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Electron paramagnetic resonance evidence of metal-ion binding sites in *Micrococcus lysodeikticus (M. luteus)* F_1-ATPase

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Evidence is presented for the presence of divalent cation binding sites in purified F_1-ATPase from *Micrococcus lysodeikticus (Micrococcus luteus)*. Electron paramagnetic resonance studies of native F_1-ATPase indicate that the enzyme binds Mn^{2+} and Cu^{2+}. Scatchard-type plot for Mn^{2+} binding to the enzyme indicates the presence of 3–4 independent and identical sites with a dissociation constant of $18.3 \cdot 10^{-6}$ M. Cu^{2+} binds to the enzyme at only one kind of site(s). This Cu^{2+} binding site(s) is characterized by a moderately ionic ligand field provided by the protein and by a tetragonal symmetry of nitrogen and/or oxigen ligands. Competition studies indicate that Mg^{2+} binds at these Mn^{2+} and Cu^{2+} binding sites.

Introduction

The water-soluble part of the ATPase-ATP synthetase complex (EC 3.6.1.3), named F_1 factor or F_1-ATPase, has been the subject of intensive studies in recent years [1–3]. These oligomeric enzymes, irrespective of their origin, require divalent cations for ATP hydrolytic activity, with Mg^{2+} and Ca^{2+} being the usual activators. Moreover, divalent cations are required for reconstitution of ATPase activity from individual subunits [4–6] and for rebinding of the enzyme to the membrane [7–9]. Also, divalent cations affect P_i binding to these proteins [10,11] as well as the P_i-ATP exchange reactions [12]. Furthermore, slow conformational changes in isolated mitochondrial F_1-ATPase are postulated to be induced by Mg^{2+} [13,14]. The remarkable effects of divalent cations on the F_1-ATPase structure and activity seem to indicate the presence of special binding sites for divalent cations on this enzyme. However, the role of divalent ions in these energy-transducing F_1-ATPases is poorly understood. Paramagnetic divalent ions have been widely used to investigate the presence of divalent cation binding sites in proteins. In this context, we have studied the interaction of Mn^{2+} and Cu^{2+} with the F_1-ATPase from the gram-positive bacterium *Micrococcus lysodeikticus* (also known as *Micrococcus luteus*) by electron paramagnetic resonance (EPR) spectroscopy in order to demonstrate the presence of metal ion binding sites in this protein. Previous work on this bacterial F_1-ATPase strongly suggested the involvement of metal ions in functional and structural properties of the enzyme [8–10,15], but direct evidence for metal-ion binding sites was absent.

Materials and Methods

Purification of F_1-ATPase

Membranes from *Micrococcus lysodeikticus (M.*

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luteus) NCTC 2665 substrain A were obtained as previously described [16]. The crude F_1-ATPase fraction was prepared by the shock wash procedure [8,16], and subsequently purified by preparative polyacrylamide gel electrophoresis [17]. The purity of the enzyme preparations was checked by gel electrophoresis in the presence and in the absence of sodium dodecyl sulfate [18]. They were shown to be more than 97% pure.

Protein concentration was determined using a specific extinction coefficient of $E^{1\%} = 6.93 \pm 0.1$ at 276 nm previously reported [19]. Scatchard plot [20] was adjusted by a linear-least-squares-regression computer program. Molar concentrations of purified F_1-ATPase are based on a molecular weight of 350000 [18].

Electron paramagnetic resonance spectroscopy

In Mn^{2+} titration, EPR measurements were performed at room temperature. Solution aliquots were introduced in 100 μl calibrated capillaries, placed in an EPR quartz tube and then inserted in the Varian E-246 quartz dewar. The dewar tail was then placed in the TE_{102} resonant cavity of the EPR spectrometer. Experiments concerning Cu^{2+} ions were conducted at low temperature (77 K). In this case, aqueous Cu^{2+}-F_1 samples were placed in 4.7 mm i.d. precision glass tubes open at both ends, frozen in liquid nitrogen and stored. To record the EPR spectrum, the sample was removed from the glass tube by warming the surface just sufficiently to allow the cylindrical sample to be pushed out of the tube. It was then placed in the EPR dewar filled with liquid nitrogen. EPR spectra were recorded by an X-band Varian E-109 spectrometer with 100 kHz modulation frequency. To calculate the experimentally observed g-factors, a magnetic field calibration was performed with a NMR gaussmeter; the microwave frequency was then determined by the relationship: $\nu = g_{DPPH}\beta H/h$, where $g_{DPPH} = 2.0036$. Paramagnetic ion concentration was determined by double integration of the EPR spectra and by comparison with suitable standards of known concentration.

Results

Manganese binding to Micrococcus lysodeikticus (M. luteus) F_1-ATPase

We used Mn^{2+} as a probe of metal binding sites in the F_1-ATPase from *M. lysodeikticus*. The EPR spectrum of Mn^{2+} in aqueous solution can be interpreted as due to spin $S = 5/2$, with $g = 2$ placed in an evironment with octahedral symmetry [21]. Owing to the nuclear spin of Mn ($I = 5/2$), each $\Delta m_s = 1$ line is split into $2I + 1 = 6$ hyperfine lines. Due to the weak spin-orbit coupling, the ion exhibits a long relaxation time and then the EPR spectrum can be observed even at room temperature (Fig. 1a). Binding of Mn^{2+} to a biological substrate results in an increase of the spin longitudinal relaxation time and the EPR linewidth becomes 4-times larger than Mn^{2+} in aqueous environment (80 G instead of 20 G). As a consequence the amplitude of the EPR signal (normally displayed as the derivative of the absorption) becomes 16-times larger. Then, the spectrum b of Fig. 1, where Mn^{2+} has been incubated with F_1-ATPase, measures essentially the free Mn^{2+}. If the total quantity of Mn^{2+} in the sample is known, the bound Mn^{2+} can be obtained by difference and the titration can thus be performed. A constant F_1-ATPase concentration of $2.57 \cdot 10^{-6}$ M was titrated with an Mn^{2+} range from $5 \cdot 10^{-6}$ to $5 \cdot 10^{-5}$ M. As shown in Fig. 2a, Mn^{2+} results bound to the enzyme. The results in the form of a Scatchard-type plot are shown in Fig. 2b. Extrapo-

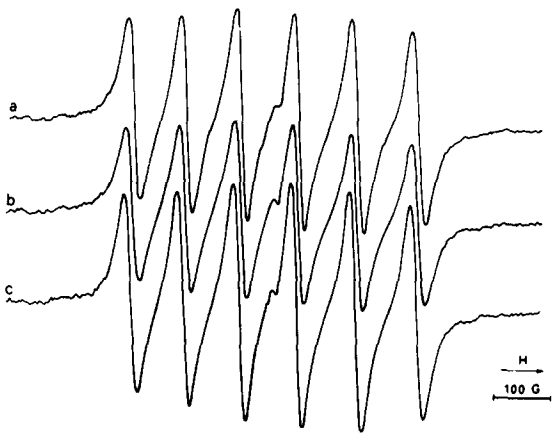

Fig. 1. Binding of Mn^{2+} to F_1-ATPase from *M. lysodeikticus*. EPR spectra at room temperature of: (a) Mn^{2+} ($2.5 \cdot 10^{-5}$ M) in aqueous solution; (b) Mn^{2+} ($2.5 \cdot 10^{-5}$ M) and F_1-ATPase ($2.57 \cdot 10^{-6}$ M); (c) Mn^{2+} ($2.5 \cdot 10^{-5}$ M) and F_1-ATPase ($2.57 \cdot 10^{-6}$ M) in the presence of Mg^{2+} ($5 \cdot 10^{-3}$ M). Microwave power level, 20 mW. Magnetic-field sweep rate, 1000 G in 8 min. Time constant, 0.128 s. Modulation amplitude, 5 G.

lation of the data indicates the presence of 3.7 equivalent and independent binding sites with a dissociation constant of 18.3 μM.

To determine whether Mg^{2+} could bind to the same sites as Mn^{2+} on the F_1-ATPase, solutions of Mn^{2+} and enzyme were incubated with Mg^{2+} and the concentration of free Mn^{2+} was followed by EPR. It was found that $5 \cdot 10^{-3}$ M Mg^{2+} completely inhibited the Mn^{2+} binding to the protein (Fig. 1c).

Owing to the presence of firmly associated adenine nucleotides in purified F_1-ATPases [22,23], questions arise as to whether the above metal-ion binding sites are contributed by tightly bound adenine nucleotides or by amino acids side chains. The enzyme preparation used in this work contains 0.5 mol ATP and 0.5 mol tightly bound ADP/mol of F_1 [24]. Since the stoichiometry of divalent cation binding differs significantly from that of tightly bound adenine nucleotides, it seems likely that divalent cation bind directly to amino acids side chains.

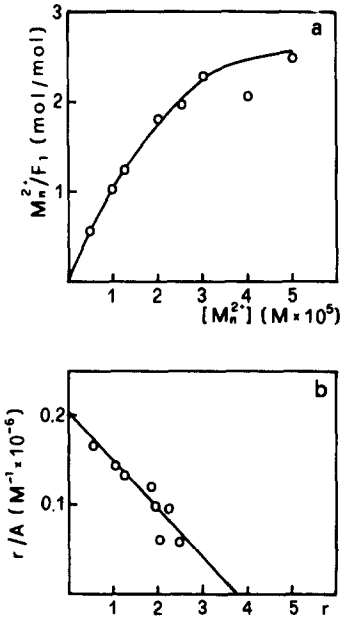

Fig. 2. Binding of Mn^{2+} to F_1-ATPase from *.M. lysodeikticus*. (a) A constant F_1-ATPase concentration of $2.57 \cdot 10^{-6}$ M was titrated with $MnCl_2$ in the concentration range of $5 \cdot 10^{-6}$–$5 \cdot 10^{-5}$ M in a final volume of 100 μl of 30 mM Tris-HCl (pH 7.5), and the EPR signal of free Mn^{2+} was measured. (b) Scatchard type plot of Mn^{2+} binding to F_1-ATPase using the data from the previous curve. *A* stands for the concentration of free Mn^{2+} and *r* represents the number of mol of Mn^{2+} bound per mol of enzyme.

Cu^{2+} binding to Micrococcus lysodeikticus (M. luteus) F_1-ATPase

In addition to the Mn^{2+} binding studies, we investigated the Cu^{2+} binding to the enzyme to gain information about the microenvironment of the metal ion binding sites. Incubation of F_1-ATPase with Cu^{2+} ions resulted in the EPR spectrum of Fig. 3a, arising from the interaction of Cu^{2+} with the enzyme. In order to correlate this Cu^{2+} binding site with the above Mn^{2+} binding site, we studied competition of these Cu^{2+} sites by Mg^{2+} ions. In the presence of Mg^{2+}, the intensity of the Cu^{2+}-F_1 factor EPR spectrum was significantly decreased, indicating that Mg^{2+} competed with Cu^{2+} for the same site(s). This Mg^{2+} competition suggests that both Mn^{2+} and Cu^{2+} bind to the same site in the protein. Fig. 3b shows one representative experiment of the Mg^{2+} competition for the Cu^{2+} binding site(s). The Cu^{2+}-F_1 factor EPR spectrum, arising from only one copper complex, displays an axial symmetry which can be interpreted in terms of the following spin Hamilto-

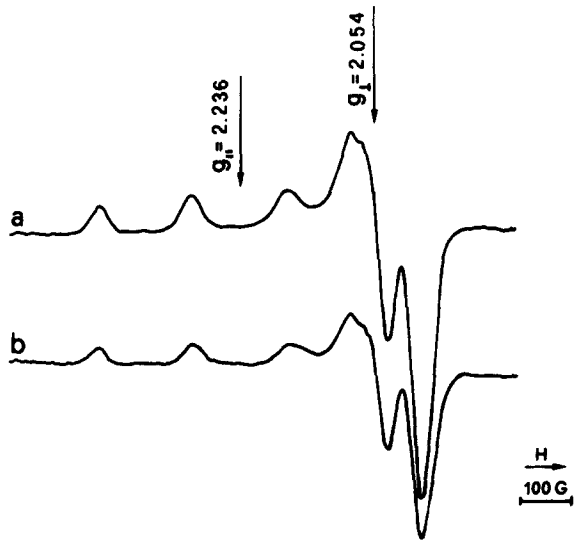

Fig. 3. Binding of Cu^{2+} to F_1-ATPase from *M. lysodeikticus*. EPR spectra at 77 K of: (a) Cu^{2+} ions (10^{-4} M) and F_1-ATPase ($4.7 \cdot 10^{-4}$ M); (b) Cu^{2+} ions (10^{-4} M) and F_1-ATPase ($4.7 \cdot 10^{-4}$ M) in the presence of Mg^{2+} ions (10^{-2} M). Magnetic-field sweep rate, 1000 G in 16 min. All other conditions as in Fig. 1.

nian [25]:

$$\mathcal{H} = \beta \{ g_{\parallel} H_z S_z + g_{\perp} (H_x S_x + H_y S_y) \} + A_{\parallel} S_z I_z + A_{\perp} (S_x I_x + S_y I_y)$$

where β is the Bohr magneton and $g_{\parallel}$ and $A_{\parallel}$ are the g-value and hyperfine components, respectively, parallel to the molecular symmetry axis; $g_{\perp}$ and $A_{\perp}$ are the g value and hyperfine components, respectively, perpendicular to the molecular symmetry axis; S is the electron spin ($S = 1/2$); and I is the nuclear spin ($I = 3/2$).

Using the standard formula:

$$\mu = \tfrac{1}{3}(g_{\parallel} + 2g_{\perp})\sqrt{S(S+1)}$$

and inserting the g values measured from the experimental spectra of Fig. 3, the magnetic moment μ can be calculated as 1.83 (in Bohr magnetons), which is in the range expected (1.75–2.20 Bohr magnetons) [26] for simple copper complexes lacking Cu–Cu interactions. For EPR spectra indicating a tetragonal environment about Cu^{2+}, as is in our case, the ratio $g_{\parallel}/A_{\parallel}$ appears to be an empirical index of tetragonal distortion of the equatorial ligands; values ranging from 105 to 135 for tetragonal structures and above this for out of plane distortions towards tetrahedral symmetry [27]. The $g_{\parallel}/A_{\parallel}$ value of 118 for Cu^{2+}-F_1 factor complex indicates that no such distortion is present.

Because the $g_{\parallel}$ and $A_{\parallel}$ values for the Cu^{2+}-F_1 complex fit the Peisach–Blumberg plots [28] for several donor sets (viz. CuN_2O_2, CuN_3O and CuN_4); then, it is quite difficult on this basis to assess which ligands are provided to the Cu^{2+} ions by the protein. Only sulfur donors are excluded by this analysis.

A measure of the metal-ligand covalency (α^2) can be obtained by using the approximate expression: $\alpha^2 = A_{\parallel}/0.036 + (g_{\parallel} - 2.0023) + 3/7(g_{\perp} - 2.0023) + 0.04$ (where A is measured in cm^{-1}), which can give an indication of the binding characteristics of the ligands [29]. The smaller the value of α^2, the greater the covalent nature of the bonding. The α^2 value for Cu^2-F_1 factor is 0.81, indicating that the average ligand field provided by the protein is moderately ionic.

Discussion

The results herein reported demonstrate conclusively the presence of high-affinity divalent cation binding sites in purified F_1-ATPase from *M. lysodeikticus (M. luteus)*. Mn^{2+} and Cu^{2+} bind to the enzyme as evidenced by EPR spectroscopy. This is to our knowledge the first work demonstrating the presence of metal ion binding sites on a bacterial F_1 factor by EPR. Direct binding of Mn^{2+} on the F_1 factor from chloroplasts (CF_1) by the EPR method has been previously reported [30,31]. These latter studies indicated the presence of 5–6 sites for Mn^{2+} binding and that Mg^{2+} and Ca^{2+} competed with Mn^{2+} for the same site in CF_1.

Furthermore, this work constitutes the first report on the study of a Cu^{2+}-F_1 factor complex by EPR. This Cu^{2+}-F_1 factor interaction, unlike Mn^{2+} binding, can provide information about the microenvironment of the metal ion binding site(s). According to the EPR parameters of the Cu^{2+}-F_1 factor complex, Cu^{2+} is ligated to oxigen and/or nitrogen ligands, but not to sulfur, in a moderately ionic environment. Competition experiments indicate that both Mn^{2+} and Cu^{2+} binding sites can be occupied by Mg^{2+} ions. This suggests that Mn^{2+} and Cu^{2+} bind to the same site(s) in the enzyme. A similar excess of Mg^{2+} in relation to Mn^{2+} and Cu^{2+} provokes complete competition of the Mn^{2+} binding and about 50% competition of the Cu^{2+} binding (compare Fig. 1c and Fig. 3b). The requirement of a different magnesium concentration to displace the manganese or copper ions from their sites can be explained by the interaction of each metal ion with a particular arrangement of the ligand groups [32], resulting in a different geometric environment and distinct binding affinities. As a matter of fact, the stability of metal complexes with ligands where oxygen and nitrogen serve as the donor atoms, as in our case, is noticeably higher for Cu^{2+} than for Mn^{2+} [32]. Taking together: (a) the above competition results; (b) previous work suggesting a role for Mg^{2+} in functional and structural properties of the *M. lysodeikticus* F_1-ATPase [8–10,15,16]; and (c) previous estimations of an apparent intracellular magnesium concentration of approx. 20 mM in this bacterium [33]; it is reasonable to suggest that

Mg^{2+} can occupy the above metal ion binding sites in the *M. lysodeikticus* F_1-ATPase under physiological conditions.

In spite of Cu^{2+} is not considered as a good probe for Mg^{2+} binding sites in proteins, evidence presented in this work suggests that Cu^{2+} and Mn^{2+} bind to the same site(s), which, in turn, can be occupied by magnesium ions. The reasons for this conclusion are: (a) the above-described Mg^{2+}-competition experiments for the Cu^{2+} and Mn^{2+} sites; and (b) the finding of only one kind of site for Cu^{2+} and Mn^{2+} ions.

On the other hand, binding of Mn^{2+} and Cu^{2+} indicate that different divalent cations are able to bind to the enzyme. In this regard, the effects of distinct metal ions on several properties of this enzyme [9,34] can be explained by direct binding to the protein of the extraneous metal ion. This latter might displace the physiological metal ion and/or occupy its site.

An interesting property shared by all the energy-transducing F_1-ATPases studied so far consists in the presence of bound adenine nucleotides [1,22,23,36,37]. The number and nature of these bound nucleotides differ among different F_1 factors and depend on the purification method used for the enzyme preparation. Previous work [24] indicates that *M. lysodeikticus* F_1-ATPase purified by gel electrophoresis, as in the present work, contains 0.5 mol ATP and 0.5 mol ADP/mol enzyme. Thus, the metal ion binding sites described in this bacterial enzyme are not located at the bound adenine nucleotides, due to differences in the stoichiometry of Mn^{2+} binding and that of bound adenine nucleotides.

In summary, the results herein reported confirm previous assumptions about the presence of divalent cation binding sites in the F_1-ATPase from *M. lysodeikticus* [34]. The occupant of these metal ion binding sites can exert a regulatory influence on the activity of the enzyme by acting as activator or inhibitor. The low number of Mn^{2+} binding sites found in this bacterial F_1 factor suggests a certain specificity in the binding. The finding of 3–4 metal binding sites for Mn^{2+} is in close relationship with the presence of three catalytic and three regulatory subuntis in this enzyme [2,8]. In this context, it has been suggested that Mg^{2+} can induce conformational changes on the F_1-ATPase from *M. lysodeikticus*, thereby regulating its activity [35].

Besides these metal ion binding sites on F_1 factors, the presence of intrinsic metal ions tightly bound to several F_1 factors has been reported. Thus, the presence of magnesium tightly bound to F_1-ATPase from *Escherichia coli* and bovine heart mitochondria has been reported [38,39]. Other metal ions, Fe [40] and Zn [41], have been tentatively identified in different F_1-ATPases, besides that of zinc in *M. lysodeikticus* F_1-ATPase [34]. However, further analysis of metal ions in this bacterial F_1-ATPase indicates the presence of 1 mol Mg/mol enzyme and small substoichiometric amounts of zinc (Mollinedo, F. and López-Moratalla, N., unpublished data).

On these grounds, it seems reasonable to suggest that F_1-ATPases, irrespective of their origin, share general properties regarding metal ions, namely: (a) the presence of tightly bound metal ions as integral parts of the enzyme; and (b) the existence of additional high-affinity metal-ion binding sites, described in this work, that may act as regulators of the enzymatic activity.

The presence of these metal ion binding sites opens a broad range of possibilities in the regulation of these energy-transducing F_1-ATPases. The elucidation of the role of these sites and their characterization will be of tremendous interest in the understanding of the molecular mechanism of ATP synthesis and hydrolysis by these enzymes. In this context, further characterization of these metal-ion binding sites as well as an extension of these studies to other F_1 systems are necessary.

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