Paramagnetic NMR spectroscopy of native and cobalt substituted manganese superoxide dismutase from *Escherichia coli*

Jean Philippe Renault[a], Catherine Verchère-Béaur[a], Irène Morgenstern-Badarau[a,*], Mario Piccioli[1,b]

[a] *Laboratoire de Chimie Bioorganique et Bioinorganique, Institut de Chimie Moléculaire d'Orsay, Université Paris-Sud, 91405 Orsay cedex, France*
[b] *Department of Chemistry, University of Florence, Via Gino Capponi 7, Florence, Italy*

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Abstract Manganese containing superoxide dismutase from *E. coli* has been investigated through paramagnetic NMR spectroscopy. The spectrum of the native form was rationalized using a $\tau_s = 3\times10^{-11}$ s for the Mn(III) ion, consistent with previous estimates from NMRD measurements. Mn(III) has been replaced by a Co(II) ion and a tentative assignment of the NMR spectrum of the Co(II)-substituted derivative has been proposed, based on T_1, chemical shifts and 1D-NOE data. The metal coordination geometry is provided by three histidines and a carboxylate group. The presence of a solvent molecule as a loosely bound fifth ligand is also proposed. The NMR data of the Co(II)-substituted derivative of *E. coli* MnSOD differs from those of Co(II)SOD from other bacterial sources. This suggests that Co(II) substitution is an efficient method to address the problem of metal ion selectivity in superoxide dismutase.

Key words: Superoxide dismutase; Manganese; Cobalt; Paramagnetic NMR; *Escherichia coli*

1. Introduction

Manganese has been shown to have structural or catalytic functions in an increasing number of protein examples [1]. As a metallic cofactor it seems to be as important as iron in the redox processes associated with dioxygen metabolism: oxygen production by plant photosystems [2], superoxide [3] or hydrogen peroxide [4] dismutation and dioxygenation of aromatic compounds [5]. Manganese containing superoxide dismutase, an antioxidant enzyme found in mitochondria of eukaryotic cells and in various bacteria [6], is one of the earlier and better characterized mangano-enzymes. X-ray diffraction [7–11], EPR [12], magnetic susceptibility [13], relaxometry [14], MCD and visible absorption [15] studies have been performed either on the Mn(III) or on the Mn(II) enzymes.

To the best of our knowledge, no NMR studies have been reported on these enzymes with the exception of some preliminary work on the cambialistic Fe/Mn SOD from *Propionibacterium freudenreichii* sp. *shermanii* (*P. shermanii*) [16]. The size of the protein and the presence of paramagnetic metal ions prevents the straightforward use of NMR spectroscopy to investigate this enzyme; NMR spectroscopy of paramagnetic systems has nevertheless proved to be an extremely efficient technique for the investigation of electronic and structural properties of metallic sites [17].

We report here the first NMR study on MnSOD from *E. coli* for which the X-ray structure is not available but which is highly homologous [6] to the well-described *T. thermophilus* SOD [10]. Fig. 1 shows a schematic drawing of the active site of MnSOD as it occurs in the *T. thermophilus* isoenzyme. The reduced, native protein is not suitable for NMR studies because of the long electron relaxation time of Mn(II). Although the oxidized form Mn(III) has shorter electron relaxation times, the situation is expected to be less favorable with respect to other metal ions, such as Co(II) and Ni(II), which have previously been shown to be efficient probes for paramagnetic NMR spectroscopy. This article deals with the NMR investigation of the oxidized form of the native protein as well as of its Co(II)-substituted derivative. We will show in this article that the replacement of the native manganese ion with Co(II) is a very promising tool for studying the active site of this protein.

This study will be useful to compare structural properties of the closely related enzymes FeSOD and MnSOD, for which the specificity and/or the selectivity of the metal ion are yet to be addressed [11]. As the NMR spectrum of Fe(II)SOD has already been reported [18], the spectra of Mn(III)SOD which we report here for the first time and the characterization of its Co(II)-substituted derivative will constitute the background for further NMR structural studies on these important enzymes.

2. Materials and methods

2.1. Sample preparation

Manganese superoxide dismutase from *E. coli* was purchased from Sigma Chemical Company and used without further purification. It showed no EPR signal and thus manganese was considered to be in the native state, Mn(III) [12].

2.2. Metal substitution

Substitution of manganese by cobalt was carried out at 4°C, using the competition method developed by Ose and Fridovich [19]. The pH of a 0.5 mM solution of the protein in Tris-HCl (5 mM buffer, pH 7.8) was gradually lowered by adding a solution of Tris-HCl (50 mM, pH 3), guanidinium chloride (1 M) and cobalt chloride (50 mM). After a 25 times dilution of the protein solution, a pH of 3.6 was achieved. The solution was dialysed against 20 volumes of a 0.1 M $CoCl_2$, 0.1 M guanidinium chloride solution for 10 h. The protein solution then became a bright pink in color. By cautious titration with sodium hydroxide, the pH was then increased to 7.8. The cobalt-hydroxide precipitate was removed by centrifugation, the solution was dialysed against 20 volumes of a Tris-HCl (5 mM, pH 7.8) and

*Corresponding author. Fax: (33) 169157281.
E-mail: imorgens@icmo.u-psud.fr

[1]Corresponding author. Fax: (39) (55) 2757555.
E-mail: mario@riscl.lrm.fi.cnr.it

Abbreviations: SOD, superoxide dismutase; MnSOD, manganese containing superoxide dismutase; FeSOD, iron containing superoxide dismutase

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EDTA (1 mM) solution for 36 h, and concentrated in an amicon-type filtration apparatus, with a cut off of 5000 M_r. The resulting Co-substituted MnSOD (40% of the initial protein) had the light pink color described previously by Fridovich ($\varepsilon_{550} = 350\ M^{-1}\ cm^{-1}$ [19]).

2.3. NMR spectroscopy

All spectra were recorded on a Bruker MSL 200 operating at a magnetic field of 4.7 T. They were recorded using the superWEFT pulse sequence (180°-τ-90°-AQ) [20]. Recycle delay (AQ) and the delay between pulses (τ) were 43 ms and 35 ms respectively for Mn(III)-SOD and 83 ms and 45 ms, respectively for Co(II) SOD. They were adjusted to optimize the suppression of the strong solvent signal. From 8 to 12 transients per second were acquired using a spectral window of 50 kHz. Reported spectra were obtained after approximately 4×10^5 scans for both adducts. Steady-state 1D-NOE experiments tailored for paramagnetic signals [21] were performed using the previously reported methodology [22]. The selective irradiation of signals A and B was accomplished through selective, rectangular pulses of 44 ms and 26 ms, respectively. Selective irradiation was applied during the τ delay of a superWEFT pulse sequence. The NOE difference spectra were obtained with the procedure previously described [22]. Approximately 2×10^6 scans for each NOE difference experiment were used, corresponding to 48–62 h of experimental time. T_1 measurements were obtained using the inversion-recovery method [23]. The delay between the 180° and the 90° pulses was varied from 40 ms to 0.4 ms in the case of Mn(III)SOD and from 150 ms to 5 ms in the case of Co(II)SOD. Signal intensities were used to estimate the time constant of the exponential recovery.

NMR sample volumes were approximately 0.3 ml. Concentrations were ca. 1 mM for Mn(III)SOD and ca. 0.7 mM for Co(II)SOD.

Proton chemical shifts were calibrated by assigning the H_2O signal the value of 4.81 ppm at 298 K with respect to sodium, 4,4-dimethyl-4-silapentane-1-sulfonate (DSS).

3. Results and discussion

3.1. Mn(III)SOD

The spectrum of native Mn(III)SOD is reported in Fig. 2. Two relatively sharp signals, A and C, were observed in the lowfield region 10–20 ppm while two broad features, D and E, were observed at highfield values of −7 and −13 ppm, respectively. In addition a minor signal B was observed.

The three downfield shifted signals disappeared when the spectrum was recorded in D_2O solution. T_1 values of signals A and C were estimated to be around 5 ms and 10 ms, respectively. The analysis of the highfield shifted signals was hampered by baseline distortion due to the bulk envelope of diamagnetic signals. Nevertheless it is likely that the signal at −7 ppm (D) is composed of two resonances while three resonances may be responsible for the signal at −13 ppm (E). They were largely unaffected by exchange in D_2O. Inversion-recovery experiments on these broad features show that these signals, which cannot be resolved through these series of experiments, have T_1 values shorter than 1 ms.

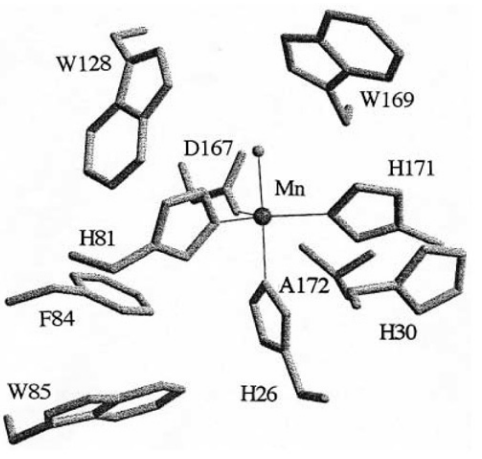

Fig. 1. Schematic drawing of the active site of *T. thermophilus* MnSOD as obtained from the X-ray structure [10] (the amino acid numbers are taken from the *E. coli* MnSOD sequence [25]).

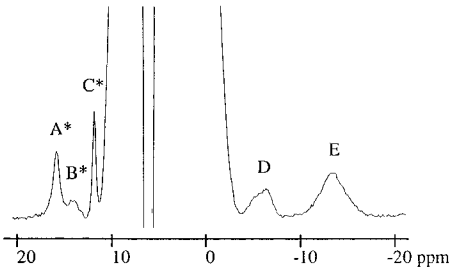

Fig. 2. 200 MHz, ^{1}H-NMR spectrum of Mn(III)SOD. The spectrum was recorded at 298 K. The sample was dissolved in 50 mM Tris buffer solution, pH 7.8. Signals marked by asterisks disappear in D_2O solution.

T_1 values of signals A, C, D and E suggest that they belong to residues affected by the electron relaxation of the S = 2 spin state of Mn(III). If a τ_s of 3×10^{-11} s is considered for Mn(III) [14] then in accordance with the Solomon equations [24], signals with T_1 values in the range 5–10 ms are expected to arise from protons within a 65–75 nm sphere centered on the metal ion, while T_1 values shorter than 1 ms are expected for protons within a corresponding 50 nm sphere. Two features are therefore apparent:

(i) The downfield shifted signals A and C could be assigned to NH protons of residues not directly bound to Mn(III) but within a 80 nm sphere from the metal. Furthermore, as a hyperfine shift induced by Mn(III) on protons of residues not directly bound to the metal ion is expected to be almost negligible, chemical shift values of 16 ppm for A and 12 ppm for C are consistent with what is expected for a His HNε2 and His HNδ1 signals. These usually lie in the 17–10 ppm region. If we take the structure of the *T. thermophilus* MnSOD as a model the most likely candidates for signals A and C are the HN protons of His-32 (His-30 in the numbering of the *E. coli* MnSOD sequence [25]).

(ii) For the upfield shifted resonances, both hyperfine shifts and fast relaxation rates account for protons of residues directly bound to the Mn(III) ion. Indeed such highfield shifts have often been observed in NMR spectra of Mn(III) complexes and explained by a through bond spin density delocalization [26–28].

Although unambiguous assignments cannot be given, these spectra provide, to the best of our knowledge, the first evidence of NMR signals of a Mn(III) active site.

3.2. Co(II)-substituted SOD

The ^{1}H-NMR spectrum of Co(II)-substituted MnSOD (Co-SOD) is shown in Fig. 3. Seven hyperfine, downfield shifted signals (A–G) were observed in the range 75–20 ppm while several resonances, which were partially suppressed or inverted in the one-dimensional spectrum of Fig. 3, were observed between +20 and −10 ppm. The upfield part of the spectrum shows a complex envelope of signals (at least 5) in the range from 0 ppm to −10 ppm, a strong peak (H) at ca.

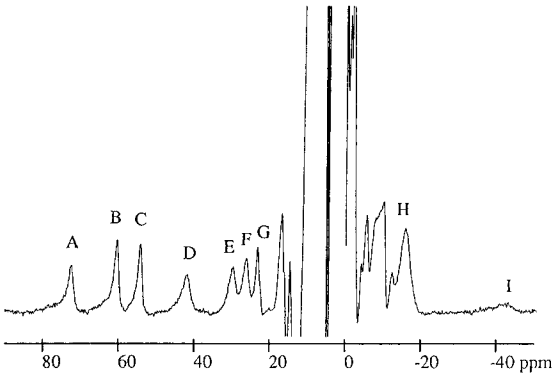

Fig. 3. 200 MHz, ^{1}H-NMR spectrum of Co(II)-substituted MnSOD. The spectrum was recorded at 298 K. The sample was dissolved in 50 mM phosphate buffer solution, pH 7.8. Labeled signals are discussed in the text.

−15 ppm, probably composed of two overlapped resonances, and a broad, barely detectable signal (I) at about −40 ppm.

Three different sets of signals may be identified in Fig. 3, according to their different T_1 values estimated by means of inversion-recovery experiments: (i) the four most downfield shifted signals as well as the upfield shifted signals H and I have T_1 values shorter than 5 ms; (ii) signals E, F and G, which were observed in the range 35–20 ppm, display T_1 values ranging from 25 to 35 ms; (iii) signals that were shifted in the range +20/−10 ppm experience a hyperfine shift up to ca. ±20 ppm and their T_1 values have been estimated to be larger than 60 ms.

We could not reach the protein concentration required for extensive multidimensional studies which will be attempted in the future; nevertheless the literature available on Co(II)-substituted proteins and on SODs allows us to propose a tentative assignment of the ^{1}H-NMR spectra of CoSOD [29,30]. Hereafter we will discuss a possible assignment of some of the observed hyperfine shifted resonances and we will use chemical shift and relaxation data to discuss the coordination geometry of Co(II) ion.

3.3. Assignment

Signals occurring in the range 90–50 ppm in the NMR spectra of Co(II)-substituted proteins are usually observed for meta-like protons of coordinated histidines [29]. When histidines are bound to the metal through the Nε2 atoms only one meta-like signal, exchangeable in D_2O solutions, is expected for each His group, while two meta-like signals occur if the metal coordination is provided by the Nδ1 atom. Given that the electron spin of the Co(II) ion is S=3/2 and that the molecular weight of the protein is large, ortho-like protons should be broadened beyond detection. We therefore expect two observable signals from each His group coordinated through Nε2 and one signal for each His coordinated through Nδ1. In the present system, only the three signals A, B and C were observed in that range, with T_1 values of 4 ms for signal A and between 2 and 4 ms for signals B and C. We concluded that signals A, B and C belong to the NHδ1 of three metal-bound histidines which all coordinate to the Co(II) ion through the Nδ 1 atom.

βCH_2 signals of Co(II)-bound aspartate residues usually fall in the region 70–30 ppm, i.e. slightly less shifted with respect to the meta-like His protons. In the case of Co(II)-substituted proteins such resonances are usually observed within a narrow range, as in the case of cobalt-substituted copper-zinc superoxide dismutase in which copper is removed and cobalt(II) replaces the native zinc ion [31]. The Cys-112-Asp mutant of Co(II)azurin [32] is another example, although differences in shifts of about 40 ppm between geminal protons of a glutamate residue coordinated to Co(II) have been found in Fe-Co uteroferrin [33]. On the other hand, the metal-proton distance of βCH_2 of aspartate groups coordinated to a metal ion is expected to be shorter than that of a meta-like His proton. Linewidths and relaxation rates of such signals are expected to be consistently larger than those of meta-like His protons, independent of the coordination number. As signal D has the shortest T_1 value and largest linewidth in the region 80–30 ppm, it most likely originates from one of the βCH_2 protons of the coordinated Asp-167. Other possibilities for such an assignment might be signals C or E; while the former signal has been attributed to one of the His ligands, signal E has a T_1 longer than 15 ms and therefore cannot be considered an Asp-167 Hβ signal. The geminal partner of signal D may not have been observed because of (i) significant line broadening due to proximity of the metal ion; (ii) an unusually small shift which leaves the signal covered by the diamagnetic resonances; (iii) possible overlap with one of the hyperfine shifted signals. From the integrated intensities of the downfield shifted resonances none of the A–F signals shows evidence of two-fold intensity which rules out the possibility of the missing signal being overlapped by some other resonance.

In order to obtain further insight into the assignment of

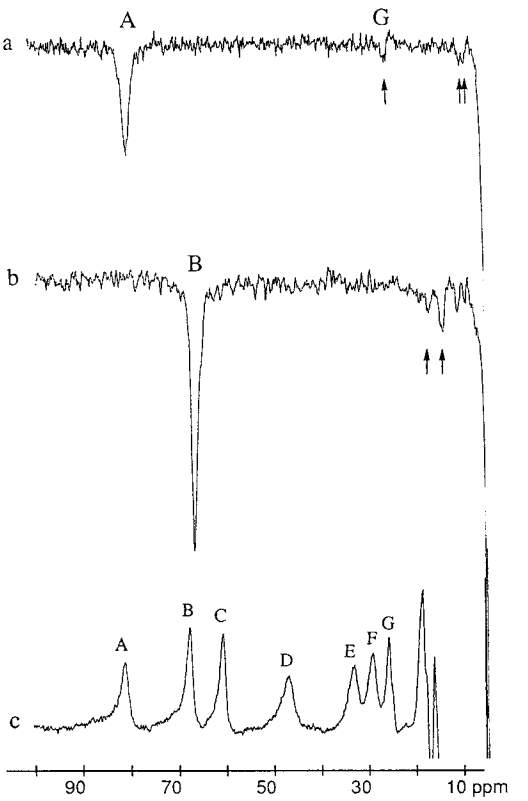

Fig. 4. 1D-NOE difference spectra, recorded in H_2O solution. Spectra were recorded at 200 MHz, 298 K. Sample was dissolved in 50 mM phosphate buffer solution, pH 7.8. Traces a and b refer to the irradiation of signals A and B, respectively. The reference spectrum is also reported in trace c.

signals, 1D-NOE experiments were performed using selective irradiation of the two most shifted signals, A and B. 1D-NOE difference spectra are shown in Fig. 4. Selective irradiation of signal A (Fig. 4a) gives rise to a significant NOE to signal G as well as to two other signals lying inside the diamagnetic region. Selective irradiation of signal B (Fig. 4b) gives rise to NOEs with two signals in the region 15–10 ppm. The three signals which undergo NOEs upon irradiation of signal A as well as the two signals which are dipole-dipole coupled to signal B most probably belong to residues which are near in space to the imidazole HN proton of two of the three metal-bound histidines. They are also relatively near in space to the metal ion. This is certainly the case for signal G (connected to A), for which relaxation rates suggest it is attributable to a proton within a 60–50 nm sphere centered on the Co(II) ion [29].

As a matter of speculation, if the pattern of the hyperfine shifts observed in *E. coli* Fe(II)SOD by L. Que Jr. and coworkers [18] is conserved in the presently investigated system, the most shifted signal A could be assigned to His-26 by comparison of the MnSOD sequence [25] with the FeSOD example [34]. Within this hypothesis, signal G arises either from one of the ring protons of Phe-84, from Trp-85, or from Ala-172. With regard to signals E and F, their shifts and relaxation properties are similar to those of signal G. Therefore they are likely to arise from protons of non-coordinated residues in the proximity of the metal ion.

A comment is necessary regarding the broad signals H and I observed at −15 and −40 ppm. Comparative analysis of relaxation rates shows that these signals are expected to be closer to the metal ion than both meta-like His protons and Hβ Asp protons. It is highly unlikely that signals H and I belong to ortho-like His protons, as a large upfield shift from contact interaction is not expected in imidazole ring protons. The protons closest to the Co(II) ion among the non-coordinated residues are the $H\beta_2$ protons of Trp-169 and $H\xi_2$ of Trp-128 which are at 37 and 34 nm respectively (see Fig. 1). Signal I may be most reasonably attributed to these protons. Signal H may be due to a methyl group at less than 50 nm from the metal ion, according to its integration and T_1 value. The Ala-172 residue contains such a methyl group.

3.4. Metal coordination geometry

The hyperfine interaction with the S=3/2 spin state of Co(II) is obviously the dominant mechanism involved in the nuclear relaxation of protons in the immediate proximity to the metal ion [35]. Such an interaction is usually dominated by dipolar relaxation which may be related to the metal-proton distance by the Solomon equation [24]. Although the occurrence of ligand-centered contribution to relaxation is such that T_1 values may not be trivially related to the r^{-6} Solomon relationship [35], their analysis still provides valuable information on the coordination number of the Co(II) ion. This is because the electronic correlation time τ_s (the dominant contribution to τ_c in Solomon equation) is, for Co(II) ions, dependent on the coordination geometry of the metal ion. T_1 values for His HNε2 of Co(II) coordinated histidines in the active site of metalloproteins span from 1.4 ms (observed in the tetrahedral site of cobalt substituted copper, zinc superoxide dismutase in which the copper ion is in the reduced state ($Cu(I)_2Co_2SOD$) [30,36]) to 20 ms (observed in the five coordinated site of the thiocyanate adduct of cobalt-substituted carbonic anhydrase [37]). The present data suggest therefore a distorted tetrahedral geometry with, possibly, a loosely bound water molecule coordinating as a fifth ligand. Indeed, although the T_1 values measured here are quite far from what is normally observed in 'purely' five-coordinated chromophores, the T_1 values of meta-like His protons are longer by almost a factor of 2 than in the case of $Cu(I)_2Co(II)_2SOD$ [30].

The hyperfine shifts of signals attributed to non-coordinated residues are of interest in this analysis, as it is known that five-coordinated chromophores have a larger anisotropy of the magnetic susceptibility tensor than four-coordinated chromophores [29,37]. The larger the anisotropy of the magnetic susceptibility tensor, the larger the pseudocontact shifts expected. In the case of *E. coli* CoSOD a significant pseudocontact contribution to the overall hyperfine shift is observed which is strongly indicative of some contribution from a fifth ligand to the coordination sphere. This has been previously discussed in other systems such as Co_2Co_2SOD in which several signals are observed in the range +30/−30 ppm [30,38]. They arise from the pentacoordinated Co(II) replacing the native copper ion and strongly resemble those observed in the presently investigated protein in terms of both shift and relaxation properties. A similar situation is encountered in the active site of Co(II)-substituted azurin [32]. In this example, the large spreading of pseudocontact shifted resonances has been interpreted as originating from a distorted tetrahedral site, weakly interacting with a fifth ligand provided by Met-121 residue [32,39]. We suggest here that, in the case of Co(II)SOD, the interaction of Co(II) with a water molecule acting as a fifth ligand is even weaker than that observed for the axial fifth ligand of Co(II)-substituted azurins, as indicated by T_1 values.

All described FeSOD and MnSOD have their metallic cofactor bound to three histidines. In the case of the *E. coli* MnSOD protein sequence these are His-26, His-81, His-171 and one aspartate, Asp-167 [6]. In most cases, Mn or Fe ions are five-coordinated [7–11] as shown in Fig. 1 but the fifth ligand, a solvent molecule, is missing in the crystallographic structure of *P. ovalis* FeSOD [40]. Meier and coworkers have recently reported the NMR spectrum of the Co(II)-substituted, cambialistic Fe/MnSOD from *P. shermanii* [16]. When comparing the ^{1}H-NMR spectra of the two derivatives, we observe that all hyperfine shifted signals in the spectrum of *P. shermanii* CoSOD also occur in the one of *E. coli* CoSOD derivative, with very similar shifts and linewidths. Additional signals, namely A, D and possibly F, are observed for *E. coli* CoSOD. In our case, an analysis of NMR parameters has been attempted suggesting that the water molecule observed in the X-ray structure of *T. thermophilus* MnSOD is loosely bound in the cobalt-substituted MnSOD of *E. coli*.

4. Conclusion

The present NMR work has shown for the first time the NMR spectra of an Mn(III)SOD complex. Both exchangeable and non-exchangeable signals were observed and their relaxation times analyzed in an attempt to rationalize the observed NMR signals.

As expected, the replacement of Mn(III) with Co(II) gives rise to a derivative which is more suitable for NMR studies.

The extensive literature on Co(II)-substituted proteins has allowed us to make some tentative assignments based on chemical shift, relaxation properties, and some 1D-NOE experiments. It is shown that the active site of Co(II)-substituted MnSOD from *E. coli* is very similar to that of the *T. thermophilus* isoenzyme [10] and that slight differences between the two chromophores exist in the metal-oxygen distance of the coordinated water molecule.

The reported data may be approximately compared with that reported by Meier and coworkers for the Co(II) derivative of the cambialistic Fe/MnSOD from *P. shermanii*. In the latter case, a smaller number of downfield shifted signals are observed, which were interpreted as originating from a four-coordinated Co(II) ion. Although previous NMR investigation of *P. shermanii* SOD did not identify the nature of coordinating residues, the comparison of the two NMR spectra suggests that the active site of *E. coli* MnSOD and that of *P. shermanii* FeSOD are different. If confirmed, this would prove to be a very interesting feature as, up to now, all available X-ray structures of FeSOD and MnSOD have revealed very similar geometries at the active site. This evidence of structural inequivalence between cambialistic SOD and *E. coli* SOD demonstrates, purely on the basis of paramagnetic NMR spectroscopy of Co(II)-substituted derivatives, the efficiency of Co(II) substitution as a tool for the study of these enzymes. This may prove to be very interesting in addressing differences between 'cambialistic' SOD and other SODs with regard to metal selectivity.

Indeed, even if Fe(II)SOD [18] and Mn(III)SOD (present article) can be investigated by NMR, we believe that an 'alternative' spectroscopic probe like Co(II) ion will allow a fine comparison of the active site structure of both FeSOD and MnSOD. This might be crucial to understand the metal selectivity in superoxide dismutases.

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