Influence of nucleotides on the secondary structure and on the thermal stability of mitochondrial F_1 visualized by infrared spectroscopy

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Abstract **We have studied the secondary structure of mitochondrial F_1 using infrared spectroscopy. Our results show that in the absence of added nucleotides this complex contains similar percentages of α-helices, β-structures and reverse turns (30%, 28% and 31%, respectively). The influence of ADP and ATP on the different types of secondary structure was determined; when all the nucleotide-binding sites were occupied, small but reproducible changes were observed, corresponding to a decrease in β-structure and an increase in α-helix and reverse turns. The effect of nucleotide binding on the thermal stability of F_1 was also studied; the thermal denaturation temperature, 55°C, was increased by 11°C and 7°C by ATP and ADP, respectively. These results indicate that nucleotide binding affects the secondary structure of F_1, stabilizing the complex.**

Key words: ATP synthase; F_1-ATPase; Secondary structure; Fourier transform infrared spectroscopy

1. Introduction

F_0F_1-H^+ type ATPases found in the membranes of mitochondria, chloroplasts and bacteria use an electrochemical gradient of protons generated by the electron transfer chain to synthesize ATP. The enzyme can also hydrolyze ATP and form an electrochemical gradient of protons. The enzyme consists of two major domains: one membrane-embedded F_0 part, which functions as a proton channel, and a hydrophilic F_1 part which contains the nucleotide-binding sites [1–6]. The F_1 part is composed of five different subunits in a stoichiometry of $3\alpha:3\beta:1\gamma:1\delta:1\varepsilon$ [7,8], which could hydrolize but not synthesize ATP. F_1 has been studied extensively with respect to its nucleotide binding sites and its structure at 2.8 Å resolution has recently been determined [9]. It is generally accepted that there are six nucleotide-binding sites localized at the α/β interfaces which fall into two categories: three high affinity binding sites have been associated with a regulatory function and are located mostly in α-subunits; and three loose or readily exchangeable sites, predominantly located in β-subunits, which have been named catalytic sites. However, despite all this information there is still little agreement about the role and function of these nucleotide binding sites. In this work we report data on the secondary structure of the soluble hydrophilic F_1 part from bovine heart mitochondria by Fourier-transform infrared spectroscopy (FTIR), related to nucleotide binding to the F_1 complex. Infrared spectroscopy has proven to be a powerful technique for the characterization of the secondary structure of proteins, due to the specific hydrogen bonding of the C = O and N–H groups for each kind of structure [10]. The infrared amide I region, which almost entirely reflects the C = O stretching vibrations of the peptide bonds that constitute the backbone structure, is sensitive to small variations in molecular geometry and hydrogen bonding patterns of proteins, and has been widely used in studies of the secondary structure and conformational changes of proteins [10–13].

Except for one report related to the effect of phospholipids on mitochondrial ATPase [14], FTIR has not been applied to study this high molecular weight complex enzyme. Mitochondrial F_1 contains 2 to 4 mol of tightly bound endogenous adenine nucleotides, depending on the isolation procedures [15,16]. The primary question we addressed was whether we could see conformational differences in F_1 with extra sites filled with nucleotides, or with ADP instead of ATP. We have also used the FTIR technique to investigate the influence of nucleotides on the conformational stability of the F_1 complex against thermal denaturation.

2. Materials and methods

2.1. Sample preparation

F_1 was prepared according to the method of Knowles and Penefsky [17]. Isolated F_1 was dissolved at 16 mg/ml (44 μM) in a buffer containing 50 mM HEPES pH 7.0, 2 mM EDTA and 17.6% glycerol. ATPase activity of this preparation, measured as described in [18] was 93 μmol ATP/(mg protein · min) at 30°C. After removal of loosely bound adenine nucleotides our preparation still retained three bound nucleotides, about two ATP and one ADP molecules per F_1 complex, in accordance with Edel et al. [16]. Experiments were performed incubating the F_1 preparation (44 μM) for 10 min at room temperature with the indicated concentrations of ADP or ATP. Taking into account the values of dissociation constants for nucleotides reported in [19], incubation with 100 μM ADP or ATP led to an enzyme that contained one additional nucleotide per F_1, whereas incubation with 2 mM ATP or ADP led to an enzyme that contained six bound nucleotides. Samples were placed in a 6-μm path length CaF_2 IR cells with tin spacers. The cell was thermostatized at the desired temperature.

2.2. Infrared spectroscopy

IR spectra were obtained on a Mattson Polaris FTIR spectrometer equipped with a MCT detector. A total of 1000 scans were averaged for each sample, automatically apodized with a triangular function and Fourier-transformed to give a spectrum at a resolution of 2 cm^{-1} with the data encoded every 0.5 cm^{-1}. The instrument was continuously purged with dry air (dew point better than −40°C). A sample shuttle was used to allow a block averaging collection in order to minimize

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Abbreviations: FTIR, Fourier transform infrared.

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water vapor bands. The spectra of the buffer to be subtracted were collected under the same scanning conditions and temperatures as the sample ones. The criteria for a good subtraction was the removal of the water band near 2130 cm^{-1}, and the obtention of a flat line between 1800 and 2000 cm^{-1}. In the series of spectra obtained at increasing temperatures, from 20 to 95°C, samples were allowed to stabilize for 10 min at each temperature before data collection. The spectra were deconvolved using the programs developed by Moffat et al. [20], using a half-bandwidth of 20 cm^{-1} and a k factor of 2.4. In order to measure the relative areas of the amide I band components, deconvoluted spectra were curve-fitted by means of a least-squares iterative program.

3. Results and discussion

3.1. Characterization of the F_1 infrared spectrum

Fig. 1A and 1B show the infrared absorbance and deconvoluted spectra of the F_1 moiety in the 1750–1490 cm^{-1} region after subtraction of the buffer spectrum, at 20°C. The bands between 1615 and 1700 cm^{-1} correspond to the amide I region with a maximum at 1655 cm^{-1}, whereas the amide II bands appear between 1500–1600 cm^{-1} with a main peak at 1549 cm^{-1}. In the deconvoluted spectrum (Fig. 1B) the major band centred at 1655 cm^{-1} is assigned mainly to α-helices [10–12,21]. The band at 1636 cm^{-1} corresponds to β-structures [10,11], whereas the small band at 1625 cm^{-1}, although difficult to assign, should

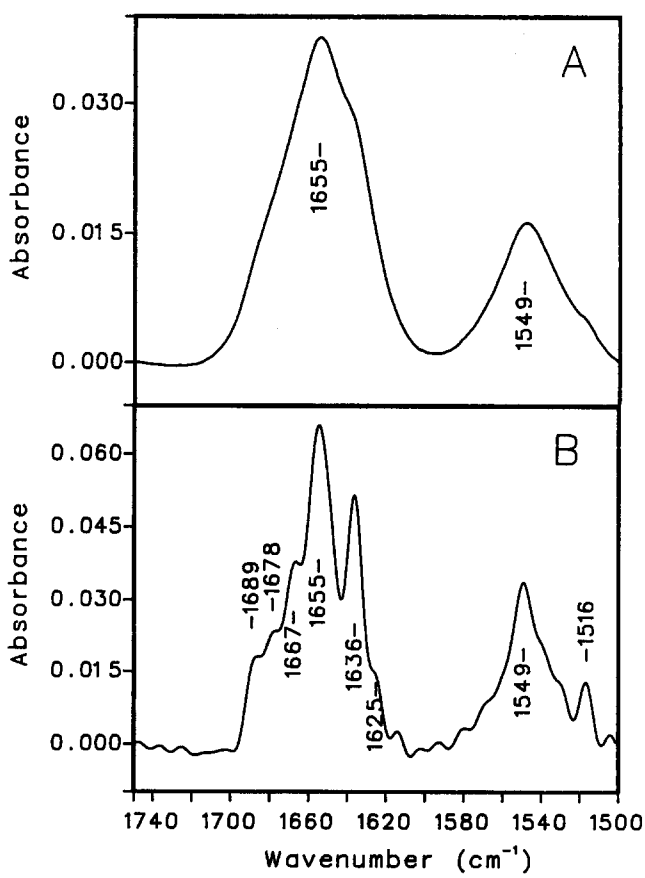

Fig. 1. Infrared absorbance spectrum (Panel A) and deconvoluted spectrum (Panel B) of mitochondrial F1 in 50 mM HEPES pH 7.0, 2 mM EDTA and 20% glycerol, recorded at 20°C. F_1 concentration, 44 μM. The absorbance spectrum was obtained by subtraction of the buffer spectrum from the sample spectrum, by coadding 1000 scans in blocks of 20 of the shuttle accessory, in order to minimize the water vapor. Deconvolution was done using a bandwidth of 20 cm^{-1} and a k factor of 2.4.

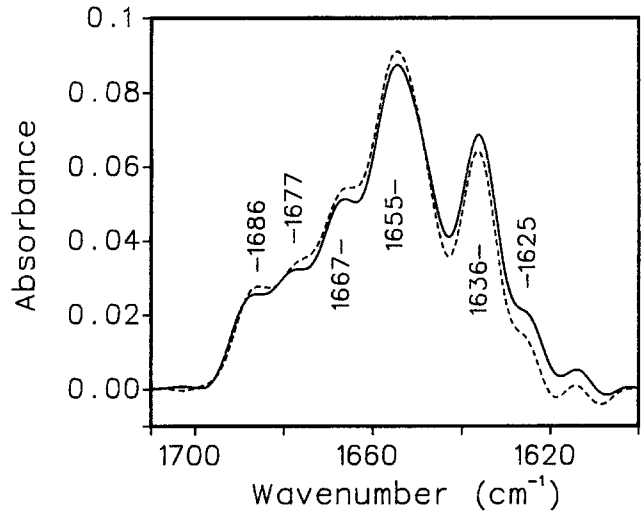

Fig. 2. Deconvoluted infrared spectra of mitochondrial F_1 (——) and F_1 plus ATP (--) in the amide I region. Spectra were obtained at 20°C. F_1 concentration 44 μM; ATP 2 mM.

correspond to a different type of β-structure and it is generally attributed to the formation of intermolecular hydrogen bonds [10–12]. Finally, the three components at high wavenumbers, 1689 cm^{-1}, 1678 cm^{-1} and 1667 cm^{-1} can mainly be assigned to reverse turns [10,11]. However, some weak contribution from β-structures is possible. Curve-fitting procedures were carried out with the deconvoluted amide I spectrum of F_1 using two independent samples (data not shown). Seven bands in the region between 1620–1700 cm^{-1} were fitted to the deconvoluted spectrum. The contribution due to unordered structures is likely to be largely hidden underneath the main band at 1655 cm^{-1}. Thus, a band at 1649 cm^{-1}, which is not apparent in the deconvoluted spectrum, was included in the curve-fitted spectrum and ascribed to unordered structures, in accordance with the well-accepted assignment for this region [10,11,22]. The overall quantification of the secondary structure relative to each band can be summarized as being 30% α-helices, 28% β-structures, 31% total reverse turns and 11% unordered segments.

The three-dimensional structure of F1-ATPase from mitochondria has recently been reported by Abrahams et al. [9] at 2.8 Å resolution using X-ray diffraction. In principle, our results with FTIR are in agreement with this latest structural determination in the sense that both techniques predict a similar content of secondary structure components with regard to helical structures, β-sheets and turns, without a clear preponderance of any of them. The inclusion of the band at 1649 cm^{-1} (random conformation) makes our quantitative estimation more consistent with the results derived from the work of Abrahams et al. [9].

3.2. Effect of nucleotide binding on the F1 secondary structure

In order to determine whether the binding of different ligands promotes structural changes in the F1 moiety, ATP or ADP (2 mM) were added to the complex. This concentration of ATP (or ADP) led to an occupancy of all six nucleotide binding sites present in F1 [19] and this sample was compared with the F1 sample purified by us, which already contained

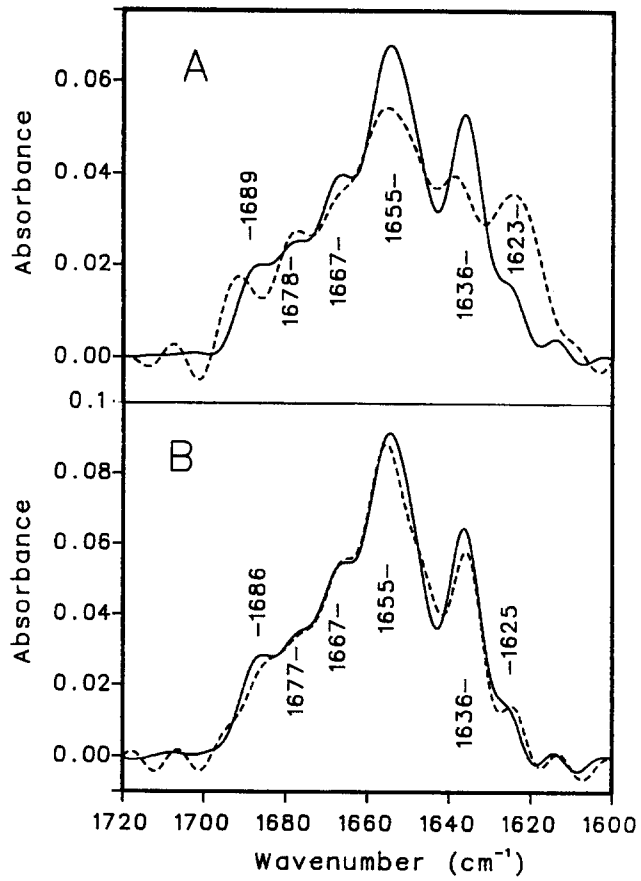

Fig. 3. Deconvoluted infrared spectra of F_1 (Panel A) and F_1 plus ATP (Panel B). Spectra were recorded at 20°C (——) and 60°C (--). The concentration of F_1 was 44 μM, that of ATP 2 mM.

three bound nucleotides (see methods). Addition of these ligands induced small (less than 6% of the toal F1 absorbance) but highly reproducible changes in the amide I infrared spectrum. As an example, Fig. 2 compares the deconvoluted spectrum of F1 in the amide I region with the spectrum obtained upon addition of an excess of ATP (final concentration 2 mM) to the F_1 sample (44 μM), at 20°C. Besides other minor differences, addition of ATP to F1 (as well as addition of ADP) produces a decrease in the absorbance band at 1636 cm^{-1} and an increase in absorbance at 1655, 1667, 1677 and 1686 cm^{-1}. Presumably, nucleotide binding to F1 induces local conformational changes which may originate from a decrease in the β-structure content with a parallel formation of reverse turns and α-helical structures. However, since the changes we observe are relatively small compared to the overall ATPase absorbance, no large reorganization of F1 protein domains is expected to occur as a consequence of nucleotide binding. Goormaghtigh et al. [23] also found very minor changes in the overall structure of the plasma membrane H^+-ATPase using FTIR spectroscopy.

3.3. *Effect of nucleotide binding on the thermal stability of F1*

We have investigated the influence of ATP and ADP binding on the thermal denaturation processes that occur in F1. Fig. 3A shows the deconvoluted amide I region corresponding to F1 (44 μM) in aqueous buffer at 20 and 60°C. The spectrum at high temperature exhibits several changes compared to the one at 20°C. The main features are a decrease in the 1655 cm^{-1} and 1636 cm^{-1} bands accompanied by an increase in the band intensity at 1623 cm^{-1} and the absorption at 1649 cm^{-1}. These changes can be interpreted as a heat-induced transformation of α helices and β structures into unordered conformation (band at 1649 cm^{-1}) and especially into some kind of β-extended chains which appear between 1620 and 1625 cm^{-1}. This last band is commonly found in the spectra of thermally denatured proteins and it is assigned to intermolecular β-sheet formation, probably as a consequence of aggregation [24]. Fig. 3B shows a comparison of the FTIR spectra of F_1 (44 μM) incubated with 2 mM ATP also at 20 and 60°C. A similar interpretation holds for this sample, although the differences between both temperatures are smaller compared to F_1, in particular for the decreased intensity of the 1625 cm^{-1} band. The absorption due to random structures which appears at 1649 cm^{-1} in the spectrum at 60°C in Fig. 3A, is not detectable in the deconvoluted spectra taken at this temperature in Fig. 3B because its contribution is less important and it appears everlapped by the main band at 1655 cm^{-1}. Thus, all the data suggest that addition of an excess of ATP stabilizes the complex against heat denaturation. In order to observe the evolution of the conformational changes caused upon heating the protein complex, FTIR spectra of the different samples were taken in the temperature range between 20 and 90°C. Fig. 4 shows plots of the entensity ratios of the peaks at 1623 cm^{-1} (indicative mainly of β-extended chains) and 1655 cm^{-1} (indicative of α helices) as a function of temperature, for F_1 samples with and without an excess of ADP or ATP. It can be observed that the relative amount of these structures hardly varies for F_1 supplemented with an excess of ATP or ADP up to 50°C, whereas this ratio starts to increase above 40°C for F_1 without addition of nucleotides. F_1 shows a temperature of denaturation ($T_{1/2}$) of 55°C, whereas both ATP and ADP promote a large increase in the thermal stability of F_1, raising the temperature of denaturation from 55 to 66°C (ATP) or to 62°C (ADP). This result indicates that the occupancy of

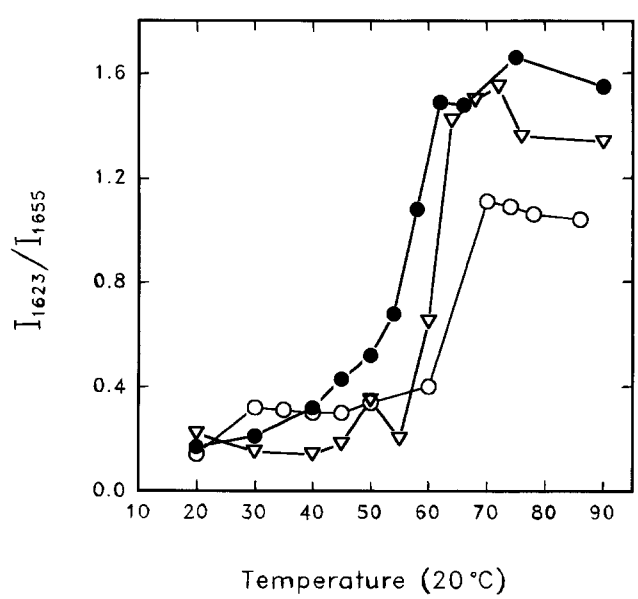

Fig. 4. Plot of the intensity ratios at 1623 cm^{-1} over 1655 cm^{-1} as a function of temperature from 20 to 90°C. (●) F_1; (▽) F_1 plus ADP and (○) F_1 plus ATP. Concentration of F_1 44 μM; ADP 2 mM; ATP 2 mM.

the six nucleotide binding sites by ATP or ADP, renders the F_1 complex more stable towards thermal denaturation.

We also investigated whether binding of just one additional nucleotide to the starting F_1 preparation was able to promote this thermal stability. Binding of about one additional ATP per F_1 did not produce any appreciable effect on the thermal stability of F_1, whereas the binding of about one ADP slightly increased the temperature of denaturation by 2°C (from 55 to 57°C) (data not shown). It is likely that ADP is more tightly bound than ATP to the fourth site (i.e. the second non-catalytic site according to the scheme of Edel et al. [16], located on an α-subunit [9]), thus rendering the complex more stable towards temperature increase. ADP bound at this site causes partial inhibition of the ATPase activity [18]. However, it is remarkable that the simple rise from four (the three nucleotides originally bound + 1 added ADP or ATP per F_1) to six bound nucleotides per F_1 complex, originates the increase in the thermal stability described above, especially for ATP. This effect on heat stability may be mostly due to binding of nucleotide at the third α-site. Therefore, our results suggest that ATP bound to this specific site may well act as a stronger compacting element, inducing an increase in stability. Considerable effects of binding of ATP to a low-affinity non-catalytic site on the kinetics of ATP hydrolysis by F_1 have also been reported [25,26].

In general, the more nucleotide binding sites are filled, the more stable the complex becomes. This increased stability is probably due to the mode of interaction of nucleotides with the protein moiety. According to Abrahams et al. [9], all six nucleotide sites have similar characteristics, involving a P-loop and at least two amino acid side chains from the neighbouring subunit. Thus, nucleotide binding increases thermal stability, probably by tightening the bonds between neighbouring α and β subunits.

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References

[1] Boyer, P.D. (1993) Biochim. Biophys. Acta 1140, 215–250.
[2] Pedersen, P.L. and Amzel, L.M. (1993) J. Biol. Chem. 268, 9937–9940.
[3] Capaldi, R.A., Aggeler, R., Turina, P. and Wilkens, S. (1994) Trends Biochem. Sci. 19, 284–289.
[4] Cross, R.L. (1988) J. Bioenerg. Biomembr. 20, 395–405.
[5] Senior, A.E. (1990) Annu. Rev. Biophys. Chem. 19, 7–41.
[6] Penefsky, H.S. and Cross, R.L. (1991) Adv. Enzymol. 64, 173–214.
[7] Todd, R.D., Giesenbeck, T.A. and Douglas, M.G. (1980) J. Biol. Chem. 255, 5461–5467.
[8] Stutterheim, E., Henneke, M.A.C. and Berden, J.A. (1981) Biochim. Biophys. Acta 634, 271–278.
[9] Abrahams, J.P., Leslie, A.G.W., Lutter, R. and Walker, J.E. (1994) Nature 370, 621–628.
[10] Surewicz, W.K. and Mantsch, H.H. (1988) Biochim. Biophys. Acta 952, 115–130.
[11] Byler, D.M. and Susi, H. (1986) Biopolymers 25, 469–487.
[12] Arrondo, J.L., Muga, A., Castresana, J. and Goñi, F. (1993) Prog. Biophys. Molec. Biol. 59, 23–56.
[13] Kauppinen, J.K., Moffat, D.J., Mantsch, H.H. and Cameron, D.G. (1981) Appl. Spectr. 35, 271–276.
[14] Sala, F.D., Loregian, A., Lippe, G., Bertoli, E. and Tanfani, F. (1993) FEBS Lett. 336, 477–480.
[15] Berden, J.A., Hartog, A.F. and Edel, C.M. (1991) Biochim. Biophys. Acta 1057, 151–156.
[16] Edel, C.M., Hartog, A.F. and Berden, J.A. (1995) Biochim. Biophys. Acta 1229, 103–114.
[17] Knowles, A.F. and Penefsky, H.S. (1972) J. Biol. Chem. 247, 6617–6623.
[18] Edel, C.M., Hartog, A.F. and Berden, J.A. (1992) Biochim. Biophys. Acta 1101, 329–338.
[19] Wagenvoord, R.J., Kemp, A. and Slater, E.C. (1980) Biochim. Biophys. Acta. 593, 204–211.
[20] Moffat, D.J., Kauppinen, J.K., Cameron, D.G., Mantsch, H.H. and Jones, R.N. (1986) in: Computer Programs for Infrared Spectrophotometry, NRCC Bulletin 18, National Research Council of Canada, Ottawa, Canada.
[21] Krimm, S. and Dwivedi, A.M. (1982) Science 216, 407–408.
[22] Dong, A., Huang, P. and Caughey, W.S. (1990) Biochemistry 29, 3303–3308.
[23] Goormaghtigh, E., Vigneron, L., Scarborough, G.A. and Ruysschaert, J.M. (1994) J. Biol. Chem. 269, 27409–27413.
[24] Casal, H.I., Kohler, U. and Mantsch, H.H. (1988) Biochim. Biophys. Acta 957, 11–20.
[25] Jault, J.M. and Allison, W.S. (1993) J. Biol. Chem. 268, 1558–1566.
[26] Edel, C.M., Hartog, A.F. and Berden, J.A. (1993) Biochim. Biophys. Acta 1142, 327–335.