A NOVEL Co(II) BINDING SITE IN COPPER-FREE SUPEROXIDE DISMUTASE

Evidence for binding of cobalt at the copper binding site

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1. Introduction

Copper–zinc superoxide dismutases from eukaryotic organisms have been extensively studied with reference to the role of the metals in the catalytic and structural properties of the protein. Metal deletion and/or substitution is a major tool in this respect. The zinc, which is apparently relevant to the structural stability of the protein, has been replaced by divalent cobalt, cadmium, mercury and copper to give still active enzyme derivatives [1–3]. The copper has been shown to be essential to the enzyme activity [4] and is less prone than zinc to replacement by other metals: only an inactive Ag^+ derivative has been reported to date [5].

Here we present evidence for the presence, in the copper-free, zinc containing bovine superoxide dismutase, of a cobalt binding site that is likely to correspond to the copper binding site. This evidence was obtained from the differential properties (stoichiometry of binding and spectra) of cobalt derivatives of apo and copper-free superoxide dismutase.

2. Materials and method

Bovine holo, apo and copper-free superoxide dismutases were prepared by the procedures described [2,4,6]. Zinc, cobalt and copper contents were determined by atomic absorption spectrometry (Hilger and Watts Atomspek, model H 1170). Absorption spectra were obtained with a Beckman UV 5230 recording spectrophotometer. Electron paramagnetic resonance spectra were recorded at liquid helium temperature using a Varian E 9 EPR spectrometer equipped with an Air Products and Chemicals LT-3-110 liquid-transfer Cryo-Tip refrigerator with automatic temperature controller.

3. Results and discussion

Apo superoxide dismutase in 0.1 M acetate buffer (pH 5.4) has been shown [2] to bind 2 equiv. Co(II), giving rise to a well-defined optical spectrum with a maximum at 583 nm, which has been assigned to cobalt bound to the zinc site [1,7,8]. The same spectrum is obtained by the exchange procedure [1,7], involving dialysis against excess Co(II) in acetate buffer (pH 5.4) of the zinc containing copper-free protein. In this case, however, the exchanged cobalt is generally <2 Co(II)/mol protein. In both cases the cobalt derivative binds 2 Cu(II) at the native position with recovery of catalytic activity.

Addition of 2 equiv. Co(II) to the copper-free or holo proteins in acetate buffer, and incubation over 24 h had no effect on the visible absorption spectrum. After dialysis against acetate buffer, only 0.1 equiv. Co(II)/mol protein were recovered. It is therefore conclusive that, in 0.1 M acetate buffer (pH 5.4) there is a specific binding of 2 Co(II) at the zinc sites.

On the other hand, in 0.1 M phosphate buffer (pH 7.4) the apo protein binds ~4 equiv. Co(II)/mol protein. At Co/protein ratios <2, in a spectrophotometric titration (fig.1A(a,b), the spectroscopic features are typical of cobalt bound to the zinc site

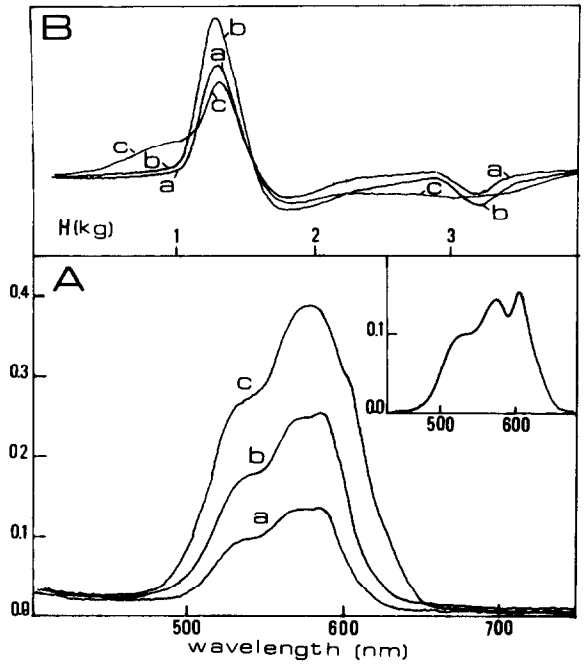

Fig.1. Optical (A) and EPR (B) titration of 0.33 mM apo superoxide dismutase in 0.1 M phosphate buffer (pH 7.4). CO(II)/protein ratios: (a) 1; (b) 2; (c) 3.4. Spectra (a) and (b) were recorded immediately after the metal addition; spectrum (c) was recorded after 4 h incubation at room temperature. Insert: calculated difference optical spectrum (spectrum (c) minus spectrum (b)). The EPR spectra were recorded at 10 K, with: field modulation amplitude 10 G; microwave frequency ~9.25 GHz; microwave power 10 mW.

[1,2,7], but when >2 equiv. Co(II) are added, a different line shape is obtained (fig.1A(c)). Difference optical spectrum (fig.1A insert) shows that the species formed at higher Co/protein ratios has different absorption maximum, indicating a new binding site.

The same titration is shown in the EPR spectra of fig.1B. On addition of 1 Co(II)/protein, the EPR signal at $g \simeq 4$ typical of the cobalt bound to the zinc site [7,8] appears (curve a). Increasing the Co(II)/protein ratio up to 2 produces an increase of the signal without affecting the shape of the line (fig.1B(b)). On the other hand, when the ratio is >2, the signal centered at $g \simeq 4$ decreases and a broad line appears at lower fields (fig.1B(c)). This behavior is indicative of magnetic interaction between Co(II) ions in two different sites, such as expected if the binding site occupied at Co(II)/protein ratios >2 is in close proximity of the zinc site.

In view of the possibility that the second site be the site that binds copper in the native protein, a series of experiments was carried out with the zinc containing copper-free superoxide dismutase. In fact, a sample of this protein in 0.1 M phosphate buffer (pH 7.4) bound ~2 equiv. Co(II) in a slow process which took hours to reach completion at room temperature. The bound cobalt was not removed by dialysis against 0.1 M phosphate buffer (pH 7.4) and gave rise to optical and EPR spectra (fig.2 spectra (a)) that are well distinct from those described [1,2,7,8]. In particular the optical spectrum corresponds to that shown in fig.1A insert, with $\epsilon = 330\ M^{-1}\ cm^{-1}$ at 605 nm, and suggests binding in a tetrahedral coordination geometry. The corresponding EPR spectrum (fig.2B(a)) shows the presence of at least two high spin cobalt species, that are clearly resolved only at lower fields. One of them displays a broad signal with poorly resolved hyperfine structure, of about 100 Gs splitting, centered at $g \simeq 6$. The other species is characterized by a much sharper line at $g \simeq 6.9$. Though other g-values can not be assigned on the basis of the

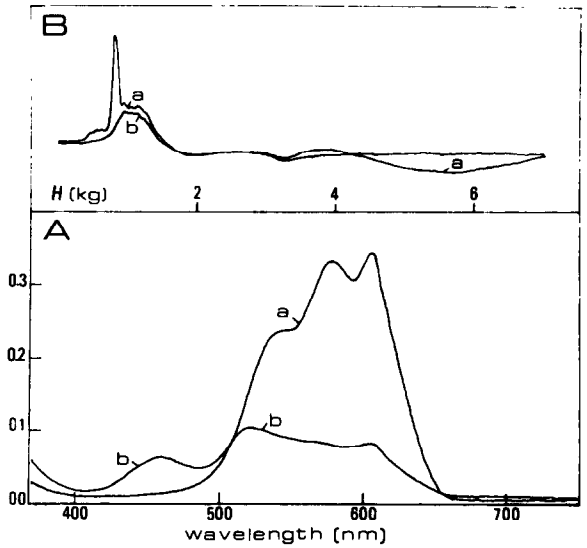

Fig.2. Optical (panel A) and EPR (panel B) spectra of Co(II) derivatives of copper-free superoxide dismutase: protein 0.57 mM; Co(II) 1.04 mM; (a) in 0.1 M phosphate buffer (pH 7.4); (b) in water. EPR spectra were recorded in the same conditions as in fig.1.

present resolution, the value of the highest g for both species is indicative of a more distorted geometry with respect to the Co(II) binding sites in superoxide dismutase described [7,8].

The spectral properties were found to depend on the presence of phosphate. In fact removal of phosphate by dialysis against water caused drastic changes in the optical and EPR spectra (fig.2, spectra (b)). In particular, in the EPR spectrum (fig.2B(b)) the broad signal with $g \simeq 6$ and hyperfine structure is still present, although with some modifications, suggesting that the heterogeneity observed in spectrum (a) was due to a mixture of two cobalt signals, that are likely to reflect different coordinations in the presence of water or phosphate. In fact, changing the pH with phosphate buffer from pH 7.4–5.8, did not significantly alter the optical spectrum, while raising the pH of water solutions of the protein up to pH 7.4 by addition of NaOH, did not produce the spectrum of the protein in phosphate buffer.

Also the binding process was found to be phosphate dependent. In 0.1 M Tris–acetate buffer (pH 7.4) a maximum of 1.4 equiv. Co(II) were bound in a very slow process. Furthermore the derivative had spectroscopic feature similar to that of the 'water' species and developed the intense absorption band at 605 nm on addition of 0.1 M phosphate.

The sensitivity of this Co(II) derivative to the presence of phosphate, which was irrelevant to the properties of the cobalt species described [1,7], is further evidence for an exposed location of the new cobalt binding site, such as actually corresponds to the original copper binding site. Moreover copper recombination with the copper-free protein was drastically influenced by the presence of cobalt in this site. To two different samples of the Co(II)-derivative saturated with 1 equiv. Co(II) and 1.8 Co(II)/protein, respectively, 1 equiv. Cu(II) was added. After 120 min incubation at room temperature only the first sample showed complete reconstitution of the native site as judged by EPR spectrum. In the second sample the EPR signal of copper cleary showed that copper was not bound to the native site.

All these data suggest that the binding site of the copper in superoxide dismutase can, in particular conditions, accept the Co(II) ion. These results seem to be particularly interesting in view of the fact that the copper binding site appears to be pentacoordinate from X-ray crystallography data [9] and to date only the tetrahedral [10] copper blue sites [11] seemed to be suitable for cobalt replacement.

Further studies of spectral and chemical characterization of this novel Co(II) derivative of copper–zinc superoxide dismutase are in progress.

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