Replacement of Mn(III) with Cu(II) in *Bacillus stearothermophilus* superoxide dismutase

Similarity of the active site to the zinc site of copper/zinc superoxide dismutase

Joe V. Bannister+, Alessandro Desideri and Giuseppe Rotilio

+*Inorganic Chemistry Laboratory, University of Oxford, Oxford OX1 3QR, England,* and *CNR Centre for Molecular Biology, Institute of Biological Chemistry, University of Rome, Rome, Italy*

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Copper(II) was substituted for manganese(III) in *Bacillus stearothermophilus* Mn-superoxide dismutase. The (EPR) spectrum of the Cu(II) is distinctly rhombic, overlapping that of Cu(II) replacing zinc in copper/zinc superoxide dismutase. The copper-cyanide complex of the bacterial enzyme gives an EPR spectrum nearly identical to the cyanide-copper complex of the Cu/Zn enzyme, indicating three nitrogens as metal ligands. These results support the suggestion that metal coordination in *B. stearothermophilus* Mn-dismutase is a tetrahedral arrangement of three imidazoles and a carboxylate group and indicate copper as a good spectroscopic probe for studying the active site of Mn- and Fe-superoxide dismutases.

Manganese superoxide dismutase *Active site* *Copper binding* Bacillus stearothermophilus

1. INTRODUCTION

Metal replacement or substitution has been widely used to probe the metal binding site of metalloproteins. The technique has been particularly useful for studying the active site of proteins containing a diamagnetic species such as zinc. More recently it has been applied to probe the metal binding site of Cu/Zn superoxide dismutases. The zinc site has been extensively probed with cobalt [1] and lately the same metal has been used to study the copper site [2].

The superoxide dismutases are a family of metalloenzymes containing either copper, manganese or iron at the active site. In contrast to the Cu-containing enzyme, the Mn- and the Fe-containing superoxide dismutases have not been extensively studied by metal replacement experiments. Although a number of reports have appeared concerning metal substitution in these enzymes, none presented detailed evidence concerning the metal binding site. Metal removal from either the Mn- or Fe-containing superoxide dismutase is a very tedious procedure because of the instability of the aproprotein. Moreover spectroscopic data from a potentially informative probe might be difficult to interpret because no complete structural data are available for this class of enzyme. The X-ray structures of 2 Fe-superoxide dismutases have been published to a satisfactory degree of resolution [3,4], while analogous information on the Mn-superoxide dismutase is still preliminary [5]. In contrast complete amino acid sequence have been determined for Mn-superoxide dismutases [6–8], whilst no amino acid sequence has yet been determined for any Fe-superoxide dismutases. It seemed therefore interesting to study a series of metal-substituted derivatives of *Bacillus stearothermophilus* Mn-superoxide dismutase. This report deals with substitution of the Mn(III) with Cu(II). Although this metal is not as frequently used as a

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paramagnetic probe as cobalt(II), a report on the EPR spectra of Cu(II)-substituted horse liver alcohol dehydrogenase has shown that a very unusual copper coordination can be analysed [9].

2. MATERIALS AND METHODS

Mn-superoxide dismutase from the thermophile *B. stearothermophilus* was a kind gift from Dr J.E. Walker (MRC Laboratory of Molecular Biology, Cambridge, England). The enzyme was further purified by chromatography on Sephadex G-100 equilibrated with 50 mM Tris-HCl, pH 7.6. The purity of the enzyme was established by gel electrophoresis in SDS and the enzyme concentration was determined from the extinction coefficient at 280 nm [7]. The enzyme was found by atomic absorption spectrophotometry to contain 1 gatom manganese per 40 kDa.

The apo-enzyme was prepared by exhaustive dialysis against 10 mM EDTA in the presence of 8 M urea. The pH of the buffer was adjusted to pH 3.8 with 1.0 M acetic acid. Excess EDTA was removed by dialysis against 8 M urea at pH 5.0. The urea removed by stepwise lowering of the concentrations from 8 M to 6 M to 4 M and finally to 2 M in the presence of 50 mM acetate, pH 5.0. Apo-enzyme was found to be unstable in the absence of urea. The Cu-substituted enzyme could be prepared either by dialysis of the apo-protein against 50 mM acetate, pH 5.0, containing 8 M urea and 10 mM copper sulphate or by dialysis of the holoprotein against 50 mM acetate, pH 5.0, containing 8 M urea and 10 mM copper sulphate. Excess copper was in both cases removed by exhaustive dialysis against 50 mM acetate, pH 5.0, containing 8 M urea and 1 mM EDTA. Both methods resulted in the enzyme containing 1 gatom copper per 40 kDa. The enzyme reconstituted with copper was found to be devoid of superoxide dismutase activity, determined polarographically [10].

EPR spectra at Q- and X-band frequencies were recorded on a Varian E9 spectrometer equipped with the variable temperature accessory.

3. RESULTS AND DISCUSSION

Native Mn-superoxide dismutase from *B. stearothermophilus* does not exhibit any EPR signal. This absence is explained by the trivalent oxidation state of the metal ion [11]. Denaturation of the native protein by the addition of HCl results in an EPR spectrum similar to that of Mn(II) in water. The dialysis of native protein in buffer containing urea against an excess of $CuSO_4$ results in the binding of the Cu(II) to the protein as detected by EPR. Addition of HCl to this derivative does not result in any EPR signal due to the Mn(II). This indicates that the Cu(II) displaced all the manganese from the protein, most likely by binding to the native metal site. The apoprotein reconstituted with $CuSO_4$ gave identical Cu(II) EPR spectra, suggesting that in this case copper also binds to the native metal site. The X-band EPR spectrum of the Cu(II)-protein is shown in fig.1a. The spectrum has a rhombic lineshape and is characterised by a relatively narrow hyperfine structure in the lower magnetic field region and a barely resolved one in the high magnetic field region. The 35 GHz spectrum (inset) clearly resolved the 3 absorptions partially overlapped in the 9 GHz spectrum indicating that a single highly rhombic species is bound to the protein. Changing the pH in the range 5–10 did not affect the EPR spectrum.

The spectral features of the copper site are quite unusual suggesting specific binding to a site with a significant degree of distortion from simple symmetry. The relatively high g_1 value suggests a tetrahedral coordination (table 1) even though a distorted pentacoordinated structure cannot be excluded. The same indication is also given by the quite small value of A_1. In a tetrahedral geometry the odd electron is a mixture of 3d and 4p orbitals, which have anisotropic hyperfine term of opposite sign, leading to a decrease in the A_1 value [12]. An almost identical EPR spectrum (see table 1 and [3]) is displayed by the Cu(II) substituted in the tetrahedral zinc site of Cu/Zn superoxide dismutase.

The bound copper was found not to be affected by ferrocyanide and was not perturbed by the addition of azide even in large excess indicating that the metal is not easily accessible to solvent molecules or to redox reagents. The lack of reactivity with ferrocyanide and azide is in line with the absence of superoxide dismutase activity. A similar result was obtained with Cu/Zn superoxide dismutase when copper is bound to the zinc site [14].

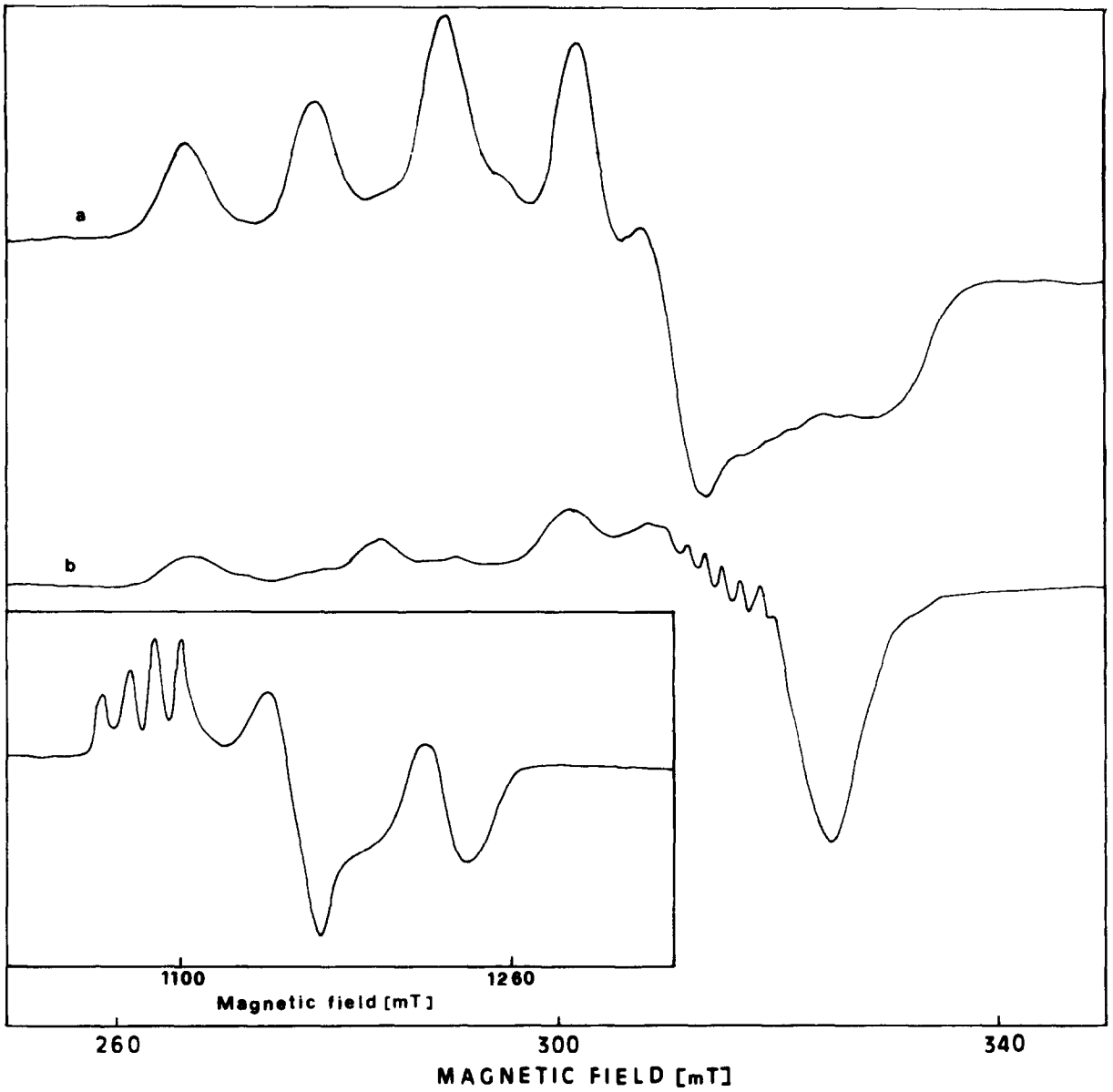

Fig.1. X-band EPR spectrum of 0.8 mM Cu(II)-substituted superoxide dismutase in 50 mM acetate buffer, pH 5.0 (a); in the presence of stoichiometric amounts of KCN at pH 9.5 (b); EPR conditions: microwave frequency, 9.15 GHz; microwave power, 20 mW; modulation amplitude 1.0 mT, 100 K. Inset: Q-band EPR spectrum of sample a. EPR conditions as above except frequency = 35.4 GHz.

Cyanide on the other hand was capable of entering the first coordination sphere of the metal giving rise to a complex with an EPR spectrum very reminiscent of that already described for the copper-cyanide complex of copper/zinc superoxide dismutase [14]. The copper assumed a tetragonal symmetry as suggested by the axial EPR spectrum (fig.1b) characterized by a lower g_1 and a higher A_1 value than in the untreated sample (table 1). This spectrum displays a super-hyperfine structure consisting of 7 equally spaced lines (about 1.5 mT) in the perpendicular region. By

Table 1

EPR parameters of Cu (II) in enzymes and model compounds of known structure

	g_1	g_2	g_3	A_1 (mT)	A_2 (mT)	A_3 (mT)	Ref.
Cu(II)Zn(II) bovine superoxide dismutase (4 Im, 1 H_2O)	2.26	2.11	2.02	13.0	–	–	14
Cu(I)Cu(II) bovine superoxide dismutase[a] (3 Im, 1 COO^-)	2.31	2.13	2.03	10.5	–	–	13
Azurin[a] (2 Im, 1 SCH_3, 1 S^-	2.26	2.05	2.05	6.00	–	–	16
CuN_2Cl_2[a]	2.30	2.06	2.06	11.4	–	–	17
(Cu, Zn)-5-Me-Sal-i-Pr[a] (2N,2O)	2.27	2.13	2.03	10.5	–	–	18
Cu(II) superoxide dismutase from *B. stearothermophilus*	2.30	2.16	2.03	11.8	–	4.0	this work
Cu(II) superoxide dismutase from *B. stearothermophilus* + CN^-	2.23	2.04	2.04	17.1	–	–	this work

[a] Tetrahedral

analogy to the nearly identical result obtained with the Cu/Zn enzyme [14], 3 magnetically equivalent nitrogen atoms can be suggested as in-plane ligands.

This conclusion is in line with the already mentioned close similarity of the EPR spectrum of Cu(II) containing *B. stearothermophilus* superoxide dismutase, to that of Cu(II) bound to the zinc site of the Cu/Zn enzyme. In the latter enzyme zinc is coordinated to 3 imidazoles and a carboxylate group [15]. A very recent paper [5] on structural homology of Mn- and Fe-superoxide dismutases suggests, on the basis of available electron density maps of this class of enzymes, that the metal ligands in the *B. stearothermophilus* enzyme may be His 26, His 81, Asp 163 and His 167. Our results on the Cu(II)-containing enzyme confirm this assignment and indicate that the 3-dimensional arrangement of these ligands is very likely to be the same distorted tetrahedron as for Zn in the Cu/Zn enzyme [15]. It is also evident that such an arrangement, while allowing exchange with external molecules in the case of Mn and Fe, perhaps via a water molecule coordinated in the fifth position [3], make the copper less available for solvent access. It should be clear that there can be some distortion of the site from the native to the Cu(II) substituted protein and that this cannot be assessed by the methods used here.

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