Effects of Fe(III) binding to the nucleotide-independent site of F_1-ATPase: enzyme thermostability and response to activating anions

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Abstract Mitochondrial F_1-ATPase was induced in different conformations by binding of specific ligands, such as nucleotides. Then, Fourier transform infrared spectroscopy (FT-IR) and kinetic analyses were run to evaluate the structural and functional effects of Fe(III) binding to the nucleotide-independent site. Binding of one equivalent of Fe(III) induced a localised stabilising effect on the F_1-ATPase structure destabilised by a high concentration of NaCl, through rearrangements of the ionic network essential for the maintenance of enzyme tertiary and/or quaternary structure. Concomitantly, a lower response of ATPase activity to activating anions was observed. Both FT-IR and kinetic data were in accordance with the hypothesis of the Fe(III) site location near one of the catalytic sites, i.e. at the α/β subunit interface. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: F_1-ATPase; Fe(III) binding site; Fourier transform infrared spectroscopy; Anion activation

1. Introduction

F_1-ATPase, the catalytic moiety of F_0F_1-ATP synthase, is an asymmetric assembly with stoichiometry $3\alpha:3\beta:1\gamma:1\delta:1\varepsilon$ and it is the only part of the enzyme well conserved [1]. As established by X-ray crystallography, the large α and β subunits contain six nucleotide binding sites and are arranged alternately around the γ subunit [2]. This latter interacts with δ and ε subunits forming a foot [3], which contacts the c-ring of F_0 and couples the transmembrane proton motive force to catalysis. According to the binding change mechanism [4], the prominent feature of the enzyme catalysis is the sequential participation of the three catalytic sites located in β subunits driven by the rotation of γ subunit, relative to $\alpha_3\beta_3$, which takes each of the three catalytic sites through three different states in a concerted manner.

F_1-ATPase in its soluble form catalyses ATP hydrolysis in the presence of Mg(II), which is an essential cofactor. The mitochondrial enzyme bears two additional metal binding sites which are specific for iron and different from the Mg(II) binding sites [5]. One of the iron sites is filled with Fe(III) irrespective of the binding of nucleotides in the enzyme (nucleotide-independent site), although its location is probably close to one of the six nucleotide binding sites [5]. This site appears to be conserved considering that a site with similar properties has been recently characterised in thermophilic *Bacillus PS3* [6,7].

Iron bound in this site mediates the H_2O_2-induced inactivation of both the bacterial [6] and mitochondrial enzyme [8] and evidence has been obtained for the enzyme inactivation even upon exposure to H_2O_2 of the whole F_0F_1-ATP synthase [9] or of intact cells [10]. Nevertheless, just as occurs for the metal cofactor Mg(II) [11,12], a structural and functional role of iron may be hypothesised. The aim of this study was to investigate the effects on F_1-ATPase structure of Fe(III) binding to the conserved nucleotide-independent site by Fourier transform infrared spectroscopy (FT-IR). The structural data are accompanied by kinetic analysis.

2. Materials and methods

2.1. Materials

Deuterium oxide (99.9% ^2H_2O) was purchased by Aldrich. All other chemicals were commercial samples of the purest quality.

2.2. Preparation of F_1-ATPase

Pure soluble F_1-ATPase was prepared from beef heart mitochondria as in [13]. Before each treatment, F_1-ATPase suspension in ammonium sulphate was pelletted, dissolved and desalted to remove loose nucleotides [14].

2.3. Preparation of samples for infrared measurement

Typically [13], 2 mg of F_1-ATPase was dissolved in 1 ml of buffer A (20 mM HEPES, 200 mM NaCl, p^{2}H 8.0) or buffer B, C or D, the composition and p^{2}H of which were like those of buffer A but also contained 13 μM $FeCl_3$ (B), 5 mM ADP plus 13 μM $FeCl_3$ (C), or 1 mM β,γ-imidoadenosine 5′-triphosphate (AMP-PNP) plus 13 μM $FeCl_3$ (D), all at 25°C. Differently, 2 mg of F_1-ATPase was suspended in buffer E (20 mM HEPES, p^{2}H 8.0) or F which also contained 13 μM $FeCl_3$. The samples, which were treated and analysed by Perkin-Elmer 1760-x FT-IR spectrometer as in [15], are referred to as F_1 (buffer A), 1Fe(III)-loaded F_1 (buffer B), 1Fe(III)-loaded ADP/F_1 (buffer C), 1Fe(III)-loaded AMP-PNP/F_1 (buffer D), HEPES/F_1 (buffer E) and 1Fe(III)-loaded HEPES/F_1 (buffer F), respectively.

2.4. Analytical procedures

ATPase activity was assayed spectrophotometrically using an ATP-regenerating system as in [16] except that in some experiments high NaCl concentration was also added (see Fig. 3). The specific activity at 37°C in the pool without activating anions of both F_1 and 1Fe(III)-loaded F_1 was 35 ± 3 U/mg protein and 62 ± 4 U/mg protein in the

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Abbreviations: FT-IR, Fourier transform infrared spectroscopy; AMP-PNP, β,γ-imidoadenosine 5′-triphosphate

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presence and absence of NaCl, respectively. Nucleotides were analysed by HPLC as in [17]. Iron content was evaluated using the ferene method [5]. Protein concentration was determined by the Lowry or the bicinchoninic acid method as in [17,18].

3. Results and discussion

3.1. Thermal stability of F_1-ATPase loaded with Fe(III) in the absence or presence of loose nucleotides

In a previous paper, in which FT-IR spectroscopy was used, we showed that treatment of F_1-ATPase with saturating concentrations of ADP or AMP-PNP in the absence of Mg(II) does not markedly affect the secondary structure, although it does significantly reduce the exchange of amide hydrogens with deuterium (H/D) and increase the enzyme thermal stability. These phenomena have been proposed to be a consequence of changes in the tertiary and/or quaternary structure of F_1-ATPase, which would be more compact in the presence of nucleotides [13].

In the present study the same technique was used to investigate the structure behaviour of F_1-ATPase after Fe(III) binding to the nucleotide-independent site. The enzyme was solved at high salt concentration, to mimic both the mitochondrial environment and crystal growth conditions [2]. The structural effect consequent on the binding of 1 equivalent of Fe(III) was evaluated in the absence of Mg(II) under three different protein conformations: (1) F_1-ATPase containing only 3 mol of tightly bound nucleotides/mol of enzyme, which are located in two α and one β subunits [14] (F_1 and 1Fe(III)-loaded F_1); (2) F_1-ATPase hysteretically inhibited by saturating ADP (ADP/F_1 and 1Fe(III)-loaded ADP/F_1); (3) potentially 'fully active' F_1-ATPase, obtained by saturating AMP-PNP, the non-hydrolysable analogue of ATP (AMP-PNP/F_1 and 1Fe(III)-loaded AMP-PNP/F_1).

Considering that FT-IR analyses are carried out after the H/D exchange, Fe(III) binding capacity and electron paramagnetic resonance (EPR) spectra of the enzyme loaded with Fe(III) in ^2H_2O were evaluated. The data confirm that under all the conditions the enzyme's ability to bind 1 equivalent of Fe(III) is conserved and that the Fe(III) binding site exhibits a geometry equal to that in H_2O buffers, which we previously defined [5] (data not shown).

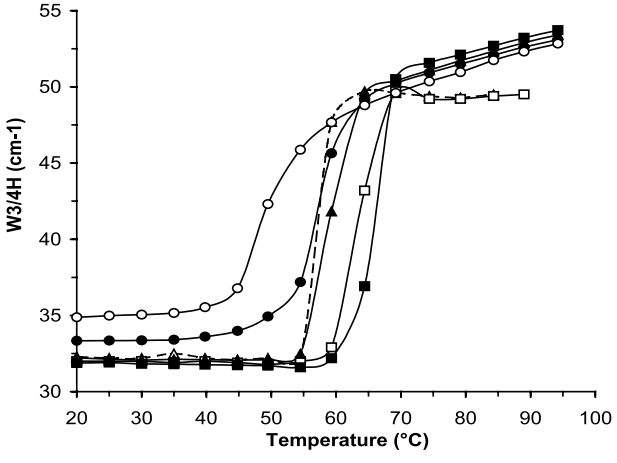

Fig. 1. Effect of Fe(III) and/or nucleotides on NaCl-destabilised F_1-ATPase. For explanation of the symbols see Section 2. Open circles: F_1; closed circles: 1Fe(III)-loaded F_1; open triangles and dotted line: ADP/F_1; closed triangles: 1Fe(III)-loaded ADP/F_1; open squares: AMP-PNP/F_1; closed squares: 1Fe(III)-loaded AMP-PNP/F_1. Thermal denaturation curves were obtained by monitoring the amide I′ band width calculated at three quarters of the amide I′ band height (W3/4H) as a function of temperature. The data reported are from original spectra repeated three times.

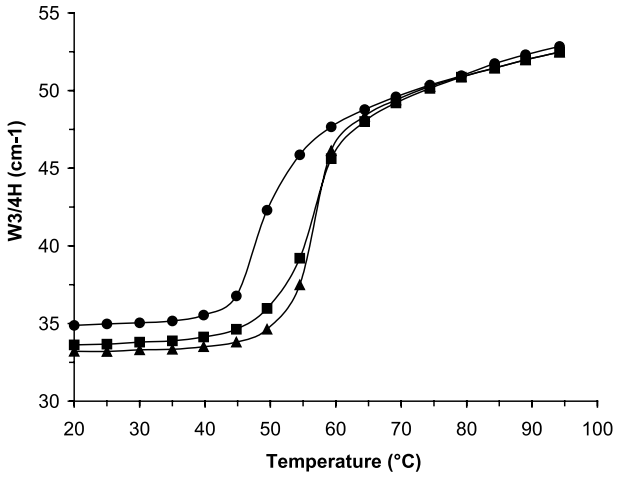

Fig. 2. Effect of high NaCl concentration on F_1-ATPase thermostability. For explanation of the symbols see Section 2. Circles: F_1; triangles: HEPES/F_1; squares: 1 Fe(III)-loaded HEPES/F_1. Thermal denaturation curves were obtained by monitoring the amide I′ band width calculated at three quarters of the amide I′ band height (W3/4H) as a function of temperature. The data reported are from original spectra repeated two times.

Fig. 1 shows the thermal denaturation profiles of the enzyme samples in the presence of 200 mM NaCl. According to [13], F_1 displays the lower thermal stability, while a saturating concentration of ADP or AMP-PNP strongly stabilises the enzyme, AMP-PNP exerting the higher stabilising effect. Interestingly, Fe(III) appears to have an important structural role. In fact, 1Fe(III)-loaded F_1 displayed a temperature of denaturation (T_m) similar to that of ADP/F_1, but the onset of denaturation was lower and the full thermal denaturation covered a wider range of temperatures. Moreover, 1Fe(III)-loaded ADP/F_1 and 1Fe(III)-loaded AMP-PNP/F_1 showed a T_m slightly higher than ADP/F_1 and AMP-PNP/F_1, respectively. These data indicate that Fe(III) plays an important role in the stabilisation of the enzyme structure, especially when F_1 is not saturated by nucleotides. The stabilising effect exerted by Fe(III) was much lower when F_1 was saturated by nucleotides, but still present. This finding and the fact that the thermal denaturation curves of ADP/F_1 and 1Fe(III)-loaded ADP/F_1 are different suggest that the Fe(III) binding site is different from those of nucleotides, in accordance with [5]. Furthermore, the different thermal denaturation curves of ADP/F_1 and 1Fe(III)-loaded ADP/F_1 suggest that the stabilising effect of Fe(III) might be limited only to some protein regions, probably close to the Fe(III) binding site.

The thermal stability of the enzyme in the absence of 200 mM NaCl (HEPES/F_1) and after Fe(III) binding (1Fe(III)-loaded HEPES/F_1) was also analysed (Fig. 2). As expected, the thermal stability was much higher than in the presence of the salt. The destabilising effect of high salt concentration may be ascribed to the weakening or disruption of ionic interactions important for F_1-ATPase stability. These interactions are most likely involved in the maintenance of tertiary and/or quaternary structure, since FT-IR spectra did not show

changes in the secondary structure of the enzyme exerted by 200 mM NaCl (data not shown). Contrary to Fig. 1, in the absence of NaCl Fe(III) did not significantly modify T_m, which was the same as that of HEPES/F_1.

We can summarise these findings as follows:

1. NaCl destabilises the enzyme structure (Fig. 2), most likely by weakening or disrupting some ionic interactions;
2. Fe(III) stabilises the NaCl-destabilised structure of F_1, but the structure is less stable than that induced by saturating ADP (Fig. 1);
3. Fe(III) has no relevant effect on T_m in the absence of NaCl (Fig. 2);
4. in the presence of NaCl, the stabilising effect exerted by Fe(III) on the enzyme saturated with nucleotides is much lower, but still present (Fig. 1).

In conclusion, Fe(III) appears to be able to restore or form new ionic interactions which are important for the enzyme's thermal stability at high salt concentrations. It is worth noting that this effect was obtained by incubating the enzyme in the presence of 0.13 μM $FeCl_3$, a concentration much lower than that used for NaCl (200 mM). This comparison further suggests that Fe(III) is particularly suitable for the stabilisation of the F_1 structure.

In the absence of NaCl, the inability of Fe(III) to stabilise the enzyme (Fig. 2) indicates that Fe(III) binding does not alter the network of ionic interactions and this, in turn, suggests that the Fe(III) site may be located outside or close to the network. Of the two possibilities the second seems to be the more probable. In fact, if the Fe(III) binding site is outside or too far from the ionic network, it would not be able to restore and/or to form new ionic interactions able to stabilise the protein (Fig. 2). It is known that ionic bonds are some of the rearrangements that occur throughout α and β subunits after nucleotide binding [2]. As Fe(III) and ADP exert similar structural effects, it is hypothesised that the interactions formed and/or restored by Fe(III) can involve the region facing the α/β interface [7].

3.2. *Effect of Fe(III) binding on the kinetic properties of F_1-ATPase: response to activating anions*

Although Fe(III) does not sustain the catalysis [5], functional studies were performed on the enzyme containing Fe(III). More specifically, the rate of Mg(II)-ATP hydrolysis by F_1 and 1Fe(III)-loaded F_1 was measured in the absence or in the presence of high NaCl concentrations and/or of saturating concentrations of activating anions, i.e. bicarbonate, maleate and malonate. These ligands have been proposed to modify the catalytic site interactions, thus strongly attenuating the negative cooperativity for ATP binding and slightly favouring the positive cooperativity for catalysis [19]. As shown in Fig. 3A, the maximal ATPase activity at high NaCl concentration of the enzyme not loaded with Fe(III) was about 35 U/mg in the absence or 50, 47 and 44 U/mg in the presence of bicarbonate, maleate and malonate, respectively. In contrast, when 1 Fe(III) equivalent is bound to the enzyme, the activating action of both maleate and malonate was abolished and that of bicarbonate was significantly reduced (Fig. 3B). This may be due to the fact that the Fe(III) stabilises the structure, rendering it more compact and probably less flexible.

In the absence of NaCl, the enzyme activity was significantly higher compared to that measured in the presence of NaCl (see Section 2) and, in accordance with FT-IR, no effect of Fe(III) on the kinetic was revealed (data not shown).

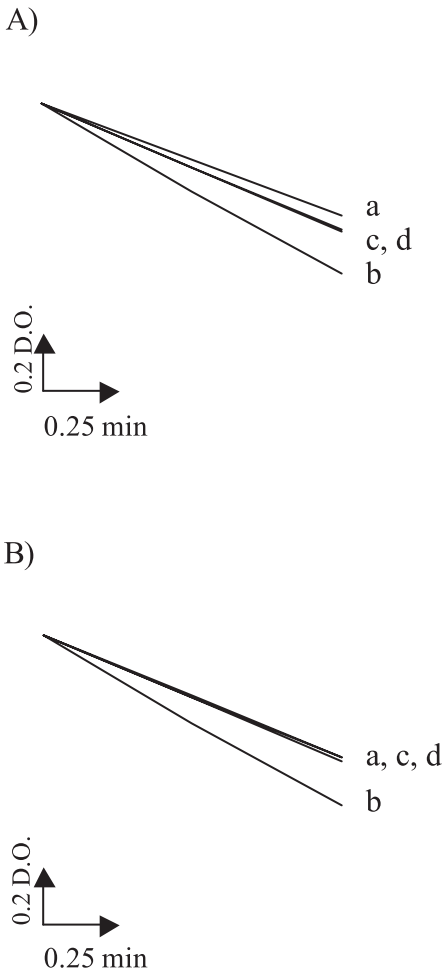

Fig. 3. Effect of activating anions on ATPase activity of F_1 (A) and 1Fe(III)-loaded F_1 (B) in the presence of NaCl. 4 μM F_1, containing three tightly bound nucleotides, was suspended in buffer A, as specified in Section 2, and incubated at 37°C with $FeCl_3$ in order to obtain 1Fe(III)-loaded F_1. After 30 min, 1–5 μg of protein was added to the assay pool, at pH 8 and 37°C, whose total salt concentration was maintained at 200 mM with NaCl, in the absence or presence of saturating concentrations of activating anions. Data are from one experiment representative of three. a: no anions; b: 30 mM HCO_3^-; c: 50 mM maleate; d: 25 mM malonate.

At high NaCl concentrations all the activating anions used showed a lower efficiency compared to the results obtained in the absence of salts [20]. Moreover, other ions which are known to act as activators, such as chromate, 2,4-dinitrophenolate [20,21] and sulphite [22,23], show no activating effect (data not shown). This is probably a consequence of the destabilising effect of the salt on the ionic interaction network, as shown by FT-IR. The adverse stabilising effect of Fe(III) binding to the enzyme further decreased the activating action of bicarbonate, maleate and malonate. Similarly, ADP, which according to FT-IR also rendered the enzyme more compact, is known to abolish the effect of these anions [20]. Thus, these results strongly suggest that the stabilising effects caused by Fe(III) and/or ADP binding were mediated by different rearrangements of the ionic network than those occurring in the absence of NaCl.

Although the number of anion binding site(s) is still under debate, it is recognised that the site(s) is (are) in the proximity of the catalytic site [2], close to which one of the two Fe(III) binding sites has been proposed to be located [5]. Thus, the finding that Fe(III) bound in the nucleotide-independent site can affect the anion binding is plausible and suggests the proximity of these sites. This is further supported by the fact that several activating anions enhance inorganic phosphate (Pi) binding to the catalytic site [24] and that Pi binding alters the Fe(III) site geometry as revealed by EPR [5].

4. Conclusions

FT-IR demonstrates that Fe(III) binding to the nucleotide-independent site stabilises the structure of F_1 destabilised by a high concentration of NaCl, a condition that mimics the mitochondrial environment. The structural effect, which is probably limited to the region close to the Fe(III) site, may form and/or restore ionic interactions at the α/β interface that are important for the maintenance of tertiary and/or quaternary structure. Kinetic data indicate that the enzyme adopting the Fe(III)-stabilised conformation shows a lower response to activating anions. This suggests that, in this conformation, the anions site(s) does (do) not have the same accessibility to the external medium or that the activating effect of anions cannot occur or not completely, as proposed in the case of the ADP-inhibited enzyme, which has a similar kinetic behaviour. In addition, these data suggest a close proximity of the Fe(III) nucleotide-independent site to the anions site(s) located near the catalytic site. This in turn supports that in mitochondrial F_1-ATPase the nucleotide-independent Fe(III) site is located at the α/β interface, in accordance with the site location on one β subunit recently demonstrated in thermophilic *Bacillus* F_1-ATPase [7].

Because Fe(III) can bind also to the whole F_0F_1-ATP synthase [9], it is possible to hypothesise that the above described stabilising effects can occur even in the native complex. A structural role of metals in many proteins is well known. In fact, metal ions have been reported to be involved in refolding processes [25] and to influence structural and functional properties [26], even of proteins lacking catalytic metals [27].

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