A NUCLEAR MAGNETIC RELAXATION STUDY OF CO(II) BOVINE SUPEROXIDE DISMUTASE
Evidence that the Co(II) site is exchange-coupled to the copper and not accessible to the solvent

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Received 29 November 1973

1. Introduction

Mammalian superoxide dismutases (SOD) contain approximately 2 Cu(II) and 2 Zn(II) per protein molecule (33 000 mol. wt. [1]). Both the metal sites can be considered essential to the enzyme, even though only the Cu(II) site is directly involved in the catalytic mechanism [2–4]. The Zn(II) site appears to have a structural role [2, 5]. A renewed interest in the zinc site of SOD has recently risen, since it has been shown that Zn(II) can be substituted by Co(II) [6, 7]. In our hands, Co(II) has only partially been substituted for Zn(II), by an exchange procedure which replaces 50% of the zinc sites with cobalt [6]. Nevertheless this derivative retains the full enzymatic activity [6]. Now we report proton magnetic relaxation measurements of the Co(II) bovine SOD.

2. Materials and methods

The cobalt enzyme was prepared as previously described [6]. Different Co(II)-substitution extents were obtained by variable exchange dialysis times. Metal analyses were performed with a Hilger and Watts Atomspek Model H1 170 atomic absorption spectrometer. X band EPR spectra were carried out as previously described [8]. The EPR detectable copper of each sample was calculated by double integration of the EPR derivative signal, in comparison to a Cu(II) EDTA standard under non-saturating conditions. Water T_1 measurements were made at 22°C by a pulsed technique using $90°-\tau-90°$ sequence at a Larmor frequency of 16.0 MHz.

3. Results and discussions

It has already been shown [9] that the enzyme-bound Cu(II) decreases the T_1 of water. If in Co(II)-bovine SOD samples the Cu(II) contribution to the water T_1 is suppressed by reduction of the copper with dithionite, one is concerned with paramagnetic effects on T_1 due only to the Co(II) center. As shown in table 1 no significant difference is observed

Table 1
T_1 as samples of reduced SOD. Concentrations are $\times 10^{-4}$ M.

Sample no.	Protein	Cu	Co(II)	T_1 (sec)
1	5.0	7.5(as Cu^+)	4.5	2.9
2	5.0	7.5(as Cu^+)	–	3.1

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Table 2
Metal content, T_1, and EPR detectable copper of samples of cobalt SOD. Concentrations are $\times 10^{-4}$ M. T_1 was measured at 22°C, EPR detectable copper at − 170°C.

Sample no.	Protein	Cu(II)	Zn(II)	Co(II)	Cu(II) $\times \frac{Zn(II)}{Zn(II)+Co(II)}$	T_1 (sec)	T_1-active Cu(II)	EPR detectable Cu(II)
1	5.4	7.8	8.6	–	7.8	0.47	7.8	7.8
2	3.7	8.0	5.0	4.6	4.2	0.75	4.6	3.5
3	3.4	5.7	2.7	4.2	2.2	1.0	3.0	1.5
4	4.1	7.6	3.4	5.1	3.0	1.0	3.0	1.7
5	4.7	7.2	2.0	6.2	1.8	1.3	1.8	1.2

between reduced cobalt–copper and reduced natural zinc–copper protein and both the values are very near to the T_1 value of the pure water. This result can be interpreted as due to a very short Co(II) spin-lattice electron relaxation time (τ_s) or to a very slow exchange of the bound water or to inaccessibility of the bulk water to the Co(II) site. However the Co(II) of bovine SOD has been shown to have tetrahedral symmetry [10], and therefore its electron relaxation time is not expected to be exceedingly short [11]. Therefore, were water bound in its coordination shell, an effect on water T_1 would have been observed, as it actually is in Co(II) carbonic anhydrase [12], which has a similar coordination geometry [10]. On the other hand it should be remarked that an effect on T_1 should be possibly observed in the case of the slow exchange of the Co(II) water ligand, because in this case the mean residence time of the protons in the first coordination sphere should be modulated by the exchange of protons between the bound and the bulk water [13]. As a consequence it is possible to assume that the Co(II) site is not accessible to the bulk water.

Table 2 shows T_1 values for bovine SOD samples containing different Co(II)/Cu(II) ratios. The T_1-active Cu(II) has been derived from T_1 values on the basis of a calibration curve (fig. 1), and therefore it has to be interpreted as the concentration of the Cu(II) practically relaxing the water protons. It is evident that the T_1-active Cu(II) does not correspond to the chemically determined copper, though it is all present as Cu(II) (see ref. [6]). In column six of table 2 are reported the amounts of Cu(II) interacting with Zn(II) calculated on the assumption that the Cu(II) is evenly distributed in proximity of Co(II) and Zn(II) sites. The good agreement between these data and

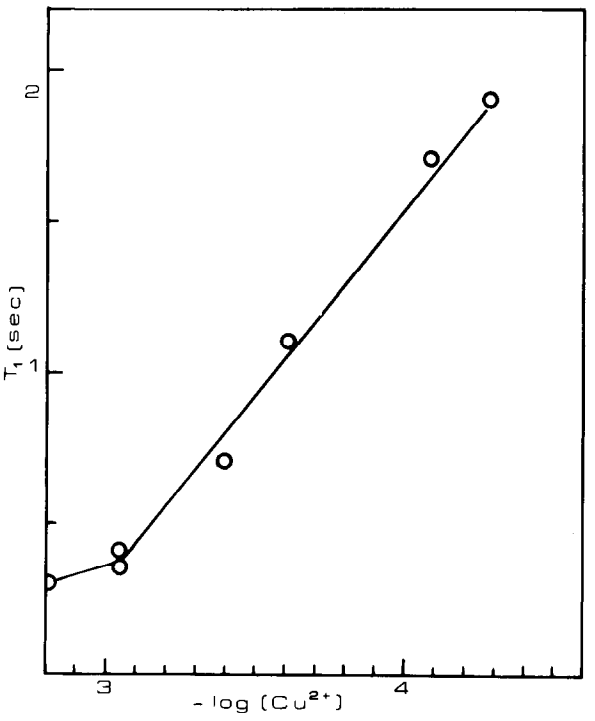

Fig. 1. Dependence of water T_1 on concentration of SOD-bound copper. Temperature : 22°C. The copper content of the protein, as determined by atomic absorption and EPR, was 1.5 atoms per SOD molecule.

those reported in the last two columns of table 2 shows that only the Cu(II) in proximity of Zn(II) is active in the relaxation of water protons and is EPR detectable. This behavior can be interpreted on the basis of an exchange coupling between the Cu(II) and Co(II) ions.

We conclude that solvent water T_1 measurements of Co(II) bovine SOD directly demonstrate that cobalt, and probably the native zinc also, does not interact with the solvent, as already suggested on the basis of indirect spectral evidence [14]. Furthermore these measurements provide on elegant way to approach the nature of the coupling of the cobalt and copper sites in the cobalt enzyme, as they can only be interpreted by assuming an anti-ferromagnetic coupling between the two centers. It is probable that copper and zinc share a common ligand in the native enzyme, so that a superexchange coupling occurs between the Cu(II) and the substituted Co(II).

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