BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **241,** 119–121 (1997)
ARTICLE NO. RC977777

Evidence of His61 Imidazolate Bridge Rupture in Reduced Crystalline Cu,Zn Superoxide Dismutase

Isabella Ascone,*[,1] Ramón Castañer,* Cataldo Tarricone,† Martino Bolognesi,‡ Maria Elena Stroppolo,§ and Alessandro Desideri§

**Laboratoire d'Utilisation du Rayonnement Electromagnétique, Faculté d'Orsay 91405 Orsay Cedex, France; † Dip. Genetica e Microbiologia, Universita' di Pavia, via Abbiategrasso 207, 27100 Pavia, Italy; ‡INFM e Centro Biotecnologie Avanzate, Universita' di Genova, via Dodecaneso 33, 16146 Genova, Italy; and §Departament of Biology, "Tor Vergata," University of Rome, Rome, Italy*

Received October 21, 1997

X-ray absorption spectroscopy has been carried out on the copper K edge in oxidized and reduced bovine Cu,Zn SOD in solution and in crystalline state. The results indicate that the copper coordination geometry is unaffected by the solution or by the crystalline state of the protein, in both oxidation states. Moreover the two oxidation states of the active copper ion are reflected under, all the experimental conditions, by distinct coordination spheres around the catalytic metal, which is four-coordinated and three-coordinated in the Cu(II) and in the Cu(I) enzyme, respectively.

Key Words: **bovine Cu,Zn superoxide dismutase, XAS; copper co-ordination; redox states; bridging ligands.**

Cu,Zn SODs are ubiquitous metallo-enzymes which catalyze the dismutation of the superoxide anion (O_2^-) trough cyclic reduction and oxidation of the copper ion which constitutes the active center [1]. The structural organization of the metal centers in the oxidized enzyme has been thoroughly characterized by crystallographic analyses of Cu,Zn SOD from different species, and shown to be fully conserved [2]. The active site is based on a binuclear metal center in which the Cu(II) and the Zn(II) ions are bridged by the imidazolate ring of residue His 61. The Zn (II) ion is coordinated by two additional histidyl and one aspartyl residue, whilst the Cu(II) ion ligands are three additional histidyl residues and one loosely bound water molecule.

Optical and NMR studies of the Cu(I), Co derivative, in which the zinc is selectively substituted by the cobalt ion, showed that the His 61 bridging imidazole is released from copper upon reduction [3,4]. Subsequently an EXAFS study of the Cu,Zn enzyme provided a direct structural evidence to support the presence of a three-coordinate copper ion in the reduced enzyme [5,6]. Rupture of the imidazolate bridge was shown to occur during catalysis and proposed to be a necessary step of the enzymatic mechanism [7]. In contrast to these observations, an X-ray crystallographic study of the reduced enzyme [8,9] suggested that the Cu(I) site coordination is virtually unchanged with respect to the oxidized state, such that the authors proposed that breaking of the Cu-Zn bridge coupled to reduction would be unnecessary for the catalytic cycle. On the other hand, the analysis of the polarized absorption spectra of crystals of the Cu,Co SOD derivative [10] showed that reduction of the Cu(II) ion induces an identical shift of the absorption bands both in the crystalline and in the soluted Cu,Co SOD, indicating that reduction of the active site copper ion causes the breaking of the Cu(I)-His61 coordination bond also in the crystalline state. In agreement with this experiment, an accurate EXAFS study, carried out in the same buffer system used for the crystallographic analysis, unambiguously showed that in solution the Cu(I) has only three-coordinated histidines with an average Cu-N distance of 1.97± 0.02 Å [11]. The three-coordinated reduced Cu(I)-Zn SOD was shown to display a characteristic and unique X-ray absorption near edge structure (XANES) spectrum.

In order to shed more light on the conflicting results reported for SOD active site Cu(I / II) ion coordination through the different spectroscopic and crystallographic techniques, we undertook a XANES investigation in solution and on microcrystals of oxidized and reduced Cu,Zn SOD. The results here reported can be unambiguously interpreted as indicative of a three-coordinated copper center in reduced Cu, Zn SOD, as opposed to the four coordinated center in the oxidized enzyme.

[1] Corresponding author: L.U.R.E., Bâtiment 209D-Centre Universitaire Paris-Sud, F-91405 Orsay Cedex, France, Fax: 33 1 64464148. E-mail: ASCONE@LURE.U-PSUD.FR.

0006-291X/97 $25.00

MATERIALS AND METHODS

Measurements were performed at Cu K-edge on 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. Experimental resolution with the Si(311) monochromator in the presence of two slits (before the monochromator and before the first ionisation chamber respectively) is about 2.3 eV [12], when the slits are set at about 300 μm. In our case we used 2 mm slits (increased by a factor two comparing to the previous work [11]) in order to irradiate a large amount of microcrystals in the sample. This choice however decreases the experimental resolution as compared to the XANES data of Murphy [11]. For proper comparison between the solution and microcrystals measurements all spectra were recorded in the same experimental conditions.

Bovine Cu,Zn SOD was purified from SIGMA lyophilized bovine Cu,Zn SOD using FPLC apparatus equipped with a HiLoad 16/60 Superdex 75 column (Pharmacia) and then onto Mono-Q 16/10 ion exchange column (Pharmacia) using a 20 mM Tris-HCl, pH 7.4. The purity of the enzyme as judged by SDS-PAGE was more than 98% [13]. The enzyme was crystallised in an orthorhombic form by vapour diffusion techniques from PEG solutions at pH 6.5, as described previously [14,10]. Microcrystals specimens (linear dimensions 10-100 μM) were mounted in a glass capillary (internal diameter 2 mM) in equilibrium with the mother liquor through the vapour phase. In order to check the absence of polarisation effects, the capillary was rotated around an axis perpendicular to the incident beam direction: no angle dependence in the XANES spectra was detected. XANES spectra were recorded following a fast procedure in order to avoid radiation damage of the protein sample (acquisition was performed at room temperature). The sample signal was detected by fluorescence using a seven-element germanium array detector (Camber Industries) [15]. The X-ray energy scale was calibrated for each single scan through to the simultaneous acquisition of the transmission spectrum of a reference copper metallic foil.

RESULTS AND DISCUSSION

Figure 1 compares the X-ray absorption near edge structure (XANES) spectra recorded at the Cu K-edge of the oxidized and reduced SOD, in solution and in the crystalline state. The data indicate that: (a) for the same redox state of Cu, Zn SOD spectra measured in solution and in the crystalline state are identical; (b) the XANES spectra of the oxidized and reduced Cu,Zn SOD are clearly distinct, and in satisfactory agreement with those previously reported [11]. The XANES spectrum of the reduced enzyme displays characteristic features which distinguish it from that of the oxidized form, the most prominent one being the appearance of a new peak at ~8983 eV due to a $1s \rightarrow 4p$ dipole allowed transition. The presence of this transition is indicative of a planar three-coordinated copper site [16].

Under the selected experimental procedures, the XANES spectra measured on CuZn SOD microcrystals are superimposable to those recorded for the enzyme in solution. A number of previous studies have examined the angular dependence of the XAS from oriented samples [17]. The problem of having bands of different intensities due to different polarization depending on crystals orientation has been overcome in the present study by use of a large number of randomly oriented

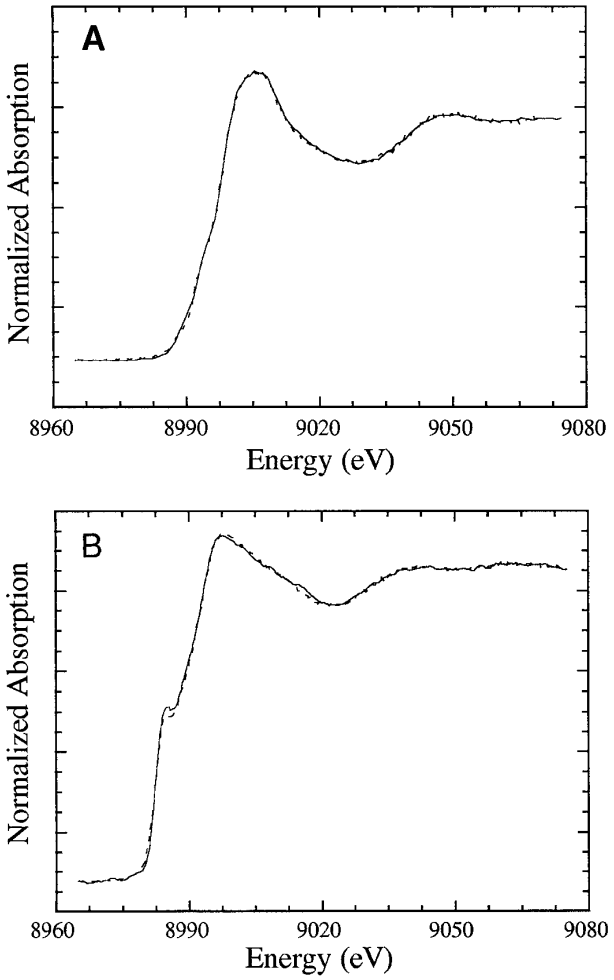

FIG. 1. (A) Normalized XANES spectra of oxidized SOD in solution (dotted line) and in crystalline state (continuous line). (B) Normalized XANES spectra of reduced SOD in solution (dotted line) and in crystalline state (continuous line).

microcrystals. This makes the samples and then the spectra measured in the crystalline state directly comparable to those recorded for the enzyme in solution.

The data there presented are the first direct evidence that: (1) the Cu ion coordination geometry is unaffected by the solution or by the crystalline state of Cu,Zn SOD in both oxidation states; (2) the two oxidation states of the active copper ion, however, are reflected by distinct coordination spheres around the catalytic metal, which is three-coordinated in the Cu(I) enzyme. In this respect the structural interpretation of the present data does not agree with the reported crystal structure of the reduced Cu,Zn SOD, reported by Rypniewsky et al. (1995). Indeed we note that in this last case of crystalline enzyme displayed a remarkable post-translational modification at residue Glu119, located in the copper ion environment, which may have substantial influence on the electrostatic and structural properties of the active site.

The results reported in this work are consistent with the atomic resolution structure observed for the binuclear metal site in the reduced yeast Cu,Zn SOD [18] and in line with the enzyme catalytic mechanism which proposes the presence of a three-coordinate Cu(I) and rupture of the imidazolate bridge, at the Cu side, upon metal reduction by the (O_2^-)substrate

ACKNOWLEDGMENTS

Financial support is gratefully acknowledged from European Union (Contract No. CHRX-CT 93-0184). R.C. acknowledges a grant under the same contract.

REFERENCES

1. Bannister, J. V., Bannister, W. H., and Rotilio, G. (1987) *CRC Crit. Rev. Biochem.* **22,** 111–180.
2. Bordo, D., Dijnovic-Carugo, K., and Bolognesi, M. (1994) *J. Mol. Biol.* **238,** 366–368.
3. McAdam, M. E., Fielden, M., Lavelle, F., Calabrese, L., Cocco, D., and Rotilio, G. (1977) *Biochem. J.* **167,** 271–274.
4. Bertini, I., Luchinat, C., and Monanni, R. (1985) *J. Am. Chem. Soc.* **107,** 2178–2179.
5. Blackburn, N. J., Hasnain, S. S., Diakun, G. P., Kmowles, P. F., Binsted, N., and Garner, D. (1984) *Biochem. J.* **219,** 985–990.
6. Blackburn, N. J., Strange, R. W., McFadden, L. M., and Hasnain, S. S. (1987) *J. Am. Chem. Soc.* **109,** 7162–7170.
7. Tainer, J. A., Getzoff, E. D., Beem, K. M., Richardson, J. S., and Richardson, D. C. (1983) *Nature* **306,** 284–287.
8. Banci, L., Bertini, I., Bruni, B., Carloni, P., Luchinat, C., Mangani, S., Oriolo, P. L., Piccioli, M., Ripniewski, W., and Wilson, K. S. (1994) *Biochemical and Biophysical Research Communications* **202**(2), 1088–1095.
9. Rypniewski, R. W., Mangani, S., Bruno, B., Orioli, P. L., Casati, M., and Wilson, K. S. (1995) *Journal Molecular Biology* **251,** 282–296.
10. Merli, A., Rossi, G. L., Djinovic-Karugo, K., Bolognesi, M., Desideri, A., and Rotilio, G. (1995) *Bioch. Biophys. Res. Comm.* **210,** 1040–1044.
11. Murphy, L. M., Strange R. W., Hasnain, S. S. (1997) *Structure* **5**(3), 371–379.
12. Goulon, J., Lemonier, M., Cortes, R., Retournard, A., and Raoux, D. (1983) *Nucl. Inst. Meth.* **208,** 625–630.
13. Cupane, A., Leone, M., Militello, V., Stroppolo, M. E., Polticelli, F., and Desideri, A. (1994) *Biochemistry* **33,** 15103–15109.
14. Djinovic, K., Coda, A., Antolini, L., Pelosi, G., Desideri, A., Falconi, M., Rotilio, G., and Bolognesi, M. (1992). *J. Mol. Biol.* **226,** 227–238.
15. Cramer, S. P., Chen, J., George, S. J., van Elp, J., Moore, J., Tensch, O., Colaresi, J., Yocum, M., Mullins, O. C., and Chen, C. T. (1992) *Nucl. Instr. and Meth.* **A319,** 285–289.
16. Kau, L. S., Spira-Solomon, D. J., Penner-Hahn, J. E., Hodgson, K. O., and Solomon, E. I. (1987) *J. Am. Chem. Soc.* **109,** 6433–6442.
17. Kutzler, F. W., Scott, R. A., Berg, J. M., Hodgson, K. O., Doniach, S., Cramer, S. P., and Chang, C. H. (1981) *J. Am. Chem. Soc.* **103,** 6083–6088.
18. Ogihara, N. L., Parge, H. E., Hart, J. P., Weiss, M. S., Goto, J. J., Crane, B. R., Tsang, J., Slater, K., Roe, J. A., Selverston, V. J., Eisemberg, D., and Tainer, J. A. (1996) *Biochemistry* **35,** 2316–2321.