Expewriment Report SC3455

Prion diseases are a class of fatal neurodegenerative disorders characterized by brain spongiosis, synaptic degeneration, microglia and astrocytes activation, neuronal loss and altered redox control. These maladies can be sporadic, iatrogenic and genetic. The etiological agent is the prion, a misfolded form of the cellular prion protein, PrP^C. PrP^C interacts with metal ions, in particular copper, through the octarepeat and non-octarepeat binding sites. The physiological implication of this interaction is still unclear, as is the role of metals in the conversion. Since prion diseases present metal dys-homeostasis and increased oxidative stress, we described the copper-binding site located in the human C-terminal domain of PrP – HuPrP(90-231) – both in the wild-type protein and in the protein carrying the pathological mutation Q212P. We used the synchrotron-based X-ray absorption fine structure technique to study the Cu(II) and Cu(I) coordination geometries in the mutant, and we compared them with those obtained using the wild-type protein. By analyzing the extended X-ray absorption fine structure and the X-ray absorption near-edge structure, we highlighted changes in copper coordination induced by the point mutation Q212P in both oxidation states. While in the wild-type protein the copper-binding site has the same structure for both Cu(II) and Cu(I), in the mutant the coordination site changes drastically from the oxidized to the reduced form of the copper ion. Copper-binding sites in the mutant resemble those obtained using peptides, confirming the loss of short- and long-range interactions. These changes probably cause alterations in copper homeostasis and, consequently, in redox control.

Materials and Methods

Protein expression, purification and samples preparation

Freshly transformed overnight culture of *E. coli* BL21 (DE3) cells (Stratagene) was added at 37°C to 2 L of ZYM-5052 auto-induction medium [57] plus ampicillin (100 µg/mL). Cells were grown in a Biostat B plus 2 L vessel (Sartorius) and harvested 18 h after inoculation. Bacterial paste was resuspended in 25 mM Tris-HCl, 5 mM EDTA, 0.8% Triton X-100, 0.4% Deoxycholic acid, 1 mM PMSF, pH 8 and lysed by French press (EmulsiFlex-C3). Inclusion bodies were separated by centrifugation (30 min, 10,000 g at 4°C), rinsed in 25 mM Tris-HCl, 5 mM EDTA, 0.8% TritonX100, pH 8, and several times in bi-distilled water. Pure inclusion bodies were solubilized in 5 volumes of 8 M GndHCl, 100 mM DTT, pH 8 and loaded onto a size-exclusion chromatography column (Superdex 200 26/60, GE). Proteins were eluted with 6 M GndHCl, 25 mM Tris-HCl, 5 mM EDTA, pH 8 at a flow rate of 2ml/min. Eluted proteins were stored at 4°C for 2 weeks for oxidation. Oxidized proteins were purified by reverse-phase (Jupiter C4, 250 mm × 21.2 mm, 300 Å pore size, Phenomenex) and separated using a linear gradient of 0-90% acetonitrile, 0.1% trifluoroacetic acid. Purified proteins were analyzed by SDS-PAGE, western blot and electrospray mass spectrometry, and then lyophilized. Refolding was performed dissolving the proteins in 8 M GndHCl and dialyzing them against 20 mM sodium acetate, pH 5.5 using a Spectrapor-membrane (MW 3000). Protein folding was analyzed by circular dichroism.

Samples with 1:1 Cu(II):HuPrP(90-231)/HuPrP(90-231, Q212P) ratio were prepared in acetate buffer. The Cu(I):HuPrP(90-231)/HuPrP(90-231, Q212P) complexes were generated reducing Cu(II) with 40 mM ascorbate. The metal:protein ratios were confirmed by using atomic absorption spectroscopy.

X-ray absorption measurements

Cu K-edge X-ray absorption spectra of HuPrP(90-231) were collected in fluorescence mode at the BM30B FAME beamline of the European Synchrotron Radiation Facility.[58] A 1.5 mM solution of Cu(II)-HuPrP(90-231) in the presence of 20 mM acetate buffer pH 5.5 was used to collect the cupric form of the protein. Sodium ascorbate was added to the solution under nitrogen atmosphere to chemically collect reduced species. All the spectra were collected at 15 K. The storage ring was running in the two-third filling mode with a typical current of 170 mA. The monochromator was equipped with a Si (111) double crystal, in which the second crystal was elastically bent to a cylindrical cross section. The energy resolution at the Cu K-edge is 0.5 eV. The X-ray photon beam was vertically focused by a Ni-Pt mirror, and dynamically sagittally focused in the horizontal size. An array detector made of 30 Ge elements of very high purity was used. The spectra were calibrated by assigning the first inflection point of the Cu foil spectrum to 8981 eV. For each sample, 8 spectra were recorded with a 7 s/point collection statistic and averaged. The collection time was 20 min for each XANES spectrum and 45 min for each EXAFS spectrum. No spectral changes were detected during the data collection.

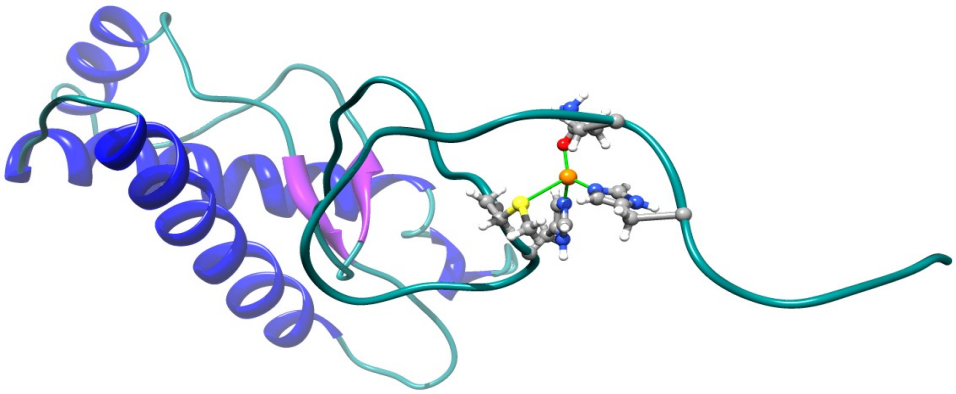

Figure 1. Schematic representation of the copper-HuPrP(90-231) complex. Blue indicates the three α-helices; purple the two β-strands and cyan the unfolded N-terminal part of the globular domain.

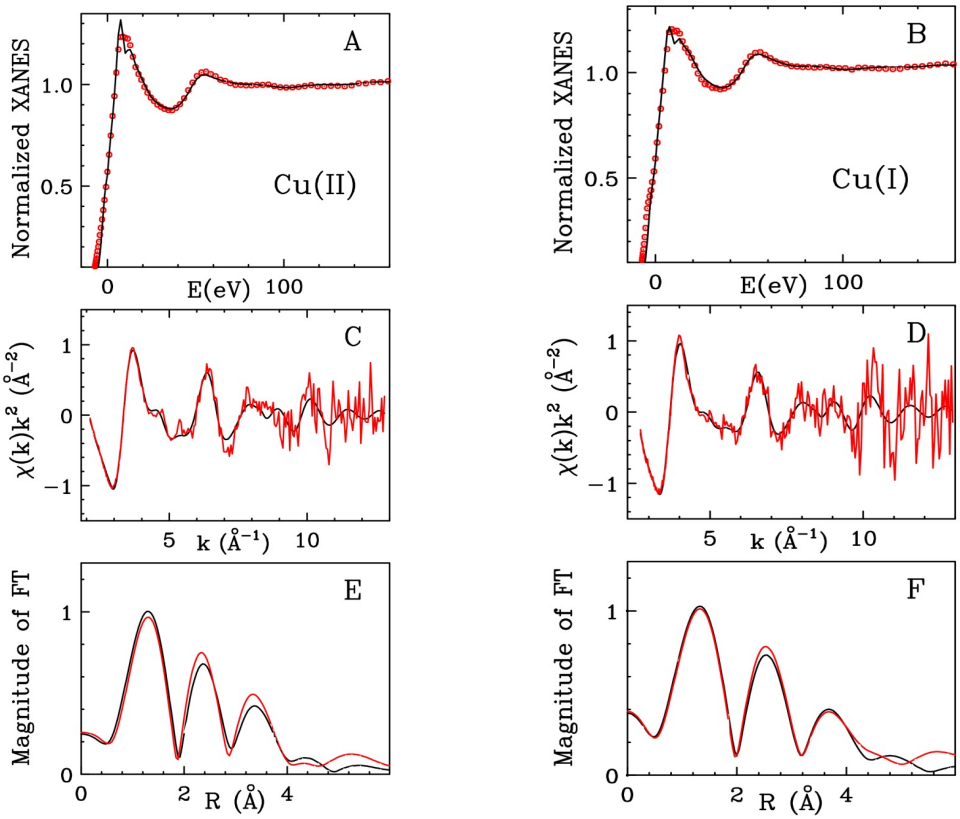

Figure 2. Comparison of the theoretical signal (black line) with experimental data (red line) of Cu K-edge of WT prion protein: XANES data of (a) Cu(II)-HuPrP(90-231) and (b) Cu(I)-HuPrP(90-231); k^2-weighted EXAFS data of (c) Cu(II)-HuPrP(90-231) and (d) Cu(I)-HuPrP(90-231). Non phase-shift-corrected Fourier transforms of the experimental data (red line) and of the total theoretical signal (black line) of (e) Cu(II)-HuPrP(90-231) and (f) Cu(I)-HuPrP(90-231).

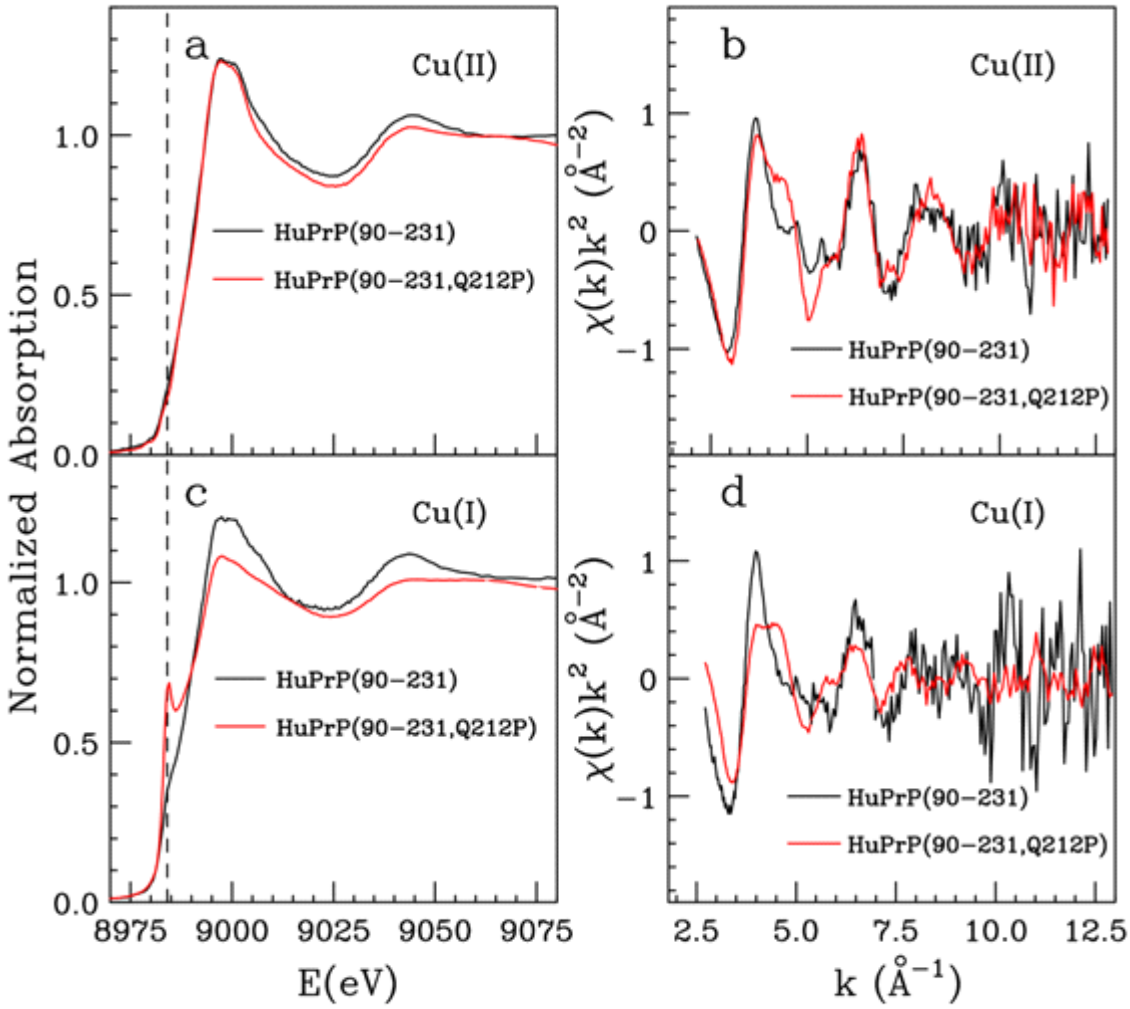

Figure 3. Cu K-edge XANES spectra for (a) Cu(II)-HuPrP(90-231) and Cu(II)-HuPrP(90-231,Q212P) and Cu K-edge k^2-weighted EXAFS data concerning (b) Cu(II)-HuPrP(90-231) and Cu(II)-HuPrP(90-231,Q212P). Cu K-edge XANES spectra for (c) Cu(I)-HuPrP(90-231) and Cu(I)-HuPrP(90-231,Q212P) and Cu K-edge k^2-weighted EXAFS data of (d) Cu(I)-HuPrP(90-231) and Cu(I)-HuPrP(90-231,Q212P).

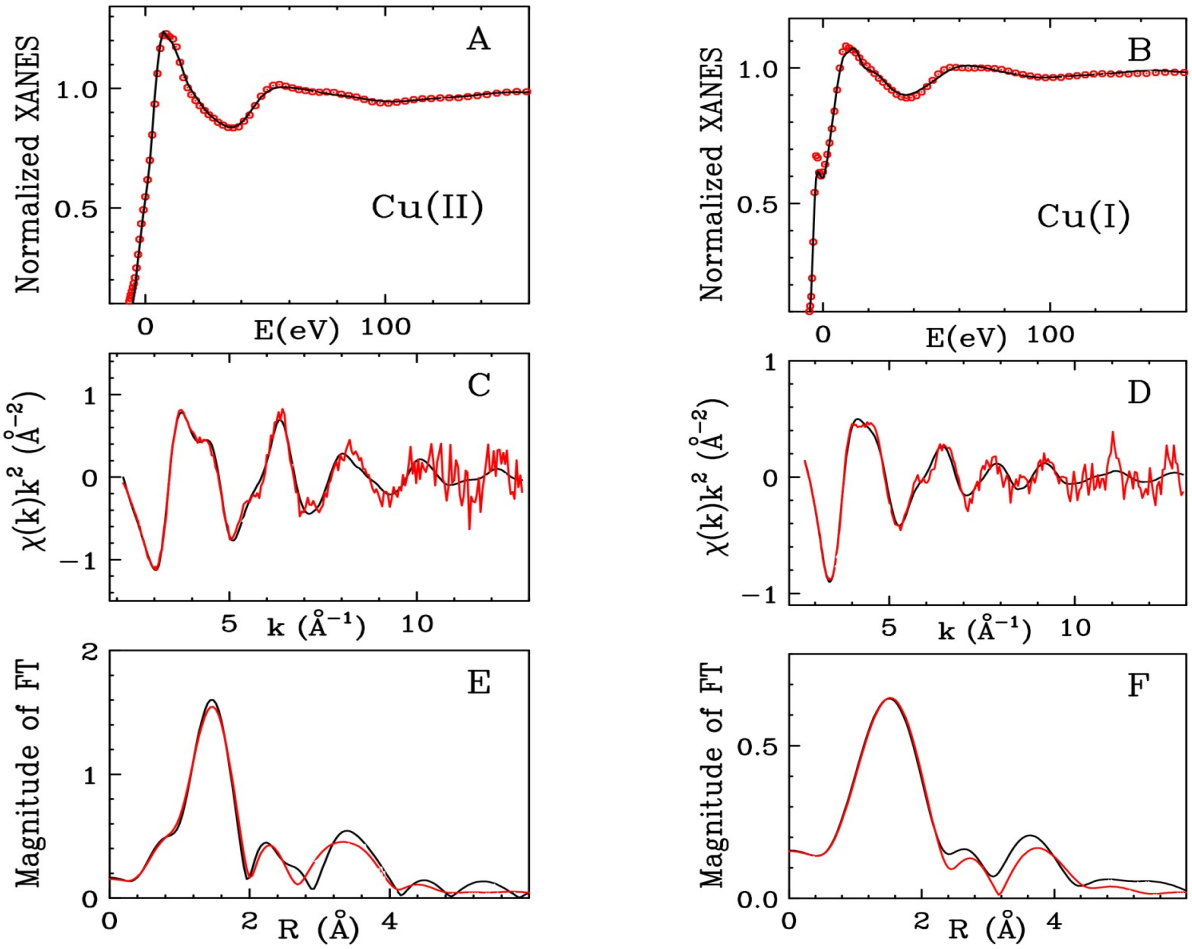

Figure 4. Comparison of the theoretical signal (black line) with experimental data (red line) of Cu K-edge of pathological mutant prion protein: XANES data of (a) Cu(II)-HuPrP(90-231,Q212P) and (b) Cu(I)-HuPrP(90-231,Q212P); k^2-weighted EXAFS data of (c) Cu(II)-HuPrP(90-231,Q212P) and (d) Cu(I)-HuPrP(90-231,Q212P). Non phase-shift-corrected Fourier transforms of the experimental data (red line) and of the total theoretical signal (black line) of (e) Cu(II)-HuPrP(90-231,Q212P) and (f) Cu(I)-HuPrP(90-231,Q212P).

Table 1 Structural parameters derived from the EXAFS analysis of Cu(II)-HuPrP(90-231), Cu(I)-HuPrP(90-231), Cu(II)-HuPrP(90-231, Q212P) and Cu(I)-HuPrP(90-231, Q212P). N is the coordination number, R is the distance and σ^2 is the Debye-Waller factor. Statistical errors are reported in parenthesis.

Cu(II)-HuPrP(90-231)			**Cu(I)-HuPrP(90-231)**			**Cu(II)-HuPrP(90-231,Q212P)**			**Cu(I)-HuPrP(90-231,Q212P)**		
N	R (Å)	σ^2 (Å^2)	N	R (Å)	σ^2 (Å^2)	N	R (Å)	σ^2 (Å^2)	N	R (Å)	σ^2 (Å^2)
2 N_{His}	1.98(2)	0.006(3)	2 N_{His}	1.98(2)	0.006(3)	1 N_{His}	2.00(2)	0.007(3)	1 N_{His}	1.99(2)	0.007(3)
2 O/N	1.98(2)	0.008(3)	2 O/N	1.99(3)	0.009(3)	3 O/N	1.99(2)	0.009(3)	1 O/N	1.99(2)	0.009(3)
1 O	2.31(3)	0.013(4)	1 O	2.32(4)	0.014(4)	1 O	2.40(4)	0.013(4)	1 S	2.28(4)	0.008(3)
1 S	3.25(4)	0.013(4)	1 S	3.26(5)	0.013(4)	1 S	3.47(4)	0.014(4)			

Table 2 Structural parameters derived from the XANES analysis of Cu(II)-HuPrP(90-231), Cu(I)-HuPrP(90-231), Cu(II)-HuPrP(90-231, Q212P) and Cu(I)-HuPrP(90-231, Q212P). N is the coordination number and R is the distance. Statistical errors are reported in parenthesis.

Cu(II)-HuPrP(90-231)		**Cu(I)-HuPrP(90-231)**		**Cu(II)-HuPrP(90-231,Q212P)**		**Cu(I)-HuPrP(90-231,Q212P)**	
N	R (Å)	N	R (Å)	N	R (Å)	N	R (Å)
2 N_{His}	1.92(6)	2 N_{His}	1.91(8)	1 N_{His}	1.91(9)	1 N_{His}	1.91(8)
2 O/N	2.06(4)	2 O/N	2.07(3)	3 O/N	1.98(4)	1 O/N	2.11(3)
1 O	2.26(9)	1 O	2.26(3)	1 O	2.23(9)	1 S	2.19(7)
1 S	3.16(12)	1 S	3.16(12)	1 S	3.14(19)		