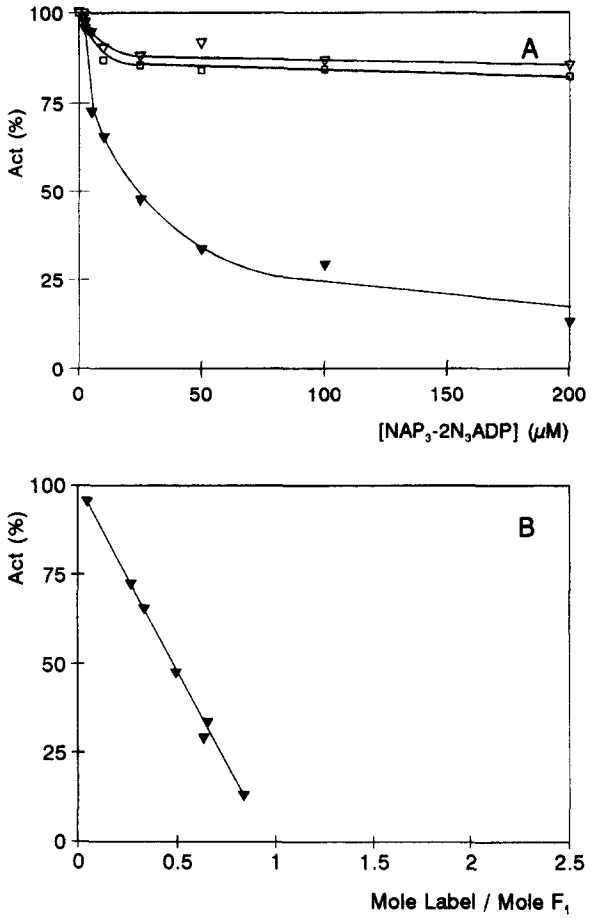

Fig. 3. (A) Inhibition of ATPase activity after incubation of $F_1(4)$ with 0–200 μM NAP_3-$2N_3ADP$ in Mg^{2+}-medium after 1 h in the dark (□), followed by removal of excess and loosely-bound label (▽) and after illumination when excess and loosely-bound label was removed before illumination (▼). (B) Inhibition of ATPase activity related to covalently bound [^{3}H]NAP_3-$2N_3ADP$ to $F_1(4)$ in mol ligand per mol F_1, determined after removal of excess and loosely-bound label and illumination in Mg-medium.

3.4. Determination of binding constants

Binding of [^{3}H]NAP_3-$2N_3ADP$ to F_1 after dark incubation was analysed by a Scatchard plot (Fig. 4) and the Hill coefficient (α_H) was determined. With $F_1(4)$ preparations in Mg^{2+}-medium about 1 mol NAP_3-$2N_3ADP$ ($n = 0.94$) was bound per mol F_1 with $K_D = 35$ μM, and $\alpha_H = 0.96$. $F_1(3)$ preparations in Mg^{2+}-medium show two binding phases: about 1 mol label ($n = 0.88$) binds with a K_D of about 7 μM and one ($n = 0.98$) with a K_D of about 23 μM. The α_H was 0.86, which points to independent sites or a slightly negative cooperativity for NAP_3-$2N_3ADP$

binding to these two sites. In EDTA-medium the Scatchard plot for binding of NAP_3-$2N_3$ADP to $F_1(3)$ shows some positive binding cooperativity for about 2 mol label per mol F_1. But the α_H is 1.20 which does not deviate much from a Hill coefficient for independent binding ($\alpha_H = 1$). When the results are treated as independent binding of the ligand (in which case the points at low concentrations are neglected), about 2 mol of label ($n = 1.98$) appear to be bound per mol F_1, with a mean K_D of 21 μM.

The affinity of NAP_3-$2N_3$ADP for one of the two high-affinity sites on $F_1(3)$ which bind this analogue, is clearly different in Mg^{2+}-medium from that in EDTA-medium. The affinity in EDTA-medium is lower and is possibly positive cooperative. In this EDTA-medium the inhibition induced by NAP_3-$2N_3$ADP measured after dark incubation is only obtained at higher label concentration and decreases when excess label is removed, so the affinity of the inhibitory non-catalytic site is decreased in EDTA-medium.

3.5. Specific labelling of a non-catalytic site with NAP_3-$2N_3$ADP

After incubation in the dark of $F_1(3)$ with [^{3}H]NAP_3-$2N_3$ADP, illumination under turnover conditions was performed to investigate whether the inhibition of ATPase activity induced by NAP_3-$2N_3$ADP is really the result of binding to a high-affinity non-catalytic site which does not exchange during turnover of the enzyme. These conditions were created by adding ATP, PEP and pyruvate kinase before illumination (see [16]). The amounts of ATP and PEP added are sufficient to allow 2 min of turnover without the formation of ADP, preventing any competition between ADP and NAP_3-$2N_3$ADP. Covalent binding of label was induced by a 20 s illumination period with a Penray UV lamp. Spectral analysis of NAP_3-$2N_3$ADP samples illuminated in this way showed that this illumination time was long enough to photolyse all the azido groups present in NAP_3-$2N_3$ADP (results not shown). After dark incubation an inhibition of about 40% was measured (Table 2) as found before. Illumination without turnover resulted in nearly complete inhibition of enzyme activity and the covalent binding of 3 mol label per mol F_1. Illumination during turnover gave 47% inhibition and 1 mol label was covalently bound per mol F_1. Removal of excess and loosely bound [^{3}H]NAP_3-$2N_3$ADP after dark incubation resulted in the residual binding of 1.8 mol label per mol F_1 and a 32% inhibition of the ATPase activity. Upon illumination without turnover, 1.6 mol per mol F_1 became covalently bound, resulting in nearly complete inhibition of ATPase activity. Upon illumination in the presence of turnover the inhibition was 38% and 0.7 mol ligand was covalently bound per mol F_1.

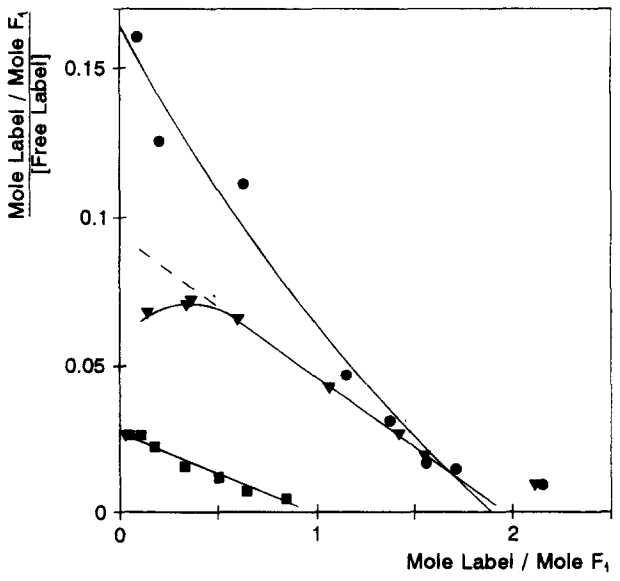

Fig. 4. Scatchard plot of [^{3}H]NAP_3-$2N_3$ADP binding to F_1 after dark incubation of $F_1(3)$ in Mg-medium (○), of $F_1(3)$ in EDTA-medium (▼) and of $F_1(4)$ in Mg-medium (□).

Table 2
Effect of incubation of $F_1(3)$ with 100 μM [^{3}H]NAP_3-$2N_3$ADP in Mg^{2+}-medium

	A		B	
	Inh. (%)	mol lab./mol F_1	Inh. (%)	mol lab./mol F_1
Dark	38±6		32±4	(1.8±0.1)
Ill.	94±2	2.98±0.2	88±4	1.60±0.05
Ill.+t.o.	47±2	1.03±0.3	38±6	0.70±0.1

The inhibition of ATPase activity and amount of covalently bound label was measured after: (1) dark incubation, (2) illumination and (3) illumination during turnover with Mg-ATP. A. Measurements performed in the presence of excess label, and B, before measurements excess and loosely bound label were removed. The number in parentheses gives the amount of non-covalently bound label. Results are the average of three experiments ± S.D.

We may conclude that $F_1(3)$ contains one binding site for NAP_3-$2N_3$ADP which does not loose or exchange the ligand during turnover. Binding of NAP_3-$2N_3$ADP at this site induces partial inhibition of the ATPase activity, both in the dark and after illumination. Therefore it can be seen as a regulatory non-catalytic site. There seems to be no difference whether NAP_3-$2N_3$ADP is covalently bound to this site or non-covalently. Without removal of excess ligand at least three sites contain bound ligand. At two sites the ligand is exchanged during turnover and at one of these two the ligand binds so weakly that it is also removed upon column centrifugation.

3.6. Inhibition of ATPase and ITPase activity

The activity assay in the previous paragraphs (at 5 mM ATP and 10 mM HCO_3^-) showed an inhibition of F_1-ATPase activity by NAP_3-$2N_3$ADP under V_{max} conditions. To determine the effect on other kinetic parameters, the activity of $F_1(3)$ was measured after incubation with NAP_3-

$2N_3$ADP using variable concentrations of ATP and ITP as substrate in the absence of HCO_3^-.

The negative cooperativity usually measured for ATPase, was not influenced by an incubation with NAP_3-$2N_3$ADP (Fig. 5A). The V_{max} of the low-affinity phase is 35% inhibited, an inhibition comparable with that measured before. The (extrapolated) V_{max} of the high-affinity phase seems less inhibited by NAP_3-$2N_3$ADP (16%), but the accuracy of this value is less than that of the V_{max} of the low-affinity phase. There is no significant effect on the K_m of both kinetic phases. When the inhibition of the ATPase activity induced by NAP_3-$2N_3$ADP is determined for each ATP concentration, this inhibition appears to be about the same for all ATP concentrations used (Fig. 5B).

For the ITPase activity of F_1 also negative cooperativity was measured, which was not affected by an incubation with NAP_3-$2N_3$ADP (Fig. 5A). The V_{max} values of both kinetic phases are not affected by NAP_3-$2N_3$ADP. On the other hand NAP_3-$2N_3$ADP induces a decrease of the affinity of the substrate when ITPase is measured: the K_m of both kinetic phases is increased by about 40%. As shown in Fig. 5B, the activity at low ITP concentrations is inhibited between 20 and 25% after an incubation with NAP_3-$2N_3$ADP (effect of increased K_m value), but this inhibition disappears at high ITP concentrations (no change in V_{max}). From the fact that occupation of this α/β site with ADP or an analogue does not affect the negative cooperativity of ATP hydrolysis it is evident that binding of ATP to this site is not responsible for the negative cooperativity. As we have shown previously [13], the binding of ATP to another α/β site, a low-affinity site with low specificity, is responsible for this phenomenon. Since the presence of ADP at the former high-affinity α/β-site causes partial inhibition, one can also state that binding of ATP to this site, always occurring during the assay in the absence of bound ADP, induces an increased rate of catalysis [contrast [23]].

3.7. Subunit identification of the binding sites for NAP_3-$2N_3$ADP on F_1-ATPase

In an attempt to analyse the sites where NAP_3-$2N_3$ADP binds covalently to F_1, samples of F_1 labelled with [^{3}H]NAP_3-$2N_3$ADP were analysed by TDAB gel electrophoresis and the amount of ^{3}H label, bound to the different subunits, was determined. Conditions were compared in which only a catalytic site (as in Fig. 3), a non-catalytic site (as in Table 2, illumination in the presence of turnover) or both sites (as in Table 2, illumination in the absence of turnover) were labelled. Fig. 6 shows the results of these experiments. Label was only found on the α and β subunits, not on the three small subunits. On the gels three small bands were visible above the α subunit, which were not present when F_1 or F_1 samples after dark incubation with [^{3}H]NAP_3-$2N_3$ADP were electrophoresed. These bands contained ^{3}H label. With the subunits of F_1 as molecular-weight markers a mass between 99–118 kDa could be determined. These bands represent crosslinks between the large subunits, α-α (107–118 kDa), α-β (104–111 kDa) and β-β (99–103 kDa), as a result of the two azido groups of NAP_3-$2N_3$ADP. The α-β crosslink is the most frequent one. These types of crosslink have also been described by Schäfer et al. [24]. The highest amount of crosslinking was found when a non-catalytic site was labelled, a site supposed to be located on the interface between an α and β subunit. Labelling of the α subunit was also found for the situations in which only a catalytic site was covalently modified. There is no clear difference in β/α labelling ratio between a catalytic and non-catalytic site. So for NAP_3-$2N_3$ADP it is not possible to distinguish between catalytic and non-catalytic sites on the basis of β/α labelling ratio as could be done for 8-azido-ATP (e.g., [8]). Analysis of labelled peptides from α and β subunits is necessary to determine at which domains

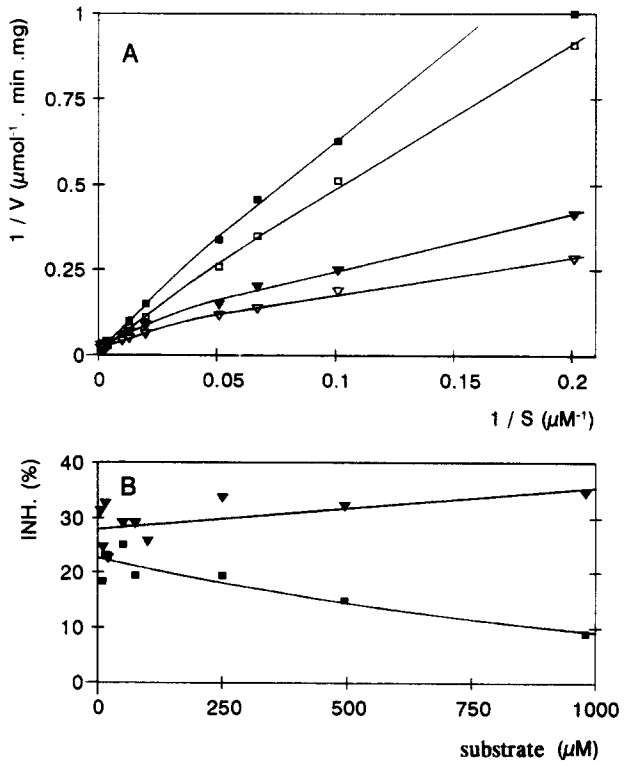

Fig. 5. (A) Lineweaver-Burk plot of ATPase and ITPase activity of F_1(3) after incubation in Mg^{2+}-medium with (▼,□) or without (▽,□) 100 μM NAP_3-$2N_3$ADP. Substrate was ATP (▽,▼) or ITP (□,□). The activities were measured in a regenerating system, following the NADH oxidation at 340 nm at 30° C in a medium containing 33 mM Tris-HCl (pH 8.0), 83 mM sucrose, 6 mM $MgCl_2$, 10 mM KCl, 2 mM PEP, 250 μM NADH, 12.5 U/ml pyruvate kinase and 4 U/ml lactate dehydrogenase. ATP and ITP concentrations were varied from 5 μM to 1 mM. ▽–▽, K_m values are 18 and 137 μM, V_{max} values 16 and 60 μmol min^{-1} mg^{-1}; ▼–▼, K_m values are 22 and 122 μM, V_{max} values 13 and 39 μmol min^{-1} mg^{-1}; □–□, K_m values are 70 and 746 μM, V_{max} values 16 and 142 μmol min^{-1} mg^{-1}; ■–■, K_m values are 97 and 1085 μM, V_{max} values 17 and 153 μmol min^{-1} mg^{-1}. (B) Inhibition of ATPase (▼) or ITPase (■) activity of F_1(3) by NAP_3-$2N_3$ADP at substrate concentrations between 5 μM and 1 mM.

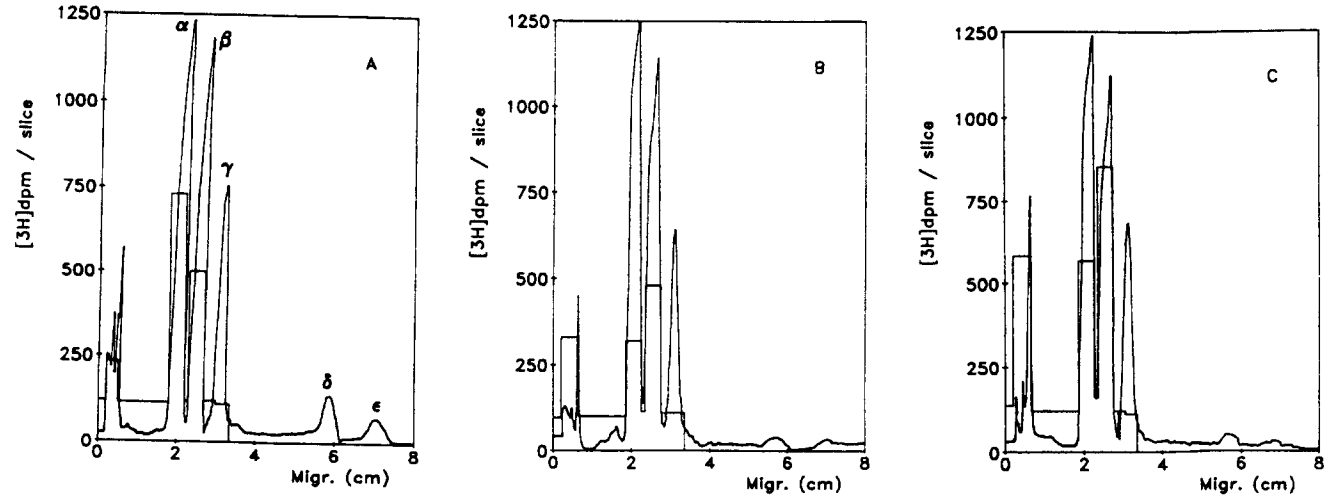

Fig. 6. TDAB gel electrophoresis of F_1 covalently modified with [^{3}H]NAP_3-$2N_3$ADP on the tight catalytic site (A), the slowly exchangeable non-catalytic site (B), or on both sites (C). Samples of 30 μg were placed on the gels. Shown are the scans of Coomassie-blue-stained gels and the ^{3}H label extracted from the gel slices after electrophoresis.

NAP_3-$2N_3$ADP covalently binds when a catalytic or non-catalytic site is modified. We may assume, however, that the $2N_3$-group of NAP_3-$2N_3$ADP, just like that of $2N_3$-ADP, mainly labels the β-subunit (at positions Tyr-345 and Tyr-368, respectively [16,33]), while the NAP_3-group mainly labels the α-subunit in both cases. This means that not only the non-catalytic sites, but also the catalytic sites are located near an interface between α- and β-subunits, in agreement with an early proposal of Williams and Coleman [34].

4. Discussion

The data reported show that the ADP analogue NAP_3-$2N_3$ADP is able to bind to at least three nucleotide binding sites on mitochondrial F_1-ATPase. Two sites bind this ligand with a sufficiently high affinity to retain it during column centrifugation: one has been characterized as a catalytic site, the other as a non-catalytic site whose occupation with NAP_3-$2N_3$ADP induces a 45% inhibition of the ATPase activity and on which no exchange occurs during a short turnover with ATP. The third and possibly fourth site have a lower affinity and do not retain label after a column centrifugation step or during turnover with Mg-ATP.

Schäfer et al. [24] used NAP_3-$2N_3$ATP as a substrate or competitive inhibitor for F_1-ATPase from PS3 and concluded that NAP_3-$2N_3$ATP binds specifically to nucleotide binding sites. Based on the different pattern of crosslink bands in comparison with the pattern obtained with NAP_3-$8N_3$ATP, Schäfer et al. concluded that with NAP_3-$2N_3$ATP labelling of both catalytic and non-catalytic sites occurs. With NAP_3-$8N_3$ATP labelling of catalytic sites only was claimed. Additional to the results with the PS3 ATPase, we have now determined the number of binding sites on mitochondrial F_1-ATPase for NAP_3-$2N_3$ADP. We could make a distinction between catalytic and non-catalytic sites on the basis of the inhibition induced by non-covalent and covalent binding of this ligand.

The high-affinity binding of NAP_3-$2N_3$ADP to F_1 is dependent on the presence of Mg^{2+} or EDTA during the treatment for removal of free and loosely bound nucleotides as well as during the incubation with the ligand. Preparation of F_1 in EDTA-medium results in an enzyme containing three bound nucleotides. Labelling of a high-affinity catalytic site and a slowly exchangeable non-catalytic site with NAP_3-$2N_3$ADP is then possible, while two of the original nucleotides remain bound. Label bound to the non-catalytic site induces partial inhibition of the ATPase activity. For the preparation of F_1 containing four bound nucleotides from an ATP-containing stock solution, Mg^{2+} must be present during ammonium sulfate precipitation and column centrifugation. With such a F_1 preparation no or very little inhibition after dark incubation with NAP_3-$2N_3$ADP was measured and only 1 mol label per mol F_1 was bound after a column centrifugation step. This 1 mol label induced total inhibition after covalent binding. Additionally, the specific activity of $F_1(4)$ preparations was 30% lower than that of $F_1(3)$. For the $F_1(4)$ preparations it may be concluded that the inhibitory non-catalytic site is already occupied with ADP and therefore high-affinity labelling with NAP_3-$2N_3$ADP of only a catalytic site is possible. For both F_1 preparations (with three or four bound nucleotides) a tightly-bound ADP is exchanged after incubation with NAP_3-$2N_3$ADP (with an apparent K_D of about 30 μM) and replaced by a tightly-bound NAP_3-$2N_3$ADP. Because both preparations show this exchange and covalent binding induces complete inhibition, the exchanged ADP was bound at a catalytic site.

With the $F_1(3)$ preparations the affinity of the slowly exchangeable non-catalytic site for NAP_3-$2N_3$ADP is several times lower in EDTA-medium than in Mg^{2+}-medium. This explains the higher label concentrations needed for maximal inhibition after dark incubation and the decrease of inhibition upon removal of excess label. For the tight binding of NAP_3-$2N_3$ADP to the slowly exchangeable non-catalytic site the presence of Mg^{2+} seems essential. The effect of EDTA on this inhibitory non-catalytic site appeared to be the same as the effect reported before by Kironde and Cross [14,15] for the binding of ADP to a slowly exchangeable non-catalytic site. The measured K_D value is higher than that for ADP (0.05 μM [15]), although the estimated rate of dissociation ($6 \cdot 10^{-4}$ s^{-1}, $t_{1/2}$ of about 20 min) is very similar. Since after 1 h of incubation no significant change in the concentration of bound ligand occurred, the system seemed to be close to equilibrium and the measured K_D value will be close to the real value, indicating that the k_{on} is not much higher than 10^2 M^{-1} s^{-1}, while for ADP binding this value is $9 \cdot 10^3$ M^{-1} s^{-1} [15].

The decrease of the affinity of NAP_3-$2N_3$ADP for the inhibitory non-catalytic site in EDTA-medium compared with Mg^{2+}-medium gives a good explanation for the higher label concentrations required for maximal inhibition after dark incubation. It remains to be explained, however, why some loss of inhibition (and of ligand bound at the non-catalytic site) is found after removal of excess NAP_3-$2N_3$ADP, while the ligand at the catalytic site remains fully bound, although the measured K_D is equal to or even higher than that of the non-catalytic site. Binding cooperativity between catalytic and non-catalytic sites, if any, cannot be a real explanation. The preferable (and simple) explanation is, however, that the measured K_D for the exchange at the tight catalytic site is actually the K_D value of the second catalytic site. When this second catalytic site binds a molecule of ligand, the two sites become equal [35]: at each moment one of them has a high affinity and the other a relatively low affinity, but they interchange. The ligand at the originally high-affinity site can dissociate and finally both sites become occupied with the added ligand. As soon as during column centrifugation one site has lost its ligand (the off-rate is relatively high), the remaining ligand is automatically tightly bound and will not dissociate any more. So, the measured K_D for the exchange of the tightly bound catalytic nucleotide is the K_D of the second catalytic site, while after passage through the column the remaining nucleotide is bound with a high affinity at the first catalytic site, resulting in a very low dissociation rate. We measure therefore a relatively high K_D for the exchange at a high-affinity site as a consequence of the well-documented negative cooperativity of binding to the catalytic sites. The ligand at the non-catalytic site, at the other hand, does not dissociate from the site during column centrifugation because of its low k_{off}, even in the presence of EDTA. In this case, therefore, the bound ligand after the column is identical with the bound ligand before the column step and the measured K_D is a real K_D, assuming that after 1 h of incubation equilibrium is obtained (see above).

From Fig. 1A one may conclude that the half-maximal inhibition of $F_1(3)$ after dark incubation with NAP_3-$2N_3$ADP in Mg-medium is obtained at a concentration between 5 and 10 μM, in agreement with the K_D of 7 μM obtained in the Scatchard plot of Fig. 4 for the site with the highest affinity. From the increase of inhibition after illumination, due to covalent binding of the analogue to the catalytic site (Fig. 1A), one may also derive that the K_D for binding of NAP_3-$2N_3$ADP to a catalytic site lies between 15 and 20 μM, again in agreement with the K_D value obtained from the Scatchard plot of Fig. 4 (K_D of 23 μM). In EDTA-medium (Fig. 1B) the binding of NAP_3-$2N_3$ADP to the non-catalytic site is clearly weaker and the K_D becomes similar and even lower than the K_D of the second catalytic site. Half-maximal inhibition after illumination is obtained at about 25 μM NAP_3-$2N_3$ADP, in agreement with the K_D found for binding to the catalytic site under these conditions (Fig. 4, K_D of 21 μM).

The curve representing mol covalently bound NAP_3-$2N_3$ADP per mol F_1 in relation with inhibition of $F_1(3)$ in Mg-medium is compared with a model based on the assumption that one non-catalytic site causing 45% inhibition and one catalytic site causing full inhibition after covalent attachment are involved in the binding (see Fig. 2A dotted lines). The upper line is based on the difference in affinity for NAP_3-$2N_3$ADP between the two sites as estimated from the Scatchard plot, the lower line is based on the assumption of equal affinity of NAP_3-$2N_3$ADP for both sites (mean K_D of 13 μM). The result suggests that some cooperativity is present by which the K_D of the (second) catalytic site decreases at increasing saturation of the non-catalytic site: the difference in affinity of both sites, visible at low label concentrations seems to change to equal affinity at higher concentrations.

The occupation of the slowly exchangeable non-catalytic site with NAP_3-2-N_3-ADP affects the ITPase activity quite differently from the ATPase activity. We have concluded earlier that at high ATP concentrations, when the exchangeable low-affinity non-catalytic binding site is occupied with ATP, the affinity of ATP (and ADP) for the catalytic sites is decreased, resulting in a higher K_m [13], without effect on V_{max}. For bovine heart F_1, the V_{max} can still be increased by anions like bicarbonate and sulfite. Since unmodified F_1 catalyses hydrolysis of ITP faster than that of ATP (at infinite substrate concentration), we may assume that with ATP as substrate the measured V_{max} is limited by the rate of dissociation of the product ADP and not by the rate of catalysis at the catalytic site, in contrast with the situation when ITP is the substrate. With a diphosphate nucleotide at the slowly exchangeable non-catalytic site the dissociation of product (ADP or IDP, respectively) is decreased and the measured V_{max} will

decrease in the case of ATP hydrolysis. But with ITP as substrate the dissociation of IDP is so fast that a decrease of this rate does not influence the V_{max}.

4.1. Hysteretic inhibition of F_1 by ADP

The inhibition of ATP hydrolysis by binding of ADP or a suitable analogue to the slowly exchangeable non-catalytic site is different from the hysteretic inhibition described by other groups [18,25] in as far as no hysteresis is detectable with our preparation. Literature data show that hysteretic inhibition occurs with preparations containing two tightly bound ADP per mol of enzyme, when a third ADP is bound. After previous depletion of the enzyme of nucleotides, followed by incubation with ADP, the hysteretic site is already occupied when two non-catalytic sites have bound an ADP, in addition to the high-affinity catalytic site [25,36]. Assuming that in the isolated enzyme both molecules of ADP are bound to non-catalytic sites (see, for example [36]), one may put the question why, starting from nucleotide-depleted enzyme, the hysteretic site is occupied when two non-catalytic sites contain ADP, while in freshly isolated enzyme occupation of three non-catalytic sites is required. Jault and Allison [25] have concluded that the hysteretic site is a non-catalytic α/β site on the basis of labelling of Tyr-368 with 2-azido-ADP. Their argument is not valid, however: under their conditions of labelling three non-catalytic sites are occupied with ligand, of which at least two are α/β-sites, and only 63% of the label is recovered in the main peak, representing the peptide with labelled Tyr-368. Since only 1 of the three sites is responsible for the hysteretic inhibition, this site may well be a different one. Hartog et al. [37] have shown previously that they could not identify the labelled peptide when only the first β-site was modified with 2-azido-ATP, although the labelling of α/β sites (Tyr-368) and catalytic β-sites (Tyr-345) was easily detected. When considering all the data in the literature, hysteretic inhibition, and especially the absence of this inhibition in the enzyme as isolated by us, cannot be explained on the basis of a model according to which three β-sites perform catalysis and three α/β-sites are non-catalytic, two containing very tightly bound nucleotides and one exhibiting a slightly lower affinity. Only one possibility is in agreement with all data, and that is that the first β-site is responsible for the hysteretic inhibition by ADP. We may assume that, in the isolated enzyme of Allison and Gautheron, this site is not occupied, since the enzyme loses bound ADP upon binding FSBA to the α/β-sites [38] and the enzyme performs single-site catalysis upon addition of substoicheiometric amounts of ATP, without pretreatment with phosphate or pyrophosphate [32], while two additional catalytic sites are still available for multi-site catalysis. Also, our own data from photolabelling studies with the

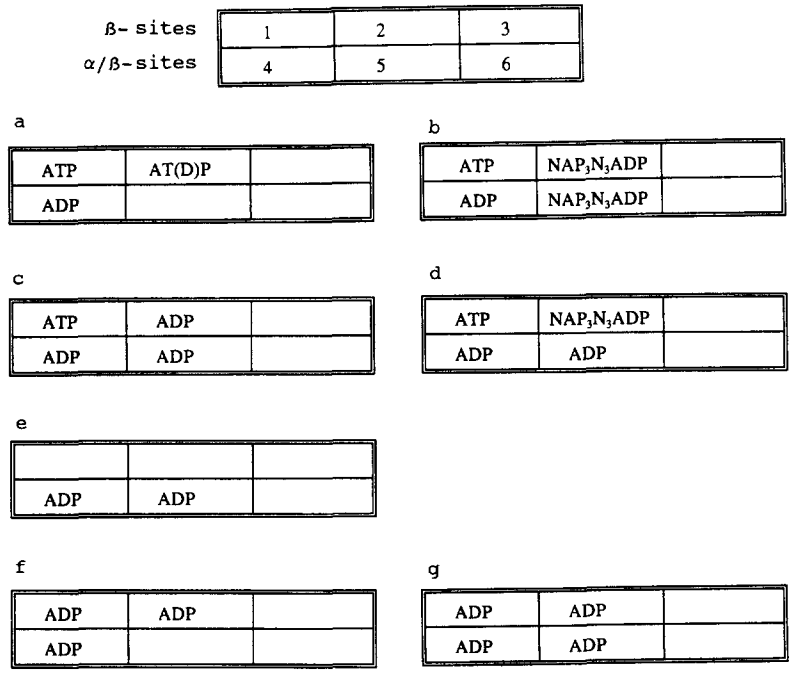

Fig. 7. Schematic representation of the occupation of the nucleotide binding sites in various preparations of F_1. Nucleotides at sites 1 and 4 are in $F_1(3)$ and $F_1(4)$ non-exchangeable, sites 2 and 3 are the two sites performing multi-site catalysis, site 5 is the slowly-exchangeable non-catalytic site and site 6 is the low-affinity non-catalytic site whose occupation with a nucleotide is responsible for the increased K_m values at high substrate concentration [13]. (a) $F_1(3)$; (b) $F_1(3)$ after incubation with NAP_3-$2N_3ADP$ in the presence of Mg^{2+}, followed by column centrifugation; (c) $F_1(4)$; (d) $F_1(4)$ after incubation with NAP_3-$2N_3ADP$ in the presence of Mg^{2+}, followed by column centrifugation; (e) F_1 preparation of Allison. ATP (0.2 mol/mol) is present at site 1 in 20% of the enzyme molecules; (f) nucleotide-depleted F_1 after incubation with ADP, followed by column centrifugation; (g) nucleotide-depleted F_1 after incubation with ADP + Mg^{2+}, followed by column centrifugation.

chloroplast ATPsynthase (F. Possmayer, A.F. Hartog, P. Gräber and J.A. Berden, unpublished observations) show that the first β-site can perform single-site catalysis. Our MF_1 preparation, however, just like the enzyme isolated in Penefsky's laboratory, requires pretreatment with phosphate or pyrophosphate to be able to perform single-site catalysis (unpublished data). We know from the data of Hartog et al. [37], confirmed by our recent experiments with the chloroplast enzyme (unpublished data) that for an active enzyme ATP has to be bound at this site. When this site becomes occupied with ADP an inactive conformation is induced after the start of ATP hydrolysis. The nucleotide-depleted enzyme binds ADP at this site (as well as at the catalytic site and the first α-β site) in the absence of Mg^{2+}. In the presence of Mg^{2+}, a fourth site binds ADP as well (see [25]). This additional binding does not affect the hysteretic inhibition, but induces a partial inhibition of the initial rate of catalysis ([25], Fig. 1A). This additional ADP, therefore, is bound to the second α/β site, causing 45% inhibition of the rate of ATP hydrolysis. After column centrifugation and a chase with ATP a small fraction of this extra ADP is lost, in agreement with the data reported in the present paper. After incubation of the nucleotide-depleted enzyme with ADP, followed by column centrifugation, the occupation of sites is very similar to that of our enzyme as described in this paper ($F_1(3)$ and $F_1(4)$) with only one difference: the first β-site is not occupied with ATP, but with ADP and this ADP causes the hysteretic inhibition. Fig. 7 shows schematically the bound nucleotides in the different preparations. The effect of ADP bound at the first β-site should not be confused with the effect of ADP bound at the tight catalytic site in the absence of phosphate. This latter ADP is removed from its site by addition of phosphate or ATP [21] and the site involved is the second β-site, the real high-affinity catalytic site that is involved in multi-site catalysis.

4.2. Comparison of the model with the new crystallographic data

The recent beautiful data on the structure of F_1 [39] confirm the location of all nucleotide binding sites on interfaces between α- and β-subunits as concluded in this paper. Considering the fact that the binding domains of the non-catalytic sites are really on the α-subunit, it seems preferable to call the sites that we identify as α/β sites, α sites. We furthermore like to suggest that the nomenclature of the three α-subunits should be different from the one proposed by Abrahams et al. [39]: the α_{TP} subunit (according to the nomenclature of these authors) seems to us to show the affinity characteristics of the low-affinity non-catalytic site (site 6 in our scheme) and should be called α_E. This implies that the α-subunit of which the nucleotide binding site is in close contact with, for instance, the β_E-subunit will have the highest interaction with this β-subunit and form a pair with it, instead of the other way round. In our model for F_1, an α/β pair exists that contains two tightly bound, non-exchangeable nucleotides, and this pair then should be the pair α/β_{TP}, consisting of the β_{TP} and the α-subunit that is called α_{DP} by Abrahams et al., but should be called α_{TP}. The finding that at one β-site the AMPPNP has been replaced by ADP, added at a low concentration, but not at the other site, suggests to us that the β-site containing ADP is the high-affinity catalytic site that is also exchanged in the experiments described in this paper. The β-site that remains occupied with AMPPNP (β_{TP}) is then the non-exchangeable (not catalytically active) β-site, site 1 in our schemes of Fig. 7. According to Abrahams et al. β_{TP} binds its substrate less tightly than β_{DP}, but one should keep in mind that the enzyme had first been treated with glycerol to remove the originally bound nucleotides and it is well known that in the glycerol-treated enzyme the two 'non-exchangeable' nucleotides are bound less firmly than in the enzyme as isolated. It should be interesting to perform, for a short time, ATP hydrolysis with the AMPPNP-incubated enzyme before crystallization and to see whether the AMPPNP is still bound at one β-subunit as predicted by our model. Although the authors of Ref. [39] place emphasis on the theoretical possibility of rotation, this possibility is certainly not realised in the isolated F_1 [40] and it is more suitable to suppose that binding of ATP to the β_E, followed by a conformational change of the binding domain, results in a conformational change of the γ-subunit and as consequence of this an opening up of the binding domain of β_{DP}, without a large change in β_{TP} (and the reverse) and that we are dealing with an alternating process, without a rotation around the γ-subunit on the time-scale of catalysis.

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