

# Influence of ADP, AMP-PNP and of depletion of nucleotides on the structural properties of F<sub>1</sub>ATPase: a Fourier transform infrared spectroscopic study

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**Abstract** Mitochondrial F<sub>1</sub>ATPase from beef heart was treated with different buffers in order to modulate the nucleotide content of the enzyme and then analysed by FT-IR spectroscopy. Treatment of F<sub>1</sub>ATPase with a buffer lacking nucleotides and glycerol led to the formation of two fractions consisting of an inactive aggregated enzyme deprived almost completely of bound nucleotides and of an active enzyme containing ATP only in the tight sites and having a structure largely accessible to the solvent and a low thermal stability. Treatment of F<sub>1</sub>ATPase with saturating ADP, which induced the hysteretic inhibition during turnover, or AMP-PNP did not affect remarkably the secondary structure of the enzyme complex but significantly increased its compactness and thermal stability. It was hypothesised that the formation of the inactive aggregated enzyme was mainly due to the destabilisation of the  $\alpha$ -subunits of F<sub>1</sub>ATPase and that the induction of the hysteretic inhibition is related to a particular conformation of the enzyme, which during turnover becomes unable to sustain catalysis.

**Key words:** F<sub>1</sub>ATPase; ATP synthase; Infrared spectroscopy; Protein structure

## 1. Introduction

Mitochondrial F<sub>1</sub>ATPase, the catalytic component of ATP synthase [1], consists of five different subunits with the stoichiometry  $3\alpha:3\beta:1\gamma:1\delta:1\epsilon$ . The enzyme, whose structure was determined by X-ray diffraction at 2.8 Å [2] and 3.6 Å [3] resolution, contains six nucleotide binding sites. Three sites, which exchange bound nucleotides rapidly during catalytic turnover, are referred to as catalytic sites. The remaining three sites exchange bound nucleotides slowly and are referred to as non catalytic sites. The role of the non catalytic sites is obscure. The catalytic and non catalytic binding sites are predominantly in  $\beta$  and  $\alpha$  subunits, respectively [2]. When the enzyme, stored

as a suspension in ammonium sulphate, is pelleted, dissolved and desalted, it retains about 3 mol of bound nucleotide/mol enzyme in tight sites which, according to [4], correspond to two non catalytic sites and one catalytic site. The remaining loose sites may be filled rapidly upon addition of exogenous nucleotides.

Among F<sub>1</sub>ATPase inhibitors, ADP, which can be substrate and inhibitor, plays a special role. In fact two types of inhibition have been described when beef heart F<sub>1</sub>ATPase is incubated in the presence of ADP prior to assay in an ATP regenerating system: one transitory inhibition caused by stoichiometric amounts of ADP added in the presence of Mg<sup>2+</sup> [5,6], and the hysteretic inhibition, which progressively develops during turnover, caused by large excess of ADP incubated without Mg<sup>2+</sup> [6,7].

In this study we analysed the secondary structure and the thermal stability of beef heart F<sub>1</sub>ATPase containing or lacking ATP in tight sites in order to gain information on its role on the enzyme's structure. F<sub>1</sub>ATPase was also treated with saturating ADP to analyse the structure involved in the hysteretic inhibition. A potentially 'fully active enzyme' obtained by treating the protein with an excess of AMP-PNP [8] was also analysed. FT-IR spectroscopy was used in this study since it is a suitable technique for the analysis of protein's secondary structure and because it can give also information on some aspects of protein's three-dimensional structure.

## 2. Materials and methods

### 2.1. Materials

Deuterium oxide (99.9% <sup>2</sup>H<sub>2</sub>O) was purchased from Aldrich. ADP, ATP, AMP-PNP were obtained from Sigma. The enzymes for F<sub>1</sub>ATPase assay were purchased from Boehringer Mannheim. All the other chemicals were commercial samples of the purest quality.

### 2.2. Preparation of F<sub>1</sub>ATPase

Pure soluble F<sub>1</sub>ATPase was prepared from beef heart mitochondria according to [9]. The purified enzyme was then suspended in 20 mM Tris-HCl pH 8.5, 200 mM NaCl, 1 mM ATP, 1 mM EDTA, 5 mM 2-mercaptoethanol and applied to a Pharmacia HiLoad 26/60 column of Sephacryl S-300 according to [2]. The eluted active F<sub>1</sub>ATPase was concentrated and stored at 4°C in 50% ammonium sulphate.

### 2.3. Analytical procedures

SDS-PAGE of F<sub>1</sub>ATPase was performed as in [10]. Protein concentration was determined as in [11].

ATPase activity was assayed spectrophotometrically at 30°C as in [12]. Average specific activity of F<sub>1</sub>ATPase preparations was 160 ± 10 μmol/min/mg.

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**Abbreviations:** FT-IR: Fourier transform infrared; Amide I': amide I band in a <sup>2</sup>H<sub>2</sub>O medium; AMP-PNP:  $\beta,\gamma$ -imidoadenosine 5'-triphosphate; ADP/F<sub>1</sub>: F<sub>1</sub>ATPase in the presence of ADP; AMP-PNP/F<sub>1</sub>: F<sub>1</sub>ATPase in the presence of AMP-PNP; sol/F<sub>1</sub>: soluble part of F<sub>1</sub>ATPase washed with a buffer in the absence of nucleotides; insol/F<sub>1</sub>: insoluble part of F<sub>1</sub>ATPase washed with a buffer in the absence of nucleotides; TMPD: temperature of maximum protein denaturation.

Analyses of adenine nucleotides were performed by HPLC on a strong anion-exchange column (Partisil SAX-10, Whatman) as in [12].

#### 2.4. Preparation of samples for infrared measurements

Typically, 2 mg of protein suspended in ammonium sulphate were centrifuged and the pellet was dissolved in 1 ml of buffer A (20 mM HEPES, 200 mM NaCl, 5 mM EDTA, p<sup>H</sup> 8.0) or buffer B (20 mM HEPES, 200 mM NaCl, 5 mM ADP, 5 mM EDTA, p<sup>H</sup> 8.0) or buffer C (20 mM HEPES, 200 mM NaCl, 1 mM AMP-PNP, 5 mM EDTA, p<sup>H</sup> 8.0) at 25°C. The buffers were prepared in a <sup>2</sup>H<sub>2</sub>O medium. The F<sub>1</sub>ATPase solution was transferred in a '30K microsep' micro concentrator (Dasit) and centrifuged at 3,000 × g and 25°C to a final volume of approximately 70–100 μl. Then 1 ml of corresponding buffer was added and the sample was concentrated again. This procedure was repeated twice. The concentrated sample was then washed in the micro concentrator with the same buffer, but without EDTA, several times. In the last washing the F<sub>1</sub>ATPase solution was concentrated to a final volume of about 40 μl.

#### 2.5. Infrared spectra

The concentrated F<sub>1</sub>ATPase samples in the final buffers were analysed using a Perkin Elmer 1760-x Fourier transform infrared spectrometer as described in [13]. Second derivative spectra were calculated over a 9-data point range (9 cm<sup>-1</sup>). Deconvolution parameters were set with the half-bandwidth at 18 cm<sup>-1</sup> and a resolution enhancement factor of 2.5. The band positions, as otherwise reported, are rounded to the nearest whole figure.

### 3. Results

#### 3.1. Conformation of F<sub>1</sub>ATPase obtained using buffer without nucleotides

The presence of nucleotides in the buffer was, so far, described as fundamental for the stability of mitochondrial isolated F<sub>1</sub>ATPase [9]. The washing of F<sub>1</sub>ATPase with a buffer lacking nucleotides and glycerol led to the formation of a soluble enzyme (sol/F<sub>1</sub>), which had hydrolytic activity, along with

a jelly protein aggregate (insol/F<sub>1</sub>), completely inactive. The soluble and insoluble F<sub>1</sub>ATPase contained 2.8 and 0.7 mol of tightly bound ATP/mol enzyme, respectively.

Fig. 1A shows the infrared spectrum of sol/F<sub>1</sub> sample. The bands at 1632, 1680 and 1671 cm<sup>-1</sup> were assigned to β-sheets. In particular, the presence of a strong peak at 1632 cm<sup>-1</sup> in conjunction with a weak one at 1680 cm<sup>-1</sup> is indicative of an anti parallel β-sheet conformation [14]. The bands at 1650 and 1645 and 1662 cm<sup>-1</sup> were assigned to an α-helix, unordered structures and turns, respectively. The amide I' component bands below 1620 cm<sup>-1</sup> are due to amino acid side-chain absorption [15], but the small shoulder at about 1619 cm<sup>-1</sup> could also contain information on protein intermolecular interactions [16,17]. The bands at 1582 and 1565 cm<sup>-1</sup> are due to absorption of carboxylate residues and the 1515 cm<sup>-1</sup> band to tyrosine residues [15].

The insol/F<sub>1</sub> spectra (Fig. 1B) show two strong bands at 1615 and 1683 cm<sup>-1</sup> which are due to protein intermolecular interactions, (protein aggregation) [13,16,17]. Fig. 1B also shows that the components of amide I' band have different intensities as compared to the spectrum of sol/F<sub>1</sub> indicating different secondary structures in the two samples. In particular, the content of unordered structures is higher in insol/F<sub>1</sub> than in sol/F<sub>1</sub> indicating that unfolding occurred to a some extent in the former sample.

#### 3.2. Conformation of F<sub>1</sub>ATPase obtained using buffer containing ADP without Mg<sup>2+</sup>

F<sub>1</sub>ATPase, when prepared with buffers containing ADP, did not give rise to the formation of jelly precipitate. In this condition (without Mg<sup>2+</sup>) the enzyme exchanged bound ATP with ADP and developed, during turnover, the hysteretic inhibition [6,7], characterised by an uninhibited initial rate which deceler-

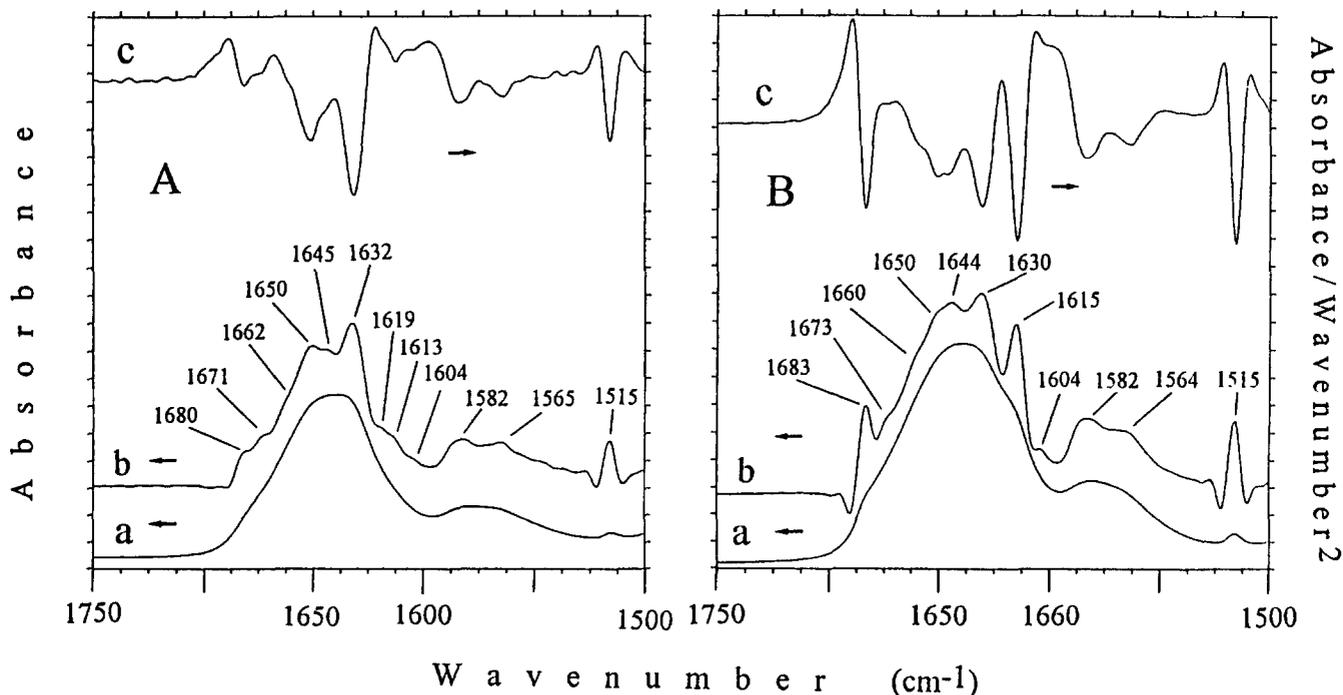


Fig. 1. Infrared spectra of soluble (sol/F<sub>1</sub>) and insoluble (insol/F<sub>1</sub>) part of F<sub>1</sub>ATPase washed with a buffer in the absence of nucleotides at 25°C. Panel (A): sol/F<sub>1</sub>; panel (B): insol/F<sub>1</sub>. (a) original absorbance spectra obtained upon subtraction of buffer A spectrum from sample spectra; (b) deconvoluted absorbance spectra; (c) second derivative spectra.

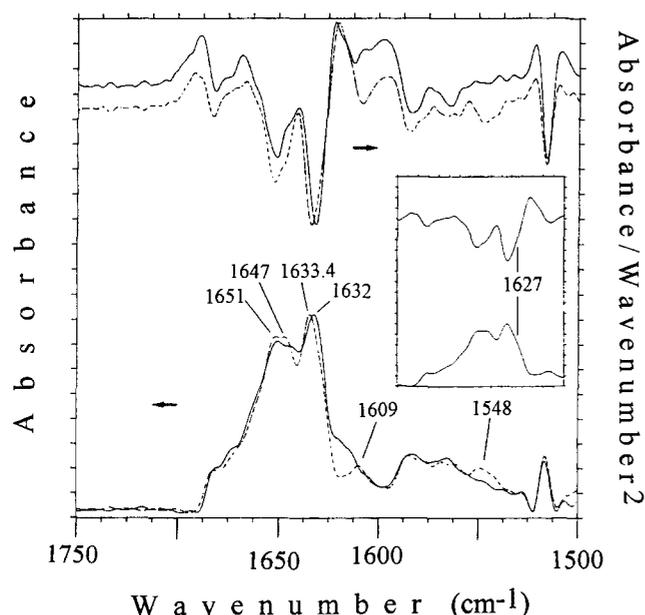


Fig. 2. Infrared spectra of ADP-treated  $F_1$  (ADP/ $F_1$ ) and sol/ $F_1$  at 25°C. Bottom: deconvoluted spectra; top: second derivative spectra. Continuous line: spectra of sol/ $F_1$ ; dashed line: spectra of ADP/ $F_1$ .

ated to an inhibited, steady-state rate. The ATP content after extensive washing with buffer B was 0.11 mol/mol of enzyme.

Spectra of proteins in  $^2H_2O$  show the amide I' component bands at lower positions and show a lower amide II band intensity (at about 1550  $cm^{-1}$ ) as compared to proteins in  $H_2O$ . The shift value and the decrease in the amide II band intensity depend on the extent of  $H \rightarrow ^2H$  exchange [18,19] which, in turn, gives information on the accessibility of the solvent to the protein and on protein compactness. Fig. 2 compares the spectra of ADP-treated  $F_1$ ATPase (ADP/ $F_1$ ) with spectra of sol/ $F_1$ . The peaks related to  $\beta$ -sheets, unordered structures and  $\alpha$ -helix shifted to 1633.4, 1647 and 1651  $cm^{-1}$ , respectively. The higher band positions found in the spectrum of ADP/ $F_1$  as compared to those of sol/ $F_1$  spectrum suggest that ADP prevents a complete isotope exchange. This is also indicated by the 1548  $cm^{-1}$  band, due to residual amide II absorption, which was instead absent in the sol/ $F_1$  sample. These findings, in turn, indicate that the protein in the presence of ADP was not completely accessible to the solvent, probably as a consequence of a more compact conformation assumed by  $F_1$ ATPase. The expanded 1700–1600  $cm^{-1}$  region in the inset shows that the 1633.4  $cm^{-1}$  peak is not symmetric showing a broad shoulder at about 1627  $cm^{-1}$  which may be related to  $\beta$ -strands that are not part of the  $\beta$ -sheet core or to an unusually strongly hydrogen bonded  $\beta$ -

Table 1  
Width of amide I' band for  $F_1$ ATPase under different conditions at 25°C

Sample	Amide I' band width at 1/2 height ( $cm^{-1}$ )	Amide I' band width at 3/4 of height ( $cm^{-1}$ )
insol/ $F_1$	63.9	42.9
sol/ $F_1$	53.0	34.5
ADP/ $F_1$	48.2	32.6
AMP-PNP/ $F_1$	47.7	32.3

sheet [16,17]. This component, which was probably present also in the spectrum of sol/ $F_1$ , is revealed in the spectrum of the ADP/ $F_1$  as a consequence of the band shift from 1632 to 1633.4  $cm^{-1}$ .

The slightly higher intensities of  $\alpha$ -helix and unordered structure bands in ADP/ $F_1$  than in the sol/ $F_1$  spectrum suggest minor differences between the overall secondary structure of the protein in the absence and in the presence of ADP.

An important difference between the sol/ $F_1$  and ADP/ $F_1$  spectra is the absence of the shoulder at about 1619  $cm^{-1}$  in the latter spectrum. We assigned this band to protein aggregation on the basis of the analysis of the amide I' band widths (Table 1), which are sensitive and increase with the occurrence of intermolecular interactions (aggregation). The absence of the 1619  $cm^{-1}$  band in the ADP/ $F_1$  spectrum indicates also that ADP prevented protein intermolecular interactions. This latter case has been indeed observed macroscopically during the preparation of  $F_1$  samples in the presence of nucleotides.

### 3.3. Effect of AMP-PNP on $F_1$ ATPase conformation

Small differences in the intensities of the amide I' component bands of ADP and AMP-PNP-treated  $F_1$ ATPase (AMP-PNP/ $F_1$ , AMP-PNP content 6 mol/mol enzyme) indicate minor dif-

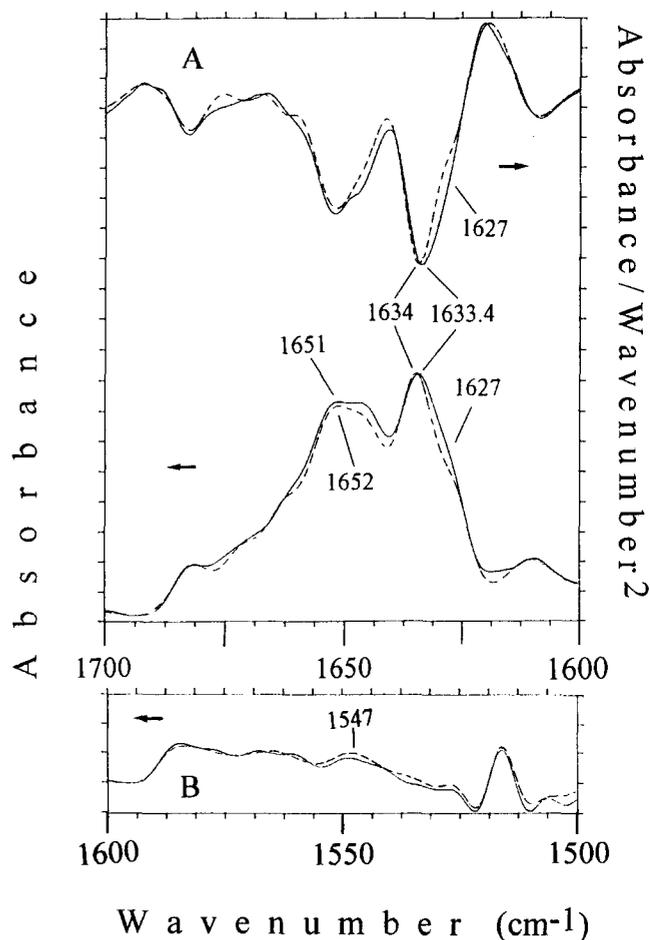


Fig. 3. Infrared spectra of ADP/ $F_1$  and AMP-PNP-treated  $F_1$  (AMP-PNP/ $F_1$ ) at 25°C. Continuous line:  $F_1$ ATPase in the presence of ADP; dashed line  $F_1$ ATPase in the presence of AMP-PNP. Panel (A) amide I' region: bottom, deconvoluted spectra; top, second derivative spectra. Panel (B) deconvoluted spectra in the 1600–1500  $cm^{-1}$  region.

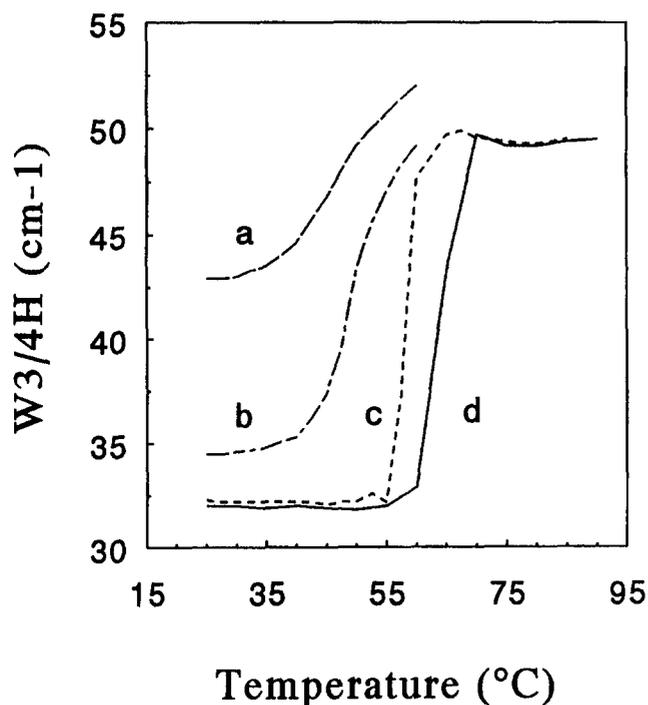


Fig. 4. Temperature-dependent changes of the width of amide I' band for  $F_1$ ATPase under different conditions. The amide I' band width was calculated at  $3/4$  of the amide I' band eight ( $W3/4H$ ). Curves (a–d) refer to  $insol/F_1$ ,  $sol/F_1$ ,  $ADP/F_1$  and  $AMP-PNP/F_1$ , respectively.

ferences between the secondary structures of the two sample preparations (Fig. 3A). A small shift from  $1633.4$  to  $1634\text{ cm}^{-1}$  ( $\beta$ -sheets), which leads to a clearer appearance of the  $1627\text{ cm}^{-1}$  band ( $\beta$ -sheets), parallels a slightly higher  $1547\text{ cm}^{-1}$  band intensity (residual amide II absorption) in the  $AMP-PNP/F_1$  spectrum (Fig. 3B). These findings indicate that in the presence of  $AMP-PNP$  the protein is less accessible to the solvent as compared to  $ADP/F_1$  suggesting, in turn, a different conformation which would be more compact in  $AMP-PNP/F_1$  than in  $ADP/F_1$ . It should be noted that even in the  $AMP-PNP/F_1$  spectrum the shoulder at about  $1619\text{ cm}^{-1}$  is absent as in the  $ADP/F_1$  spectrum, indicating that protein intermolecular interactions are inhibited also by  $AMP-PNP$ .

#### 3.4. Thermal stability of $F_1$ ATPase in the absence and in the presence of nucleotides

Fig. 4 reports the thermal denaturation profiles of  $F_1$ ATPase samples as monitored by the amide I' band-width as a function of temperature [20]. The figure shows that the temperature of maximum protein denaturation (TMPD), corresponding to the curve inflection points, occurs at about  $50$ ,  $60$  and  $65^\circ\text{C}$  in  $sol/F_1$ ,  $ADP/F_1$  and  $AMP-PNP/F_1$ , respectively. This stabilising effect of nucleotides on  $F_1$ ATPase structure probably involved also the tertiary and/or quaternary structure of the enzyme, since the secondary structure of the protein was not markedly affected by  $ADP$  or  $AMP-PNP$  (Figs. 1–3). The curves related to nucleotidestreated samples were steeper as compared to those of  $insol/F_1$  and  $sol/F_1$ , indicating that the denaturation of the former samples was more co-operative with respect to the latter ones.

#### 4. Discussion

The comparison of the spectra of differently treated  $F_1$ ATPase shows that the secondary structure of the enzyme changes slightly except in the case of  $insol/F_1$  sample which showed an increase in the content of unordered structures. The different  $F_1$ ATPase samples show a remarkable dependence of their stability and compactness on the nucleotide binding site occupancy. In fact the FT-IR spectrum of an active enzyme containing ATP only in tight sites ( $sol/F_1$ ) reveals a structure largely accessible to the solvent ( $^2\text{H}_2\text{O}$ ) (Fig. 1) with respect to the nucleotide-treated samples (Figs. 2 and 3). In accordance, the TMPD is also significantly lower in  $sol/F_1$  as compared to nucleotide-treated samples (Fig. 4). Our data clearly show that the presence of nucleotides in tight sites ( $sol/F_1$ ,  $ADP/F_1$ ,  $AMP-PNP/F_1$ ) protects the enzyme from aggregation which instead occurs in the  $insol/F_1$  sample (Fig. 1) almost completely deprived of ATP ( $0.7\text{ mol/mol}$  of enzyme). As the binding of nucleotides to  $F_1$ ATPase stabilises its structure, the removal of ATP from the tight sites could destabilise both  $\beta$  and  $\alpha$  subunits since one tight site (catalytic) and two tight sites (non-catalytic) reside in the former and latter subunits, respectively. However, since the catalytic tight site in  $\beta$  subunits shows a high affinity for ATP [4], the residual ATP of  $insol/F_1$  ( $0.7\text{ mol/mol}$  enzyme) is most probably bound to the  $\beta$ -subunits suggesting, in turn, that the aggregation of  $insol/F_1$  sample is due mainly to the destabilisation and consequent aggregation of  $\alpha$ -subunits lacking bound nucleotides. Although FT-IR spectroscopy cannot tell us if the phenomenon of intermolecular interactions (aggregation) occurs between different  $F_1$  complexes or within  $F_1$  subunits, it is very likely that it involves and/or starts from the  $\alpha$ -subunits of the enzyme since it is observed upon removal of ATP from the tight sites.

The  $1619\text{ cm}^{-1}$  shoulder in the spectrum of  $sol/F_1$  reveals that aggregation occurs to a very low extent in this sample. The presence of this band could be due to (i) contamination of the  $sol/F_1$  sample with  $insol/F_1$  and/or (ii) unfolding and aggregation of a small population of  $\alpha$ -subunits having the tight sites partially deprived of bound ATP ( $2.8\text{ mol/mol}$  enzyme).

The dependence of enzyme compactness on the nucleotide binding site observed in this study for mitochondrial  $F_1$ ATPase is consistent with the conformational changes induced by different nucleotide content in chloroplast ( $CF_1$ ) [21] and in thermophilic bacterium PS3 [22]  $F_1$ ATPases ( $TF_1$ ). The different conformation of  $ADP/F_1$ , which has been analysed before catalysis, with respect that of  $AMP-PNP/F_1$ , could represent the structural basis for the development of the hysteretic inhibition. According to Jault and Allison [6], such inhibition occurs during ATP hydrolysis because  $\text{Mg}^{2+}/\text{ADP}$  is not able to dissociate from a catalytic site. However, similar conformational changes, at least from a qualitatively point of view, seem to be induced by  $ADP$  with respect to  $AMP-PNP$  in  $CF_1$  [21] and  $TF_1$  [22], although these enzymes do not develop the hysteretic inhibition. So, it is plausible that such conformational changes are related to a common physiological role of the nucleotides bound to the enzyme rather than solely to the hysteretic inhibition. Consequently, it is reasonable to hypothesise that in mitochondrial  $F_1$ ATPase the development of the hysteretic inhibition, which is triggered by the addition of  $\text{Mg}^{2+}$  ions, is related to a very punctual conformational change around the catalytic site containing the inhibitory  $ADP$ . This should be in accord-

ance with the peculiar role that  $Mg^{2+}$  ions play in the geometry of the nucleotide binding sites in mitochondrial  $F_1ATPase$  with respect to chloroplast  $F_1ATPase$  [23].

When in a previous FT-IR spectroscopy study [24] we analysed the whole  $F_0F_1ATPase$ , we observed a dramatic change in the secondary structure as a consequence of the insertion of  $F_0$  sector into phospholipids. Conversely, in this study we observed small changes in the secondary structure of isolated  $F_1ATPase$  upon addition of different nucleotides. It is tempting to hypothesise that the changes in the secondary structure in intact ATP synthase might participate in the transmission of energy between the proton-conducting  $F_0$  and catalytic sites in  $F_1$ , while the conformational changes shown in isolated  $F_1ATPase$  might represent an important control of the catalytic activity.

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