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An X-ray absorption study of the reconstitution process of bovine Cu,Zn superoxide dismutase by Cu(I)-glutathione complex

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The Cu(I)GSH complex has recently been shown to be a good candidate for delivering copper to the active site of Cu-free Cu,Zn superoxide dismutase both in vivo and in vitro. In this work X-ray absorption spectroscopy has been used to characterize the Cu(I)GSH complex and to follow in vitro the reconstitution of Cu,Zn superoxide dismutase from the copper-free protein and this complex. The results obtained indicate that the copper is directly transferred as Cu(I) from the GSH complex into the empty copper binding site. No evidence has been obtained for a ternary complex in which the metal is bound to both GSH and the protein.

Copper-free,Zn superoxide dismutase; Glutathione; Reconstitution process; XANES; X-ray spectroscopy

1. INTRODUCTION

Cu,Zn superoxide dismutase (SOD) is an enzyme which catalyzes the dismutation of the superoxide anion to O_2 and H_2O_2 and plays an essential role in the defense of the cell against toxic derivatives of the biological activation of oxygen [1]. The three-dimensional structure of the bovine enzyme is known in detail for the oxidized form [2]. The enzyme is a dimer of identical subunits made of a rigid β-barrel core scaffolding external loops, which accommodate the active site. This site consists of a copper surrounded by four histidine residues (His-44, -46, -61, -118) in a distorted square-planar geometry approaching tetrahedral coordination. The imidazole ring of a histidine residue is a common ligand to an adjacent zinc ion, further coordinated by 2 more histidines (His-69, -78) and an aspartate (Asp-81) in a tetrahedral fashion.

The mechanism by which copper is incorporated in vivo into the active site of this enzyme is unknown. Copper-metallothionein has been successfully used in vitro for reconstitution and reactivation of several copper enzymes [3–5], but it has not been possible to reconstitute the copper-zinc SOD [6]. Recently it has been shown, by using EPR and NMR spectroscopy [7], that a very high reconstitution efficiency is obtained by adding copper to Cu-free SOD as the Cu(I)GSH complex, which had previously been suggested to be involved in copper trafficking inside copper-loaded hepotome cells [8]. Neither technique monitors the copper atom in its reduced state, but from indirect evidence it was possible to demonstrate that copper was transferred as Cu(I) and to suggest that a Cu(I)–GSH–protein ternary intermediate was active in the mechanism of transfer.

In this work we have re-investigated the reconstitution process by XANES (X-ray absorption near edge structure), a spectroscopic technique that can directly monitor the copper atom both in the reduced and oxidized state, and is currently used to investigate metalloproteins [9]. The results indicate that the metal is rapidly transferred as Cu(I) from GSH into the native copper site of the protein without forming an intermediate copper complex sharing ligands with both the protein and GSH. The copper is then slowly reoxidized to give the spectrum typical of the Cu(II),Zn(II) protein.

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Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione.

2. MATERIALS AND METHODS

Cu,Zn-SOD was isolated from bovine erythrocytes according to McCord and Fridovich [10]. The reduced form was obtained by adding dithionite to the oxidized protein. The copper-free derivative was prepared by reducing the copper with excess potassium ferrocyanide and dialyzing for 12 h at 4°C against 0.1 M phosphate buffer containing 0.05 M KCN at pH 6.0 [11]. The samples were further dialyzed for 24 h at 4°C against water. Final copper content was less than 2%.

Cu(I) complexes with GSH were prepared by adding $CuSO_4$ to a solution GSH in 0.1 M phosphate buffer at pH 7.4. Addition of Cu(II) to GSH in a 3:1 ratio causes oxidation of stoichiometric amounts of GSH to GSSG and complexation of the resulting Cu(I) with the

remaining GSH [7]. Glutathione complexes were tested by optical absorption spectra before and after XANES: measurements to verify the absence of any absorbance at 625 nm, which is diagnostic of the presence of Cu(II)GSSG. The Cu(II) complex with GSSG was prepared by adding $CuSO_4$ to a 3-fold excess of GSSG in 0.1 M phosphate buffer at pH 7.4.

In the reconstitution experiment 100 μl of 1.32 mM copper-free SOD were used. The appropriate amount of Cu(I)GSH was then added to the apo-protein in order to obtain a 1:1 ([Cu]/[available Cu binding sites]) ratio. The final concentration of copper in the Cu(I)GSH/Cu-free,Zn-SOD incubation mixture was 2.48 mM. X-ray absorption measurements were carried out at the copper K-edge using the D21 station of the DCI storage ring of LURE (Orsay, France) with a positron beam of 1.85 GeV and a maximum stored current of 310 mA. The XANES spectra of copper–glutathione complexes were obtained in both oxidation states in the range 8,950–9,150 eV with a step size of 0.5 eV. The oxidation kinetics were followed by recording the spectra of the Cu(I)GSH/Cu-free,Zn-SOD incubation mixture over 15 h. The experiment was repeated twice with a different acquisition time (37 min and 55 min) in the two sets of experiments. A tightly sealed sample-holder was used in order to slow down the oxidation rate.

Experimental resolution with the Si(311) monochromator and in the presence of two slits (before the monochromator and the first ionization chamber, respectively) was about 2 eV [12].

Spectra of protein samples were recorded in the fluorescence mode and at the same time the spectrum of a reference copper metallic foil was recorded in the transmission mode. The simultaneous acquisition of the two spectra allows the calibration of the energy scale and the exact detection of the K-edge energy shift of copper in the protein as a function of time. The contribution of the sample to the absorption can be neglected in view of the following considerations: (i) the low portion of total beam irradiating the sample, as the beam is larger than the sample and (ii) the low concentration of the absorber atom in the protein with respect to that of the metallic copper foil. Sample signal was detected by fluorescence using a plastic scintillator [13]. The fluorescence signal was filtered by a Ni metallic foil of 25 μm of thickness.

3. RESULTS AND DISCUSSION

The X-ray absorption spectra at the Cu K-edge are reported in Fig. 1a and b for the Cu(I)GSH and Cu(II)GSSG complexes. The spectrum of the reduced complex is different from that of the oxidized one and does not change for at least 3 h. This rules out its oxidation which would have produced an up-shift of the energy edge and a peak intensity variation. This result confirms that GSH is able to form a very stable complex with Cu(I) even in the presence of oxygen [7]. Addition of Cu(I)GSH to Cu-free,Zn-SOD, in a 1:1 ([Cu]/[available Cu binding sites]) ratio, gave the XANES spectrum reported in Fig. 2a. The spectrum is identical to that of the native enzyme with the copper in the reduced state recorded under the same conditions (Fig. 2B), and both are very similar to the previously reported spectrum of the native reduced enzyme [14]. In fact the shoulder on the rising part of the edge at about 8,980 eV is less pronounced in our case. This difference is due to the larger slit width used in our experiments in order to increase the intensity of the signal. The identity between the spectrum of the Cu(I)GSH/Cu-free,Zn-SOD mixture and that of the native Cu(I) enzyme (Fig. 2) indicate that the mechanism of copper transfer involves an immediate coordination to the protein in a fashion typical of the native Cu(I) enzyme. In fact, if two different copper coordination site geometries were present, the resulting spectrum would have been a mixture. As there is no evidence for the presence of the Cu(I)GSH complex (see Fig. 1a) in the spectrum of the Cu-free protein/Cu(I)GSH mixture we conclude that the transfer process is complete in 20 min, i.e. the measurement time.

NMR experiments previously recorded in the same time range [7] detected significant differences between the native Cu(I) enzyme and the reconstituted one. This result should be ascribed to some interaction involving the protein and glutathione since the identity of the XANES spectra (Fig. 2), which directly monitors the

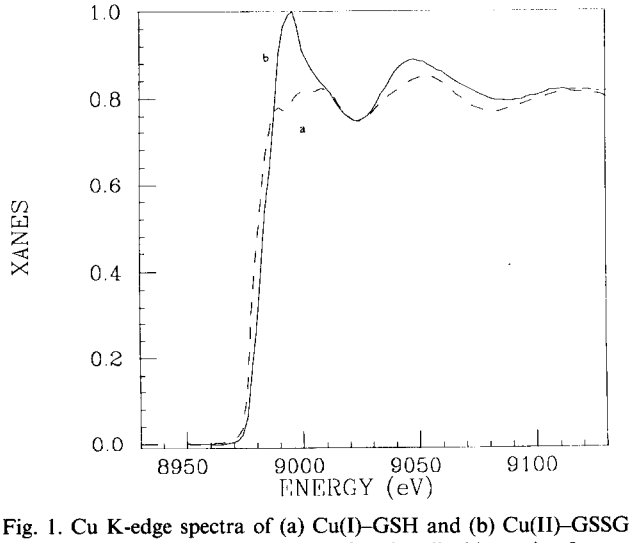

Fig. 1. Cu K-edge spectra of (a) Cu(I)–GSH and (b) Cu(II)–GSSG complexes. The samples were prepared as described in section 2; copper concentration was 28 mM. The spectra are an average of 5 different scans.

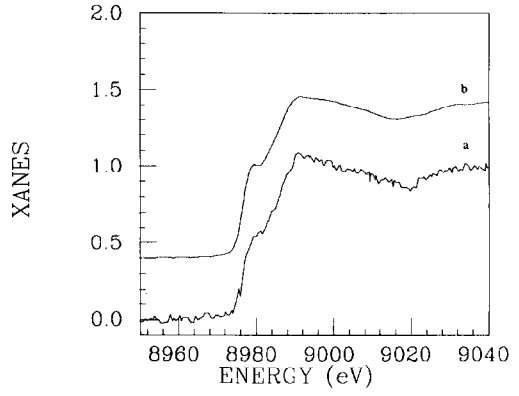

Fig. 2. Cu K-edge spectra of (a) Cu-free,Zn SOD in 0.05 M phosphate buffer, pH 7.4, immediately after addition of Cu(I)–GSH; (b) native reduced SOD in 0.05 M phosphate buffer, pH 7.4. Copper concentration was 2.5 and 3 mM for the reconstituted and the native enzyme, respectively. Spectrum a is a single scan, spectrum b is an average of 5 different scans.

Cu(I) probe, rules out the presence of a Cu(I)–GSH–protein ternary complex.

The mechanism of reconstitution can be hypothized to occur in the following way: (i) attraction of the Cu(I)GSH complex toward the active site of the protein; (ii) formation of hydrogen bonds and salt bridges, most likely through the negative carboxyl group of GSH and the positive side chains surrounding the active site of the enzyme [15]; (iii) release of the copper from the GSH moiety directly into the active site channel of the protein. This last step is likely to be facilitated by the low dielectric cage formed between the protein and glutathione.

After the transfer has occurred the copper slowly reoxidizes in air [7]. The kinetics of reoxidation can be easily followed by taking advantage of the fact that the copper of superoxide dismutase displays different X-ray absorption spectra depending on its oxidation state. In fact the position of the edge reflects the effective charge density on the absorbing atom. The XANES spectra of the reconstituted protein as a function of time are reported in Fig. 3a–l. The protein-bound Cu(I) is slowly reoxidized to Cu(II) and after 15 h is almost completely oxidized. No intermediate species between the Cu(I) and the Cu(II) states, typical of the native protein, could be detected during the process. This result confirms that copper is transferred as Cu(I) to the preformed metal binding site of the protein. This is even more clearly demonstrated by the first derivative of the spectra (Fig. 4a–l) which show the linear disappearance of the two peaks, which are diagnostic of the reduced enzyme, with the concomitant appearance of the broad band typical of the oxidized protein (Fig. 4m).

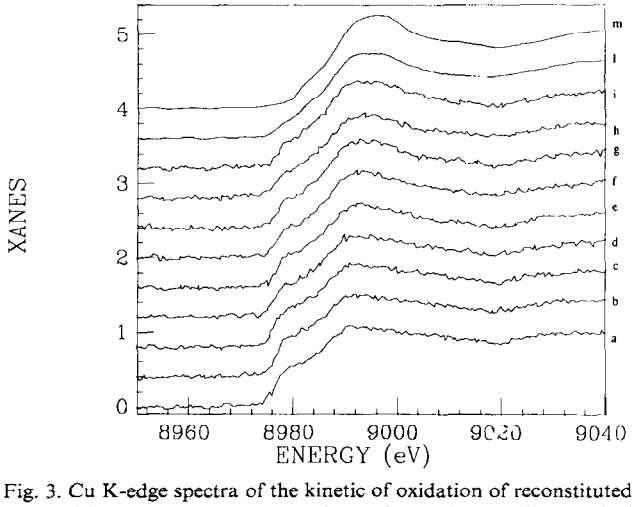

Fig. 3. Cu K-edge spectra of the kinetic of oxidation of reconstituted superoxide dismutase. Spectrum a is relative to the sample described in Fig. 2a recorded at the beginning of the reaction. Spectra b–i were recorded as a function of time: each single scan after 55 min. Spectrum l is an average of 3 different scans recorded after 15 h. Spectrum m represents the fully oxidized native protein and is an average of 5 different scans.

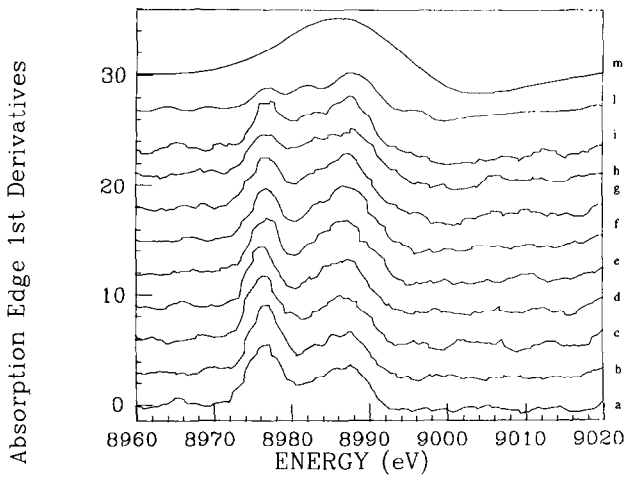

Fig. 4. First derivative of the Cu K-edge spectra reported in Fig. 3.

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