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Redox Properties of Iron in the Binding Site(s) of F_1ATPase from Mammalian Mitochondria and Thermophilic Bacterium PS3: A Comparative Study

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Iron ions in the two iron centers of beef heart mitochondrial F_1ATPase, which we have been recently characterized (*FEBS Letters* 1996, 379, 231–235), exhibit different redox properties. In fact, the ATP-dependent site is able to maintain iron in the redox state of Fe(II) even in the absence of reducing agents, whereas in the nucleotide-independent site iron is oxidized to Fe(III) upon removal of the reductant. Fe(III) ions in the two sites display different reactivity towards H_2O_2, because only Fe(III) bound in the nucleotide-independent site rapidly reacts with H_2O_2 thus mediating a 30% enzyme inactivation. Thermophilic bacterium PS3 bears one Fe(III) binding site, which takes up Fe(III) either in the absence or presence of nucleotides and is unable to maintain iron in the redox state of Fe(II) in the absence of ascorbate. Fe(III) bound in thermophilic F_1ATPase in a molar ratio 1:1 rapidly reacts with H_2O_2 mediating a 30% enzyme inactivation. These results support the presence in mitochondrial and thermophilic F_1ATPase of a conserved site involved in iron binding and in oxidative inactivation, in which iron exhibits similar redox properties. On the other hand, at variance with thermophilic F_1ATPase, the mitochondrial enzyme has the possibility of maintaining one equivalent of Fe(II) in its peculiar ATP-dependent site, besides one equivalent of Fe(III) in the conserved nucleotide-independent site. In this case mitochondrial F_1ATPase undergoes a higher inactivation (75%) upon exposure to H_2O_2. Under all conditions the inactivation is significantly prevented by PBN and DMSO but not by Cu, Zn superoxide dismutase, thus suggesting the formation of $OH^{\cdot}$ radicals as mediators of the oxidative damage. No dityrosines, carbonyls or oxidized thiols are formed. In addition, in any cases no protein fragmentation or aggregation is observed upon the treatment with H_2O_2.

Keywords: F_1ATPase, Fe(II)/Fe(III) ions, Thermophilic bacterium PS3, Ascorbic acid, Hydrogen peroxide, protein oxidative damage

Abbreviations: DMSO: dimethylsulphoxide; PBN: α-phenyl-*tert*-butylnitrone; DTNB: 5, 5'-dithiobis-(2-nitrobenzoic acid); DNPH: 2,4-dinitrophenylhydrazine

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INTRODUCTION

F-ATP synthases are the central enzymes in the energy conversion in mitochondria, chloroplasts and bacteria.[1] These enzymes are closely related in structure and mechanism. They consist of a globular catalytic domain, F_1, and an intrinsic membrane domain, F_0, linked by a stalk. When the two sectors are properly associated the complexes can use the protonmotive force to drive the synthesis of ATP or generate the protonmotive force by the hydrolysis of ATP. At variance, when F_1 is purified as a water-soluble complex, termed F_1ATPase, it only hydrolyzes ATP. F_1ATPase from prokaryotic and eukaryotic cells is composed of five subunits in the ratio $3\alpha:3\beta:1\gamma:1\delta:1\varepsilon$ and contains six binding sites for nucleotides.[2] Three sites are predominantly located in β subunits and have a catalytic role, while the other three reside in α subunits and are referred to as non-catalytic sites. Although the subunit composition of F_1ATPase is conserved, some functional and structural differences have been so far characterised. In particular, purified F_1ATPase isolated from mammalian mitochondria contains always tightly bound nucleotides, which are absolutely necessary for subunit assembly,[3] while Thermophilic bacterium PS3 F_1ATPase does not contain nucleotides.[4] Nevertheless, the conformation of both enzymes is modulated by ADP and ATP.[3,5,6] Besides, mitochondrial F_1ATPase is rapidly cold inactivated and its maximal ATPase activity is at 37°C, while thermophilic F_1ATPase is cold stable and its maximal ATPase activity is at 75°C.[7]

ATPase activity catalyzed by mitochondrial and bacterial ATPsynthases has been reported to be markedly inhibited upon exposure of cells[8,9,10] or mitochondrial membranes[11] to oxygen radical species, but the mechanisms of inhibition are controversal. In this regard, in our previous reports it is documented that a marked decrease of ATPase activity occurs upon infusion of H_2O_2 on purified mitochondrial F_1ATPase[12] and ATP synthase,[13] demonstrating that in both enzyme preparations redox-active Fe ions bound in F_1 moiety are crucial for the inactivation. Furthermore, we reported that exposure of differentiating erythroleukemia cells to non-lethal treatment with H_2O_2 produces a marked decrease of the maximal ATP synthesizing capacity, that appears to be mediated by redox reaction between the oxidant and Fe ions.[14] On the basis of the effects observed in sonicated cell homogenates both on the activity and the coupling of the enzyme complex, we can safely state that such redox reactions occur both at the level of F_1 moiety and at the level of coupling subunits. More recently, evidence has been provided that, concomitant with oxidative inactivation of ATPsynthase, severe energy impairment of cells occurs leading to loss of cell growth capacity.[15] These results strongly suggest that F_1 moiety may bind iron *in situ* and that iron may mediate enzyme inactivation, at least in erythroid cells. Furthermore, although the chemical nature of the non-heme non-FeS iron is still controversal,[16] considering that the mitochondrial concentration of this iron pool reported in non-erythroid cells[17,18] is higher than the mitochondrial F_1 content,[19] taking also into account that under pro-oxidant conditions mobilization of iron from ferritin or iron-containing proteins may occur,[18] as well as stimulation of cell iron uptake,[20] the hypothesis that Fe-ATPsynthase adducts may be formed *in vivo* is reasonable even in non-erythroid cells.

In vitro characterization of the iron binding to the enzyme complex purified from bovine heart mitochondria revealed that F_1ATPase contains two distinct Fe-binding sites located in the proximity of two nucleotide-binding sites.[21] One site is rapidly filled with Fe(III) irrespective of the presence of nucleotides (nucleotide-independent site). Conversely, the other site (ATP-dependent site) is opened up for Fe(III) uptake selectively by the conformational changes induced by binding of saturating ATP to one specific non-catalytic nucleotide-binding site, i.e. the loose site.[21] In the presence of ATP Fe(III)-binding to the two sites is cooperative, with a $Kd = 6\ \mu M$.[22]

Considering the high homology of the nucleotide-binding subunits in F_1ATPases,[23] in

this study we evaluated the Fe(III)-binding capacity of F_1ATPase from Thermophilic bacterium PS3, which resists to very extreme conditions. We obtained evidence for the presence of only one Fe(III)-binding site on such cold stable and nucleotide free enzyme. We characterized the binding site of thermophilic F_1ATPase, in comparison with the two sites of mitochondrial F_1ATPase, and we investigated the redox properties of iron ions through reaction with ascorbate, dithionite, glutathione, and H_2O_2. Based on our findings, we suggest that the difference in Fe(III)-binding capacity between mitochondrial and thermophilic F_1ATPase could be of biological relevance, considering that under Thermophilic bacterium PS3 growing conditions the oxidative stress is strongly enhanced.

MATERIALS AND METHODS

Materials

ADP, ATP, DMSO, DTNB, DNPH are obtained from Sigma; Sephadex G-50 (fine) is from Pharmacia; Chelex 100 is from Bio-Rad. Authentic dityrosine was a generous gift of Prof. S. Formisano (Department of Biology and Cellular and Molecular Pathology, University of Naples Federico II, Italy). Cu, Zn superoxide dismutase isolated from bovine erythrocytes was very kindly provided by Prof. G. Rotilio's laboratory (Department of Biology, University of Rome Tor Vergata, Italy). All the other 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, ATPase activity assay, thiol and carbonyl determinations.

Methods

Mitochondrial F_1ATPase is purified from beef heart mitochondria as in.[24] Thermophilic F_1ATPase is purified as described in.[7] In order to obtain the mitochondrial enzyme loaded with 1 equivalent of Fe(III) in the nucleotide-independent site (1Fe(III)-loaded MF_1) or the enzyme loaded with 2 equivalents of Fe(III) (2Fe(III)-loaded MF_1), 10 μM mitochondrial F_1ATPase is suspended in 0.25 M sucrose, 10 mM Tris-HCl pH 7.4 in the absence or presence of 4 mM ATP respectively and is treated at 30°C for 60 min with 40 μM $FeCl_3$.[21] Fe(III)-loaded thermophilic F_1ATPase (Fe(III)-loaded TF_1) is prepared by incubating, at different temperature and incubation time, thermophilic F_1ATPase, suspended at 10 μM in 50 mM Tris-SO_4 pH 7.4 containing or not 4 mM ADP or 4 mM ATP, with 40–80 μM $FeCl_3$. Fe bound in the enzymes is separated from free Fe(III) by passage of the samples through Sephadex G-50 (fine) centrifugation columns equilibrated with the corresponding medium. The enzymes are characterized for nucleotide content as in.[21] Reduction of bound Fe(III) ions is obtained by treating the samples with 500-fold excesses of ascorbate or dithionite for 5 min and then by passing them twice through Sephadex G-50 (fine) centrifugation columns in the absence of reducing agents. When glutathione is used, the mitochondrial enzyme is incubated up to 30 min with 500–1000-fold excesses of glutathione and then passed through Sephadex G-50 (fine) centrifugation columns as in the previous experiments. Thiol content of the enzymes before and after the oxidant treatment is determined by reaction with DTNB as in;[25] carbonyl production is assayed by reaction with DNPH as[26] and dityrosine content is measured as in.[27] Fe(II) is determined by ferene method,[21] while Fe(III) is determined by either ferene method or by following the EPR signal of Fe(III)-protein adduct at $g = 4.3$ as in.[21] ATPase activity is assayed spectrophotometrically by an ATP-regenerating system using the conditions specified in[21] for mitochondrial F_1ATPase and those reported by[28] for thermophilic F_1ATPase. SDS-PAGE is performed as in.[29]

RESULTS

Fe(III) Binding to Thermophilic F_1ATPase

Table I clearly indicates that, at variance with mitochondrial F_1ATPase, incubation of thermophilic F_1ATPase with 4-fold excesses of Fe(III), both in the absence or presence of saturating concentration of nucleotides, produces the binding of only 1 equivalent of Fe(III) in less than 5 min. Prolonged incubation of thermophilic F_1ATPase with Fe(III) does not increase the quantity of Fe(III) bound in the enzyme. Similarly, increasing the excesses of Fe(III) up to 8-fold does not change the Fe(III) binding capacity of the enzyme (data not shown). In addition, Table I shows that upon changing the Fe(III)-loading temperature of thermophilic F_1ATPase from 30°C to 70°C the quantity of iron bound in the enzyme does not change, thus indicating that the temperature-dependent conformational changes of the protein do not influence the Fe(III) binding to the enzyme. These data suggest a similarity between the Fe(III)-binding site of thermophilic F_1ATPase and the nucleotide-independent site of mitochondrial F_1ATPase, as before characterized in.[21] In accordance, EPR spectrum of Thermophilic F_1ATPase loaded with Fe(III) shows a signal at $g = 4.3$ of Fe(III)-protein adduct very similar to that we have reported[21] as characteristic of mitochondrial F_1ATPase (data not shown).

TABLE I Uptake of Fe(III) by thermophilic F_1ATPase

Additions	temperature (°C)	time (min)	bound Fe(III) (mol/mol)
A)			
none	30	5	0.2±0.1
$FeCl_3$	30	5	0.8±0.1
$FeCl_3$ + 4 mM ADP	30	5	0.8±0.1
$FeCl_3$ + 4 mM ATP	30	5	1.0±0.1
$FeCl_3$	30	120	1.1±0.2
$FeCl_3$ + 4 mM ADP	30	120	0.9±0.1
$FeCl_3$ + 4 mM ATP	30	120	0.9±0.1
B)			
$FeCl_3$	70	30	0.9±0.2
$FeCl_3$ + 4 mM ADP	70	30	0.8±0.1
$FeCl_3$ + 4 mM ATP	70	30	0.8±0.1

A) Thermophilic F_1ATPase is suspended at 10 μM in 50 mM Tris-SO_4 pH 7.4 containing or not 4 mM ADP or 4 mM ATP. The suspensions are incubated up to 120 min at 30°C with 40 μM $FeCl_3$. Aliquots are withdrawn at different times and passed through Sephadex G-50 centrifugation columns to remove unbound Fe. Fe(III) bound in the protein is then measured by ferene method as specified in Methods. B) Thermophilic F_1ATPase is incubated with $FeCl_3$ as in A), except that the incubation temperature is changed from 30 to 70°C. Fe(III) bound is analyzed by EPR, in order to avoid the passage of the samples through the Sephadex G-50 centrifugation columns.

Treatment with Ascorbate of Thermophilic and Mitochondrial F_1ATPase Loaded with 1 Equivalent of Fe(III)

Table II shows that when thermophilic F_1ATPase is loaded with 1 equivalent of Fe(III), as well as when mitochondrial F_1ATPase is loaded with 1 equivalent of Fe(III) selectively in the nucleotide-independent site,[21] and subsequently both enzymes are treated for few minutes with 500-fold excesses of ascorbate, Fe(III) is completely reduced to Fe(II). However, when the enzymes are washed out of the ascorbate by passages through Sephadex G-50 centrifugation columns, about 1 equivalent of Fe(III) bound in both proteins is detected. This result indicates that in the nucleotide-independent site of mitochondrial F_1ATPase, as well as in the iron site of thermophilic F_1ATPase, Fe(II) is oxidized to Fe(III) in the absence of an excess of ascorbate, thus suggesting that iron ions bound in these sites may have similar redox properties.

Treatment with Different Reducing Agents of Mitochondrial F_1ATPase Containing 2 Equivalents of Fe (III)

Table III shows that when mitochondrial F_1ATPase containing 2 equivalents of Fe(III) is treated with 500-fold excesses of ascorbate, the complete reduction of Fe(III) to Fe(II) occurs, demonstrating the accessibility of both sites to ascorbate. However, when ascorbate is removed, 1 equivalent of Fe(II) and 1 equivalent of Fe(III)

TABLE II Redox state of iron in the binding site of thermophilic F_1ATPase and in the nucleotide-independent site of mitochondrial F_1ATPase after addition and removal of ascorbate

Sample	Fe(III) (mol/mol)	Fe(II) (mol/mol)	Fe(III) + Fe(II) (mol/mol)
A = 1Fe (III)-loaded TF_1	0.9±0.1	n.d.	0.9±0.1
B = Sample **A** treated with ascorbate	n.d.	0.9±0.1	0.9±0.1
Sample **B** washed out of ascorbate	0.7±0.1	n.d.	0.7±0.1
C = 1Fe(III)-loaded MF_1	1.1±0.1	n.d.	1.1±0.1
D = Sample **C** treated with ascorbate	n.d.	1.1±0.1	1.1±0.1
Sample **D** washed out of ascorbate	0.9±0.1	0.1±0.1	1.0±0.1

Thermophilic and mitochondrial F_1ATPase are loaded with 1 equivalent of Fe(III) (1Fe(III)-loaded TF_1 and 1Fe(III)-loaded MF_1) and then treated with ascorbate as specified in Methods. In samples B and D the amount of Fe(II) bound in the proteins is calculated by the disappearance of the EPR signal at g = 4.3 of the Fe(III)-protein adduct detected in samples A and C, respectively. In samples washed out of ascorbate Fe(II) bound in the enzymes is determined by ferene method. Data are means±S.D. of three experiments. n.d., not detectable.

TABLE III Effects of treatments with reducing agents on the redox state of iron in the two binding sites of mitochondrial F_1ATPase

Sample	Fe(III) (mol/mol)	Fe(II) (mol/mol)	Fe(III) + Fe(II) (mol/mol)
A = 2Fe(III)-loaded MF_1	2.0±0.1	n.d.	2.0±0.1
B = Sample **A** treated with ascorbate	n.d.	2.0±0.1	2.0±0.1
Sample **B** washed out of ascorbate	1.1±0.1	0.9±0.1	2.0±0.1
C = Sample **A** treated with and subsequently washed out of glutathione	1.7±0.2	n.d.	1.7±0.2
D = Sample **A** treated with and subsequently washed out of dithionite	1.1±0.1	0.6±0.1	1.8±0.1

Mitochondrial F_1ATPase is loaded with 2 equivalents of Fe(III) (2Fe(III)-loaded MF_1) and treated with ascorbate, glutathione and dithionite as specified in Methods. In the absence of reducing agents Fe(II) bound in the enzymes is determined by ferene method, while in the presence of ascorbate Fe(II) is calculated by the disappearance of the EPR signal of the Fe(III)-protein adduct at g = 4.3. Data are means±S.D. of three experiments. n.d., not detectable.

bound in the enzyme are detected. By comparison with data of Table II this result indicates that, at variance with the nucleotide-independent site, the ATP-dependent site is able to maintain iron in the redox state of Fe(II) even in the absence of ascorbate, strongly suggesting that the redox properties of the iron ions bound in the two sites are different. Similarly, when 2Fe(III)-loaded mitochondrial F_1ATPase is treated with dithionite, more than half equivalent of Fe(II) and one equivalent of Fe(III) bound in the enzyme are detected upon dithionite removal. On the contrary, when the enzyme is treated up to 1000-fold excesses with glutathione, no Fe(II) bound in the protein is measured upon glutathione removal. These results suggest that iron ions in the ATP-dependent site are buried, so that glutathione, at variance with ascorbate and dithionite, cannot bind and reduce them, due to its greater steric hindrance.[30] The incomplete reduction of Fe(III) to Fe(II) upon dithionite addition could be due to its greater charge hindrance with respect to ascorbate.

Removal of Loose Nucleotides from Mitochondrial F_1ATPase Containing Both Fe(II) and Fe(III)

The removal of the nucleotides from the loose nucleotide-binding sites of mitochondrial F_1ATPase has been previously demonstrated[21] to

selectively induce the loss of iron ions from the ATP-dependent site. Then, with the aim to obtain more evidences that Fe(II) is bound only in the ATP-dependent site, the following experiment has been performed: the enzyme containing both Fe(II) and Fe(III) has been prepared by ascorbate treatment of the protein loaded with 2 equivalents of Fe(III), followed by washing out of ascorbate, and subsequently has been deprived of loose nucleotides. Table IV clearly shows that, upon depletion of loose nucleotides, the enzyme completely loses Fe(II), whilst it retains Fe(III), thus confirming that Fe(II) is bound in the ATP-dependent site. Table IV also shows that even the untreated enzyme isolated from mitochondria (native MF_1) contains, in addition to tightly and loose nucleotides, both Fe(II) and Fe(III). As in the case of mitochondrial F_1ATPase loaded with both Fe(II) and Fe(III), the removal of loose nucleotides from native mitochondrial F_1ATPase causes the loss of Fe(II) and not of Fe(III), indicating that Fe(II) is bound in the ATP-dependent site even in the native enzyme. On the contrary, in native thermophilic F_1ATPase only Fe(III), and no Fe(II), is detected (see Table I), further on confirming the lack of the ATP-dependent site in this enzyme.

Treatment with H_2O_2 of Mitochondrial and Thermophilic F_1ATPase

Fig. 1A and B clearly show that the redox state of iron bound in mitochondrial F_1ATPase strongly influences the susceptibility towards H_2O_2. In fact, either in the case of the enzyme containing 1 equivalent of Fe(III) in the nucleotide-independent site, or in the case of the enzyme containing two equivalents of Fe(III) a 30% decrease of the ATP hydrolysis rate is observed upon treatment with three-fold excess of H_2O_2, also after a long incubation time. Conversely, when the mitochondrial enzyme loaded with both Fe(II) and Fe(III) is treated with H_2O_2, a dramatically higher inactivation is observed (75%), while no inactivation occurs in mitochondrial F_1ATPase containing Fe(II) in the ATP-dependent site and no Fe(III) in the nucleotide-independent site (data not shown). These results indicate that selectively Fe(III) bound in the nucleotide-independent site reacts with H_2O_2 giving rise to enzyme inactivation, while iron bound in the ATP-dependent site either as Fe(III) or as Fe(II) does not react, in accordance with their different redox properties as revealed by the reductant experiments. Nevertheless, Fe(II) in the ATP-dependent site enhances the H_2O_2 inactivation mediated by Fe(III) in the nucleotide-independent site. Fig. 1C shows that in thermophilic F_1ATPase Fe(III) reacts with H_2O_2 giving rise to 30% inactivation, as in mitochondrial F_1ATPase containing only Fe(III), thus supporting again the similarity between the iron-binding site of the thermophilic F_1ATPase and the nucleotide-independent site of the mitochondrial enzyme. In addition, Fig. 1A, B and C show that under all conditions 100 mM PBN or 100 mM

TABLE IV Removal of loose nucleotides from Fe(III)/Fe(II)-loaded mitochondrial F_1ATPase and from native mitochondrial F_1ATPase affects the content of Fe(II), but not of Fe(III)

Sample	Fe(III) (mol/mol)	Fe(II) (mol/mol)	Fe(III) + Fe(II) (mol/mol)
A = Fe(III)/Fe(II)-loaded MF_1	1.1±0.1	0.6±0.1	1.7±0.1
Sample **A** depleted of loose nucleotides	0.9±0.1	n.d.	0.9±0.1
B = native MF_1	0.3±0.1	0.2±0.1	0.5±0.1
Sample **B** depleted of loose nucleotides	0.2±0.1	n.d.	0.2±0.1

Fe(III)/Fe(II)-loaded MF_1 is prepared by treating mitochondrial F_1ATPase containing 2 equivalents of Fe(III) with ascorbate, followed by ascorbate removal, as specified in Table III. Depletion of loose nucleotides is performed as in (21): in samples **A)** and **B)** the content of tight nucleotides is 2.9±0.20 mol/mol of protein. Data are means±S.D. of two independent experiments. n.d., not detectable.

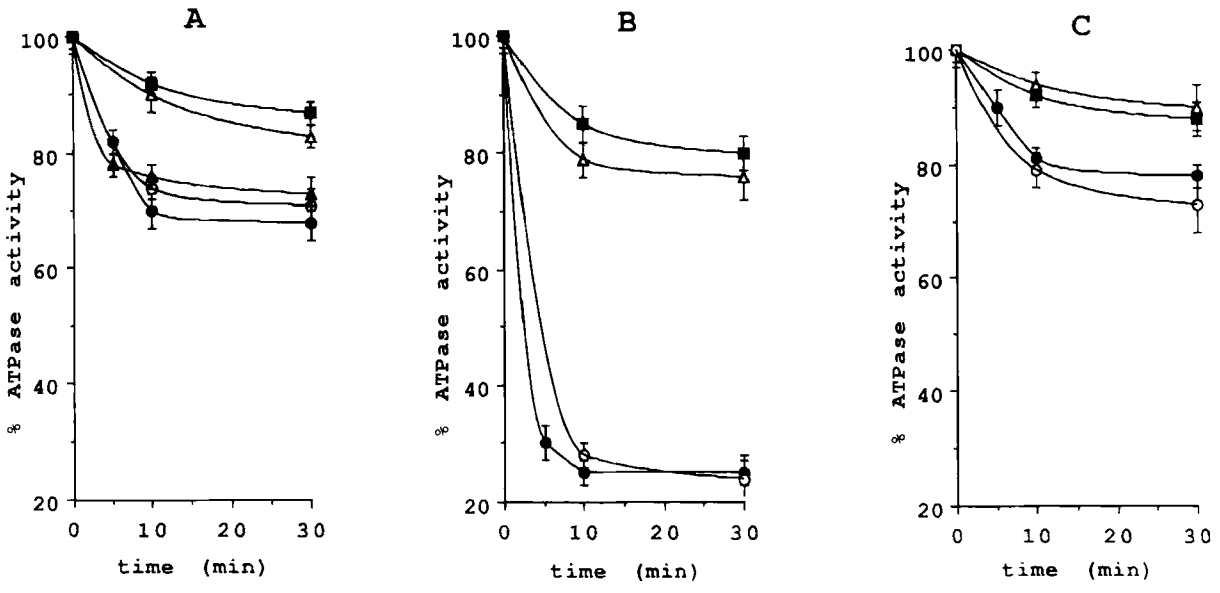

FIGURE 1 Inhibition of ATPase activity by H_2O_2: protective effects of oxygen radical scavengers. 17 μM mitochondrial F_1ATPase, loaded with 1 equivalent of Fe(III) in the nucleotide-independent site (1Fe(III)-loaded MF_1) (**A**) or 2 equivalents of Fe(III) (2Fe(III)-loaded MF_1) (**A**) or both 1 equivalent of Fe(II) and 1 equivalent of Fe(III) (Fe(III)/Fe(II)-loaded MF_1) (**B**) as in Tables II and III, is suspended in 0.25 M sucrose, 10 mM Tris-HCl pH 7.4 in the absence or presence of 4 mM ATP and incubated at 30°C with 45 μM H_2O_2. Similarly, 17 μM thermophilic F_1ATPase, loaded with 1 equivalent of Fe(III) (Fe(III)-loaded TF_1), is suspended in 50 mM Tris-SO_4 pH 7.4 and incubated at 30°C with 45 μM H_2O_2 (**C**). Aliquots of all enzyme preparations are also added with 100 mM DMSO or 100 mM PBN or 1 μM Cu,Zn superoxide dismutase. ATPase activity is assayed at different time intervals as specified in Methods. 100% ATPase activity of thermophilic F_1ATPase is 7.6 U/mg, while that of mitochondrial F_1ATPase is 80 U/mg. **A**: 1Fe(III)-loaded MF_1 incubated with H_2O_2 (▲) and 2Fe(III)-loaded MF_1 incubated with H_2O_2 in the presence of none (●) or DMSO (△) or PBN (■) or Cu,Zn superoxide dismutase (○); **B**: Fe(III)/Fe(II)-loaded MF_1 incubated with H_2O_2 in the presence of none (●) or DMSO (△) or PBN (■) or Cu,Zn superoxide dismutase (○); **C**: 1Fe(III)-loaded TF_1 incubated with H_2O_2 in the presence of none (●) or DMSO (△) or PBN (■) or Cu,Zn superoxide dismutase (○).

DMSO equally decrease the H_2O_2-dependent inactivation, while 1 μM Cu, Zn superoxide dismutase does not protect the enzymes against H_2O_2. This suggests the formation of $OH^{\bullet}$ radicals, or closely related species, but not of $O_2^{-\bullet}$, as mediators of the enzymze inactivation.

Detection of Protein Oxidation Products

SDS-PAGE of H_2O_2-treated enzyme samples (Fig. 2) indicates that either in thermophilic and mitochondrial F_1ATPase loaded with Fe(III) and in the mitochondrial enzyme loaded with both Fe(II) and Fe(III) no protein fragmentation or aggregation occurs. In addition, Fig. 2 shows that, at variance with the data reported by Belogrudov,[31] neither protein fragmentation nor aggregation is caused by the ascorbate-treatment of both mitochondrial and thermophilic F_1ATPase containing Fe(III). In accordance, no enzyme inactivation is measured upon reductant treatment (data not shown). As unbound Fe(III) is always removed in our experiments before reductant addition, this suggests that reactive oxygen species, formed due to redox cycling of protein-bound Fe(II)/ascorbate system in air, are not sufficient to inactivate or fragment the enzyme.

Determination of thiol, carbonyl and dityrosine content of the enzymes before and after the oxidant treatment reveals that both in thermophilic and mitochondrial F_1ATPase no oxidized thiols are formed upon H_2O_2 inactivation, as well as no dityrosine and very low carbonyl derivatives (about 0.1 mol of carbonyl/mol of

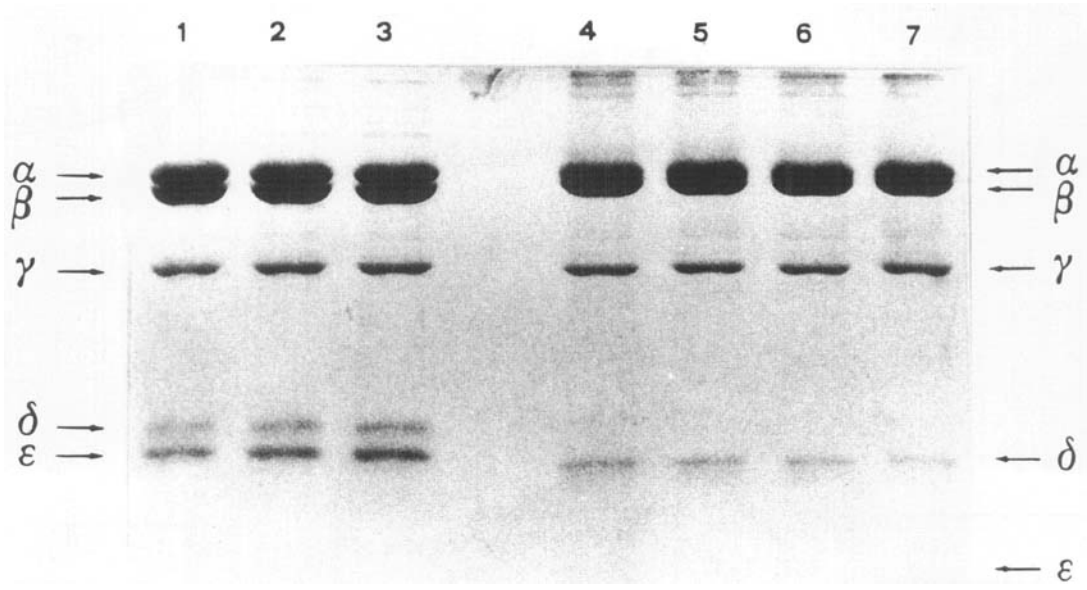

FIGURE 2 SDS-PAGE of thermophilic F_1ATPase **(A)** and mitochondrial F_1ATPase **(B)** before and after the treatment with ascorbate and/or H_2O_2. The following samples are analyzed on the 15% SDS-PAGE, using the sample buffer without β-mercaptoethanol to avoid the reduction of new formed S-S bridge. Thermophilic F_1ATPase, suspended in 50 mM Tris-SO_4 pH 7.4 and 4 mM ATP, or mitochondrial F_1ATPase, suspended in 0.25 M sucrose, 10 mM Tris-HC1 pH 7.4 and 4 mM ATP, are treated in order to obtain 1Fe(III)-loaded TF_1 (lane 1), and 2Fe(III)-loaded MF_1 (lane 4); 1Fe(III)-loaded TF_1 is then inactivated by about 30% with H_2O_2 (lane 2) or is treated with ascorbate as in Table II (lane 3); 2Fe(III)-loaded MF_1 is inactivated by about 30% with H_2O_2 (lane 5) or is treated with ascorbate to obtain Fe(III)/Fe(II)-loaded MF_1 (lane 6); Fe(III)/Fe(II)-loaded MF_1 is then inactivated by about 75% by H_2O_2 (lane 7).

enzymes) occur irrespective of the inactivation extent (data not shown).

DISCUSSION

The results reported in this study clearly indicate that both thermophilic and mitochondrial F-ATP synthases bind iron ions in the same portion of the enzymes, i.e. the catalytic moiety F_1, and that the redox properties of iron ions bound in the proteins strongly influence the susceptibility of the enzymes to the oxidative stress. Thermophilic F_1ATPase bears only one Fe binding site, which binds Fe(III) either in the absence or presence of nucleotides and is unable to maintain iron in the redox state of Fe(II) in the absence of ascorbate. Conversely, mitochondrial F_1ATPase contains two iron centers: the ATP-dependent site is able to maintain iron in the redox state of Fe(II), whereas in the nucleotide-independent site Fe(II) is oxidized to Fe(III) in the absence of reducing agents. Both enzymes undergo 30% iron-mediated inactivation upon H_2O_2 exposure when almost one Fe(III) ion is bound to one mole of enzyme. These results strongly support the hypothesis that in mitochondrial and thermophilic F_1ATPase a conserved site exists which binds iron in a redox-active form, able to react with ascorbate, as well as with H_2O_2 giving rise to enzyme inactivation.

In agreement with models of site-directed oxidative damage of proteins and with our previous suggestion about the mechanism of the oxidative inactivation of the mitochondrial F_1ATPase,[12,13] neither protein fragmentation nor aggregation is detected in both enzymes upon H_2O_2-dependent inactivation. Moreover, in the presence of $OH^{\bullet}$ scavengers a marked but

incomplete prevention is observed. Such effects are observed even in the case of the very high inactivation caused by H_2O_2 in the mitochondrial F_1ATPase binding both Fe(II) and Fe(III). Then, irrespective of the mechanism responsible for the greater extent of inactivation observed in this case, under all conditions site-specific generation of $OH^{\bullet}$ radicals or of species with similar reactivity should be involved resulting in damages, likely restricted to different portions of protein.

The amino acids undergoing oxidative damages in both enzymes are at present unknown. In fact, under all conditions no oxidized thiols and no dityrosine or carbonyl formation are detected. As for the mitochondrial enzyme, the lack of thiol oxidation could be expected considering the different location in the protein of the cysteine residues, which are far from the nucleotide-binding sites,[32] and the iron centers, which we documented to be in the proximity of the nucleotide-binding sites.[21] Conversely, the lack of dityrosine and carbonyl formation in both enzymes is somewhat surprising, as either tyrosine residues and other amino acid residues particularly sensitive to carbonyl conversion[33] are involved in the nucleotide-binding site pockets of both mitochondrial[32] and thermophilic[23] F_1ATPase.

The molecular mechanisms and the amino acid residues involved in the H_2O_2 inactivation of mitochondrial F_1ATPase and thermophilic F_1ATPase are at present under investigation in our laboratories. In particular, EPR studies of protein-associated radical species formed during the reaction of the enzymes with H_2O_2 are in progress.

The different redox properties of iron ions in the two iron-binding sites in mitochondrial F_1ATPase are in accordance with the existence of two singles Fe atoms directly coordinated by amino acid residues in the enzyme.[21] In fact, in other non-heme, non-Fe/S proteins iron atoms can have a large range of redox potential.[34] In addition, considering that previous EPR data revealed an equal geometry of the two sites,[21] it is tempting to speculate that the different redox properties of iron ions may be in part controlled by the different degree of exposure to solvent water in these sites. In support of this hypothesis, the experiments with different reducing agents indicate that the ATP-dependent site is buried in the protein, while the H_2O_2 inactivation experiments strongly suggest the nucleotide-independent site as selectively exposed to solvent water. Moreover, the hypothesis of different accessibility of the two iron-binding sites should be in accordance with the inherently different structural properties of the nucleotide-binding sites[2] of mitochondrial F_1ATPase, as the iron sites are in the proximity of them.[21]

The lack of the ATP-dependent site in thermophilic F_1ATPase may reflect the different properties of the non-catalytic nucleotide-binding sites in mitochondrial and thermophilic F_1ATPase, mainly the dramatic differences in the affinity of the nucleotide binding sites.[35] Thus it is tempting to hypothesize that ATP binding to these sites do not induce an ATP-dependent conformation in thermophilic F_1ATPase equal to that induced in mitochondrial F_1ATPase by high concentration of ATP. Moreover, thermophilic F_1ATPase, at variance with mitochondrial F_1ATPase, is able to hydrolyze Fe(III)-ATP,[36] although not so efficiently as Mg(II)-ATP. Then we can't exclude that ADP, obtained by the hydrolysis of Fe(III)-ATP, remains bound in the catalytic site, thus influencing the effects of unhydrolyzed ATP on the conformation of the enzyme. In conclusion, it may be speculated that the lack of the ATP-dependent Fe-binding site in thermophilic F_1ATPase, whose molecular basis is under investigation, could have biological relevance, mainly with respect to the ability of thermophilic F_1ATPase of resisting to very extreme conditions, where the oxidative stress is strongly enhanced.

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