Preferential Binding of Copper Ions to Superoxide Dismutase Subunits Lacking Metal Ions in the Zinc Sites

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Abstract

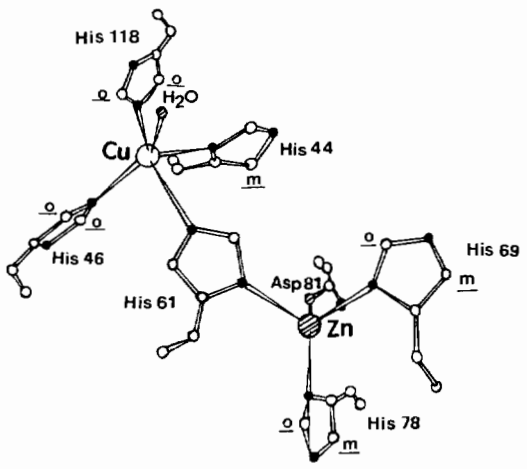
The distribution of copper in its binding sites of superoxide dismutase is followed through ^1H NMR when the zinc sites are only half filled by cobalt. It is shown that copper can bind preferentially subunits without cobalt(II) and the observation is accounted for.

Introduction

Bovine superoxide dismutase (SOD hereafter) is a dimeric enzyme of MW 32 000 containing one zinc(II) ion and one copper(II) ion in each subunit [1]. The X-ray structure, resolved at 2 Å, has shown that copper is bound to three histidines (His-44, His-46, His-118), to a water molecule, and to a fourth histidine (His-61) which is bridging with zinc(II). The latter is coordinated to two more histidines (His-69 and His-78) and to an aspartate residue (Asp-81) (Scheme 1) [2].

It is possible to prepare the demetallized protein and substitute the native metals with other metal ions [1]. The demetallized SOD binds cobalt(II) in the zinc site giving rise to E_2Co_xSOD ($x \leqslant 2$, E means empty), containing high spin cobalt(II) [3–5]. This compound gives rise to typical ^1H NMR spectra with isotropically shifted signals far away from the diamagnetic region (Fig. 1A) [6]. When copper is present magnetic coupling takes place between the two metal ions, and the electronic relaxation time of copper is dramatically reduced. As a consequence of it, it is possible also to detect the proton signals of groups coordinated to copper(II) (Fig. 1B) [7]. The spectra of Fig. 1 can be considered as finger-prints of the two species, E_2Co_2SOD, the metal derivative where cobalt(II) occupies the zinc site and Cu_2Co_2-SOD, the metal derivative where copper(II) occupies the copper site which is magnetically coupled with cobalt(II) through His-61.

The aim of the present paper is to learn whether copper prefers to bind to a subunit containing cobalt or to a subunit without cobalt, when cobalt does not saturate the zinc sites. Therefore the experiment is



Scheme 1.

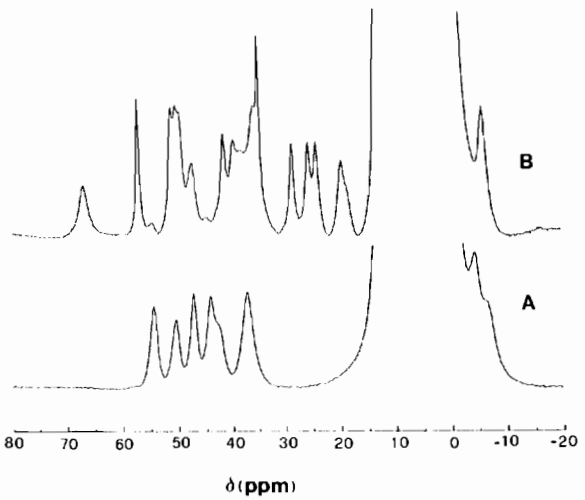


Fig. 1. 200 MHz ^1HMR spectrum of E_2Co_2SOD (A), and Cu_2Co_2SOD (B), at 296 K, in 50 mM acetate buffer, at pH 5.5. The spectra were recorded on a Bruker MSL 200, by using the modified DEFT pulse sequence in order to suppress H_2O and bulk protein signals.

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performed by using a sample containing less than 2 equivalents of cobalt(II). When copper(II) is added it may bind to a site with or without cobalt(II). By measuring the relative intensity of the ^1H NMR signals of the copper(II)–cobalt(II) and empty-cobalt(II) species at different and increasing amounts of copper(II) it has been possible to follow the disappearance of the derivative E_2Co_xSOD ($x < 2$) and the appearance of the species Cu_yCo_xSOD where y is the number of equivalents of copper at the subunit already occupied by cobalt(II). The ^1H NMR spectrum of the CuESOD subunit does not provide any detectable isotropically shifted NMR signal therefore high spin cobalt(II) may be an excellent probe to monitor the occupancy of the copper site in the subunit where cobalt(II) is present [8].

Experimental

The demetallized protein was obtained through dialysis against 0.05 M acetate buffer at pH 3.8 containing 0.01 M EDTA as described elsewhere [9]. The chelating agent was removed through extensive dialysis against 0.1 M NaCl at pH 3.8, in the same buffer and then against 0.05 M acetate. The pH of this buffer was gradually increased during the dialysis from 3.8 to 5.5. The cobalt(II) derivatives were prepared adding 0.5, 1.0 and 2.0 equivalents of cobalt(II) to the apoprotein. All these samples showed the typical ^1H NMR spectrum of E_2Co_2SOD [3]. The copper–cobalt SOD was obtained by slowly infusing small amounts of copper(II) solution [3], corresponding each time to 0.2–0.3 equivalents of copper(II). For each addition the ^1H NMR spectrum was recorded.

Results and Discussion

The initial additions of copper(II) to solutions of E_2Co_xSOD ($x < 2$) at pH 5.5 neither change the intensity of the spectrum of E_2Co_xSOD nor cause the appearance of the typical spectrum of the magnetically coupled copper(II)–cobalt(II) moiety. The amount of copper necessary for the formation of the latter derivative depends on the relative occupancy of the zinc sites by cobalt. For example in Fig. 2A the intensity (calculated by integrating the area) of the signal at 54.4 ppm chosen as typical of $E_2Co_{0.5}SOD$ is reported as a function of the equivalents of copper(II). The intensity is estimated considering the sum of the intensities of the signals at 54.4 ppm of $E_2Co_{0.5}$-SOD and at 57.0, which we have chosen as indicative of $Cu_yCo_{0.5}SOD$, as 100% (see Fig. 1). Copper binds at the sites without cobalt, until they are available, *i.e.* $\simeq 1.5$. Only when these are no longer available, does copper go into the sites containing cobalt (above

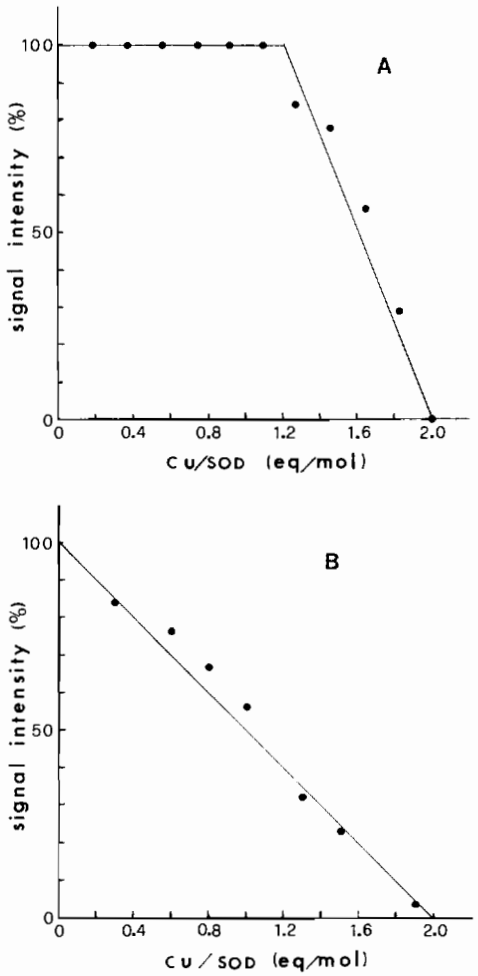

Fig. 2. Intensity of the signal at 54.5 ppm in the ^1H NMR spectra of $Cu_yCo_{0.5}SOD$ (A) and Cu_yCo_2SOD (B), as a function of the Cu/protein ratio. The intensity, estimated as a percent of the intensity of the same signal for Cu/protein ratio = 0, was measured integrating its area. The lines connecting the points are only indicative of the observed behavior.

1.5 equivalents of copper). At this stage the spectrum of the copper(II)–cobalt(II) species begins to appear and that of the copper(II)–cobalt(II) derivative begins to disappear. Obviously, when the zinc sites are completely filled with cobalt (E_2Co_2SOD), we can observe the appearance of the spectrum of Cu_yCo_2SOD from the very beginning of the titration (Fig. 2B). These data are the result of many experiments repeated with different amounts of cobalt(II).

The experimental results provide evidence of the anticooperative behavior of cobalt(II) in the binding of copper(II). This may be explained considering that the binding of copper in a site where His-61 is already bound to cobalt needs to experience a second deprotonation. On the other hand, when His-61 is free from cobalt a single deprotonation is needed

(just like when cobalt binds to the apoprotein) for the binding of the copper ion. It is possible that at higher pH, where the deprotonation of the histidine NH is easier, different results are obtained. It should be remembered that, above pH 8, copper(II) occupies both the copper and zinc sites in the same subunit rather than preferentially filling only the copper site [10].

These experiments do not provide information on the interaction between subunits. Indeed Rigo *et al.* [11, 12] and Hirose *et al.* [13] studied this problem to find out whether there is a cooperative interaction between the two copper sites or the distribution of copper is merely a statistical process. Hirose *et al.* did not observe any interaction between the copper binding sites in measurements of binding constants. Rigo *et al.* proposed that the two copper sites are thermodynamically equivalent, but the occupation of the first site lowers the activation energy of the binding of the second copper. A change in the protein tertiary structure due to the binding of the first metal causes a decrease of the energy involved in binding of the second one.

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References

1 J. S. Valentine and M. W. Pantoliano, in T. G. Spiro (ed.), 'Copper Proteins', Vol. 3, Wiley, New York, 1981, Chap. 8.
2 J. A. Tainer, E. D. Getzoff, K. M. Beem, J. S. Richardson and D. C. Richardson, *J. Mol. Biol., 160,* 181 (1982).
3 J. A. Fee, *J. Biol. Chem., 248,* 4229 (1973).
4 L. Calabrese, D. Cocco and A. Desidesi, *FEBS Lett., 106,* 142 (1979).
5 A. Desideri, D. Cocco, L. Calabrese and G. Rotilio, *Biochim. Biophys. Acta, 785,* 111 (1984).
6 I. Bertini, G. Lanini, C. Luchinat, L. Messori, R. Monnanni and A. Scozzafava, *J. Am. Chem. Soc., 107,* 4391 (1985).
7 I. Bertini, G. Lanini, C. Luchinat, M. Mancini and G. Spina, *J. Magn. Reson., 63,* 56 (1985).
8 I. Bertini and C. Luchinat, in H. B. Gray and A. B. Lever (eds.), 'NMR of Paramagnetic Molecules in Biological Systems', Benjamin/Cummings, Menlo Park, Calif., 1986.
9 M. W. Pantoliano, J. S. Valentine and L. A. Nafie, *J. Am. Chem. Soc., 104,* 6310 (1982).
10 J. S. Valentine, M. W. Pantoliano, P. J. McDonnell, A. R. Burger and S. J. Lippard, *Proc. Natl. Acad. Sci. U.S.A., 76,* 4245 (1979).
11 A. Rigo, P. Viglino, L. Calabrese, D. Cocco and G. Rotilio, *Biochem. J., 161,* 27 (1977).
12 A. Rigo, M. Terenzi, P. Viglino, L. Calabrese, D. Cocco and G. Rotilio, *Biochem. J., 161,* 31 (1977).
13 J. Hirose, K. Iwatzuka and Y. Kidani, *Biochem. Biophys. Res. Commun., 98,* 58 (1981).