Biochimica et Biophysica Acta, 635 (1981) 284—294

BBA 48023

THE INTERACTION OF NUCLEOTIDES WITH F_1-ATPase INACTIVATED WITH 4-CHLORO-7-NITROBENZOFURAZAN

R. GREGORY, D. RECKTENWALD * and B. HESS

Max-Planck-Institut für Ernährungsphysiologie, Rheinlanddamm 201, D-4600 Dortmund (F.R.G.)

(Received November 26th, 1980)

Key words: F_1-ATPase; Nucleotide binding; 4-Chloro-7-nitrobenzofurazan; (Yeast)

Summary

In common with the F_1-ATPase from other sources, yeast mitochondrial F_1-ATPase was inhibited by 4-chloro-7-nitrobenzofurazan. Total inhibition of the F_1-ATPase activity was compatible with the modification of a single tyrosine residue per F_1-ATPase molecule. Radioactive labelling experiments localized this modification on a β-subunit. The inactive modified enzyme retained the capacity to bind the photoaffinity label 8-azido-1,N^6-etheno-ATP, which has previously been shown to bind nucleotide sites of low affinity. As well, the inactive modified enzyme bound MgATP with high affinity, yielding a K_d of 14 μM. The results are consistent with the hypothesis of alternating, or cooperative, site catalysis by F_1-ATPase.

Introduction

Yeast mitochondrial F_1-ATPase catalyses the hydrolysis of ATP. In the intact mitochondria, the enzyme bound to F_0-components of the inner membrane, generates ATP, coupled to mitochondrial respiration. It has been shown that 4-chloro-7-nitrobenzofurazan (NbfCl) is a potent inhibitor of F_1-ATPase activity from bovine heart [1,2], chloroplast [3,4], and *Escherichia coli* [5,6]. The inhibition correlates with a covalent linkage of NbfCl to F_1-ATPase, and the β-subunit has been found to contain most of the Nbf label although the

* Present address: Department of Chemistry, Stanford University, Stanford, CA 94305, U.S.A.

Abbreviations: Tricine, *N*-tris(hydroxymethyl)methylglycine; Hepps, 4-(2-hydroxyethyl)-1-piperazinepropanesulphonic acid; Pipes, piperazine-*N*,*N'*-bis(2-ethanesulphonic acid); Taps, 3-[tris(hydroxymethyl)-methyl]aminopropanesulphonic acid; ϵ-N_3-ATP, 8-azido-1,N^6-etheno-ATP; NbfCl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, or 4-chloro-7-nitrobenzofurazan; SDS, sodium dodecyl sulphate.

other subunits (particularly the α-subunit) were also labelled depending on the experimental conditions [3,5,7,8]. There is evidence that a single tyrosine residue in a β-subunit of the whole F_1-ATPase molecule has been modified by NbfCl resulting in its inactivation [2]. It should be noted that primary amines and sulphydryl groups might also react with NbfCl [9—11].

In this paper it will be shown that yeast mitochondrial F_1-ATPase activity is inhibited by NbfCl, the α-, β-, γ- and δ-subunits being covalently labelled by [^{14}C]NbfCl with the β-subunit carrying most of the label. Using the NbfCl chromophore as a fluorescence label it can be shown that the inactive enzyme still binds ATP with a K_D of 14 μM.

Materials and Methods

Materials. Yeast F_1-ATPase was prepared according to the method of Takeshige et al. [12] and stored as a suspension in $(NH_4)_2SO_4$. Tricine and Pipes were obtained from Calbiochem, triethanolamine hydrochloride and nucleotide triphosphates from Boehringer Mannheim, and Hepps and Taps from Sigma Chemical Co.; other buffer salts, sucrose and $MgCl_2$ were from Merck, Darmstadt. 2-Mercaptoethanol was purchased from Schuchardt, München, Sephadex G-50 and G-25 (fine) from Pharmacia; *N*-ethylmaleimide, SDS, Coomassie Brilliant Blue R-250 and reagents for polyacrylamide gel electrophoresis from Serva Co., Heidelberg. Organic solvents came from J.T. Baker, Deventer. NbfCl was obtained from Merck, Darmstadt, while [U-^{14}C]NbfCl was purchased from Commissariat à l'Energie Atomique, Centre D'Etudes Nucléaires de Saclay, Gif-sur-Yvette, France. $H_2{}^{35}SO_4$ and [^{3}H]ATP were from Amersham, Buchler. The scintillant cocktail Quickszint 212 came from Kochlight. ϵ-N_3-ATP was kindly provided by Dr. H.-J. Schäfer (Johannes Gutenberg University, Mainz).

Methods. F_1-ATPase activity was measured by the coupled enzyme system [13] and protein concentration by the method of Lowry et al. [14]. Buffers containing triethanolamine hydrochloride, Tricine, Pipes, Hepps or Taps were adjusted to the required pH with KOH.

Rapid desalting of, and removal of excess reagents from, the enzyme solution, were performed by centrifuging through Sephadex G-50 or G-25 (fine) [15].

Photoaffinity labelling of F_1-ATPase with ϵ-N_3-ATP was done in 50 mM Hepps buffer (pH 8.0) containing approx. 100 μM ϵ-N_3-ATP and 1—2 mg F_1-ATPase/ml. Irradiation of samples (in 0.5 ml quartz fluorescence cuvettes) was carried out by placing them for 45 min in the middle of the floor of an ultraviolet cabinet (original Hanaŭ Fluotest, Type 5201, 80 W), with full ultraviolet illumination. The fluorescent photolabelled subunit was detected visually using an ultraviolet Mineral lamp for illumination, after SDS-polyacrylamide gel electrophoresis [16] of the F_1-ATPase sample on 10% gels. The position of the fluorescent band was marked and the gel then stained for protein to enable correlation of the fluorescence with a particular subunit.

The NbfCl reaction with F_1-ATPase was performed at room temperature in the dark, with approx. 100 μM NbfCl and 1—2 mg F_1-ATPase/ml, unless described otherwise. Stock solutions of 8.3 mM NbfCl in ethanol were prepared and samples added to the F_1-ATPase solution. For detection of subunits label-

led with [^{14}C]NbfCl, 250 μg F_1-ATPase was dissolved in 250 μl buffer, desalted, then [^{14}C]NbfCl (3 mM in ethanol) was added to a final concentration of 100 μM. The F_1-ATPase concentration was approx. 2.5 μM. Unreacted NbfCl was removed by centrifugation of samples through buffer-equilibrated Sephadex G-25 gel. Aliquots were taken for SDS-polyacrylamide gel electrophoresis. After staining and destaining the gels, protein bands were excised in the form of 1 mm slices. These were extracted with 1 ml 3% SDS for 24 h at 60°C [17], cooled, and 10 ml Quickzint 212 scintillant cocktail was added for counting of ^{14}C activity in a Searle Liquid Scintillation Counter.

The effect of the reaction of NbfCl with F_1-ATPase on a subsequent photoaffinity labelling of the complex with ϵ-N_3-ATP, was examined by reacting NbfCl and F_1-ATPase. Then, after desalting in 50 mM Hepps buffer (pH 8.0) the protein solution was used for photoaffinity labelling.

ATP binding to the modified F_1-ATPase was studied with [^{3}H]ATP in the presence of $^{35}SO_4^{2-}$, serving as a marker for non-bound low molecular weight ligands. After mixing the Nbf-F_1-ATPase with both radioactive ligands, low molecular weight components were removed by repeated centrifugation through Sephadex G-25 gel as described previously [15,18]. After three centrifugation steps sulphate radioactivity was removed to background level (less than 1% of initial counts) and the percentage of the remaining ATP was determined.

Spectra were measured in a Beckmann 5230 spectrophotometer and fluorescence spectra in a Perkin-Elmer MPF3 fluorimeter. Changes of the equilibrium fluorescence of the Nbf-F_1-ATPase in the presence of nucleotide triphosphates and Mg^{2+} were measured using a SLM 8000 photon-counting spectrofluorimeter equipped with a Hewlett-Packard 9815 calculator and a 9862A plotter for better resolution of intensity changes. CD spectra were obtained with a Jobin Yvon Dichrographe III.

Stopped-flow fluorescence measurements were carried out using a Durrum stopped-flow apparatus with an argon laser as excitation light source as described elsewhere [19].

Results

The reaction of F_1-ATPase with NbfCl

Following the tyrosyl modification mechanism given by Ferguson et al. [2], we analysed the reaction of F_1-ATPase from yeast with NbfCl by recording the absorbance change at 385 nm and the change in enzyme activity. Within 30 min more than 90% of the activity was lost. Activity loss and absorbance change could be described by a single exponential, when a large excess of NbfCl over F_1-ATPase was used as illustrated in Fig. 1, confirming the results of Ferguson et al. [2] obtained with bovine heart F_1-ATPase. With 15 μM F_1-ATPase and 0.3 mM NbfCl in 50 mM triethanolamine, 4 mM EDTA, 200 mM sucrose, pH 7.5, we found rate constants of 0.099 min^{-1} and 0.12 min^{-1} for the absorbance change and the activity loss, respectively. The two almost identical rate constants suggest that the activity loss correlates with the modification of the tyrosine residues. The amplitude of the absorbance change at 385 nm in this experiment was 0.167. Using an extinction coefficient of 11 600 M^{-1}.

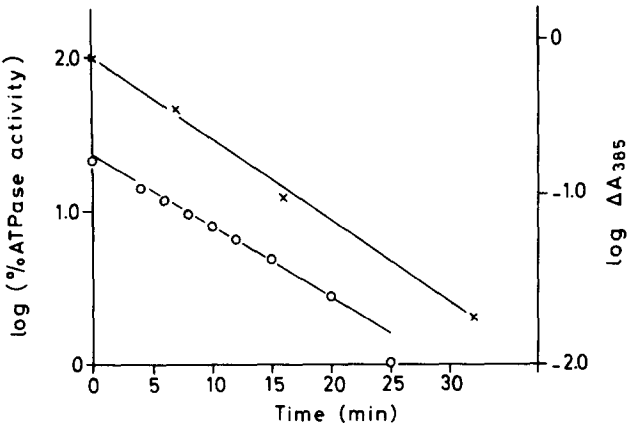

Fig. 1. Kinetics of inactivation of F_1-ATPase by NbfCl. Shown are semilog plots against time of percent ATPase activity (x———x), and of the absorbance charge at 385 nm relative to the endpoint absorbance obtained from a regression analysis (○———○).

cm^{-1} for tyrosyl-*O*-Nbf [2] it can be computed that 0.96 Nbf per F_1-ATPase have caused a complete loss of the ATPase activity.

These experiments confirm the observation made with ATPase from different sources [2,6], that the modification of one single tyrosine residue in F_1-ATPase leads to a complete loss of the ATPase activity. In agreement with the results of Ferguson et al. [2], ATPase activity could be fully restored with 5 mM dithioerythritol, when added to ATPase assays made during the incubation with NbfCl.

NbfCl can react with amino groups in proteins to form a fluorescent-NH-Nbf derivative with an absorbance maximum at approx. 470 nm and a fluorescence emission maximum at 520 nm upon excitation at 470 nm [7,11]. When 1 μM yeast F_1-ATPase was incubated with 100 μM NbfCl at pH 7.5, fluorescence (excitation at 468 nm, emission at 520 nm) was visible within 5 min of mixing, and increased with time. In another experiment, F_1-ATPase was incubated with 250 μM NbfCl at pH 8.4; after 30 h, the sample was desalted into 20 mM Hepps, 5 mM $MgSO_4$, pH 7.7. The solution showed an absorbance maximum at 480 nm and a fluorescence emission peak at 520 nm upon excitation at 480 nm, confirming earlier results obtained with bovine heart F_1-ATPase [7].

Labelling of F_1-ATPase with [^{14}C]NbfCl

The stoicheiometry of binding of Nbf to F_1-ATPase was determined using [^{14}C]NbfCl, as described in Materials and Methods. The incubation medium was 50 mM triethanolamine-HCl, 200 mM sucrose, 4 mM EDTA (pH 7.5). After 30 and 60 min, samples were centrifuged twice through Sephadex G-25 (fine); this removed completely the unbound isotope. At the same time, further samples were preincubated for 5 min with 2 mM dithioerythritol before centrifugation. At 30 min, 64% of the F_1-ATPase activity had been lost and 1.24 mol Nbf were bound per mol F_1-ATPase. The dithioerythritol treatment removed 0.78 mol Nbf/mol F_1-ATPase (and restored full activity). After 60 min, only 19% of the enzymic activity remained, and there were 1.99 mol Nbf bound per

mol enzyme, of which dithioerythritol removed 1.10 mol Nbf/mol enzyme.

The subunits involved in the reaction were identified by SDS electrophoresis of ^{14}C-labelled F_1-ATPase. Most of the radioactivity was found in the β-subunit, although the α- and δ-subunits were also labelled as shown in Fig. 2a, where the position of the radioactive δ-subunit is not correlated by a densitogram peak because of the small amount of the sample used. Control experiments identified this position. Usually, the ($\alpha + \beta$) region contained approx. 84% of the bound label: the above dithioerythritol treatment at 30 min caused a 70% reduction in the label found in the ($\alpha + \beta$) region.

It is known that sulphydryl reagents split the labile -O-Nbf bond [2]. Besides, it can be shown that the -S-Nbf bond can also be cleaved. This was demonstrated by reacting either 50 μM 2-mercaptoethanol or 50 μM reduced glutathione with 200 μM NbfCl in phosphate buffer, pH 7.5. An absorption maximum formed at 420 nm, indicative of the -S-Nbf derivative [10]. Addition of 1 mM dithioerythritol caused the immediate bleaching of the yellow solutions, followed by the gradual appearance of an absorption maximum at 470 nm. The lability of -S-Nbf bonds to sulphydryl reagents was also demonstrated by Cantley et al. [20]. The reaction with sulphydryl reagents can thus be utilized to determine which of the radioactively labelled subunits contains an -O-Nbf or -S-Nbf bond.

With this in mind, the SDS electrophoresis was repeated with 2-mercaptoethanol present during the preparation of the sample (100°C for 2—5 min, in 1% SDS/1% 2-mercaptoethanol). As shown in Fig. 2b, a significant amount of label is lost from the β-subunit, all of the label on the δ-subunit is removed, while the radioactive counts on the α-subunit are relatively unaffected (see cpm scale). Approx. 70% of the label was lost from the ($\alpha + \beta$) region. Further experiments showed that prior reaction of F_1-ATPase with 1 mM *N*-ethylmaleimide reduced labelling of the δ-subunit by 96—99%, whereas labelling of the ($\alpha + \beta$)-subunits was not significantly affected. The δ-subunit of yeast F_1-ATPase has an accessible sulphydryl group (unpublished observation), and it is most likely, that NbfCl reacts with this group. That label which is resistant to 2-mercaptoethanol is assumed to be bound via the more stable -NH-Nbf bond.

At a higher pH, the degree of labelling of F_1-ATPase is expected to increase, as further reactive groups are titrated. This is demonstrated in Fig. 2c which shows the labelling pattern obtained at pH 8.4. Noteworthy is the fact that the γ-subunit now contains significant Nbf label, in addition to the α-, β- and δ-subunits although the β-subunit still carries more label than the other subunits. As noted above, the -*S*-Nbf derivative has an absorbance maximum at 420 nm and some overlap occurs with that of the -*O*-Nbf peak at 385 nm [20]. However, we can exclude the possibility that any such interference drastically alters the above spectrophotometric observations for the inactivation of F_1-ATPase by NbfCl. Thus, when F_1-ATPase was reacted firstly with 1 mM *N*-ethylmaleimide for 20 min at pH 7.5 (to block free sulphydryl groups), and secondly with NbfCl under the usual conditions, less than 5% of F_1-ATPase activity remained after 30 min, agreeing closely with the control, and from the change in absorbance at 385 nm with the extinction coefficient given above, it could be calculated that 1.2 mol -*O*-Nbf derivative had formed per mol F_1-ATPase in 30 min (compared with 0.96 for the native enzyme, as was described earlier).

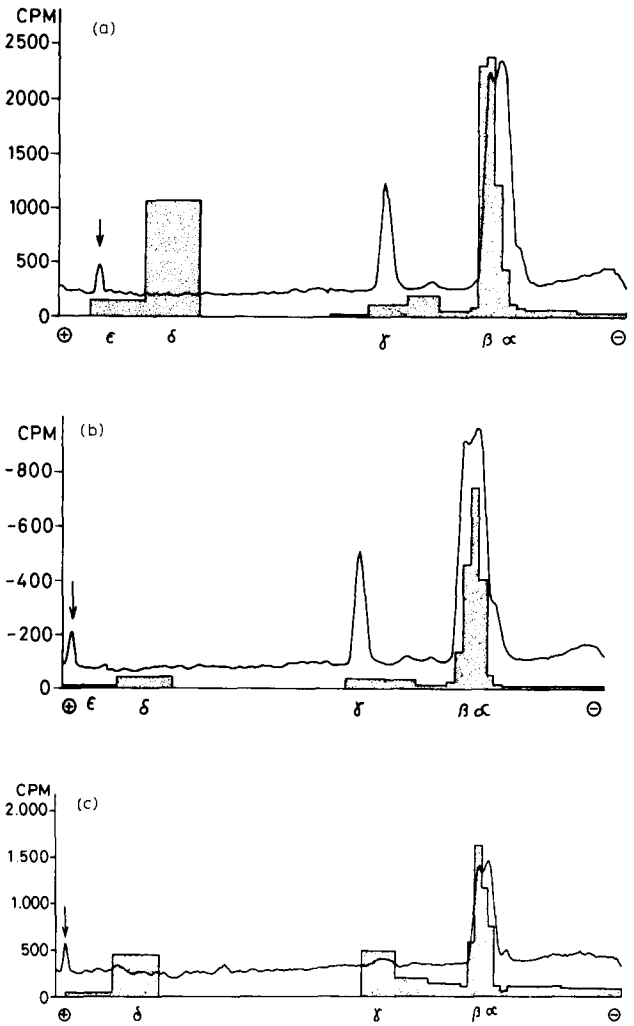

Fig. 2. The distribution of [^{14}C]Nbf in the subunits of F_1-ATPase. ———, absorbance at 540 nm of the stained gel and the shaded areas indicate radioactivity. (a) The F_1-ATPase (1 mg/ml) was incubated for 1 h with [^{14}C]NbfCl in 50 mM Hepps, 15 mM $MgCl_2$, 200 mM sucrose, pH 7.5. After the NbfCl reaction, excess NbfCl was removed by twice desalting in 10 mM sodium phosphate, pH 7.5. SDS-polyacrylamide gel electrophoresis was carried out in the absence of 2-mercaptoethanol. The sample contained approx. 30 μg ATPase. The δ- and ε-subunits are not visible at this loading of the gel. The arrow indicates the position reached by the marker dye. (b) Sample size and conditions are identical to those in (a) except that 1% 2-mercaptoethanol was present during preparation of the sample for electrophoresis. The arrow indicates the position reached by the marker dye. (c) Conditions are the same as in (a) except that the incubation buffer was 50 mM Taps, 15 mM $MgCl_2$, 200 mM sucrose (pH 8.4) and the sample size was approx. 20 μg. The arrow indicates the position reached by the marker dye.

The influence of labelling on nucleotide-binding sites

It has been shown that there are different classes of nucleotide-binding sites or states on F_1-ATPase of low and high affinity [21,22]. The following experiments demonstrate their sensitivity toward labelling.

Low-affinity binding sites. To test whether the modified enzyme could bind

nucleotides with low affinity, the photoaffinity analogue, ϵ-N_3-ATP, was used. This compound has been shown to bind to sites with low affinity [23]. To determine whether the binding of this compound to F_1-ATPase was blocked by prior reaction of F_1-ATPase with NbfCl, F_1-ATPase was first incubated for 90 min with NbfCl at pH 7.5 and then reacted with ϵ-N_3-ATP in the light.

After twice desalting the solution, the fluorescence characteristics were measured. Excitation at 468 nm caused emission at 520 nm (characteristic of -NH-Nbf) while excitation at 325 nm gave an emission peak at 405 nm, which is characteristic of the photolabel; -NH-Nbf does not emit at 405 nm when excited at 325 nm. Thus, the F_1-ATPase was doubly labelled. After SDS electrophoresis, a fluorescent band was found corresponding to the β-subunit. The fluorescent band was not observed in a control experiment where the photolabel was omitted. This result shows that low-affinity nucleotide-binding sites are not lost when NbfCl binds to F_1-ATPase.

High-affinity binding sites. The inactive fluorescent -NH-Nbf derivative of F_1-ATPase is also used to test the state of the high-affinity binding sites for nucleotides. Since ATP is able to bind to both low- and high-affinity nucleotide-binding sites (see Ref. 21), its affinity was analysed by making use of the observation of its influence on the fluorescent Nbf-F_1-ATPase as an internal label bound to a β-subunit. 15 μM F_1-ATPase was reacted with 0.3 mM NbfCl at pH 7.5 for 2 h, the solution was desalted twice into 50 mM Tris/sulfate, 2 mM EDTA (pH 7.4), and fluorescence measurements taken. Upon addition of 16 μM MgATP, a 10.8% increase in fluorescence was observed. The increase was only 2.8% if Mg^{2+} was omitted. In the presence of 25 mM Mg^{2+} alone, an increase of 3.3% was observed. The experiment clearly demonstrates that in the presence of Mg^{2+}, the fluorescence of Nbf-F_1-ATPase is sensitive to ATP binding.

To further characterize the nucleotide-binding sites involved in the observed fluorescence change, stopped-flow experiments were performed. This allowed the determination of the dissociation constant using the change of the fluorescence amplitudes at different ATP concentrations. Fig. 3 shows a reaction progress curve obtained after mixing ATP with Nbf-F_1-ATPase in the presence of 5 mM Mg^{2+}. The figure shows a fast increase with a rate constant of 0.5 s^{-1} followed by a much slower change. The rate constant was independent of the ATP concentration over the range 0—50 μM ATP. This observation excludes a second-order association step and suggests a step subsequent to the binding for the observed fluorescence change.

The dissociation constant for ATP in the complex reaction was calculated from a reciprocal plot of the amplitude of the total fluorescence change against ATP concentration. The results can be represented schematically as follows:

$$\underbrace{\text{NbfE} + \text{AdN} \rightleftharpoons \text{NbfE} \cdot \text{Adn} \overset{0.5\,s^{-1}}{\rightleftharpoons} \text{Nbf}^{*}\text{E} \cdot \text{Adn}}_{K_D = 14\,\mu\text{M}}$$

where NbfE is NbfCl-inactivated F_1-ATPase, AdN is adenine nucleotide, and * denotes a different conformation of the species. Computation yields a value of 14.1 ± 0.5 μM for the dissociation constant and a value of 10.8 ± 0.3% for

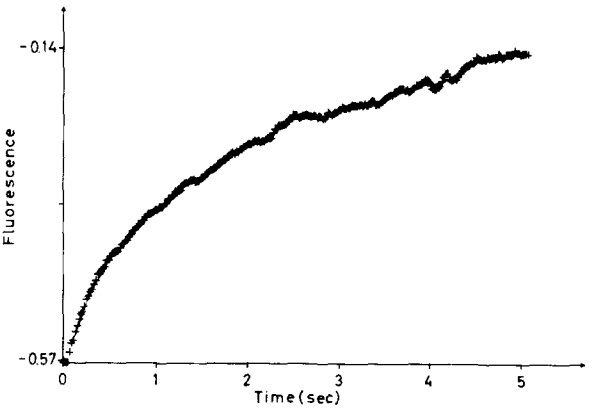

Fig. 3. Fluorescence change upon mixing NbfCl-modified F_1-ATPase with MgATP. One syringe of the stopped-flow apparatus contained 50 μM ATP, 5 mM $MgSO_4$ in 10 mM Taps, 10 mM Pipes buffer, pH 7.6, and the other syringe held 1 μM Nbf-F_1-ATPase and 5 mM $MgSO_4$ in the same buffer. 10 μM F_1-ATPase had been previously incubated at room temperature for 18 h with 200 μM NbfCl in 30 mM Tricine, 15 mM $MgCl_2$, pH 8.4, then the solution desalted and diluted for the stopped-flow experiment. The y-axis shows the voltage change registered on the Nicolet, which is proportional to fluorescence.

the maximum fluorescence change based upon the fluorescence in the absence of ATP. The experiments show that inactive Nbf-F_1-ATPase can bind ATP with high affinity followed by a first-order change with a rate constant of 0.5 s^{-1}.

To substantiate further the finding that Nbf-F_1-ATPase can bind MgATP tightly, a binding experiment with [^{3}H]ATP in the presence of $^{35}SO_4^{2-}$ was carried out as described in Materials and Methods. We found (Fig. 4) that approx. 40% of the total added [^{3}H]ATP is bound to the modified F_1-ATPase,

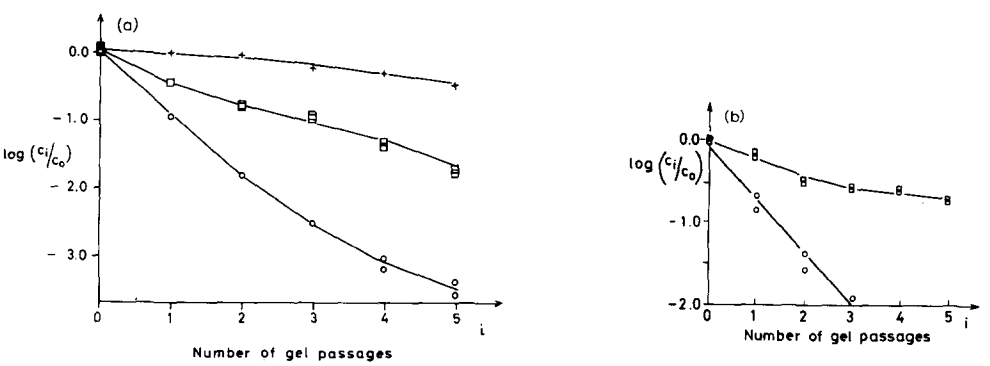

Fig. 4. Dilution of low molecular weight solutes on repeated centrifugation through Sephadex G-25 in 50 mM Hepps (pH 7.8). (a) The figure (reproduced from Ref. 18) shows the control experiment using F_1-ATPase. A 1 mg sample of F_1-ATPase after desalting, was mixed with [^{3}H]ATP and $^{35}SO_4^{2-}$, and centrifuged several times through Sephadex G-25. In each centrifugate, the ATPase activity was determined and an aliquot (10 or 20 μl) was taken for scintillation counting as described in Ref. 18. Enzyme activity (c) is expressed in c_i/c_0. Conditions at the start of the experiment: +———+, 3 μM F_1-ATPase; □———□, 20 μM [^{3}H]ATP approx. 100 000 cpm ^{3}H; ○———○, 15 000 cpm $^{35}SO_4^{2-}$. (b) The figure shows an experiment similar to that described in (a) but using Nbf-F_1-ATPase. The enzyme in 50 mM triethanolamine-HCl, 200 mM sucrose, 4 mM EDTA (pH 7.5) was treated with NbfCl for 3 h as described in Materials and Methods. After desalting into 50 mM Hepps, 5 mM $MgCl_2$ (pH 8.0), the above isotope mixture was added and the sample centrifuged several times through Sephadex: □———□, [^{3}H]ATP; ○———○, $^{35}SO_4^{2-}$.

thus confirming the finding that tight ATP-binding sites are not lost by modifying yeast F_1-ATPase with NbfCl.

Furthermore, the possibility of gross structural changes induced by the labelling was excluded by analysis of the circular dichroism spectrum of the doubly labelled F_1-ATPase after reaction first with NbfCl and then photolabelling with ϵ-N_3-ATP. We found a spectrum with a value for $\Delta\epsilon$ of 16 000 at 220 nm, well in comparison with native F_1-ATPase giving a $\Delta\epsilon$ of 15 600.

From these experiments we conclude that Nbf-F_1-ATPase still binds nucleotides at its low-affinity site as well as MgATP at its high-affinity binding site.

Discussion

In common with the F_1-ATPase from bovine heart [1], chloroplast [3] and *E. coli* [5], the yeast enzyme activity is also inhibited by NbfCl. The spectrophotometric results are consistent with the total inactivation of F_1-ATPase by covalent binding to a tyrosine residue of approx. one Nbf moiety per F_1-ATPase molecule, indicating a highly cooperative function of the multicomponent molecule.

Radioactive labelling showed that the β-subunit was labelled most strongly, but that the α-subunit and the δ-subunit also bound some NbfCl at pH 7.5. The labelling of the α-subunit was most likely to be via an -NH-Nbf derivative because of the absence of any significant change in the amount of label bound when polyacrylamide gel samples were prepared in the absence or presence of 2-mercaptoethanol. The labelling of the δ-subunit was concluded to be via an -S-Nbf derivative because (i) the labelling is prevented by prior reaction of F_1-ATPase with *N*-ethylmaleimide; (ii) the label is removed by 2-mercaptoethanol, which can act on -S-Nbf bonds as well as acting on -O-Nbf bonds, and (iii) the δ-subunit contains an accessible sulphydryl group (unpublished observation). These facts taken together with the similarity of the results from the comparative experiments with native and *N*-ethylmaleimide-reacted F_1-ATPase, argue that this minor labelling did not influence the inactivation of F_1-ATPase by NbfCl and that this loss of activity reflects events in the β-subunits.

The labelling pattern is pH dependent as evidenced by significant labelling of the γ-subunit if the reaction is carried out at pH 8.4. The labelling pattern is in agreement with those for the other enzyme sources noted above, where the β-subunit was found to be labelled most [3,5,7].

Although the spectrophotometric data imply full inactivation of F_1-ATPase by covalent modification of one tyrosine residue per F_1-ATPase, the [^{14}C]-NbfCl binding data show that the enzyme can bind more that one Nbf moiety. Thus, the F_1-ATPase bound 1.24 mol Nbf/mol and approx. 2 mol Nbf/mol in 30 min and 60 min, respectively, although full inactivation had not occurred over this time. The SDS electrophoresis showed that most of the label was bound to the β-subunit; because it was not always easy to separate clearly the α- and β-subunits, reference is made to combined (α + β)-subunits. In the above-mentioned experiment, the (α + β)-subunits at 30 min contained approx. 84% of the bound label. Further treatment of this Nbf-F_1-ATPase sample with 2 mM dithioerythritol restored the F_1-ATPase activity to the control level, corresponding to a depletion of 0.78 mol Nbf/mol F_1-ATPase. It was estimated that

approx. 0.73 mol Nbf/mol F_1-ATPase was removed from the ($\alpha + \beta$)-subunits by this treatment. A small amount (12%) of the label on the δ-subunit was also removed by dithioerythritol. Full depletion required the more severe conditions of sample preparation for SDS electrophoresis. The results imply that the inactivation of F_1-ATPase was the result of the covalent modification of a group in ($\alpha + \beta$) (i.e., the β-subunit) to produce a labile Nbf moiety. Ferguson et al. [2] have listed spectroscopic evidence that sulphydryl reagents remove the Nbf moiety from the labile tyrosyl-O-Nbf bond, whereas the more stable -NH-Nbf bonds are not affected [2,7]. It is most likely then, that our results show the removal of the Nbf moiety by dithioerythritol or 2-mercaptoethanol from an Nbf-substituted tyrosine on the β-subunit. The absence of SH groups in the yeast β-subunit (unpublished observation) excludes the involvement of -S-Nbf bonds.

It is assumed that the dithioerythritol-resistant label (0.46 mol Nbf/mol at 30 min and 0.89 mol Nbf/mol enzyme at 60 min) represents the more stable -NH-Nbf moiety (as well as possibly a small amount of -S-Nbf, see the discussion above with respect to the δ-subunit). The slow formation with time of -NH-Nbf (fluorescence data) when F_1-ATPase is incubated with NbfCl, is consistent with this.

A most interesting result was the finding that the covalent binding of NbfCl to yeast F_1-ATPase to produce an inactive enzyme did not prevent binding of MgATP to tight binding sites, nor did it prevent the binding of ϵ-N_3-ATP to low-affinity nucleotide-binding sites (both on the β-subunit), suggesting that these sites are not directly involved in the NbfCl reaction. Both nucleotide-binding experiments were done with F_1-ATPase which had reacted with NbfCl for a time sufficiently long to allow for substantial formation of the -NH-Nbf derivative. According to Ferguson et al. [7], this product forms upon alkalinization via a slow intramolecular transfer of the Nbf moiety from the tyrosine to an amino group, from which it is clear that these two reactive groups must be situated very close together. In contrast, work with F_1-ATPase from bovine heart mitochondria and *E. coli* showed that prior reaction of F_1-ATPase with NbfCl did prevent photoaffinity labelling of the β-subunit (but not of the α-subunit) by a different photoaffinity label, *N*-4-azido-2-nitrophenyl-γ-aminobutyryl-ADP or -ATP [6,24], a result which may be explained by steric differences after labelling.

For the first time, a stopped-flow kinetic analysis of nucleotide binding has been performed on the inactive F_1-ATPase. The stopped-flow fluorescence results obtained with MgATP binding to Nbf-F_1-ATPase indicate that the modified enzyme is able to bind nucleotide and undergo a conformational change, but the Nbf moiety prevents actual hydrolysis of ATP. A study of aurovertin fluorescence of NbfCl-modified bovine heart F_1-ATPase led to the same conclusion [25]. Thus, the Nbf-modified ATPase lends itself to nucleotide-binding studies.

The fact that one Nbf moiety per F_1-ATPase suffices to inhibit almost all ATPase activity, agrees with the proposed model of the enzyme where ATP hydrolysis occurs via an alternating site mechanism [6,18,24,26,27]. The binding of only one NbfCl to a tyrosine on a β-subunit, at or close to one site, would clearly be sufficient to inhibit the whole of the cyclical catalytic process, while

leaving other sites free. It is interesting to note that an alternating site model has been proposed for *E. coli* F_1-ATPase on the basis of experiments with dicyclohexylcarbodiimide which also binds to the β-subunit to inactivate the enzyme [28].

Acknowledgments

We wish to thank Mrs. M. Böhm for providing us with the yeast F_1-ATPase, Dr. J. Watters for measuring the CD spectra as well as Dr. Schäfer for a generous gift of ϵ-N_3-ATP. Helpful discussions with Drs. R. Jürss and M. Engelhard are gratefully acknowledged.

References

1 Ferguson, S.J., Lloyd, W.J. and Radda, G.K. (1974) Biochem. Soc. Trans. 2, 501—502
2 Ferguson, S.J., Lloyd, W.J., Lyons, M.H. and Radda, G.K. (1975) Eur. J. Biochem. 54, 117—126
3 Deters, D. and Nelson, N. (1974) Fed. Proc. 33, 602
4 Girault, G. and Galmiche, J.M. (1977) Eur. J. Biochem. 77, 501—510
5 Nelson, N., Kanner, B.I. and Gutnick, D.L. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 2720—2724
6 Lunardi, J., Satre, M., Bof, M. and Vignais, P.V. (1979) Biochemistry 18, 5310—5316
7 Ferguson, S.J., Lloyd, W.J. and Radda, G.K. (1975) Eur. J. Biochem. 54, 127—133
8 Deters, D.W., Racker, E., Nelson, N. and Nelson, H. (1975) J. Biol. Chem. 250, 1041—1047
9 Ghosh, P.B. and Whitehouse, M.W. (1968) Biochem. J. 108, 155—156
10 Birkett, D.J., Price, N.C., Radda, G.K. and Salmon, A.G. (1970) FEBS Lett. 6, 346—348
11 Aboderin, A.A., Boedefeld, E. and Luisi, P.L. (1973) Biochim. Biophys. Acta 328, 20—30
12 Takeshige, K., Hess, B., Böhm, M. and Zimmermann-Telschow, H. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 1605—1622
13 Pullmann, M.E., Penefsky, H.S., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322—3329
14 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
15 Neal, M.W. and Florini, J.R. (1973) Anal. Biochem. 55, 328—330
16 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
17 Senior, A.E. (1975) Biochemistry 14, 660—664
18 Recktenwald, D. and Hess, B. (1980) Biochim. Biophys. Acta 592, 377—384
19 Jürss, R., Prinz, H. and Maelicke, A. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 1064—1068
20 Cantley, L.C., Gelles, J. and Josephson, L. (1978) Biochemistry 17, 418—425
21 Harris, D.A. (1978) Biochim. Biophys. Acta 463, 245—273
22 Recktenwald, D. (1978) Ph.D. Thesis, University of Bochum
23 Gregory, R., Recktenwald, D., Hess, B., Schäfer, H.-J., Scheurich, P. and Dose, K. (1979) FEBS Lett. 108, 253—256
24 Lunardi, J. and Vignais, P.V. (1979) FEBS Lett. 102, 23—28
25 Ferguson, S.J., Lloyd, W.J., Radda, G.K. and Slater, E.C. (1976) Biochim. Biophys. Acta 430, 189—193
26 Moudrianakis, E.N. and Adolfsen, R. (1975) in Electron Transfer Chains and Oxidative Phosphorylation (Quagliariello, E., Papa, S., Palmieri, F., Slater, E.C. and Siliprandi, N., eds.), pp. 373—378, North Holland/Elsevier, Amsterdam
27 Kyalar, C., Rosing, J. and Boyer, P.D. (1977) J. Biol. Chem. 252, 2486—2491
28 Satre, M., Lunardi, J., Pougeois, R. and Vignais, P.V. (1979) Biochemistry 18, 3134—3140