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Effect of inhibitor binding to β subunits of F_1ATPase on enzyme thermostability: a kinetic and FT-IR spectroscopic analysis

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Abstract FT-IR analysis shows that treatment of F_1ATPase with the inhibitors DCCD and Nbf-Cl, in the presence of saturating concentrations of ADP and AMP-PNP and in the absence of Mg^{2+}, does not modify the secondary structure of the enzyme, but significantly modifies its compactness and thermal stability, although to different extents. Nbf-Cl causes a significant increase in stabilisation, in addition to that induced by nucleotides, while DCCD is less effective in this regard. Determination by HPLC of the exchange rate, in the absence of Mg^{2+}, of tightly bound nucleotides of F_1ATPase treated with the two inhibitors shows that DCCD does not significantly affect the exchange rate of ADP with AMP-PNP and vice versa in catalytic and non-catalytic tight sites, while Nbf-Cl selectively reduces the enzyme's capacity to exchange ADP bound in the tight catalytic site. It is suggested that the effects of DCCD, unlike those of Nbf-Cl, are closely related to the presence or absence of Mg^{2+}.

Key words: F_1-ATPase; Chemical labeling; 7-Chloro-4-nitrobenz-2-oxa-1,3-diazole; Dicyclohexylcarbodiimide; Infrared spectroscopy; Protein structure

1. Introduction

F_1ATPase, the catalytic component of ATP synthase [1], consists of five different subunits with the stoichiometry $3\alpha:3\beta:1\gamma:1\delta:1\varepsilon$. The mitochondrial enzyme, the structure of which was determined by X-ray diffraction at 2.8 Å [2], contains six nucleotide binding sites. The three catalytic sites located in β subunits exchange bound nucleotides rapidly during catalytic turnover, while the three non-catalytic sites on α subunits exchange nucleotides very slowly [3]. When the enzyme is subjected to repeated ammonium sulphate precipitation, it retains three bound nucleotides in tight sites, two non-catalytic and one catalytic [4]. According to the binding change mechanism [5], the prominent feature of F_1 catalysis is the sequential participation of the three catalytic sites (tri-site catalysis) driven by the rotation of the $\alpha_3\beta_3$ aggregate relative to the γ subunit, probably in a clockwise direction during ATP hydrolysis and in a counterclockwise direction during ATP synthesis [6,7].

ATP synthases are inhibited by various classes of inhibitors, which may react with the whole enzyme or with the F_1 portion only. A wide variety of inhibitors is known for mitochondrial, chloroplast and bacterial F_1. In this study, we chose two covalent inhibitors, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (Nbf-Cl) and dicyclohexylcarbodiimide (DCCD), which bind to β subunits. In mitochondrial F_1ATPase Nbf-Cl reacts with Tyr-311 [8], which is located on the surface at 5.5 Å from the terminal phosphate of the nucleotides in the catalytic site [2]. Conversely, DCCD binds to Glu-199 [9], which is located in the conical tunnel leading to the catalytic sites [2]. Both inhibitors have the peculiarity of leading to a greater degree of inhibition of Mg^{2+}-ATP hydrolysis than synthesis [10,11]. The almost complete inhibition of Mg^{2+}-ATP hydrolysis requires the binding of 1 mol of Nbf-Cl/mol [12] and 2–3 mol of DCCD/mol of enzyme [9,13]. It has been reported that in mitochondrial enzyme this inhibition is due to the reduction and not to the abolition of the trisite catalytic capacity for Mg^{2+}-ATP hydrolysis: the hypothesis is that both inhibitors can prevent the binding changes necessary for the release of Mg^{2+}-ADP and phosphate from the catalytic sites [10]. Conversely, other studies indicate that DCCD-inactivated enzyme behave differently from Nbf-Cl-modified F_1ATPase [13,14].

To obtain further insight into the mechanism of action of these inhibitors, in this study we used Fourier transform infrared (FT-IR) spectroscopy to analyse the secondary structure and the thermal stability of beef heart F_1ATPase upon treatment with DCCD and Nbf-Cl. The FT-IR technique has already been used with success to determine the conformational changes of F_1ATPase induced by saturating concentrations of ADP or AMP-PNP, a non-hydrolysable analogue of ATP [15]. The rate of exchange of nucleotides bound in tight sites in the enzyme modified with both inhibitors has also been analysed. Since it is known that DCCD and Nbf-Cl effects are largely modulated, although in different ways, by Mg^{2+} and nucleotides [12,13], FT-IR and nucleotide exchange experiments were performed in the absence of Mg^{2+}. The absence of Mg^{2+} is the same condition previously used in FT-IR analysis of F_1ATPase [15]. Results indicate that, in this condition, Nbf-Cl influences the nucleotide exchange rate, and the FT-IR spectra, unlike DCCD.

2. Materials and methods

2.1. Materials

Deuterium oxide (99.9% ^2H_2O) was purchased from Aldrich. ADP, ATP, AMP-PNP, DCCD and Nbf-Cl were from Sigma. The enzymes for F_1ATPase assay were purchased from Boehringer Mannheim. All other chemicals were commercial samples of the purest quality.

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Abbreviations: FT-IR, Fourier transform infrared; Amide I′, amide I band in a ^2H_2O medium; DCCD, dicyclohexylcarbodiimide; Nbf-Cl, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; AMP-PNP, β,γ-imidoadenosine 5′-triphosphate

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2.2. Preparation of F_1ATPase

Pure soluble F_1ATPase was prepared from beef heart mitochondria as previously described [15]. The purified enzyme was treated with 5 mM EDTA as in [16] and was stored at 4°C in ammonium sulphate suspension in 2 mM EDTA.

2.3. Chemical modification of purified F_1ATPase

F_1ATPase was dissolved at 2 mg/ml in 20 mM HEPES, 200 mM NaCl, pH 8.0 and added with 0.15 mM Nbf-Cl or 0.5 mM DCCD at 25°C. Incubation was prolonged until >90% of the Mg^{2+}-ATP hydrolysis rate was inhibited with both DCCD and Nbf-Cl. Reactions with inhibitors were terminated by precipitation with ammonium sulphate. In these conditions, each mol of F_1ATPase incorporated more than 1 mol of DCCD/mol of enzyme, as reported [9,10,13], and about 1 mol of Nbf-Cl, as reported [12]. The Nbf-Cl reaction was followed by monitoring the increase in absorbance at 385 nm of the Nbf-Cl-F_1ATPase chromophore, as in [12].

2.4. Analytical procedures

The Mg^{2+}-ATP hydrolysis rate was assayed spectrophotometrically at 30°C, as in [15]. Average specific activity of F_1ATPase preparations was 120 ± 10 μmol/min/mg. Protein concentration was determined as in [17].

2.5. Preparation of samples for infrared measurement

Typically [15], 2 mg of F_1ATPase was dissolved in 1 ml of buffer A (20 mM HEPES, 200 mM NaCl, p^2H 8.0) or buffer B, C, D, E and F, the composition and p^2H of which were like those of buffer A but also contained 5 mM ADP (B), 1 mM AMP-PNP (C), 5 mM ADP plus 0.15 mM Nbf-Cl (D), 1 mM AMP-PNP plus 0.15 mM Nbf-Cl (E) or 0.5 mM DCCD (F), all at 25°C. F_1ATPase treated with buffers A, B, or C was transferred in a '30K microsep' microconcentrator (Dasit) and centrifuged at 3000×*g* and 25°C to a final volume of approximately 60 μl. Then 200 μl of corresponding buffer was added and the sample was concentrated again. This procedure was repeated several times. In the last wash, the F_1ATPase solution was concentrated to a final volume of about 40 μl and analysed by FT-IR spectroscopy. Samples obtained after several washes with buffers A–C are referred to throughout the paper as sol/F_1, ADP/F_1, and AMP-PNP/F_1, respectively, as in [15]. Sol/F_1 contained 3 mol of tightly bound nucleotides/mol enzyme, as in [15].

For FT-IR analysis of F_1ATPase treated with buffers containing the inhibitors the enzyme was washed, as described above, as follows: F_1ATPase in buffer D with buffer B in order to obtain Nbf-Cl/F_1/ADP; F_1ATPase in buffer E with buffer C in order to obtain Nbf-Cl/F_1/AMP-PNP; F_1ATPase in buffer F with buffers B or C in order to obtain DCCD/F_1/ADP or DCCD/F_1/AMP-PNP respectively. To check the ability to exchange nucleotides, Nbf-Cl/F_1/ADP or Nbf-Cl/F_1/AMP-PNP were washed with buffer C or B respectively. The resulting samples are referred to as Nbf-Cl/F_1/ADP/AMP-PNP or Nbf-Cl/F_1/AMP-PNP/ADP.

2.6. Infrared spectra

The concentrated F_1ATPase samples in the final buffers were analysed using a Perkin-Elmer 1760-x FT-IR spectrometer as described [15]. Deconvolution parameters were set with a half-bandwidth at 18 cm^{-1} and a resolution enhancement factor of 2.5.

2.7. Preparation of samples for tightly bound nucleotide exchange experiments

F_1ATPase, treated or otherwise with the inhibitors as described in Section 2.3, was suspended in 20 mM HEPES, 200 mM NaCl, pH 8.0, and incubated for 3 h with 5 mM ADP or 1 mM AMP-PNP. Loosely bound nucleotides were then removed by two precipitation/dissolution cycles using 2.2 M $(NH_4)_2SO_4$. The enzyme containing tightly bound ADP was incubated with 1 mM AMP-PNP and vice versa, that containing tightly bound AMP-PNP was incubated with 5 mM ADP. At different incubation times aliquots were removed and applied to centrifuge columns equilibrated with 20 mM HEPES, 200 mM NaCl, pH 8.0, in the presence of Mg^{2+}, as in [4]. When specified, the samples were then incubated with 1 mM Mg^{2+}-ATP for 1 min and applied to a second centrifuge column as in [10,18]. In all samples, loosely bound nucleotides were then removed as described above, and tightly bound ADP and AMP-PNP were determined by HPLC on a strong anion-exchange column (Partisil SAX-10, Whatman), as in [15].

3. Results

In a previous paper, in which FT-IR spectroscopy was used [15], we showed that treatment of F_1ATPase with saturating ADP or AMP-PNP does not remarkably affect the secondary structure of the enzyme complex, although it does significantly reduce the hydrogens with deuterium (H/D) exchange and increase enzyme thermal stability. We also observed a correlation between the extent of H/D exchange and the thermal denaturation curves: the lower the H/D exchange, the higher the thermal stability [15]. In the present study, conducted with the same technique, we observed a similar behaviour: treatment of F_1ATPase with the inhibitors DCCD and Nbf-Cl in the presence of saturating concentrations of ADP or AMP-PNP did not modify the enzyme secondary structure (Fig. 1), but did significantly modify the ability of H/D exchange (Fig. 1) and enzyme thermal stability (Figs. 2 and 3), although to different extents. Fig. 1 shows, as an example, the deconvoluted spectra of F_1ATPase treated with saturating ADP in the absence and in the presence of DCCD or Nbf-Cl. The secondary structure of F_1ATPase in the presence of different nucleotides has already been described [15]. Comparison of the spectra of Fig. 1 shows that treatment of F_1ATPase with DCCD or Nbf-Cl does not greatly affect the secondary structure of the enzyme, as shown by the almost superimposable amide I′ bands (1700–1600 cm^{-1}). DCCD, and more markedly Nbf-Cl, has the effect of reducing H/D exchange as shown by the higher amide II band intensity (1548 cm^{-1}) and the slightly higher positions of β-sheet bands (1634 cm^{-1}) ([15] and references therein). Fig. 2A,B shows the thermal denaturation profiles of F_1ATPase samples treated with DCCD and Nbf-Cl, respectively. In particular, Fig. 2A shows that DCCD binding to the enzyme has very little additive effect on the thermostability of F_1ATPase induced by ADP

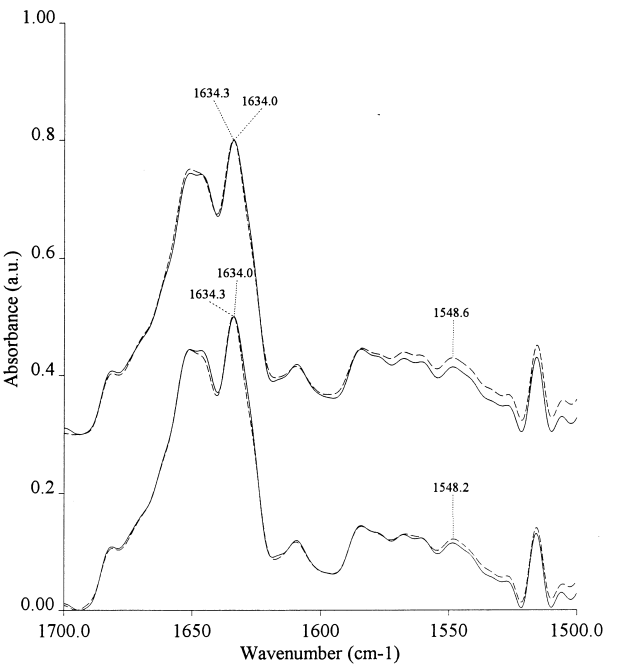

Fig. 1. Deconvoluted spectra of ADP/F_1 in presence of DCCD or Nbf-Cl at 25°C. Upper traces: continuous and dashed lines refer to ADP/F_1 and ADP/F_1/Nbf-Cl, respectively. Lower traces: continuous and dashed lines refer to ADP/F_1 and ADP/F_1/DCCD, respectively.

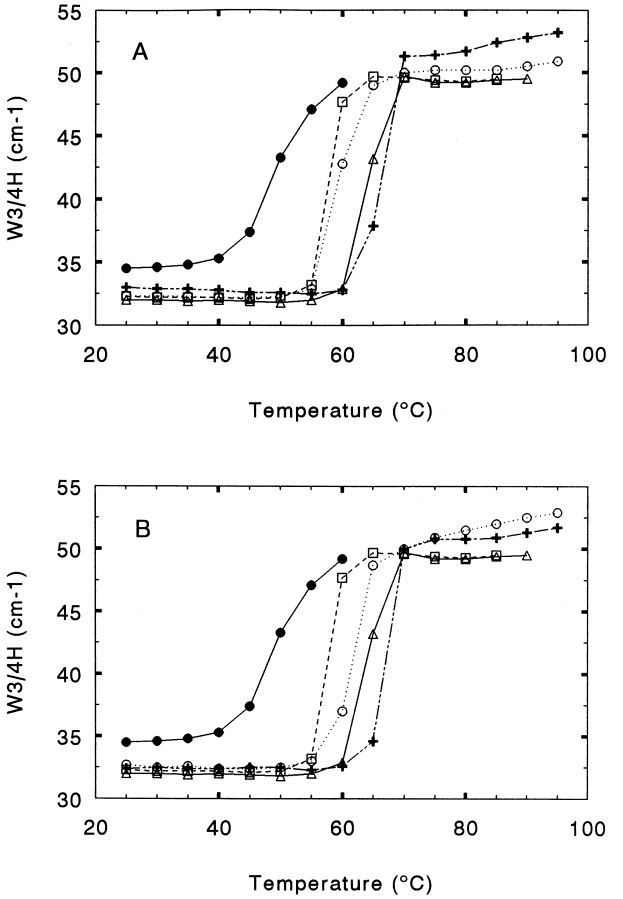

Fig. 2. Thermal denaturation curves of native F_1, DCCD-treated F_1 and Nbf-C-treated F_1. For explanation of the symbols see Section 2. A: Full circles: Sol/F_1. Open triangles and open squares: AMP-PNP/F_1 and ADP/F_1, respectively. Open circles and crosses: DCCD/F_1/ADP and DCCD/F_1/AMP-PNP, respectively. Thermal denaturation curves obtained by monitoring the amide I′ band width calculated at 3/4 of amide I′ band height (W3/4H) as a function of temperature [15]. B: Full circles: Sol/F_1. Open triangles and open squares: AMP-PNP/F_1 and ADP/F_1, respectively. Open circles and crosses: Nbf-Cl/F_1/ADP and Nbf-Cl/F_1/AMP-PNP, respectively.

and AMP-PNP. Fig. 2B shows that Nbf-Cl treatment, unlike that with DCCD, does have a significant additive effect on the stability induced by the nucleotide, although a more pronounced effect is seen in the presence of ADP (i.e. T_m increase of ~5°C) than with AMP-PNP (i.e. T_m increase of ~3°C). The high thermal stability and low H/D exchange induced by nucleotides and inhibitors may have different explanations, i.e. changes in secondary structure composition, and/or changes in the tertiary and/or quaternary structure of the enzyme. As the secondary structure composition of F_1ATPase is not markedly affected by nucleotides and inhibitors, it is possible that the above observed phenomena are due to changes in the tertiary and/or quaternary structure of F_1ATPase which would be more compact in the presence of the above substances.

In these experiments, the stable ATP analogue AMP-PNP was used in order to avoid the slow hydrolysis rate of ATP which was observed to take place even in our conditions, i.e. the absence of Mg^{2+} and the presence of inhibitors, during the long time necessary for the FT-IR sample preparation (see Section 2). ATP hydrolysis is revealed to occur by the thermal denaturation curve of F_1ATPase treated with buffer containing a saturating concentration of ATP, which is the same as that of F_1ATPase treated with buffer containing a saturating concentration of ADP, and this hypothesis was confirmed by analysis of nucleotides bound to F_1ATPase (data not shown). The same stable analogue AMP-PNP instead of ATP has been successfully used in an X-ray/crystallisation study [2].

In Fig. 3, FT-IR thermal denaturation analysis shows that the thermostability induced by nucleotides in the Nbf-Cl-modified enzyme is a completely reversible effect, as seen in the native enzyme. In fact, upon exchange of ADP for AMP-PNP and vice versa the thermal denaturation patterns overlap.

Since FT-IR data suggested that ATP hydrolysis and tightly bound nucleotide exchange may occur even in Nbf-Cl- or DCCD-treated F_1ATPase, we followed the rate of nucleotide exchange by HPLC.

Fig. 4 shows the exchange rate of ADP with AMP-PNP and vice versa in the absence and in the presence of DCCD or Nbf-Cl. Comparison of Fig. 4A with Fig. 4B shows that DCCD does not have any effect on the exchange rate of nucleotides in catalytic and non-catalytic tight sites, nor on the total quantity of nucleotides tightly bound in F_1, i.e. about 3 mol/mol. Conversely, enzyme modification with Nbf-Cl decreases the exchange rate of ADP with AMP-PNP (Fig. 4C), so that the complete exchange is made only after several hours, while the reversal exchange rate of bound AMP-PNP with ADP is not affected.

The effect on the decreased rate of ADP exchange may have different causes: (1) release of ADP from the catalytic tight site, (2) release of ADP from the two non-catalytic tight sites, (3) both types of release. To discriminate among these possibilities, the Nbf-Cl-treated enzyme, which had already exchanged ADP with AMP-PNP, was incubated for 1 min with saturating Mg^{2+}-ATP. This condition activates nucleotide release only at the catalytic sites [18]. Fig. 4C shows that this treatment markedly slows the initial binding of AMP-PNP compared to the control. These data indicate that the

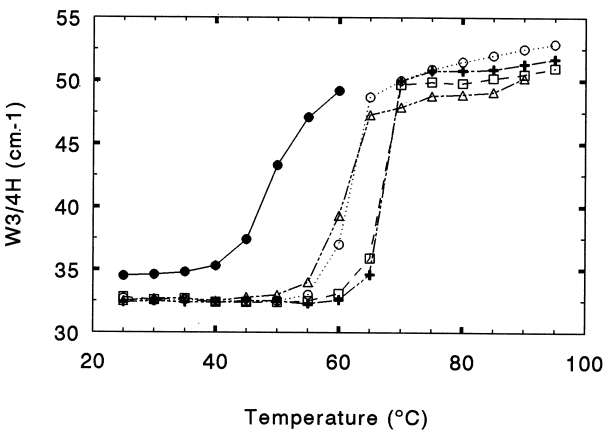

Fig. 3. Thermal denaturation curves of native F_1 and Nbf-Cl-treated F_1 on nucleotide exchange. For explanation of the symbols see Section 2. Full circles, open circles, crosses, open squares and open triangles: Sol/F_1, Nbf-Cl/F_1/ADP, Nbf-Cl/F_1/AMP-PNP, Nbf-Cl/F_1/ADP/AMP-PNP and Nbf-Cl/F_1/AMP-PNP/ADP, respectively. Thermal denaturation curves obtained by monitoring amide I′ band width calculated at 3/4 of amide I′ band height (W3/4H) as a function of temperature [15].

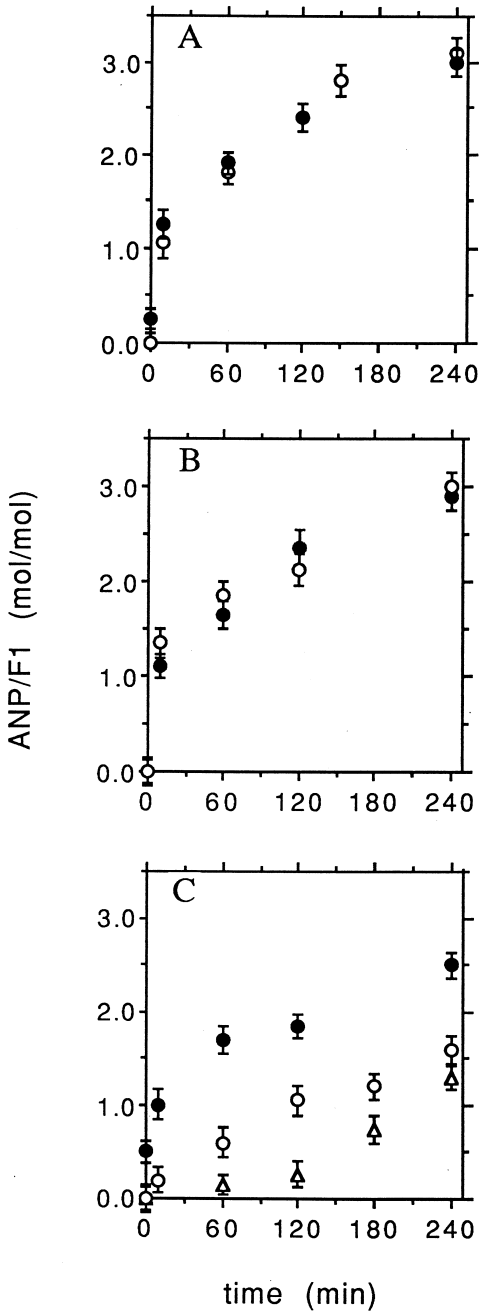

Fig. 4. Exchange rate of tightly bound nucleotides in native F_1, DCCD-treated F_1 and Nbf-Cl-treated F_1. Native F_1 (A), DCCD-treated F_1 (B) and Nbf-Cl-treated F_1 (C) were incubated with 5 mM ADP (○, △) or 1 mM AMP-PNP (●) as described in Section 2. At $t=0$, samples were subjected to two precipitation/dissolution cycles using 2.2 M $(NH_4)_2SO_4$ and subsequentially incubated with 1 mM AMP-PNP (○, △) or 5 mM ADP (●). At the times indicated, aliquots were removed, applied to centrifuge columns, with (△) or without (○,●) subsequent exposure to 1 mM Mg^{2+}-ATP for 1 min. Tightly bound ADP and AMP-PNP were determined, as specified in Section 2. Data are means ± S.D. of three experiments.

first possibility mentioned above is correct, and that reduced ADP exchange with AMP-PNP is definitely related to the catalytic site.

4. Discussion

The data presented in this paper indicate that the two inhibitors DCCD and Nbf-Cl, in the absence of Mg^{2+}, do not modify the secondary structure of F_1ATPase, but affect to different degrees its thermostability and compactness (tertiary and/or quaternary structure). Moreover, the effect of the two inhibitors on the exchange rate of tight nucleotides does not overlap. Comparison between spectroscopic and kinetic data suggests that the selective decrease in the exchange rate of ADP bound in the tight catalytic site induced by Nbf-Cl (Fig. 4C) is related to the more pronounced compactness of the modified enzyme in the presence of ADP, compared with the native enzyme (Figs. 1 and 2B). Moreover, as a strongly reduced capacity to release ADP from the catalytic site in the Nbf-Cl-modified enzyme has also been observed in the presence of Mg^{2+} [10], it may be concluded that the effects of Nbf-Cl are not strictly dependent on the presence of Mg^{2+} in the catalytic sites. This is in accordance with the independence of Nbf-Cl and Mg^{2+} binding sites [19].

The effect of Nbf-Cl may be compared with that recently suggested for azide, another inhibitor of Mg^{2+}-ATP hydrolysis of F_1ATPase [6]. The β subunit containing bound Mg^{2+}-ADP and azide is unable to sustain any round of ATP hydrolysis in a clockwise direction, but can synthesise ATP by rotation in a counterclockwise direction [6]. However, Nbf-Cl, unlike azide, can bind to the β subunit, probably in the open state, as reported in *Escherichia coli* F_1ATPase [20], and not in the closed state as azide [6] and its binding also occurs in the absence of nucleotides and Mg^{2+} [12].

As described in the experiments of Kandpla et al. [10], DCCD, like Nbf-Cl, inhibits Mg^{2+}-ATP hydrolysis by reducing trisite catalysis, so that the three catalytic sites continue to act in sequence, although at a reduced rate. Other experiments have also indicated that, in the presence of Mg^{2+}, DCCD [13], unlike Nbf-Cl [12], inhibits the changes in the fluorescence of the aurovertin-F_1 complex induced by ATP and ADP, thus indicating that DCCD prevents the conformational changes induced by these nucleotides, at least those which are propagated to the aurovertin site. Surprisingly, we observed no decrease in the exchange rate at the tight catalytic site, and our FT-IR analysis indicates that DCCD does not interfere with the stabilisation induced by nucleotides (Fig. 2A). However, both results may be related to our particular experimental conditions, i.e. the absence of Mg^{2+}. It is suggested that the inhibition induced by DCCD on ATP hydrolytic activity depends on the presence of Mg^{2+} in the catalytic sites, the localisation of which is very close to the amino acid residue modified by DCCD [9,19]. It is therefore tempting to hypothesise that DCCD selectively reduces the exchange rate of Mg^{2+}-nucleotide complexes. Moreover, the lack of influence of DCCD on the exchange rate at the tight non-catalytic sites fits with a previous report [3], according to which no effect of DCCD on the exchange of ADP bound in the loose non-catalytic site was observed even in the absence of Mg^{2+}.

In conclusion, the different effects induced by NBf-Cl and DCCD on the thermostability and nucleotide exchange rate of F_1ATPase may be related to the different location of the two inhibitors on the surface of β subunits, i.e. βTyr-311 (Nbf-Cl) [8] and βGlu-199 (DCCD) [9]. They may also explain the different relationship existing between the binding stoichiometry and the percentage inactivation of Nbf-Cl compared to

DCCD [9,12], the effect of which appears to be highly dependent on Mg^{2+}.

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