ADENINE NUCLEOTIDE BINDING SITES IN CHEMICALLY MODIFIED F_1-ATPase

Inhibitory effect of 4-chloro-7-nitrobenzofurazan on photolabeling by arylazido nucleotides

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1. Introduction

The mitochondrial F_1-ATPase is composed of 5 nonidentical subunits designated $\alpha, \beta, \gamma, \delta, \epsilon$ in the order of decreasing molecular weight. There are at least 2 monomers of the high molecular weight subunits α and β per F_1-ATPase. Inhibition of F_1-ATPase by an analogue of adenine, 4-chloro-7-nitrobenzofurazan at neutral pH involves the binding of this reagent to a single tyrosine residue of F_1-ATPase in an unidentified subunit. Full inhibition requires the incorporation of 1 mol Nbf/mol F_1-ATPase. At alkaline pH, Nbf shifts to a nitrogen residue which has been located in subunit β [1,2]. Although it is generally accepted that Nbf modifies the interaction of ADP or ATP with the catalytic site of the enzyme, no direct evidence for inhibition of ADP or ATP binding by Nbf has been reported. In an attempt to better understand the mechanism of action of Nbf on ADP/ATP binding as well as the interaction of subunits, we have used photoactivable derivatives of ADP and ATP, namely *N*-4-azido-2-nitrophenyl-γ-aminobutyryl ADP and the corresponding ATP analogue. It was reported [3] that both analogues are able to bind covalently to F_1-ATPase, most likely by displacing loosely-bound ADP and ATP. Two arylazido derivatives are bound per F_1-ATPase. The bound arylazido derivatives are equally distributed in the α and β subunits of F_1-ATPase [4].

Abbreviations: Nbf, 4-chloro-7-nitrobenzofurazan or 7-chloro-4-nitrobenzo-2-oxo-1,3 diazole; arylazido-ADP (ATP), N-4-azido-2-nitrophenyl-γ-aminobutyryl ADP (ATP)

Here we show that binding of 1 mol Nbf to 1 mol F_1-ATPase at neutral pH prevents the covalent photolabeling of subunit β by arylazido-ADP or arylazido-ATP, but not that of subunit α. When this F_1-ATPase, both photolabeled and Nbf-modified, is treated by dithiothreitol to remove Nbf, the resulting protein is photolabeled essentially in subunit α. This enzyme has no ATPase activity, which indicates that modification of only one α subunit of F_1-ATPase produces total inactivation. This observation complements other data that show that modification of only one β-subunit in F_1-ATPase either by Nbf [1] or by phenylglyoxal [5] also abolishes enzymatic activity, and strongly suggests that F_1-ATPase exhibits half-site reactivity.

2. Materials and methods

2.1. *Materials*

[4-^{3}H]Aminobutyric acid (45 Ci/mmol) and [^{14}C]-Nbf-Cl) (109 mCi/mmol) were obtained from the Commissariat à l'Energie Atomique, Saclay and were diluted before use. [^{3}H]Arylazido-ADP and [^{3}H]arylazido-ATP were synthesized by the method in [6] slightly modified [4].

F_1-ATPase was prepared according to [7] and stored at 4°C as an ammonium sulfate precipitate. Before use, the suspension was centrifuged and the pellet solubilized in a buffered medium containing 200 mM suscrose, 20 mM Tris–acetate and 2 mM EDTA (final pH 7.5) to obtain 5–8 mg protein/ml. The F_1-ATPase solution was finally desalted by filtration through a small Sephadex G-50 (fine) column equilibrated with the same buffer.

2 2 *Methods*

ATPase activity was assayed with a regenerating system containing 40 mM Tris–HCl, 10 mM ATP, 5 mM $MgCl_2$, 40 μg/ml pyruvate kinase and 4 mM phosphoenolpyruvate (final pH 8) The incubation was done at 30°C for 5 min and terminated by addition of cold trichloracetic acid The phosphate released by ATP hydrolysis was measured in trichloracetic extracts according to [8] Protein concentration was determined according to [9] using bovine serum albumin as a standard Sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis was performed as in [10] using a 10% acrylamide gel Gel staining, destaining and slicing were processed as in [4] In photolabeling assays, usually 0 1 mg F_1-ATPase in 0 1 ml sucrose, Tris–acetate, EDTA medium was incubated with [^{3}H]arylazido-ADP or [^{3}H]arylazido-ATP in a small stopped glass tube that was rotated horizontally at 150 rev /min in a water bath at 20°C and illuminated with visible light for 30 min (10 cm from a 250 W Osram lamp equipped with a filter to cut off radiation below 300 nm) The final concentration of [^{3}H]-arylazido-ADP or -ATP used was between 130 and 160 μM (cf section 3) Following photoirradiation, the samples were incubated in the dark with 6 mM ATP or ADP to displace the non-covalently bound arylazido-nucleotide The remaining bound radioactivity was determined by the elution–centrifugation method in [11] A 1 ml plastic syringe equipped with a porous polyethylene disk was filled with 1 ml Sephadex G-50 (fine) equilibrated with the sucrose–Tris acetate, EDTA buffer mentioned above The packed syringe was placed in a centrifuge tube and spun at low speed to remove excess of buffer A small sample of F_1-ATPase photolabeled with [^{3}H]arylazido-ADP was applied to the top of the Sephadex column The eluate contained > 95% of the bound [^{3}H]arylazido-ADP Controls for radioactivity background were performed by adding a large excess of ADP or ATP to samples before photoirradiation (10 mM)

Inhibition and labeling by [^{14}C]Nbf was performed by incubating samples of F_1-ATPase dissolved in a medium made of 0 2 M sucrose, 10 mM triethanolamine, 2 mM EDTA (final pH 7 5) with Nbf at 30°C for various periods of time, the incubation was carried out in the dark to prevent photolytic decomposition [1] Excess Nbf was removed by the above elution–centrifugation method [11]

3 Results

3 1 *Inhibition of F_1-ATPase by Nbf binding stoichiometry of [^{14}C]Nbf*

Preliminary studies had shown, in agreement with [12], that the time course of inhibition of F_1-ATPase by Nbf follows pseudo-first-order kinetics for different concentrations of Nbf The number of Nbf molecules reacting per active site of F_1-ATPase to form an inactive complex was calculated, following [13], by plotting the log of the inactivation half-time against the log of Nbf concentration and found to be equal to unity As shown in fig 1, full inactivation of F_1-ATPase by Nbf was accompanied by the binding of 1 mol [^{14}C]Nbf to 1 mol F_1-ATPase, corroborating the spectrophotometric data of Nbf binding [1] All these data suggest that there is one catalytic site per F_1-ATPase The bound [^{14}C]Nbf (> 90%) could be displaced by dithiothreitol with concomitant recovery of ATPase activity, provided the amount of bound [^{14}C]Nbf was < 1 mol/mol F_1-ATPase When the amount of bound [^{14}C]Nbf was > 1 mol/mol F_1-ATPase (by increasing the period of incubation with [^{14}C]Nbf), the stoichiometric excess of bound [^{14}C]-Nbf could be removed by dithiothreitol only with great difficulty (cf insert fig 1)

3 2 *Effect of Nbf on the covalent binding of photoactivable derivatives of ADP or ATP to F_1-ATPase*

Upon photoirradiation with F_1-ATPase, arylazido-ADP and arylazido-ATP are found to bind covalently to the α and β subunits of the enzyme, most likely to sites from which bound nucleotides can freely exchange with ADP and ATP of the medium (or their arylazido analogues) Covalent photolabeling by arylazido-ADP or arylazido-ATP is accompanied by inactivation of F_1-ATPase, full inactivation requiring the binding of 2 mol derivative/mol F_1-ATPase The binding stoichiometry to the α and β subunits is in a ratio of ~1 1 [4]

Figure 2 and table 1 show that Nbf only interferes with the binding of arylazido-ADP to subunit β For the results of fig 2, samples of F_1-ATPase were incubated with increasing concentrations of Nbf at pH 7 5 in order to inhibit the ATPase activity to various extents, [^{3}H]arylazido-ADP was then added to each sample and photoirradiation begun The concentration of [^{3}H]arylazido-ADP used was sufficient to almost

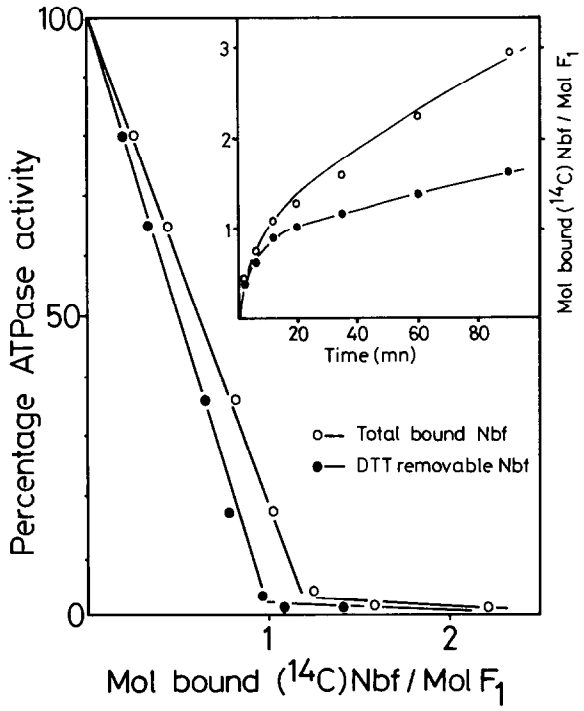

Fig. 1. Relation between the binding of [^{14}C]Nbf to F_1-ATPase and ATPase inactivation by the bound Nbf. F_1-ATPase (0.8 mg) was incubated in the presence of 77 μM [^{14}C]Nbf at 30°C in 1 ml 200 mM sucrose, 10 mM triethanolamine and 2 mM EDTA, final pH 7.5. At time intervals and ≤ 90 min (cf. insert), 0.1 ml aliquots of the incubation mixture were filtered through small Sephadex columns equilibrated with the same above medium, except that Nbf was omitted. (cf. section 2). The bound [^{14}C]Nbf and the ATPase activity were assayed on small aliquots (5–10 μl) of the filtrates. Then dithiothreitol (DTT) was added to 1 mM final conc. to the remaining fractions and let to react for 1 h at 20°C in the dark. The samples were again filtered on Sephadex and the ^{14}C radioactivity was assayed in the filtrates. The two curves shown in the figure refer to the total amount of bound [^{14}C]-Nbf (o——o) and to the amount of bound [^{14}C]Nbf removed by dithiothreitol (DTT) (•——•). The insert gives the time-course of incorporation of [^{14}C]Nbf (total) and DTT removable in F_1-ATPase.

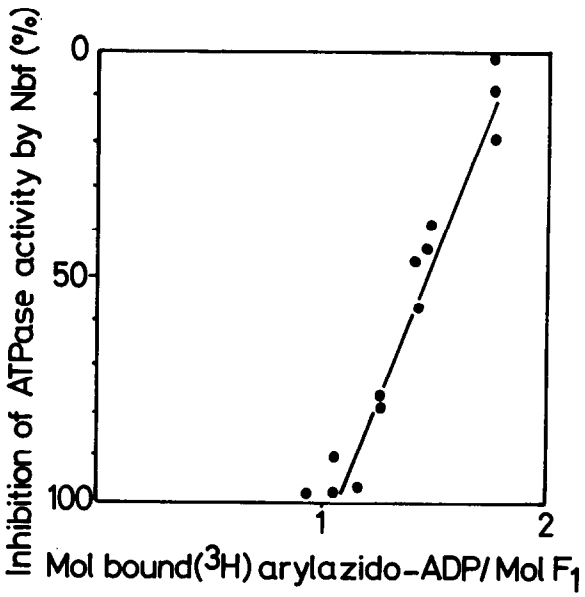

Fig. 2. Effect of preincubation with Nbf on the covalent photolabeling of F_1-ATPase by [^{3}H]arylazido-ADP. F_1-ATPase was incubated at 30°C for 30 min in series of tubes at 1 mg/ml final conc. with increasing concentrations of Nbf (5–150 μM) in 0.1 ml 200 mM sucrose, 30 mM Tris–acetate and 2 mM EDTA (final pH 7.5). After incubation, the mixture was filtered through small columns of Sephadex equilibrated with the same medium (without Nbf) to remove the non-reacted Nbf (cf. section 2). A small aliquot (5 μl) was used for determination of the ATPase activity. [^{3}H]Arylazido-ADP was added to the remaining fraction at 150 μM final conc. and the mixture was photoirradiated at 20°C for 30 min. Under these conditions the photolabeled control F_1-ATPase (without Nbf) was inactivated to 80%; its content in bound [^{3}H]aryl-azido-ADP was 1.75 mol/mol. In the figure the ordinate corresponds to the inhibitory effect of increasing concentrations of Nbf on the ATPase activity (an effect which is directly related to the amount of bound Nbf (cf. fig.1) and the abscissa corresponds to the amount of bound [^{3}H]arylazido-ADP in the different samples of Nbf-modified ATPase.

totally inactivate the native F_1-ATPase after photoirradiation and to bring about the binding of an amount of arylazido-ADP close to the maximum, i.e. 2 mol/mol enzyme (see fig.2). Addition of increasing concentrations of Nbf decreased the amount of bound arylazido-ADP. Addition of Nbf, at a concentration yielding full inhibition of F_1-ATPase and corresponding to the binding of 1 mol Nbf/mol F_1-ATPase (cf. fig.1), prevented the binding of 1 mol arylazido-ADP out of the 2 mol which can bind to the native enzyme. Arylazido-ATP behaved in the same way as arylazido-ADP.

For the results presented in table 1, Nbf was added to a sample of F_1-ATPase at pH 7.5 in excess of the amount required for full inhibition. The non-reacted Nbf was eliminated by gel filtration on Sephadex G-50. The activity of F_1-ATPase, which was almost

Table 1
Effect of Nbf on the hydrolytic activity of F_1-ATPase and on the binding of [^{3}H]arylazido-ADP to F_1-ATPase

Additions	% Activity before photo-irradiation	% Activity after photo-irradiation	% Activity after treatment with DTT	Bound [^{3}H]arylazido-ADP (mol/mol F_1)
F_1	100	99	98	–
F_1 + Nbf	2	2	74	–
F_1 + [^{3}H]aryl-azido-ADP	100	30	30	1 65
F_1 + Nbf + [^{3}H]-arylazido-ADP	2	1	10	0 90

F_1-ATPase was incubated for 30 min at 30°C at 1 mg/ml final conc in 0 1 ml 200 mM sucrose, 30 mM Tris–acetate and 2 mM EDTA either in the absence or presence of 150 μM Nbf (final pH 7 5) After incubation, the medium was passed on a small Sephadex column (cf section 2) to remove the non-reacted Nbf A small aliquot (5 μl) of the filtrate was used for determination of ATPase activity, the remaining fraction was supplemented with 160 μM [^{3}H]arylazido-ADP and photoirradiated for 30 min at 20°C The photolabeled sample was applied to a small Sephadex column, as above, to remove the non-covalently bound [^{3}H]-arylazido-ADP A small aliquot (10 μl) of the filtrate was taken for assaying the ATPase activity and the amount of bound [^{3}H]-arylazido-ADP Then dithiothreitol (DTT) was added to 1 mM final conc and let to react in the dark for 1 h at 20°C to remove the bound Nbf Another determination of ATPase activity was done after dithiothreitol incubation Data are corrected for the loss due to the different samplings

100% abolished after this treatment, was recovered to ~74% after addition of dithiothreitol (table 1, line 2) Another sample of F_1-ATPase was photo-irradiated in the presence of arylazido-ADP and inactivated by photolabeling to 70%, the bound aryl-azido-ADP was 1 65 mol/mol F_1-ATPase (table 1, line 3) When photoirradiation in the presence of arylazido-ADP was performed on F_1-ATPase previously inhibited by Nbf, and then dithiothreitol added to remove the bound Nbf, dithiothreitol treatment did not reactivate the enzymic activity, though the amount of bound arylazido-ADP was decreased to 0 90 mol/mol F_1-ATPase, i e , to ~1/2 the value found in the control F_1-ATPase in the absence of Nbf Exactly similar results were obtained when arylazido-ADP was replaced by arylazido-ATP The data of fig 2 and table 1 show that F_1-ATPase modified by Nbf has the capacity of binding 1 mol photoactivable derivative instead of 2, as in the case of the native enzyme

3 3 *Characterization by SDS–polyacrylamide gel electrophoresis of the subunit which binds arylazido derivatives in Nbf-modified F_1-ATPase*

A sample of F_1-ATPase modified by Nbf was photolabeled by [^{3}H]arylazido-ADP Another sample of native F_1-ATPase was photolabeled under similar conditions The two samples were then subjected to SDS–polyacrylamide gel electrophoresis As shown in fig 3, [^{3}H]arylazido-ADP binds both to α and β subunits in the native F_1-ATPase, but predominantly to the α subunit in the Nbf-modified ATPase No significant difference in the labeling by arylazido-ADP was found when the experiment was carried out in the presence of $MgCl_2$ instead of EDTA The above labeling experiment repeated with [^{3}H]arylazido-ATP in the presence of EDTA yielded the same type of radioactivity pattern

4 Discussion

The α and β subunits of F_1-ATPase possess adenine nucleotide binding sites, as shown by the use of photoactivable derivatives of ADP and ATP [4,14] and of the adenine analogue, *p*-fluorosulfonylbenzoyl-5′-adenosine [15] Since adenine nucleotides protect against inactivation by the above reagents, it was postulated that those reagents react with an adenine nucleotide binding site of the F_1-ATPase, possibly located in subunit β Data reported here show unambiguously that Nbf prevents the photolabeling of subunit β by arylazido-ADP and arylazido-ATP, and thus interferes with the binding of ADP and ATP to subunit β

Two other points of interest are raised here the

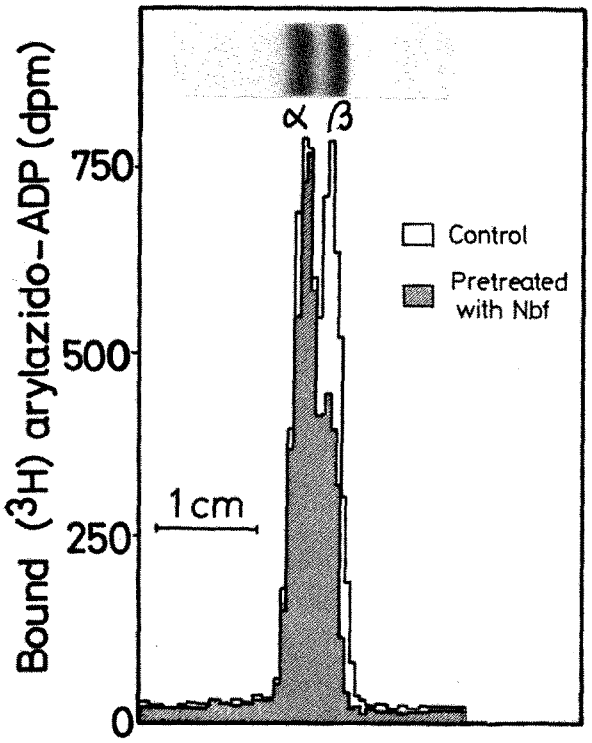

Fig.3. SDS–polyacrylamide gel electrophoresis of F_1-ATPase photolabeled with [^{3}H]arylazido-ADP after pretreatment with Nbf. A sample (50 μg) of F_1-ATPase was preincubated with 100 μM Nbf at 30°C for 30 min in 0.1 ml 200 mM sucrose, 30 mM Tris–acetate and 2 mM EDTA (pH 7.5). This preincubation yielded a 90% inactivation. The non-reacted Nbf was eliminated by filtration on a small column of Sephadex as in section 2. The filtrate, which contained the Nbf-modified F_1-ATPase, was recovered, to be photoirradiated with 130 μM [^{3}H]arylazido-ADP at 20°C for 30 min. A control sample of F_1-ATPase (non-treated by Nbf) was photoirradiated with [^{3}H]arylazido-ADP, under the same conditions. Identical aliquots (10 μg) of the two enzyme samples were subjected to SDS–gel electrophoresis. In the figure, the hatched columns correspond to the ^{3}H radioactivity present in F_1-ATPase pretreated by Nbf. The non-hatched columns correspond to the ^{3}H radioactivity present in the control ATPase.

selective photolabeling of subunit α by arylazido-ADP or -ATP; and the loss of F_1-ATPase activity by photolabeling of only one α subunit. Photolabeling of subunit α in F_1-ATPase can be achieved provided that subunit β be protected against photolabeling by the binding of Nbf. As Nbf is easily removable by dithiothreitol, the following sequence of reactions is required for selective photolabeling of subunit α:

1. Incubation of F_1-ATPase in the presence of Nbf at neutral pH;
2. Photoirradiation in the presence of arylazido-ADP or -ATP;
3. Removal of bound Nbf by dithiothreitol.

Full inactivation of F_1-ATPase results from the binding of arylazido-ADP or -ATP to only one α subunit. It is known that binding of Nbf [1] or phenylglyoxal [5] to only one β subunit of F_1-ATPase also causes full inactivation of the enzyme; thus despite the presence of $\geqslant 2$ subunits β, the modification of only one β subunit is sufficient to fully inactivate the enzyme, possibly by conformational change of the active site on the other subunits. The same observation extends to subunit α, indicating that both α and β subunits play a strategic role in F_1-ATPase activity and that F_1-ATPase may belong to the class of enzymes exhibiting half-site reactivity [16,17].

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