Characterization of the binding of Fe(III) to F_1ATPase from bovine heart mitochondria

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Abstract The binding of Fe(III) to F_1ATPase purified from beef heart mitochondria has been characterized by chemical analyses and EPR spectroscopy. F_1ATPase binds 2 mol of Fe(III)/mol of protein selectively in the presence of saturating concentrations of ATP. In the absence of nucleotides or in the presence of either saturating ADP or limiting ATP concentrations, the enzyme binds 1 equivalent of Fe(III). F_1ATPase pretreated with 5′-*p*-fluorosulfonylbenzoyladenosine, that selectively modifies the non-catalytic sites, binds only 1 mol of Fe(III)/mol of protein in the presence of either saturating ATP or ADP. Fe(III)-loaded F_1ATPase containing either 1 or 2 equivalents of Fe(III) show identical EPR signals at $g = 4.3$. The signals are not perturbed by the binding of nucleotides to the enzyme while they are altered by phosphate addition. These results indicate that F_1ATPase contains two distinct Fe(III)-binding sites, which differ from nucleotide-binding sites, and that one of these sites is opened up for Fe(III) uptake by conformational changes induced by binding of ATP to the loose non-catalytic site.

Key words: F_1ATPase: Metal-binding site; Electron paramagnetic resonance; Iron

1. Introduction

F_1ATPase is the catalytic component of F_0F_1ATPsynthase, which consists of 5 different subunits with the stoichiometry $3\alpha:3\beta:1\gamma:1\delta:1\varepsilon$ [1]. The three α-subunits and three β-subunits are arranged alternatively around a central domain containing the γ-subunit [2]. α-and β-subunits contain six nucleotide-binding sites, three of which have a catalytic role. The other three sites do not rapidly exchange bound nucleotides during ATP hydrolysis and are referred to as non-catalytic sites. Two of the non-catalytic and one of the catalytic sites bind nucleotides very tightly with respect to the remaining three sites, which are named loose sites [3]. Different experimental approaches have established that ATP or ADP binding induces distinct conformations of these sites, that are transmitted over long distance [4,5].

Till now little is known about the properties of the metal-binding sites in this protein complex. In particular, mitochondrial F_1ATPase is documented to have two tight [6,7] and one 'easily exchangeable' [8] binding sites for divalent metal cations, that are different from the metal-nucleotide-binding sites. However, their location and their role are far to be completely elucidated. Fe(II) behaves differently from the other metals. In fact, beef heart F_1ATPase is reported to bind Fe(II) up to 2 mol/mol of protein in the presence of ATP, without displacing Mg(II) [6].

In a previous work, we reported [9] that beef heart F_1ATPase is able to bind Fe(III) up to 2 mol/mol of protein in the presence of saturating concentrations of ATP, without affecting catalysis as previously shown for Fe(II) [6]. We further demonstrated that iron ions bound in F_1 moiety mediate the oxidative inactivation of the enzyme due to treatment with H_2O_2 of either purified F_0F_1ATPsynthase complex [10] or intact cells [11], besides isolated F_1ATPase [9]. The capacity of iron bound in the protein to undergo to redox-cycling with H_2O_2 prompted us to study Fe(III) binding to F_1ATPase as a critical factor favouring the inactivation of the enzyme under peroxidative conditions. In fact, although iron ions do not affect per se the enzyme activity, they are able to react with H_2O_2, leading to site-directed generation of more oxidant radical species. Furthermore, Fe(III) binding to F_1ATPase may have a crucial role in mediating the oxidative attack to the enzyme complex under physiopathological conditions associated to overproduction of H_2O_2 as well as to iron dismetabolism, like aging, ischemia-reperfusion, or iron overload.

In this study, we characterize the Fe(III)-binding sites of beef heart F_1ATPase by chemical analyses and EPR spectroscopy. In particular, the enzyme has been loaded with Fe(III) selectively affecting the occupancy of nucleotide-binding sites with ATP or ADP. The results indicate the presence of two iron-binding sites, which differ from the nucleotide-binding sites. One of these sites is accessible for iron only in the presence of an excess of ATP.

2. Materials and methods

2.1. Materials

ADP, ATP, FSBA are obtained from Sigma; Sephadex G-50 (fine) is from Pharmacia; the enzymes for ATP hydrolysis are purchased from Boehringer Mannheim; Chelex 100 is from Bio-Rad. Commercial standard solutions of iron are from J.T. Baker. Centricon-30 concentrators are from Amicon. All the others chemicals used are commercial samples of the purest quality.

Iron-deprived buffer solutions, prepared by treatment with metal-chelating resin (Chelex-100), are used in all procedures, except during enzyme purification and ATPase activity assay.

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Abbreviations: EPR, electron paramagnetic resonance; FSBA, 5′-*p*-fluorosulfonylbenzoyladenosine; CDTA, trans-1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid; HPLC, high-performance liquid chromatography; I-LN-DF_1, iron-loose nucleotide-depleted F_1ATPase.

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2.2. Preparations of F_1ATPase

Purified F_1ATPase is usually prepared from beef heart mitochondria according to [12]. The native enzyme contains 0.5 ± 0.1 mol of iron per mol of protein. When specified, a F_1ATPase preparation purified according to [13] is used, which is almost completely devoid of loose and tight nucleotides.

Iron-Loose Nucleotides-Depleted F_1ATPase (I-LN-DF_1) is prepared by suspending the native enzyme in 0.25 M sucrose, 10 mM Tris–HCl pH 7.4. Depletion of iron is obtained by incubation with 5 mM EDTA pH 7.4 for 1 h at 30°C while loose-nucleotides removal is performed by successive precipitations (at least 2 ×) with 50% ammonium sulfate or by repeated washings (at least 3 ×) through centricon-30 concentrators.

FSBA-treated F_1ATPase is obtained by treating 1 mg/ml of I-LN-DF_1, suspended in 50 mM Tris–HCl pH 7 containing 2 mM CDTA, with 1.2 mM FSBA at 25°C according to [14]. When residual activity is ~15%, the FSBA-treated enzyme is centrifuged through Sephadex G-50 (fine) columns in 50 mM Tris–HCl pH 7.4 and subsequently concentrated through centricon-30 concentrators.

Fe(III)-loaded F_1ATPase is prepared by suspending I-LN-DF_1 in 0.25 M sucrose, 10 mM Tris–HCl pH 7.4 in the absence or in the presence of 4 mM ATP, or 4 mM ADP, or 70 μM ATP. After suitable standing, the suspensions are incubated at 30°C with 5-fold excess of $FeCl_3$. Fe(III) bound in the enzyme is separated from free Fe(III) by passage of the samples through Sephadex G-50 (fine) centrifugation columns equilibrated in 0.25 M sucrose, 10 mM Tris–HCl pH 7.4 containing or not 4 mM ATP, or 4 mM ADP, or 70 μM ATP. To remove loose nucleotides from Fe(III)-loaded F_1ATPase, the enzyme is passed through successive Sephadex G-50 (fine) centrifugation columns equilibrated in 0.25 sucrose, 10 mM Tris–HCl pH 7.4; whereas to remove also tight nucleotides, the protein is passed through a Sephadex G-50 (fine) column equilibrated in 100 mM Tris–sulfate pH 8 containing 50% glycerol [15].

2.3. Analytical procedures

ATPase activity is assayed spectrophotometrically at 340 nm and 30°C using an ATP-regenerating system [16]. Average specific activity of F_1ATPase is 75 ± 10 μmol/min/mg.

Analysis of iron is carried out by either atomic absorption as in [9] and spectrophotometrically using ferene method [17]. In the former case, samples of the different F_1ATPase preparations (1–4 μM) are diluted 5 × with water, boiled 5 min and centrifuged 10 min at 11,000 × *g*. The supernatants are added with HNO_3 (0.3% w/v) before iron analysis. Calibration curve is made using a commercial solution of iron: increasing amounts of iron (0.09–0.36 μM) are added to the proper media used for the enzyme suspensions previously diluted 5 × with water. The iron solutions are then added with HNO_3 (0.3% w/v). In the latter case, iron analysis is carried out directly by adding 30 μl of the different enzyme preparations (10–30 μM) to 130 μl of 0.05 M acetate buffer pH 4.8 containing 5 M guanidine hydrochloride, 2 mM ferene and 30 mM ascorbic acid. Calibration curve with 1–75 μM iron is made using a commercial solution of iron. A good agreement between the two methods is obtained using either solutions of Fe(III)-ATP complex in 0.25 M sucrose, 10 mM Tris–HCl, 4 mM ATP pH 7.4 (coefficient of correlation 0.99) or samples of the different enzyme preparations (coefficient of correlation 0.90). Therefore, chemical analysis with ferene is preferred as it requires fewer sample manupulations.

EPR spectra are carried out on a Bruker ESP 300 × band spectrometer operating at 9 GHz with 100 kHz field modulation equipped with a Bruker Low Temperature System. Fe(III) amount bound in the enzyme is calculated by double integration of the signal of Fe(III)-protein adduct. The resulting numerical value is compared with that obtained from solutions of Fe(III)-ATP complex (10–140 μM). A good agreement is observed among the data obtained by EPR and by the other two methods used for iron analysis: values differ less than 10%.

Nucleotides are analysed by HPLC as in [16]. Protein concentration is determinated as in [18].

3. Results and discussion

3.1. The binding of Fe(III) to F_1ATPase is affected by saturating concentrations of ATP

F_1ATPase isolated from mitochondria contains substoichiometric amounts of iron (see section 2). To avoid interference with the endogenous iron, the interaction of F_1ATPase with Fe(III) has been carried out on an enzyme in which all the iron has been depleted by EDTA [9]. In addition, to avoid the conformational changes produced by the ATP and ADP binding to nucleotide sites [4,19], both the unbound and loosely bound nucleotides have been removed from the enzyme. F_1ATPase sample so obtained is named Iron-Loose Nucleotide-Depleted F_1ATPase (I-LN-DF_1) and contains 2.9 ± 0.2 mol of nucleotides per mol of protein, which are bound, according to [3], in the two tight non-catalytic sites and in the tight catalytic site.

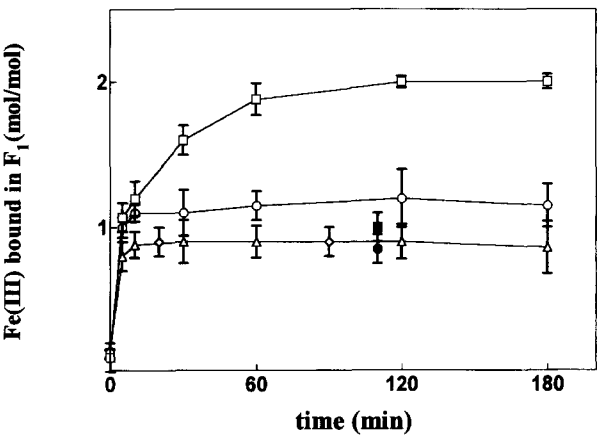

Fig. 1. Effects of nucleotides on Fe(III) binding to F_1ATPase. I-LN-DF_1 is suspended at 10 μM in 0.25 M sucrose, 10 mM Tris–HCl pH 7.4 containing 4 mM ATP (□) or 4 mM ADP (○) or 70 μM ATP (△) or none (◇). After suitable standing, the suspensions are incubated up to 180 min at 30°C with 50 μM $FeCl_3$ and aliquots are withdrawn at different times to measure Fe(III) bound in the enzyme. 5 min is the shortest time winterval because the relatively long period of time required to remove unbound Fe(III). Experiments are performed using I-LN-DF_1 pretreated with FSBA, as specified under section 2, and incubated with $FeCl_3$ in the presence of 4 mM ATP (■) or 4 mM ADP (●). The lines are only intended to show the progress of the experiments. Data are mean ± S.D. of three different experiments in which Fe(III) determinations are in duplicate.

Fig. 1 shows the time course of Fe(III) binding to I-LN-DF_1 under different conditions. Fe(III) binding never affects the catalytic activity of the enzyme. Addition of 5-fold excess of Fe(III)-Tris and no nucleotides to I-LN-DF_1 produces the binding of 1 mol of Fe(III)/mol of protein in <5 min. Addition of 5-fold excess of Fe(III)-nucleotide to I-LN-DF_1 previously incubated with either limiting concentrations of ATP (70 μM), able to fill selectively the catalytic sites [20], or saturating concentrations of ADP (4 mM), able to fill all nucleotide sites, again produces the binding of 1 equivalent of Fe(III) to the enzyme in less than 5 min. Addition of 5-fold excess of Fe(III)-ATP to I-LN-DF_1 preincubated with saturating concentrations of ATP (4 mM) produces binding of 2 mol of Fe(III)/mol of protein as already reported [9]. In the latter case, the binding of Fe(III) proceeds in two distinct phases, a rapid one (5 min) and a slow one (60 min) in which F_1ATPase binds the 1st and the 2nd equivalent of iron respectively. Addition of saturating concentrations of ATP to I-LN-DF_1, left to incubate 60 min with a 5-fold excess of Fe(III)-Tris, produces the binding of the 2nd equivalent of Fe(III) to the enzyme (data not shown). Addition of Fe(III)-nucleotide complexes and saturating ATP

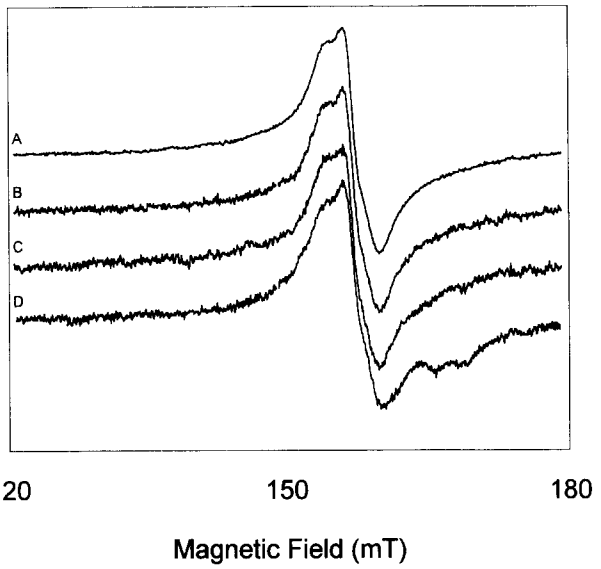

Fig. 2. EPR spectra of F_1ATPase loaded with Fe(III). I-LN-DF_1 is suspended at 20 μM in 0.25 M sucrose, 10 mM Tris–HCl pH 7.4 and incubated for 60 min at 30°C with 50 μM $FeCl_3$ (C). Spectra A and B are obtained upon Fe(III) loading of I-LN-DF_1 in the presence of saturating concentrations of ATP (A) or ADP (B). F_1ATPase, devoid of both loose and tight nucleotides (nucleotide content 0.4 mol/mol), is suspended at 13 μM in 0.25 M sucrose, 10 mM Tris–HCl pH 7.4 containing 5% glycerol and incubated for 60 min at 30°C with 40 μM $FeCl_3$(D). Spectra B and C are amplified two times while spectrum (D) is amplified three times, with respect to (A). EPR spectra of Fe(III)-ATP and Fe(III)-ADP are qualitatively well different from the spectra of Fe(III)-loaded F_1ATPase (data not shown). EPR conditions: T = 100 K, microwave power was 20 mW, frequency = 9.44 GHz.

or ADP to I-LN-DF_1 reacted with the nucleotide analog FSBA, able to selectively and irreversibly bind to the non-catalytic sites [14], produces the binding of only 1 mol of Fe(III)/mol of protein, even after 2 h incubation (Fig. 1).

These results indicate that the 2nd Fe(III) equivalent is found in F_1ATPase only when ATP has bound to non-catalytic sites, although the order of the addition of Fe(III) and saturating concentrations of ATP is not important. In fact, when these sites are filled with FSBA, ATP is not any more able to favor the binding of the 2nd equivalent of iron.

Furthermore, Fe(III) appears able to bind to the protein in the presence of an excess of Mg(II). In fact, even when the incubation with 5-fold excess of Fe(III)-ATP is performed in the presence of 50-fold excess of Mg(II)-ATP, 2 mol of Fe(III)/mol of protein binds to I-LN-DF_1 preincubated with saturating concentrations of ATP.

Fig. 2 shows the EPR spectra of I-LN-DF_1 and of a F_1ATPase preparation devoid of loose and tight nucleotides, which have been loaded with Fe(III) in the absence or in the presence of nucleotides. The signal is identical in all samples, even when iron, provided as Fe(III)-Tris, is added to the enzyme devoid of nucleotides. The signal is characterized by an absorption at g = 4.3 due to a high spin (S = 5/2) Fe(III) centre in a distorted tetragonal ligand field. Double integration of the spectra show that either in the absence of nucleotides or in the presence of saturating ADP the enzyme has bound only half the amount of Fe(III) compared with the quantity bound in the presence of saturating ATP, in line with the data of Fig. 1. The spectra are not perturbed by the presence of tight and loose nucleotides, indicating that nucleotides do not directly associate to the metal in both sites. Then, the results of Figs. 1 and 2 clearly indicate that binding of ATP to non-catalytic sites is needed to induce the conformational changes in the enzyme structure making accessible the second site for Fe(III) binding.

Addition of large amounts of phosphate to I-LN-DF_1 loaded with Fe(III) alters in an identical way the geometry of the two Fe(III) sites. In fact, Fig. 3 shows that the same EPR spectral changes occur in I-LN-DF_1 loaded with 1 or 2 mol Fe(III)/mol of protein. However, phosphate does not detach the iron from the protein as evaluated by double integration of the EPR signal.

F_1ATPase contains two distinct phosphate-binding sites [21], one of which has been documented to be located at a catalytic site [22]. Since it has been reported that phosphate, under conditions comparable to those of Fig. 3, does not produce conformational changes transmitted over long distances [5,19], the spectral changes of the Fe(III)-protein adducts induced by phosphate are attributed to a direct interaction of the anion with the metal ions, implying that one of the two iron-binding sites is located in the proximity of a catalytic site. Nothing may be said concerning the location of the second iron centre. However, the identity of the iron EPR spectra indicate an equal geometry of the two sites. It may be suggested that the second iron centre is located in the proximity of a nucleotide site, since inspection of the recently available X-ray structure [2] indicates that the surrounding of the nucleotide sites has similar geometry.

3.2. *Fe(III) is a poor cofactor for ATP hydrolysis catalyzed by F_1ATPase*

The efficiency of Fe(III) compared with Mg(II) as cofactor for ATP hydrolysis reaction has been checked using saturating concentrations of ATP (2.5–8 mM) and a metal:ATP ratio of 0.5, to avoid free metals as well as to minimize metal-free ATP. Under these conditions, a mononuclear metal-ATP complex is reported to be mostly formed, even with Fe(III) [23]. The results reported in Table 1 clearly show that the ATPase activity of I-LN-DF_1 in the presence of Fe(III) is very much lower than in the presence of Mg(II) and comparable to that observed in

Table 1
Relative capacities of Fe(III) and Mg(II) to support ATPase activity of F_1ATPase

Metal ion in the assay	ATP hydrolysis rate (μmol/min/mg protein)
Mg(II)	50 ± 3
Fe(III)	0.020 ± 0.015
None	0.008 ± 0.003

I-LN-DF_1 is suspended at 0.004 μM in 50 mM Tris–HCl pH 8, 1.25 mM $MgCl_2$ and 2.5 mM ATP. After 1–5 min, aliquots are withdrawn, added with 4% perchloric acid and centrifuged 10 min at 10,000 × g. The supernatants are then used for the determination of inorganic phosphate as in [29]. When no metal or Fe(III) is added to the assay mixture, I-LN-DF_1 is 18 μM and ATP varies from 2.5 to 8 mM while Fe(III):ATP ratio is maintained 0.5:1. After 10–60 min, aliquots are withdrawn, treated with 4% perchloric acid, and ATP and ADP in the supernatants are assayed by HPLC. Control experiments have shown that under these conditions F_1ATPase binds 2 Fe(III) equivalents. Temperature is 30°C. Neither Fe(III) or Mg(II) catalyze non-enzymatic ATP hydrolysis. Values represent mean ± S.D. of four experiments.

the absence of the metal ions. This finding excludes the production of a high concentration of ADP affecting the enzyme conformation, thereby confirming that the binding of the 2nd Fe(III) equivalent actually occurs because of conformational change induced by saturating concentrations of ATP.

The very low rate of ATP hydrolysis catalyzed by F_1ATPase in the presence of Fe(III) (Table 1) is in contrast with the result reported by Schuster and coworkers [24], but Fe(III) ion was already reported to be unable to activate F_1ATPase [25] because of its ionic radius, which is too small for coordinating correctly the phosphate oxygens of the nucleotide. In this regard, more recently it has been reported that the metal–ATP interactions existing in Fe(III)-ATP are very different with respect to other complexes, like Mg(II)-ATP, Mn(II)-ATP, etc. [23]. Specifically, Mg(II) and other divalent cations interact strongly with the phosphate moiety and indirectly with the adenine moiety [26]. In contrast, Fe(III) is directly coordinated both to the phosphate chain and the N-7 ring position of adenine [23].

3.3. *ATP-dependent binding of Fe(III) is reverted by loose nucleotide removal whereas nucleotide replacement by the catalytic cycle does not affect Fe(III) content*

Depletion of loose nucleotides from an enzyme previously loaded with 2 equivalents of Fe(III) induces the loss of 1 Fe(III) equivalent. Further depletion of tight nucleotides does not alter the Fe(III) content (Table 2, part A). This result shows that removal of ATP from the loose non-catalytic site induces the loss of the 2nd Fe(III) equivalent, indicating that the conformational changes produced by the detachment of ATP from the loose non-catalytic site, or by the binding of ATP to such site, modulate the binding of Fe(III) to the second site. In line with this result, is the recent finding that binding of ATP or ADP to such site is most responsible for the different conformations assumed by F_1ATPase in the presence of saturating ATP or ADP [19].

Incubation of the enzyme previously loaded with 2 equivalents of Fe(III) with large quantities of Mg(II)-ATP does not affect the Fe(III) content of the enzyme (Table 2, part B). This indicates that replacement of nucleotides bound in the enzyme with Mg(II)-ATP during the burst of catalytic cycle does not cause the displacement of Fe(III) bound in the protein, in line with EPR data showing that Fe(III) ions do not directly associate to nucleotides in both sites.

Table 2
Effects of nucleotide removal or replacement on Fe(III) content of Fe(III)-loaded F_1ATPase

Sample		Fe(III) content (mol/mol)
(A)		
(a)	2FE(III)-loaded F_1ATPase depleted of loose nucleotides	1.0 ± 0.1
(b)	sample a depleted of tight nucleotides	0.9 ± 0.1
(c)	1FE(III)-loaded F_1ATPase depleted of loose and tight nucleotides	0.9 ± 0.1
(B)		
(d)	2Fe(III)-loaded F_1ATPase added with Mg(II)-ATP, t = 10 min	2.0 ± 0.1
	t = 30 min	2.0 ± 0.1
	t = 60 min	1.9 ± 0.1

(A) 2Fe(III)-loaded F_1ATPase and 1Fe(III)-loaded F_1ATPase are prepared by treating I-LN-DF_1 with $FeCl_3$ in presence of 4 mM ATP or 70 μM ATP, respectively. Removal of loose and tight nucleotides are carried out as specified under section 2: in sample a nucleotide content is 2.9 ± 0.20 while in samples b and c nucleotide content is 0.6 ± 0.05 mol/mol of protein. (B) 2Fe(III)-loaded F_1ATPase is suspended at 10 μM in 0.25 M sucrose, 10 mM Tris–HCl pH 7.4, 4 mM ATP and incubated with 2 mM Mg(II) for 1 h at 30°C. Data are mean ± S.D. of three different experiments in which Fe(III) are in duplicate.

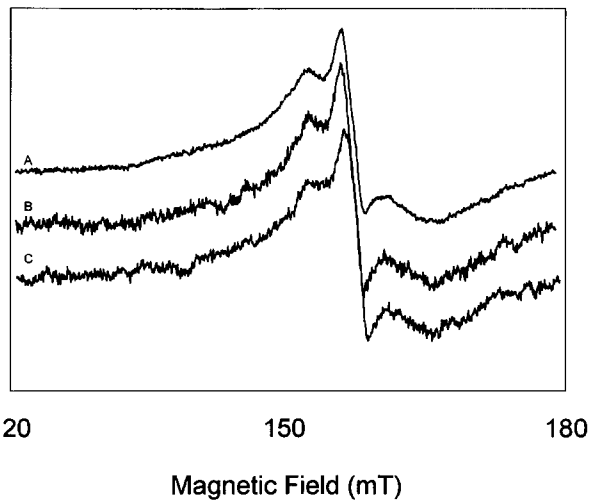

Fig. 3. Effects of phosphate on the line shape of the EPR spectra of Fe(III)-loaded F_1ATPase. The samples, which had been used to obtain the spectra A, B and C reported in Fig. 3, are thawed and incubated for 15 min at 30°C with 10 mM inorganic phosphate. Setting conditions as in Fig. 3. Spectra B and C are amplified two times with respect to A.

4. Conclusion

This study demonstrates that beef heart F_1ATPase contains two distinct iron-binding sites, which differ from the nucleotide-binding sites. One site is rapidly filled irrespective of the presence of nucleotides. Conversely, the second site is opened up for Fe(III) uptake selectively by conformational changes due to ATP binding to the loose non-catalytic site. Perturbation of the iron EPR signal by phosphate indicates that one site is located in the proximity of the catalytic site-binding phosphate. Furthermore, EPR experiments show that an equal geometry is exhibited by the two iron-binding sites, thus suggesting as location of the iron centres the region close to nucleotide-binding sites. The existence of two iron centres in the proximity of two among the six nucleotide-binding sites is in accordance with the inherent asymmetry of such sites [2]. In line with our results, it has recently been found [27] that the reticulocyte endosome H^+-ATPase, a close cousin of the F_0F_1ATPsynthase [28], contains iron-binding sites located near the nucleotide sites. The authors have reported evidence that such enzyme is involved in the pathway of iron transport across the endosomal membranes. Then, on the basis of the similarity of the two proteins as well as of the location of the iron-binding sites next to the nucleotide sites in both F_1ATPase and H^+-ATPase, the possibility is suggested that F_0F_1ATPsynthase may have a role in the mitochondrial iron homeostasis.

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