Influence of ADP, AMP-PNP and of depletion of nucleotides on the structural properties of F_1ATPase: a Fourier transform infrared spectroscopic study

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Abstract **Mitochondrial F_1ATPase 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_1ATPase 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_1ATPase 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 α-subunits of F_1ATPase 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_1ATPase; ATP synthase; Infrared spectroscopy; Protein structure

1. Introduction

Mitochondrial F_1ATPase, the catalytic component of ATP synthase [1], consists of five different subunits with the stochiometry $3\alpha:3\beta:1\gamma:1\delta:1\varepsilon$. The enzyme, which 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 β and α subunits, respectively [2]. When the enzyme, stored as a suspension in ammonium sulphate, is pelletted, 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_1ATPase 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_1ATPase 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^{2+} [5,6], and the hysteretic inhibition, which progressively develops during turnover, caused by large excess of ADP incubated without Mg^{2+} [6,7].

In this study we analysed the secondary structure and the thermal stability of beef heart F_1ATPase containing or lacking ATP in tight sites in order to gain information on its role on the enzyme's structure. F_1ATPase 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% ^2H_2O) was purchased from Aldrich. ADP, ATP, AMP-PNP were obtained from Sigma. The enzymes for F_1ATPase assay were purchased from Boehringer Mannheim. All the other chemicals were commercial samples of the purest quality.

2.2. Preparation of F_1ATPase

Pure soluble F_1ATPase 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_1ATPase was concentrated and stored at 4°C in 50% ammonium sulphate.

2.3. Analytical procedures

SDS-PAGE of F_1ATPase was performed as in [10]. Protein concentration was determined as in [11].

ATPase activity was assayed spectrofotometrically at 30°C as in [12]. Average specific activity of F_1ATPase preparations was 160 ± 10 μmol/min/mg.

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Abbreviations: FT-IR: Fourier transform infrared; Amide I': amide I band in a ^2H_2O medium; AMP-PNP: β,γ-imidoadenosine 5'-triphosphate; ADP/F_1: F_1ATPase in the presence of ADP; AMP-PNP/F_1: F_1ATPase in the presence of AMP-PNP; sol/F_1: soluble part of F_1ATPase washed with a buffer in the absence of nucleotides; insol/F_1: insoluble part of F_1ATPase washed with a buffer in the absence of nucleotides; TMPD: temperature of maximum protein denaturation.

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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, p²H 8.0) or buffer B (20 mM HEPES, 200 mM NaCl, 5 mM ADP, 5 mM EDTA, p²H 8.0) or buffer C (20 mM HEPES, 200 mM NaCl, 1 mM AMP-PNP, 5 mM EDTA, p²H 8.0) at 25°C. The buffers were prepared in a ^2H_2O medium. The F_1ATPase 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_1ATPase solution was concentrated to a final volume of about 40 μl.

2.5. Infrared spectra

The concentrated F_1ATPase 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^{-1}). Deconvolution parameters were set with the half-bandwidth at 18 cm^{-1} 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_1ATPase 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_1ATPase [9]. The washing of F_1ATPase with a buffer lacking nucleotides and glycerol led to the formation of a soluble enzyme (sol/F_1), which had hydrolytic activity, along with a jelly protein aggregate (insol/F_1), completely inactive. The soluble and insoluble F_1ATPase contained 2.8 an 0.7 mol of tightly bound ATP/mol enzyme, respectively.

Fig. 1A shows the infrared spectrum of sol/F_1 sample. The bands at 1632, 1680 and 1671 cm^{-1} were assigned to β-sheets. In particular, the presence of a strong peak at 1632 cm^{-1} in conjunction with a weak one at 1680 cm^{-1} is indicative of an anti parallel β-sheet conformation [14]. The bands at 1650 and 1645 and 1662 cm^{-1} were assigned to a α-helix, unordered structures and turns, respectively. The amide I′ component bands below 1620 cm^{-1} are due to amino acid side-chain absorption [15], but the small shoulder at about 1619 cm^{-1} could also contain information on protein intermolecular interactions [16,17]. The bands at 1582 and 1565 cm^{-1} are due to absorption of carboxylate residues and the 1515 cm^{-1} band to tyrosine residues [15].

The insol/F_1 spectra (Fig. 1B) show two strong bands at 1615 and 1683 cm^{-1} 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_1 indicating different secondary structures in the two samples. In particular, the content of unordered structures is higher in insol/F_1 than in sol/F_1 indicating that unfolding occurred to a some extent in the former sample.

3.2. Conformation of F_1ATPase obtained using buffer containing ADP without Mg^{2+}

F_1ATPase, when prepared with buffers containing ADP, did not give rise to the formation of jelly precipitate. In this condition (without Mg^{2+}) the enzyme exchanged bound ATP with ADP and developed, during turnover, the hysteretic inhibition [6,7], characterised by an uninhibited initial rate which deceler-

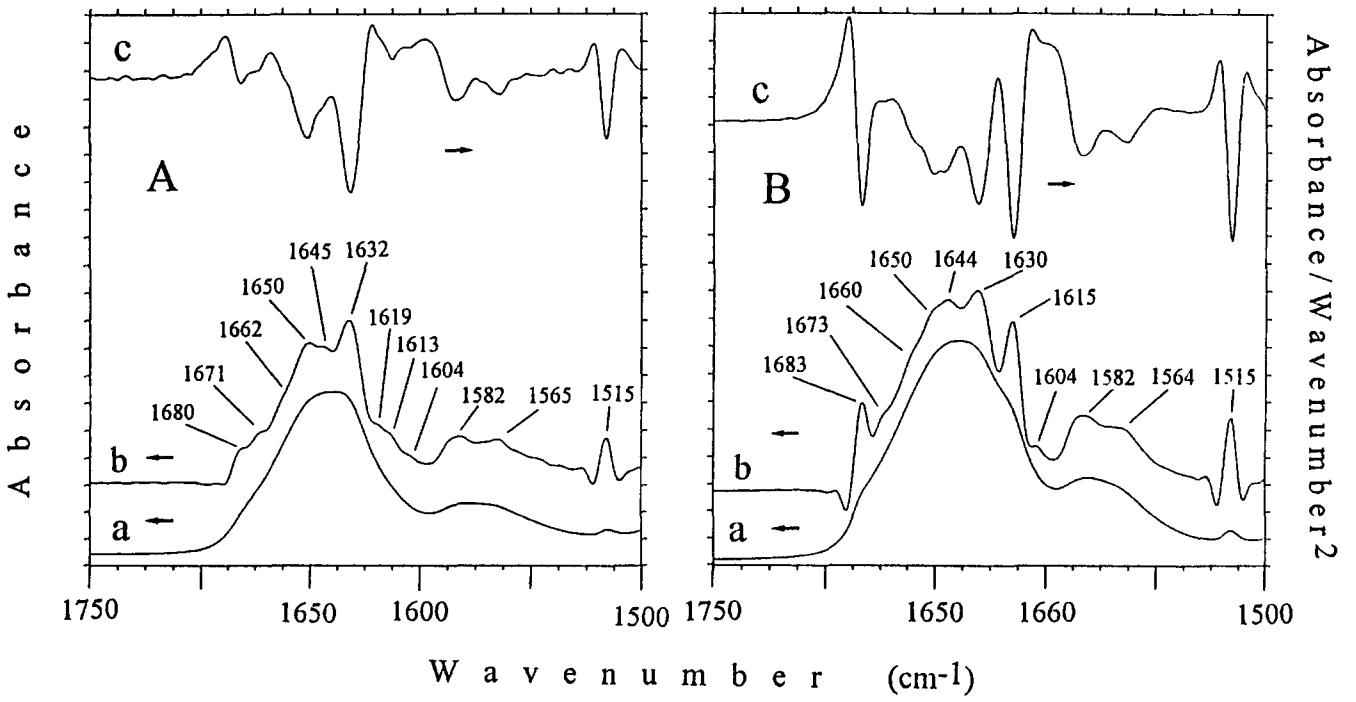

Fig. 1. Infrared spectra of soluble (sol/F_1) and insoluble (insol/F_1) part of F_1ATPase washed with a buffer in the absence of nucleotides at 25°C. Panel (A): sol/F_1; panel (B): insol/F_1. (a) original absorbance spectra obtained upon subtraction of buffer A spectrum from sample spectra; (b) deconvoluted absorbance spectra; (c) second derivative spectra.

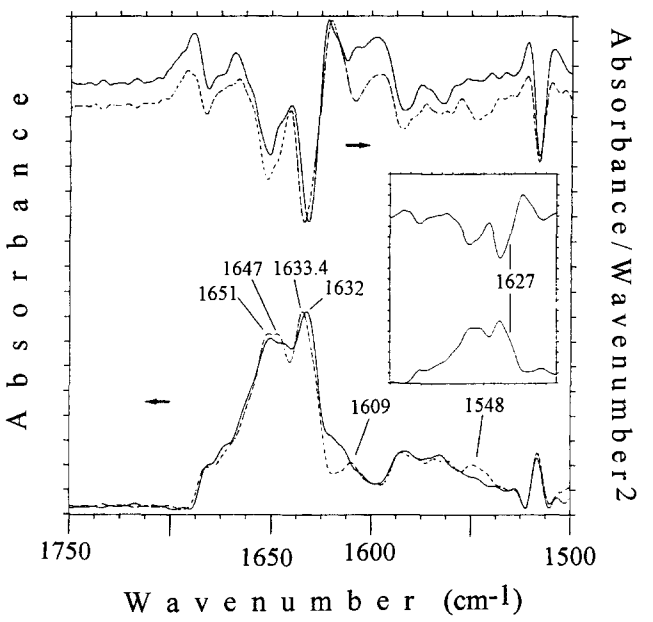

Fig. 2. Infrared spectra of ADP-treated F_1 (ADP/F_1) and sol/F_1 at 25°C. Bottom: deconvoluted spectra; top: second derivative spectra. Continuous line: spectra of sol/F_1; dashed line: spectra of ADP/F_1.

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→^{2}H 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_1ATPase (ADP/F_1) with spectra of sol/F_1. The peaks related to β-sheets, unordered structures and α-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_1ATPase. 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 β-strands that are not part of the β-sheet core or to an unusually strongly hydrogen bonded β-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}.

Table 1
Width of amide I′ band for F_1ATPase 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

The slightly higher intensities of α-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_1ATPase conformation

Small differences in the intensities of the amide I′ component bands of ADP and AMP-PNP-treated F_1ATPase (AMP-PNP/F_1, AMP-PNP content 6 mol/mol enzyme) indicate minor dif-

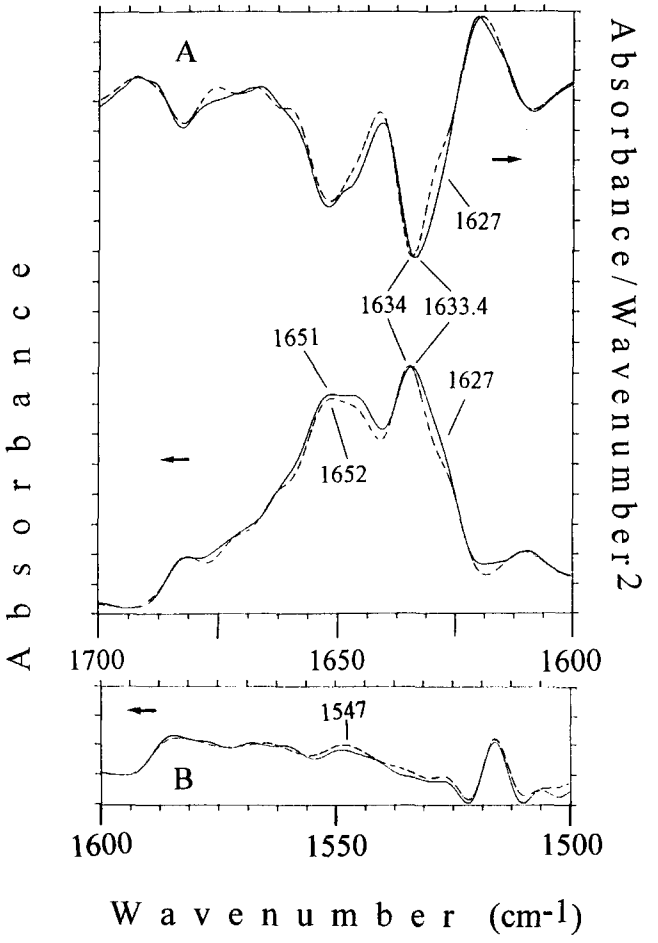

Fig. 3. Infrared spectra of ADP/F_1 and AMP-PNP-treated F_1 (AMP-PNP/F_1) at 25°C. Continuous line: F_1ATPase in the presence of ADP; dashed line F_1ATPase in the presence of AMP-PNP. Panel (A) amide I′ region: bottom, deconvoluted spectra; top, second derivative spectra. Panel (B) deconvoluted spectra in the 1600–1500 cm^{-1} region.

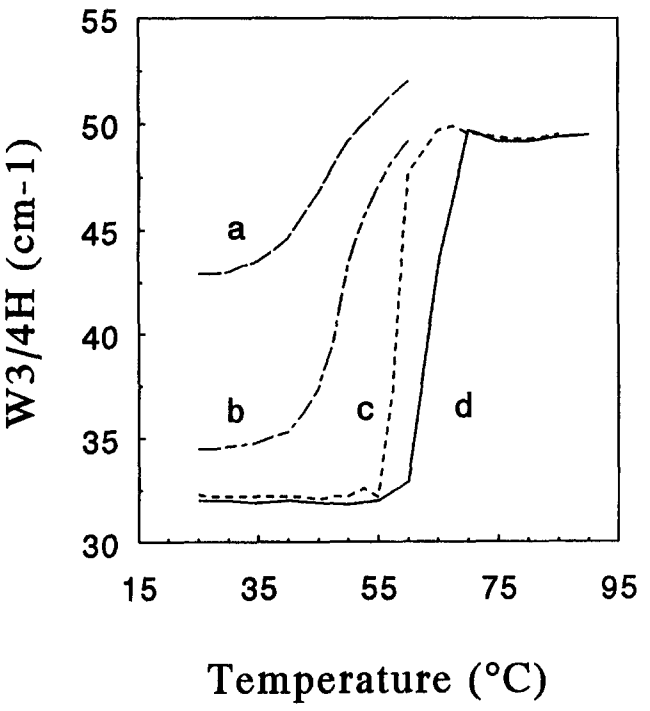

Fig. 4. Temperature-dependent changes of the width of amide I′ band for F_1ATPase under different conditions. The amide I′ band width was calculated at 3/4 of the amide I′ band eight (W3/4H). Curves (a–d) refer to insol/F_1, sol/F_1, ADP/F_1 and AMP-PNP/F_1, respectively.

ferences between the secondary structures of the two sample preparations (Fig. 3A). A small shift from 1633.4 to 1634 cm^{-1} (β-sheets), which leads to a clearer appearance of the 1627 cm^{-1} band (β-sheets), parallels a slightly higher 1547 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 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_1ATPase in the absence and in the presence of nucleotides

Fig. 4 reports the thermal denaturation profiles of F_1ATPase 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°C in sol/F_1, ADP/F_1 and AMP-PNP/F_1, respectively. This stabilising effect of nucleotides on F_1ATPase 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_1ATPase 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_1ATPase 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 (^2H_2O) (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 mol/mol of enzyme). As the binding of nucleotides to F_1ATPase stabilises its structure, the removal of ATP from the tight sites could destabilise both β and α 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 β subunits shows an high affinity for ATP [4], the residual ATP of insol/F_1 (0.7 mol/mol enzyme) is most probably bound to the β-subunits suggesting, in turn, that the aggregation of insol/F_1 sample is due mainly to the destabilisation and consequent aggregation of α-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 α-subunits of the enzyme since it is observed upon removal of ATP from the tight sites.

The 1619 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 α-subunits having the tight sites partially deprived of bound ATP (2.8 mol/mol enzyme).

The dependence of enzyme compactness on the nucleotide binding site observed in this study for mitochondrial F_1ATPase is consistent with the conformational changes induced by different nucleotide content in chloroplast (CF_1) [21] and in termophilic bacterium PS3 [22] F_1ATPases (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 Mg^{2+}/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_1ATPase the development of the hysteretic inhibition, which is triggered by the addition of 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_oF_1ATPase, we observed a dramatic change in the secondary structure as a consequence of the insertion of F_o 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_o 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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References

[1] Penefsky, H.S. and Cross, R.L. (1991) Adv. Enzymol. 64, 173–214.
[2] Abrahams, J.P., Leslie, A.G.W., Lutter, R. and Walker, J.E. (1994) Nature 370, 621–628.
[3] Bianchet, M., Ysern, X., Hullihen, J., Pedersen, P.L. and Amzel, M.L. (1991) J. Biol. Chem. 266, 21197–21201.
[4] Kironde, F.A.S. and Cross, R.L. (1986) J. Biol. Chem. 261, 12544–12549.
[5] Chernyak, B.V. and Cross, R.L. (1992) Arch. Biochem. Biophys. 295, 247–252.
[6] Joult, J-M. and Allison, W.S. (1994) J. Biol. Chem. 269, 319–325.
[7] Di Pietro, A., Penin, F., Godinot, C. and Gautheron, D.C. (1980) Biochemistry, 19, 5671–5678.
[8] Capaldi, R.A., Aggeler, R., Turina, P. and Wilkens, S. (1994) TIBS 19, 284–289.
[9] Horstmann, L.L. and Racker, E. (1970) J. Biol. Chem. 245, 1336.
[10] Laemmli, U.K. (1970) Nature 227, 680–685.
[11] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[12] Di Pietro, A., Penin, F., Julliard, J.H., Godinot, C. and Gautheron, D.C. (1980), Biochem. Biophys. Res. Commun. 152, 1319–1325.
[13] Banecki, B., Zylicz, M., Bertoli, E. and Tanfani, F. (1992) J. Biol. Chem. 25052–25058.
[14] Muga, A., Cistola, D.P. and Mantsch, H.H. (1993) Biochim. Biophys. Acta 1162, 291–296.
[15] Chirgadze, Y.N., Fedorow, O.W. and Trushina, N.P. (1975) Biopolymers 14, 679–694.
[16] Jackson, M. and Mantsch, H.H. (1991) Biochim. Biophys. Acta 1078, 231–235.
[17] Jackson, M. and Mantsch, H.H. (1992) Biochim. Biophys. Acta 1118, 139–143.
[18] Osborne, H.B. and Nabedryk-Viala, E. (1982) Methods Enzymol. 88, 676–680.
[19] Haris, P.I., Lee, D.C. and Chapman, D. (1986) Biochim. Biophys. Acta 874, 255–265.
[20] Fernandez-Ballester, G., Castresana, J., Arrondo, J.L.R., Ferragut, J.A. and Gonzales-Ros, J.M. (1992) Biochem. J. 288, 421–426.
[21] Wang, Z.Y., Freire, E. and McCarty, R.E. (1993) J. Biol. Chem. 268, 20785–20790.
[22] Ohta, S., Nakanishi, M., Tsuboi, M., Yoshida, M. and Kagawa, Y. (1978), Biochem. Biophys. Res. Commun. 80, 929–935.
[23] Burgard, S., Nett, J.H., Sauer, H.E., Kagawa, Y., Schafer, H.J., Wise, J.G., Vogel, P.D. and Trommer, W.E. (1994) J. Biol. Chem. 269, 17815–17819.
[24] Dabbeni-Sala, F., Loregian, A., Lippe, G., Bertoli, E. and Tanfani, F. (1993) FEBS Lett. 336, 477–480.