EXAFS analysis of a human Cu,Zn SOD isoform focused using non-denaturing gel electrophoresis

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Abstract. Isoelectric point isoforms of a metalloprotein, copper-zinc superoxide dismutase (CuZnSOD), separated on electrophoresis gels were analyzed using X-ray Absorption Spectroscopy. Mutations of this protein are involved in familial cases of amyotrophic lateral sclerosis. The toxicity of mutants could be related to defects in the metallation state. Our purpose is to establish analytical protocols to study metallation state of protein isoforms such as those from CuZnSOD. We previously highlighted differences in the copper oxidation state between CuZnSOD isoforms using XANES. Here, we present the first results for EXAFS analyses performed at Cu and Zn K-edge on the majority expressed isoform of human CuZnSOD separated on electrophoresis gels.

1. Introduction
X-ray Absorption Spectroscopy (XAS) is a powerful analytical technique which enables the study of an element of interest at the atomic level. This multielemental technique can be applied to the analysis of metalloproteins in order to understand the importance of the metallic heteroelement in its structure and properties.

Usually, XAS analysis of metalloproteins is performed in solution or protein crystals. In this study, our purpose was to set up new analytical protocols to prepare and analyze metalloprotein isoforms separated by gel electrophoresis under non-denaturing conditions. Analyses of distinct metalloprotein isoforms variability could elucidate their nature better than assessing a protein bulk.

Copper-zinc superoxide dismutase (CuZnSOD) is an enzyme containing two atoms of copper and two atoms of zinc. These heteroelements are of high importance, Cu being involved in its catalytic activity and Zn being involved in the stability of its three-dimensional structure.
Mutants of this metalloenzyme were highlighted in familial cases of a neurodegenerative disease, amyotrophic lateral sclerosis. The etiology of this disease remains unknown; however it is commonly accepted that mutants CuZnSOD present a gain of toxic function. This new function could be linked with defects in the metallation state of mutants CuZnSOD [1-5]. Hence, there is a need of assessing this metallation state.

To fulfill this prospect, we developed a preparative protocol to separate CuZnSOD isoforms using non-denaturing conditions to avoid Cu and Zn loss because these metals are not covalently bound to the protein. We used isoelectric focusing polyacrylamide gel electrophoresis which separates proteins in a pH gradient according to their isoelectric point (pI). We previously performed XANES analysis on these samples, highlighting differences in the Cu oxidation state between isoforms [6]. In this paper, EXAFS analyses were directly performed on gels. Measurements were carried out on BM30b/FAME beamline at the European Synchrotron Radiation Facility (ESRF).

2. Materials and Methods

2.1. Material and Reagents

Human CuZnSOD was purchased from Sigma. Reagents and materials for gel electrophoresis were purchased from BioRad. Metal-free water is provided using a Millipore Synergy 185 UV Ultrapure water system.

2.2. Isoelectric focusing

Lyophilized human CuZnSOD was rehydrated with metal-free water. 17 cm immobilized pH gradients (IPG) gel strips, with a pH range of 3.9-5.1, were used to separate human CuZnSOD isoforms. Gels rehydration and isoelectric focusing were performed as previously published [6].

Prior to EXAFS analysis, strips were cut in a 1.7 cm band centered on the isofrom of pI 4.94 of human CuZnSOD which is the majority expressed isoform. Excess mineral oil was removed using filter paper. Strips were cryofixed in liquid nitrogen and stored in cryotubes placed in a liquid nitrogen container.

2.3. EXAFS measurements

Experiments were performed on the beamline BM30b-FAME at the ESRF. This beamline is specialized in the X-ray Absorption Spectroscopy applied to environmental, material and biological sciences [7]. A double crystal monochromator Si(220) selected the energy of the incident beam, which was calibrated using a Cu foil. The flux of photons at the sample was about $5 \times 10^{12}$ ph.s$^{-1}$ at 12 keV. Measurements were performed in fluorescence mode using a thirty elements germanium solid-state detector placed at 90° respectively to the incident beam. Sample is placed at 45° to the incident beam and the detector.

Absorption spectra were recorded at the Cu and Zn K-edges. Samples were maintained at 5-20K using a flow helium cryostat. At the Zn K-edge, each spectrum was run during 47.4 min. 19 spectra were recorded and averaged to extract EXAFS. At the Cu K-edge, acquisition time of each spectrum was 43.1 min. 13 spectra were recorded and averaged for EXAFS analysis. The spectra shape remained unchanged during the repeated acquisitions, suggesting that there was no oxidation nor reduction phenomenon occurring during the analysis.

3. Results and Discussion

In this paper, we present the results of the EXAFS spectra at the Cu and Zn K-edges of the majoritary expressed isoform of human CuZnSOD, focused at pI 4.94. Spectra backgrounds were substracted using Athena software and data were analyzed using Artemis software, fitted in q space and k$^2$ weighted [8].

The FEFF file [9] used for the fit was provided by the structure 1E9P from the Protein Data Bank. This structure was obtained by crystallographic study of bovine CuZnSOD [10]. The resort to the
structure of a bovine CuZnSOD to fit spectra from human CuZnSOD is approved by the sequence homology of this protein between species [11]. In CuZnSOD, Zn is bound to one aspartate (Asp) and three histidines (His). Cu can be present either in the Cu(II) oxidation state, surrounded by four ligands His and a molecule of water, or in the Cu(I) oxidation state which is surrounded by only three His.

Results are summarized in Figure 1 and Table 1. For the spectrum at Zn K-edge, which has a measurement uncertainty (in k) of 0.00059, we obtained an R-factor of 0.00400. For the spectrum at Cu K-edge, with an uncertainty of 0.00056, we obtained an R factor of 0.00406. The values obtained for ∆E₀ were respectively of 4.48 eV and of 3.55 eV.

Figure 1: EXAFS (left) and Fourier Transforms (right) of the isoform at pI 4.94 of human CuZnSOD (dot-line) and the fit associated (dashes) at the Zn K-edge (upper curves) and at the Cu K-edge (lower curves) (in this case only for the two first shells).

| EXAFS Zn K-edge                  | EXAFS Cu K-edge                  |
|----------------------------------|----------------------------------|
| Single scattering                | Single scattering                |
| Multiple scattering              | Multiple scattering              |
| O (Asp) 1.88 ± 0.067 0.001       | O (water) 2.41 ± 0.024 0.0096     |
| O 2.62 ±                         | N (His) 3.88 ± 0.007 0.0055       |
| (Asp) 1 0.019 0.0065             | C (His) 3.88 ± 0.017 0.0084       |
| N 2.01 ±                         | C (His) 3.88 ± 0.016 0.0084       |
| C 2.88 ± 0.069 0.0064            | [+ C N [+] 3.10 ± 0.0065         |
| C 3.00 ± 0.277 0.0058            | C (His) 3.88 ± 0.017 0.0084       |
| C 3.10 ± 0.277 0.0058            | [+ C N [+] 3.1521 0.0058         |

Table 1: Zinc and Copper K-edge EXAFS parameters of the isoform at pI 4.94 of human CuZnSOD.

At the Zn K-edge, we obtained values in accordance with data previously published on bovine CuZnSOD in solution [12] demonstrating the feasibility of EXAFS analysis on proteins separated on electrophoresis gels.

The data analysis at the Cu K-edge was more difficult due to the presence of a mixed redox state of Cu(II) and Cu(I), previously highlighted using XANES measurements [6]. After several attempts, we succeeded in fitting the two first shells using a variable degenerescence value and mathematical
correlations between distance of the multiple scattering path and distances of the simple scattering paths. The proportion of oxidized form is majoritary (88% for the last fit). The values obtained are also in accordance with data published [12], except for the distance of the water molecule which is known to be labile [12-16].

4. Conclusion
In this paper, we presented the first EXAFS analysis of a metalloprotein separated on an electrophoresis gel. Analyses were performed at the Zn and Cu K-edge on CuZnSOD. Data obtained were in accordance with the literature. Analysis at the Cu K-edge was more complex because of the presence of a mixture of two oxidation states.

In the next experiments, we consider spreading out this fit to the third shell to refine the parameters and applying this protocol of sample preparation and analysis on mutants CuZnSOD. The determination of the metallic site structure of CuZnSOD could highlight structural differences between CuZnSOD isoforms and enlighten processes involved in the toxicity of mutant CuZnSOD in familial cases of amyotrophic lateral sclerosis.

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