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Demonstration of two exchangeable non-catalytic and two cooperative catalytic sites in isolated bovine heart mitochondrial F_1, using the photoaffinity labels [2-^{3}H]8-azido-ATP and [2-^{3}H]8-azido-ADP

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The photoreactive nucleotides [2-^{3}H]8-azido-ATP and [2-^{3}H]8-azido-ADP could be used to label the nucleotide binding sites on isolated mitochondrial F_1-ATPase to a maximum of 4 mol of nucleotide per mol F_1, also when the F_1 was depleted of tightly bound nucleotides. At a photolabel concentration of 300–1000 μM, label was found on both α and β subunits in a typically 1:3 ratio, independent of the total amount bound. Under these conditions the covalent binding of two nucleotides is needed for full inactivation (Wagenvoord, R.J., Van der Kraan, I. and Kemp, A. (1977) Biochim. Biophys. Acta 460, 17–24). At lower concentrations of [2-^{3}H]8-azido-ATP (20 μM), it was found that covalent binding of only 1 mol of nucleotide per mole F_1 was required for complete inactivation to take place indicating catalytic site cooperativity in the mechanism of ATP hydrolysis. Under those conditions, radioactivity was only found on the β subunits, which would indicate that the catalytic site is located on a β subunit and that a second site is located on the α/β interface. It is found that four out of the six nucleotide binding sites are exchangeable and can be labelled with 8-azido-AT(D)P, i.e., two catalytic sites and two non-catalytic sites.

Introduction

F_1-ATPase consists of five different subunits with a stoichiometry of α_3, β_3, γ, δ, ε [1]. Little is known about the localisation of the minor subunits (γ, δ, ε). It has been concluded [2,3] that α and β subunits alternate to form a hexagonal quaternary structure. The six nucleotide binding sites are located on the α- and β subunits [4–6]. The number of catalytic sites is still uncertain, although data in the recent literature indicate the presence of two or three catalytic binding sites. Experiments by Cross et al. [7] give evidence for at least two catalytic sites. Arguments have been brought forward for the existence of three catalytic sites [5,6,8,9], whereas the other three sites should be non-exchangeable. On the other hand, the presence of exchangeable regulatory binding sites has been convincingly demonstrated [10–13]. While the total number of catalytic sites on F_1 is a matter of debate, it is also uncertain whether or not they are involved in an independent site catalysis or cooperate in a sequential dual or triple site mechanism.

Isolated F_1-ATPase contains two to three nucleotides tightly bound to the enzyme (nucleotides still present on F_1 after repeated gel filtration

Abbreviations: 8-N-ATP, 8-nitreno-adenosine-5′-triphosphate; Mes, 4-morpholineethanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid; NAP_3, 3′*O*-[3-[*N*-(4-azido-2-nitrophenyl)-amino]propionyl.

on a Penefsky column or ammonium sulphate precipitation [4,14–16]). The function of the tight nucleotide-binding sites is still not clear. It has been assumed [17] that they exert a regulatory function, but it is not excluded that they are (partially) involved in catalysis.

In order to establish the different types of nucleotide binding site in F_1, we made use of the photoaffinity-labelling technique. Furthermore, we investigated whether the catalytic sites involved operate independently of each other or not.

Materials and Methods

Bovine heart mitochondrial F_1-ATPase was isolated according to the method described by Knowles and Penefsky [18] and stored in liquid nitrogen in medium containing 10 mM Tris-HCl, 4 mM ATP, 250 mM sucrose and 4 mM EDTA (pH 7.5). The ATPase activity of the F_1 preparations (at high concentrations of ATP) was about 40 μmol P_i liberated per min per mg F_1 in the absence of an activating anion and 100–140 μmol/min per mg in the presence of 10 mM bicarbonate. Prior to use, F_1 was filtered four times on a fast centrifugation column with Sephadex G-50 coarse (a Penefsky column, 5×1 cm [19]) equilibrated in the same medium, except that ATP had been omitted (medium A). ATP-hydrolysis activity was measured spectrophotometrically. The assays were performed at 30°C in the presence of an ATP-regenerating system in a medium comprising 83 mM sucrose/33 mM Tris-HCl (pH 8.0)/10 mM $KHCO_3$/6 mM $MgCl_2$/5 mM ATP/0.5 mM phospho*enol*pyruvate/250 μM NADH/2.5 U pyruvate kinase per ml/2 U lactate dehydrogenase per ml. The oxidation of NADH was followed at 340 nm on a Zeiss M4QIII spectrophotometer. Tightly bound nucleotides were extracted from F_1 with 5.2% perchloric acid followed by neutralisation with 0.6 M Mops/0.6 M KOH [20]. ATP and ADP contents were measured with the luciferine/luciferase system. In the experiment where the release of tightly bound nucleotides was measured upon LiCl treatment, ATP and ADP were measured fluorimetrically by following the formation of NADPH after addition of hexokinase and glucose-6-phosphate dehydrogenase in the presence of glucose and $NADP^+$ The ADP content was determined after preceding phosphorylation of the ADP with phospho*enol*-pyruvate and pyruvate kinase. In the case of the fluorimetric determination the pyruvate kinase was inactivated by heat denaturation (3 min, 95°C). Subsequently the amount of ATP was measured again. After subtraction of the initial amount of ATP (determined prior to the phosphorylation) the amount of ADP could be calculated.

[2-^{3}H]8-azido-ATP and [2-^{3}H]8-azido-ADP were synthesized from [2-^{3}H]ATP (Amersham) as described by Schäfer [21] for the non-labelled compounds. [β-^{32}P]2-azido-ADP was synthesized by analogy of a method described by Hoard and Ott [22] using 10 mCi [^{32}P]Pi (New England Nuclear) and starting with 2-azido-AMP. 30% of the 2-azido-AMP was chemically transformed into the labelled compound, which was phosphorylated to [β-^{32}P]2-azido-ATP using the phospho*enol*-pyruvate/pyruvatekinase system. 2-azido-AMP was synthesized from 2-chloroadenosine mainly as described by Schaefer and Thomas [23] and Sowa and Ouchi [24]. The complete synthesis procedure will be published elsewhere. Photoaffinity labelling was carried out in medium A or in the same medium containing 6 mM $MgCl_2$ instead of EDTA (medium B). Changing of the medium and removal of photolysed unbound ligands were performed on a Penefsky column (5×1 cm).

Irradiation was performed at room temperature at 360 nm using a CAMAG universal ultraviolet lamp. Protein concentrations were determined as described by Lowry et al. [25], using bovine serum albumin as a standard. SDS-urea polyacrylamide gel electrophoresis was performed on 7% polyacrylamide gels according to a slightly modified version of that described by Swank and Munkres [26]: the differences were that (a) the buffer medium for the gel was freshly prepared each time, (b) SDS was added as a solid to the upper buffer compartment, and (c) the ratio of acrylamide/bisacrylamide was 30 : 1. After staining and destaining, the α/β region was cut into 1 mm slices using a Mickle gel slicer. After extraction of the radioactivity from the gel slices with a 9 : 1 protosol/H_2O solution (20 h at 37°C), the mix was neutralised by addition of acetic acid. After addition of scintillation cocktail (Packard 299), the amount of radioactivity was measured in a

Packard Tricarb liquid scintillation spectrophotometer. In case of [β-^{32}P]2-N-AT(D)P-labelled F_1, the gels were directly cut into slices after electrophoresis. Radioactivity was liberated by oxidation of the gel slices with 0.5 ml H_2O_2 (30%) (overnight at 60°C). After the addition of 1 ml H_2O, the amount of radioactivity was measured as Cerenkov radiation in the same scintillation spectrophotometer. The resulting peak diagram of the radioactivity in the gel slices was analysed by using a newly developed computer program. This program is based on the assumption that after electrophoresis α and β subunits have identical tailing characteristics in the gel. For the determination of the distribution of radioactivity over α and β subunits, the front side of the radioactivity peak in both subunits was approximated by a Gaussian distribution, whereas the other (tail) side was approximated by a polynomal function. After integration of both curves, the ratio of label bound to the α and the β subunits, respectively, could be calculated.

ATP, phospho*enol*pyruvate, pyruvate kinase, lactate dehydrogenase and NADH were purchased from Boehringer, Mannheim, luciferine/luciferase (ATP Monitoring Reagent) was from LKB Wallac. All other chemicals used were of analytical grade.

Results

Photoaffinity labelling of F_1 in EDTA-containing medium (medium A) with 500 μM [2-^{3}H]8-azido-ATP in four subsequent irradiation steps resulted in the binding of 1.4 mol nucleotide per mol F_1 (Table I). Subsequent labelling in Mg^{2+}-containing medium (medium B) with 500 μM [2-^{3}H]8-azido-ADP resulted in a further binding of 2 mol label per mol F_1. Under these conditions F_1 still contains ATP and ADP tightly bound to the enzyme [4]. After removal of these tightly bound nucleotides with LiCl at low pH (Refs. 27 and 28; see below), subsequent labelling with [2-^{3}H]8-azido-ATP resulted in additional binding to a total amount of 4 mol nucleotide per mol F_1.

In the same table it is shown that treatment of F_1 with LiCl prior to photolabelling, thereby removing the tightly bound nucleotides to less than 0.6 mol per mol F_1, did not change the amounts of [2-^{3}H]8-azido-ATP and [2-^{3}H]8-azido-ADP that could be bound after several irradiation steps in the presence of EDTA. Identical results were obtained when the labelling was performed in the reverse order of addition of nucleotides. The binding of 8-azido-nucleotides to the F_1 could be inhibited by the addition of AT(D)P, in agreement with earlier data by Wagenvoord et al. [29].

The effect of treatment of F_1 with high concentrations of LiCl on the tightly bound nucleotides was studied in more detail in order to investigate whether the tightly bound nucleotides were really removed. From Table II it can be seen that the isolated F_1 contained 3 mol tightly bound nucleotides per mol F_1. Upon treatment with 0.8 M LiCl at pH 6.1, a time-dependent release of both tightly bound ATP and ADP to 0.67 mol nucleotide per mol F_1 was observed. A comparable

TABLE I

PHOTOLABELLING OF F_1-ATPase WITH [2-^{3}H]8-AZIDO-AT(D)P

Irradiation of F_1 (dissolved in medium A) at 500 μM [2-^{3}H]8-azido-ATP (specific activity 57000 dpm/nmol) in four subsequent irradiation steps was followed by two irradiation steps at 500 μM [2-^{3}H]8-azido-ADP (specific activity, 57000 dpm/nmol) in medium B. Subsequently, the labelled F_1 was treated with 0.8 M LiCl for 4 min in the following medium: 50 mM Mes-NaOH (pH 6.1)/4 mM EDTA/10% glycerol to remove tightly bound nucleotides (to less than 0.7 AT(D)P per mol F_1). Hereafter labelling was carried out at 500 μM [2-^{3}H]8-azido-ATP in medium A in one irradiation step (upper part of the table). Irradiation of F_1 (dissolved in medium A) after pretreatment of F_1 with LiCl in the glycerol-containing medium to reduce the amount of tightly bound AT(D)P (to less than 0.6 mol/mol F_1). Irradiation was performed with [2-^{3}H]8-azido-ATP (specific activity, 87000 dpm/nmol) in medium A in two irradiation steps and subsequently with [2-^{3}H]8-azido-ADP (specific activity, 57000 dpm/nmol) in the same medium in two irradiation steps. The concentration of photolabel was varied between 350 and 500 μM, the irradiation steps took 50 min each (lower part of the table).

Irradiation medium	Photolabel	Bound label (mol/mol F_1)		
		α-subunits	β-subunits	total
A	[2-^{3}H]8-azido-ATP	0.35	1.06	1.41
B	[2-^{3}H]8-azido-ADP	0.93	2.62	3.55
A	[2-^{3}H]8-azido-ATP	0.97	3.07	4.04
A	[2-^{3}H]8-azido-ATP	0.35	1.28	1.63
A	[2-^{3}H]8-azido-ADP	0.88	2.80	3.68

TABLE II

RELEASE OF TIGHTLY-BOUND NUCLEOTIDES UPON INCUBATION AT 0.8 M LiCl

Tightly bound nucleotides were removed by incubating isolated F_1 (dissolved in glycerol-containing medium, see Table I) with 0.8 M LiCl. After 0, 3 and 6 min the incubation was stopped by gel filtration on a Penefsky column (with Sephadex G-50 coarse equilibrated in medium (A) thereby simultaneously removing released nucleotides and LiCl. Extraction and determination of the amount of remaining bound nucleotides were carried out as described in Materials and Methods. Prior to the LiCl incubation, removal of loosely bound and medium nucleotides was performed by a 3-fold gel filtration on a Penefsky column in the glycerol-containing medium.

Incubation time (min)	Amount of tightly-bound nucleotides (mol/mol F_1)		
	ATP	ADP	total
0	1.87	1.16	3.03
3	0.44	0.73	1.17
6	0.47	0.20	0.67

amount of tightly bound nucleotides was likewise measured on F_1 when the LiCl treatment was carried out after covalent binding of nitreno-ATP and nitreno-ADP to the exchangeable sites (Table I).

Following the exact amounts of tightly bound nucleotides during the labelling experiments, it was found that after 2 mol of 8-N-ATP were bound per mole of F_1, still 2.6 mol tightly bound nucleotides were present on the F_1 (Table III).

However, after the additional binding of about 2 mol 8-N-ADP per mol F_1, only two tightly bound nucleotides were present (about one ATP and one ADP), making the total number of demonstrated adenine nucleotides 6. Subsequent LiCl treatment and labelling with [2-^{3}H]8-azido-AT(D)P resulted in the earlier demonstrated maximum of about 4 mol label per mol F_1. If, however, the additional labelling was performed with [β-^{32}P]2-azido-AT(D)P, extra amounts of 0.8 (2-azido-ADP) or 1.5 (2-azido-ATP) could be covalently bound (Table III). In case of the 2-azido-nucleotides, the radioactivity was found on the β-subunits (not shown). The maximal amount of 4 mol 8-N-AT(D)P per mol F_1 was also found when the F_1 used had been depleted of nucleotides by means of filtration through a Sephadex G-50 coarse column equilibrated in 50% glycerol (Herweijer, M.A., unpublished results). So it can be concluded that 4 mol 8-N-AT(D)P can be bound per mol F_1 and that the tight nucleotide

TABLE III

PHOTOLABELLING OF F_1-ATPase WITH [2-^{3}H]8-AZIDO-AT(D)P AND [β-^{32}P]2-AZIDO-AT(D)P

Irradiation of F_1 (dissolved in medium A) at 1000 μM [2-^{3}H]8-azido-ATP (specific activity, 90000 dpm/nmol) in three subsequent irradiation steps was followed by two irradiation steps at 1000 μM [2-^{3}H]8-azido-ADP (specific activity, 90000 dpm/nmol) in medium B. Subsequently, the labelled F_1 was treated with 0.8 M LiCl for 4 min in the glycerol-containing medium (see Table I) to remove tightly bound nucleotides (upper part of the table). Hereafter, a separate labelling was carried out in medium B in one irradiation step with the four different 8- and 2-azido-analogues. The irradiation steps took 30 min each. The concentration of photolabel in the last step was 500 μM. The amount of extra bound label after LiCl-treatment is indicated as 'extra label'. Before labelling, after the first labelling with 8-azido-ATP and 8-azido-ADP as well as after LiCl treatment, samples were drawn to determine the amount of tightly bound AT(D)P with the luciferine/luciferase system.

Irradiation medium	Photolabel/ treatment	Bound label (mol/mol F_1)	Extra label after LiCl treatment	Tightly bound nucleotides		
				ATP	ADP	total
	none			1.60	2.25	3.85
A	[2-^{3}H]8-azido-ATP	2.0		0.87	1.75	2.62
B	[2-^{3}H]8-azido-ADP	3.8		0.81	1.16	1.97
	LiCl			0.23	0.57	0.80
B	[2-^{3}H]8-azido-ATP	4.3	0.5			
B	[2-^{3}H]8-azido-ADP	4.2	0.4			
B	[β-^{32}P]2-azido-ATP	5.3	1.5			
B	[β-^{32}P]2-azido-ADP	4.6	0.8			

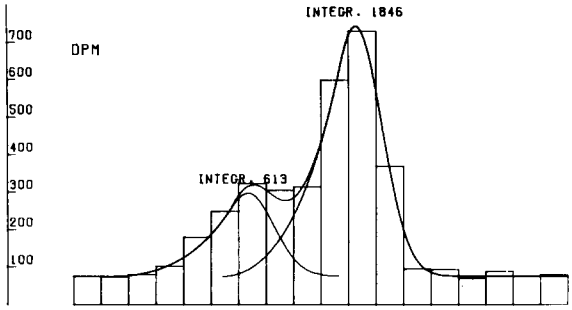

Fig. 1. Distribution of radioactivity over α and β subunits after labelling F_1 (7.4 mg/ml) with 500 μM [2-^{3}H]8-azido-ATP (specific activity, 70000 dpm/nmol) in medium A. A sample of 27 μg of the labelled F_1 was applied to the SDS-urea polyacrylamide gel and electrophoresed. Bars represent the amount of radioactivity extracted from gel slices in the α/β region. The computerized analysis is used to determine the distribution over α and β subunits. The two peaks of the radioactivity corresponded with the A560 scan of the stained gels (not shown).

binding sites can only be labelled with 2-azido-AT(D)P.

In Table I it can be seen that α and β subunits were both labelled with [2-^{3}H]8-azido-ATP, the β's containing three times as much label as the α subunits. Fig. 1 shows a representative result of a computer analysis used for the determination of the distribution of the label over α and β subunits, in which the 1 : 3 ratio can be noticed. The distribution of label was found to be independent of the amount of label bound and independent of the presence of Mg^{2+} during irradiation. This holds for the whole range from 0 to 4 mol 8-N-AT(D)P per mol F_1 that could be bound. It should be kept in mind that this constancy of the distribution is measured under conditions that the concentration of nucleotides during the incubation is kept high (300–1000 μM).

When these high concentrations (300–1000 μM) of [2-^{3}H]8-azido-ATP or [2-^{3}H]8-azido-ADP were used for the labelling of F_1, a maximal covalent binding of 2 mol per mol F_1 was found under the defined conditions (Tables I and III, Fig. 2). This maximal binding of two mol nucleotide per mol F_1 under the defined conditions was needed for complete inactivation of the enzyme (Fig. 3). The linear relationship between bound label and extent of inactivation has also been found by Wagenvoord et al. [4,29]. A consequence of the ratio of 1 : 3 found here is that, when the maximal amount of 2

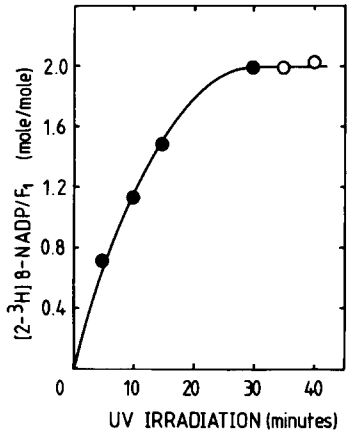

Fig. 2. Determination of the maximal amount of covalently bound 8-N-ADP after repeated irradiation of F_1 (2.0 mg/ml) with 1 mM [2-^{3}H]8-azido-ADP (specific activity, 90000 dpm/nmol) in medium B, (●) label bound in the first irradiation step, (○) label bound in the second irradiation step. Comparable results were obtained when 2-azido-ATP was used in the presence of EDTA. UV, ultraviolet.

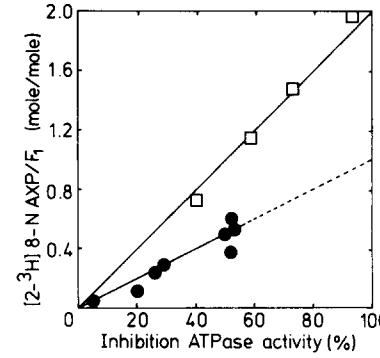

Fig. 3. Relation between percentage of inactivation of ATPase activity and amount of bound [2-^{3}H]8-N-AT(D)P (mol/mol F_1). Irradiation was performed at 1000 μM [2-^{3}H]8-azido-ADP (specific activity, 90000 dpm/nmol) in medium B (□) (F_1 2.0 mg/ml). Inactivation was also performed at 20 μM [2-^{3}H]8-azido-ATP (specific activity, 70000 dpm/nmol) in medium A (●) (starting concentration F_1 6.4 mg/ml). This latter inactivation was performed in eight subsequent irradiation steps of 30 min each. After each step non-bound photolabel was removed by gel filtration on a Penefsky column and samples were drawn to determine the amount of label bound, its distribution over α and β subunits, as well as its ATPase activity. In all cases the ATPase activity was compared to the activity of a control sample in which the photolabel was omitted.

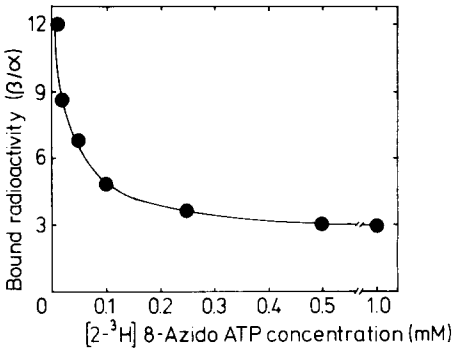

Fig. 4. Effect of the concentration of photoaffinity label on the distribution of radioactivity over α and β subunits. Irradiation of F_1 dissolved in medium A was performed with varying concentrations of [2-^{3}H]8-azido-ATP (specific activity, 70000 dpm/nmol). At 5, 10 and 50 μM label F_1 was irradiated in three subsequent steps of 30 min and at 100 and 250 μM label F_1 was irradiated for 30 min. At 500 and 1000 μM photolabel F_1 was irradiated for 15 min. Total amount of bound label varied between 0.3 and 1.0 mol of photolabel per mol F_1. The distribution was determined by using the computerized analysis.

mol nucleotide analogue is bound to the enzyme, 1.5 mol label is bound to the β and 0.5 to the α subunit. To account for this at full inhibition of the enzyme, two interpretations are possible: one assuming that two identical sites are labelled, and that these are located in such a way that the activated nitreno group has a 3-fold higher possibility for reaction with a residue on the β subunit than with a residue on the α-subunit. The second possibility is that two distinct types of binding site are labelled under these conditions: one located on the β subunit and the other type located on the interface between α and β subunits. Binding of 8-azido-AT(D)P to this latter type would then result in labelling of α and β subunits to the same extent. In the second interpretation it should be possible to find conditions under which these sites can be distinguished.

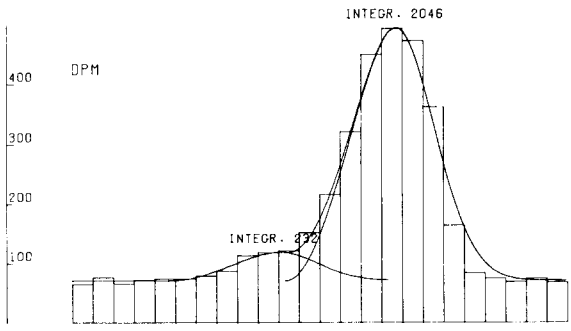

Fig. 5. Distribution of radioactivity over α and β subunits after labelling of F_1 with 20 μM [2-^{3}H]8-azido-ATP (specific activity, 70000 dpm/nmol) in medium A after seven subsequent irradiation steps of 30 min each (F_1 1.3 mg/ml). For further details see legend to Fig. 1.

On the basis of a possible difference in K_d values of the two types of site for the [2-^{3}H]8-azido-ATP, experiments were performed with decreasing concentrations of photolabel. In Fig. 4, it can be seen that labelling experiments at lower concentrations of photolabel resulted in preferential labelling of the β subunit. At the lowest concentration used (5 μM), more than 90% of the label was bound to the β subunits. An example of a computer analysis of the distribution of radioactivity over α and β subunits after labelling at a low concentration of photolabel (20 μM) is shown in Fig. 5. It can be concluded that indeed two types of binding site can be distinguished. Since a comparable labelling efficiency for both sites may be assumed (due to the fact that the distribution of the covalently bound label over the two sites is independent of the amount of bound label), the data in Fig. 4 can be used to estimate the K_d values of both sites for 8-azido-ATP. The β binding site then has a K_d value of about 20 μM, whereas the interface binding site has a K_d value of about 100 μM. In the same figure it can be seen that at higher concentrations of photolabel a ratio of 1 : 3 for the distribution of radioactivity was found, which is in agreement with the ratio found in the experiments presented in Table I.

Since the experiments discussed so far clearly point to the existence of two types of exchangeable binding site, we investigated a possible catalytic involvement of the two separate types of site. When relatively high concentrations of photolabel were used, F_1 was fully inhibited when 2 mol label were bound per mole of F_1. When, however, F_1 was illuminated in the presence of 20 μM [2-^{3}H]8-azido-ATP, a linear relationship was found between bound label and inactivation, extrapolating to one mol label bound at complete inactivation as can be seen in Fig. 3. As the reactive nitreno radical can also react with surrounding

water molecules, several irradiation steps were needed in this case to obtain enough binding of the photolabel. After seven irradiation steps, however, the control sample started to be substantially inactivated so the relative inactivation of the last sample by the covalent binding of nitreno-ATP could not be determined accurately enough any more. The radioactivity in all samples of this experiment was mainly bound to the β subunit. This is presented in Fig. 5 and corresponds to the results in Fig. 4 for a concentration of 20 μM of the photolabel.

It can be concluded that the labelling of only one site, located on the β subunit with [2-^{3}H]8-azido-ATP, is required for the complete inactivation of the enzyme.

Discussion

In this paper it is shown that in isolated F_1 a total of four binding sites can be labelled with 8-azido-AT(D)P: about 2 mol 8-azido-ATP in the presence of EDTA and 2 mol 8-azido-ADP in the presence of Mg^{2+} or EDTA in agreement with previous results by Wagenvoord [4] (Tables I and III). It can therefore be concluded that F_1 contains at least four exchangeable nucleotide binding sites [30]. It was additionally shown that depletion of tightly bound nucleotides during or prior to labelling had no effect on the maximal amount of bound label leading to the conclusion that the two remaining binding sites cannot be labelled with 8-azido-nucleotides. Comparable results were found for the closely related 8-bromo-ADP, which could not replace tightly bound ATP under conditions where ADP could do so [31]. In contrast, it is demonstrated in this paper that at least part of the two socalled non-exchangeable nucleotide binding sites can be labelled with the 2-azido-analogues (Table III). It has been suggested [32] that an 'anti' configuration of the nucleotide is required for binding to these tight nucleotide binding sites, whereas 8-azido-nucleotides are assumed to be almost completely in the 'syn' configuration. Labelling experiments with 2-azido-ADP, which is expected to be in this 'anti' configuration, indicate that it is also possible to label tight sites on CF_1 [32,33].

It is clearly demonstrated in Fig. 2 that under the defined conditions (8-azido-ADP, Mg^{2+}) a maximal amount of 2 mol label per mol F_1 can be covalently bound. Similar results were obtained with 8-azido-ATP/EDTA, this is also illustrated in the Tables I and III. At the high concentration of analogue used in the experiment of Fig. 2 (1000 μM), 2 mol label were also needed to completely inactivate the F_1-ATPase activity (Fig. 3). From this, and from the findings that (a) at these concentrations α and β subunits are labelled in a typical 1 : 3 ratio and (b) lowering the photolabel concentration resulted in the preferential labelling of the β-subunit (Fig. 4), it is concluded that two distinct binding sites are present on the F_1: one located on the β subunit with a relatively high affinity and one located on the α/β interface with a lower affinity. In the α/β interface binding site, the nitreno radical is supposed to have about an equal chance to react with the α or the β subunit. If both the β site and the α/β interface site are labelled equally, the ratio of radioactivity bound to α/β is 1 : 3. The distribution of the label over the two subunits differs from that reported previously by Wagenvoord [4,29]. This discrepancy can be attributed to the improved separation of α and β subunits on the SDS-urea gels in combination with the more accurate approximation of label distribution over both subunits with the computer analysis. It has to be added that in a single case a label distribution lower than 3 was found.

The same distribution of label ($\alpha/\beta = 1 : 3$) was also found by Sloothaak et al. working with membrane-bound F_1 [34]. Localisation experiments by Hollemans et al. [35], in which F_1 was used which was labelled at 1600 μM [2-^{3}H]8-azido-ATP, showed that besides three amino acid residues near each other (at the supposedly catalytic site), the N-terminus of the β subunits was labelled too. This is in agreement with the proposed two binding sites per β subunit. The possibility of interface binding sites is supported in the literature, such as the cross-linking of α and β subunits with the photoaffinity label 3'-arylazido-8-azido-ATP [36] and experiments by Williams and Coleman [37] with 2'-*o*-(4-benzoyl) benzoyl ATP. Recent binding studies on isolated β subunits [38] showed that 2 mol nucleotide can be bound per mol β, which suggests that the interface binding site is preserved in the isolated subunit.

It has to be kept in mind that the distinction of the two sites is based on labelling with 8-azido-nucleotides; labelling of these two sites with other analogues might result in a completely different distribution of the label over α and β subunits, due to other orientations of the photoreactive group. With the photoaffinity label [^{3}H]NAP$_3$-ADP, however, comparable results as with the 8-azido-nucleotides were found [39]: α and β subunits of F_1 were labelled in a 1 : 3 ratio and after preincubation with ADP, the ratio was 1 : 1, suggesting that after binding of ADP to a β site only the proposed interface site is labelled.

The 8-azido-nucleotides might be very important because of their ability to differentiate between the two types of exchangeable binding sites. In this paper we show that not only a differentiation on the basis of the localisation of the binding sites can be made, but also a functional difference can be demonstrated between the β binding site and the interface binding site.

Since a linear relationship was found for labelling vs. inactivation (Fig. 3), it could be excluded that at higher concentrations of photolabel the two labelled sites are both catalytic and operate in an unindependent site mechanism, because in that case a hyperbolic relationship would be expected, not a linear one. So, actually two possibilities remained: (1) the two labelled sites are both catalytic and operate independently of each other, or (2) only one of the two sites is catalytic and the other was simply co-labelled when high concentrations of photolabel were used. Since the location of the two sites is different, it might be expected that the second possibility is more probable.

The finding that binding of 8-N-ATP only to the β-site caused full inhibition of the hydrolytic activity of the enzyme (Fig. 3) indicates that this site is catalytic. It cannot be excluded that this site is regulatory but the specific labelling of a regulatory site is not likely to result in the complete inactivation of the enzyme, but should possibly influence its kinetic parameters. Since under conditions when both sites are labelled equally (high concentrations of ligand) the relationship between bound 8-N-ATP and inhibition is linear, full inhibition being reached when 2 mol nucleotides are bound, one has to conclude furthermore that binding of 8-N-ATP to the interface site does not influence the hydrolytic activity of F_1 when measured under V_{max} conditions. Indeed, it was found in our laboratory that the inhibition of ATP hydrolysis with submitochondrial particles, inhibited for 50% with 8-azido-ATP (under conditions whereby a 1 : 3 ratio of label bound to $\alpha : \beta$ was found) when measured under V_{max} conditions, was considerably larger at low ATP concentrations [34].

The experimental finding that 1 mol photolabel per mol F_1 is sufficient for complete inhibition of the ATPase activity indicates that, when more than one catalytic site is present, the individual sites cannot be operative without the involvement of the other(s), as is the case in an alternating site, or in a sequential mechanism in which changes in conformation are passed on between the catalytic sites involved. A sequential mechanism for the hydrolysis of ATP is also supported by experiments with ATPase inhibitors such as 7-chloro-4-nitro-benzo-2-oxo-1,3-diazol (NDB-Cl). Only one molecule of this inhibitor is needed per F_1 for full inactivation of the hydrolytic activity and, what is more, it has been demonstrated that the inhibition is based on binding of the inhibitor to the β-Tyr 311 [40]. This Tyr 311 is also one of the three amino acid residues lying near each other which were labelled by [2-^{3}H]8-azido-ATP, which strongly suggests that it is the catalytic site that is located in this area of the β subunit. The interface binding site then would be located at the N-terminus of the β subunit [35]. The stoichiometry of 1 mol [2-^{3}H]8-N-ATP per mol F_1 required for full inactivation found by us was found earlier with [^{14}C]8-azido-ATP on BF_1 isolated from *Micrococcus luteus* [41], and in mitochondrial F_1 with the phosphate analogue 4-azido-3-nitrophenylphosphate [42] bound to the phosphate binding site.

The question remains how many catalytic sites are present in F_1. Labelling with high concentrations of 8-azido-ATP/ EDTA, 8-azido-ADP/ EDTA or 8-azido-ADP/Mg^{2+} resulted in the same 1 : 3 ratio for the distribution of radioactivity. This indicates that two types of site (one β site and one α/β interface site) are labelled when 2 mol label are bound. Subsequent covalent binding of another 2 mol label resulted also in the labelling of two distinct sites with a ratio of label bound to α/β of

1 : 3, hereby not affecting the ratio of the total amount of label. So, when the maximum of four molecules of 8-N-AT(D)P) per mol F_1 was bound, it could be concluded that two β-sites and two α/β-sites were labelled. Consequently, the two residual sites, which contain tightly bound nucleotides and are inaccessible to 8-azido-AT(D)P, are also located on a β subunit and an α/β interface. This statement is not contradicted by the fact that in the case of the labelling of these sites with [β-^{32}P]2-azido-AT(D)P all radioactivity is found on the β-subunits, because of the different location of the photoreactive azido-group. In fact, in all labelling experiments performed in our laboratory with [β-^{32}P]2-azido-AT(D)P, all radioactivity was found on the β-subunits [30,43,44]. Labelling experiments with the 2-azido-analogue on CF_1 also resulted in the labelling of only the β-subunits [32,33].

In this paper the presence of (at least) two cooperative catalytic (β) sites is demonstrated, whereas the labelling of only one site is required for the complete inhibition of the F_1-ATPase activity. Furthermore, in this paper it is shown that the 2-azido analogues can also be used to label part of the (so-called non-exchangeable) tight nucleotide binding sites. This, however, does not necessarily imply that F_1 contains a third catalytic (β)site, but this cannot be excluded. The presence of one distinct β-subunit is in agreement with results of DCCD-binding studies to the F_1 [45].

The presence of three cooperative catalytic sites as postulated by Gresser et al. [9] is based on kinetic data, i.e., three K_m values for hydrolysis of ATP could be distinguished. Taking into account that the turnover of the first site as described by Gresser et al. [9] is at least 1000-times faster than the rate of single site catalysis described by Cross et al. [7] one has to conclude that in fact four K_m values can be distinguished. Such a kinetic pattern is in good agreement with the presence of two catalytic and two non-catalytic (but exchangeable) binding sites, when the two non-catalytic sites are regulatory, affecting the affinity of the catalytic sites for substrate and product, as has been proposed by Recktenwald and Hess [12] and Stutterheim et al. [46].

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