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# 3D local structure around copper site of rabbit prion-related protein: Quantitative determination by XANES spectroscopy combined with multiple-scattering calculations

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## HIGHLIGHTS

- The first structure of the metal ion binding site in RaPrP fifth copper-binding site.
- Quantitative determination by XANES spectroscopy combined with *ab initio* calculations.
- Provide a proof of the roles of copper in prion conformation conversions.
- Provide a proof of the molecular mechanisms of prion-involved diseases.

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## ABSTRACT

Prion-related protein (PrP), a cell-surface copper-binding glycoprotein, is considered to be responsible for a number of transmissible spongiform encephalopathies (TSEs). The structural conversion of PrP from the normal cellular isoform ($PrP^{C}$) to the post-translationally modified form ($PrP^{Sc}$) is thought to be relevant to $Cu^{2+}$ binding to histidine residues. Rabbits are one of the few mammalian species that appear to be resistant to TSEs, because of the structural characteristics of the rabbit prion protein ($RaPrP^{C}$) itself. Here we determined the three-dimensional local structure around the C-terminal high-affinity copper-binding sites using X-ray absorption near-edge structure combined with *ab initio* calculations in the framework of the multiple-scattering (MS) theory. Result shows that two amino acid resides, Gln97 and Met108, and two histidine residues, His95 and His110, are involved in binding this copper(II) ion. It might help us understand the roles of copper in prion conformation conversions, and the molecular mechanisms of prion-involved diseases.



## 1. Introduction

Transmissible spongiform encephalopathies (TES), including Kuru and Creutzfeldt–Jakob Disease (CJD) in humans and bovine spongiform encephalopathy (BSE) in cattle, are neurodegenerative diseases caused by prion-related protein (PrP) (Prusiner, 1997). Scrapie prion protein ($PrP^{Sc}$), a disease-associated isoform of normal cellular prion protein ($PrP^{C}$), has been identified as the infectious agent (Prusiner, 1998). $PrP^{Sc}$ is able to convert normal $PrP^{C}$ proteins into the infectious isoform by changing their conformation (Griffith, 1967). As it has been reported (Brown et al., 1997; Stockel et al., 1998), PrP has the high affinity of binding copper(II) ion, and the metal ion induces secondary structural changes destabilizing the native fold of $PrP^{C}$ (Pan et al., 1993; Yunan et al., 2011). A role for

*Abbreviations:* PrP, prion-related protein; TSE, transmissible spongiform encephalopathies; $PrP^{C}$, cellular prion protein; $PrP^{Sc}$, scrapie prion protein; $RaPrP^{C}$, rabbit prion protein; XANES, X-ray absorption near-edge structure; EXAFS, extended X-ray absorption fine structure; MS, multiple scattering; MXAN, Minuit XANES, a software package to reconstruct 3D structures from XANES spectra; XAS, X-ray absorption spectra; MT, muffin-tin; FT, Fourier transform

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$Cu^{2+}$ as a cofactor in prion disease should not be ruled out, indeed, Cu(II) interaction is believed to be the one that has been correlated with physiological impairments linked to the disease (Morante et al., 2004).

Rabbit is one of the few mammalian species reported to be resistant to TSE as a result of the inability of the conformational conversion of rabbit prion (RaPrP) (Wen et al., 2010a, 2010b). It has been pointed out that the conformation stability of RaPrP is mostly due to the structural feature of the RaPrP (Vorberg et al., 2003). However, few researches have been done on copper binding to C-terminal globular region, and even fewer on TSE-resistant species. Thus, interpretation of three-dimensional structure of copper binding sites in RaPrP is very important and necessary, which might obtain the particular structural properties of the protein and provide an essential insight into the molecular mechanism of the immunity to TSE.

Here, we used X-ray absorption near-edge structure (XANES) technique to determine the three-dimensional (3D) local structure of high-affinity copper-binding sites in rabbit recombinant $PrP^{91-228}$ which lacks the octapeptide repeat region in N-terminal. XANES is one of the most suitable tools for reconstructing the local structure around metal binding sites in metalloproteins (Stone et al., 2005; Wu et al., 2005). It has the fingerprints capability and is able to supply atomic level information about coordinated atomic species. We use a package named Minuit XANES (MXAN) to exact information from XANES spectra to reproduce 3D structures (Benfatto et al., 2003). The geometry parameters are determined by fitting the experimental data to the phenomenological model implemented in the MXAN package and many tests on the biological structures have been performed using this novel package (Hayakawa et al., 2004; Benfatto et al., 2004; Zhang et al., 2010).

## 2. Materials and methods

### 2.1. Protein expression and purification

The construct was generated by a standard PCR-based cloning method. The amplified gene fragment coding for $RaPrP^{C}$-(91–228) was inserted into the vector pET30a via NdeI and XhoI, The recombinant proteins were expressed in *Escherichia coli* Bl21(DE3) cells and purified using dialysis. The other details of plasmid construction, protein expression and purification were conducted as described previously (Wen et al., 2010a).

### 2.2. X-ray absorption spectra (XAS) collection

The Cu K-edge X-ray absorption spectra were collected at the X-ray Absorption Fine Structure station (Beam line 14 W) of Shanghai Synchrotron Radiation Facility (SSRF) in the fluorescence mode at room temperature. The storage ring was operating at the typical energy of 3.5 GeV with the current ranging from 200 to 300 mA during a time span of 12 h. Energy calibration was carried out with a copper foil. A Si [311] double crystal monochromator and a focusing mirror with a cut-off energy of 22.5 keV were used throughout the experiment. Experiments were performed on samples in solution kept in a 1.2-mm-thick Teflon spacer cell sealed by Kapton films. The incident beam intensity was monitored using ionization chamber filled by 25% argon-doped nitrogen mixture and the fluorescence signal was collected by a fluorescence ionization chamber filled with argon gas.

The sample contained about 1.89 mM $RaPrP^{C90-228}$ fragment in 20 mM HAc/NaAc buffer with pH 5.5 and the copper concentration was kept sub-stoichiometric to certify that binding occurred at a single high-affinity site, with the ratio $Cu^{2+}$:$PrP^{C}$=0.8:1. A blank sample with 2 mM $CuAc_2$ was measured for contrast. To ensure the liability of the experiment, each sample was measured twice in sequence in the same experimental conditions.

### 2.3. XANES data analysis

We use the package MXAN to extract the geometrical information around the metal site from XANES spectrum. The software is based on the accurate analysis of multiple scattering (MS) events and fits the reconstructed spectrum from *ab initio* calculation to the experiment data by changing relevant geometrical parameters of the atom sites around the absorbing atom.

The X-ray photo-absorption cross-section is calculated using the full multiple scattering scheme in the framework of the muffin-tin (MT) approximation for the shape of the potential (Wu et al., 1996). In this case, the exchange and correlation part of the potential are determined on the basis of the local density approximation of the self energy. This method is based on the evaluation of the scattering path operator and its effectiveness has been successfully tested over the years in a wide number of different applications. The MT radii were chosen according to the Norman criterion. The convergent atomic cluster contains 40 atoms from 4 amino acid residues within 7 Å from copper ion, and the spectrum is convergent within this cluster.

### 2.4. EXAFS data check

As there is no crystal structure, the initial structure we used for XANES calculation might not be reasonable. In that case, the final conclusion may not be so convincing. So, we used information obtained from the MXAN fitting as initial model for EXAFS analysis to check the result. The *k*-weighted Cu EXAFS spectra $\chi(k)$ were refined with the software Artemis, using a least-squares refinement algorithm. The signal noise ratio of EXAFS spectrum is not so good in reason of low sample concentration; as a result, we just fit atoms less than 3 Å from copper ion.

## 3. Results and discussions

### 3.1. X-ray absorption spectroscopy analysis

In Fig. 1a, we compared the Cu K-edge XAS spectra of $CuAc_2$ (black curve) and $RaPrP^{90-238}$ (red curve). The Fourier transforms (FT) are also shown (Fig. 1b). They have the same absorption edge position, which stands for the same oxidation state. The shape of two spectra present significant differences, suggesting the different arrangements of scattering atoms around copper ions in $CuAc_2$ and RaPrP protein. Compared to $CuAc_2$, data of the RaPrP sample exhibits a longer distance between atoms of the nearest coordination shell and the photo-absorber. Klewpatinond et al. (2008) reported that His95 and His110, called the fifth binding site, have the highest binding affinity to copper ions, and taking into account our experimental condition, the $Cu^{2+}$ ions probably bind this site. The broad weak peaks between 3 and 4 Å of the FT (Fig. 1b) support the presence of imidazole rings (Hasnain et al., 2001; Morante et al., 2004). Compared with the spectrum of human PrP reported by Hasnain et al. (2001), there are some resemblances in the phase and amplitude. So the first coordination shell atoms may be the same, and a Cu–S bond is considered. Thus, it can be predicted the $Cu^{2+}$ binding site in RaPrP contains His95, His110, Met108 as well as one oxygen-donating residue close to His95, which might be Glu97.

### 3.2. MXAN calculations and EXAFS check

The choice of the initial geometry around the absorber is a crucial point of MXAN analysis. Due to the flexible nature of the N-terminal of RaPrP and the paramagnetic nature of $Cu^{2+}$, there is no crystallographic or NMR structures of the loop region in $RaPrP^{90-121}$. Lacking high resolution data for $RaPrP^{90-121}$, the only indications about possible metal binding sites come from the knowledge of the peptide amino acidic sequence and considerations above summarized. Among the many structures we considered, the theory spectrum of a penta-coordinate model had the best agreement with experimental spectrum, which contained two histidine residues, one methionine residue, one glutamic acid residue and one water molecule.

Fig. 2a shows the best fit (red circles) of the experimental XANES spectra (black curve) of Cu–$RaPrP^{90-228}$. As we can see, the result shows an excellent agreement with experimental data, it provides a good model with the right positions of coordination atoms. The main refined critical distances of the best fit are summarized in Table 1. The structure obtained by data of the best fit is shown in Fig. 2b.

Fig. 3 shows the results of EXAFS fit. It is a great agreement in the window, that is between 1 Å and 3 Å. The EXAFS check prove that the model provided by MXAN is correct.

### 3.3. Structure analysis

This manuscript presents the first 3D structure of copper binding sites in the loop region of $RaPrP^{C}$ and an additional demonstration of the relevance of MXAN calculations for geometrical characterization of metalloproteins where high resolution crystallographic data or NMR structures are missing. Compared with the human PrP (Hasnain et al., 2001), both coordination of amino acid residues and bonds are substantially different. The bond lengths of RaPrP are much larger, so the interaction between copper ion and protein may be weaker. The conformation change is

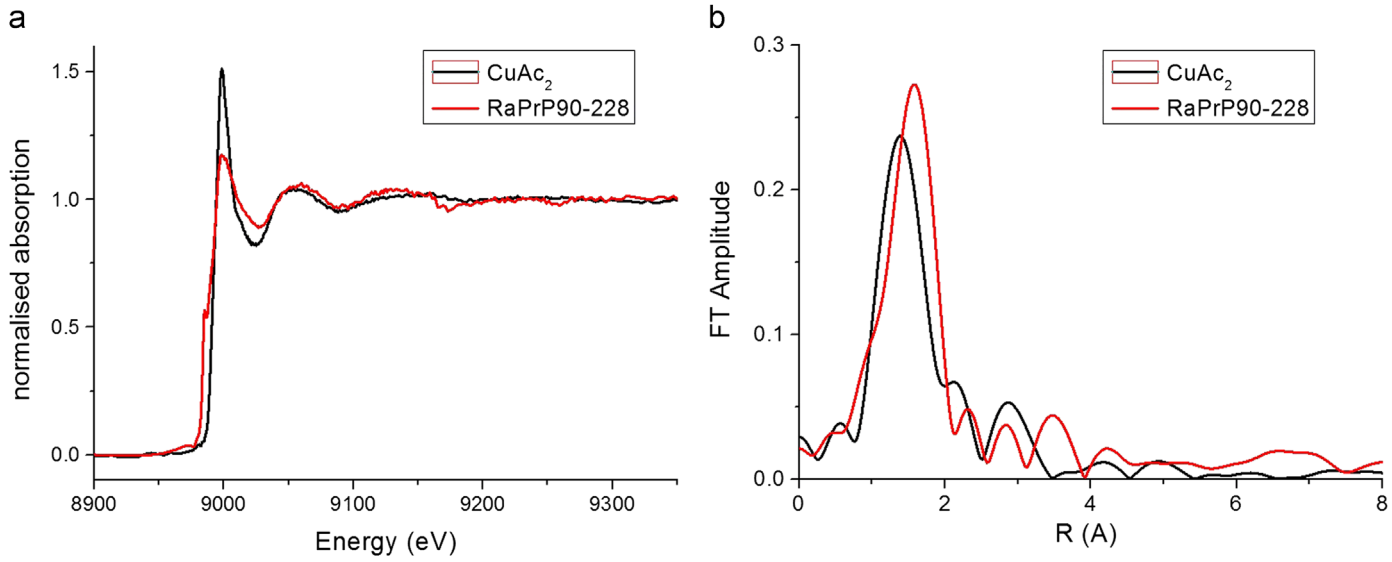


**Fig. 1.** Cu K-edge X-ray absorption data (a) and the Fourier transforms (b) for $RaPrP^{90-228}$ (red curve) compared with $CuAc_2$ (black curve). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

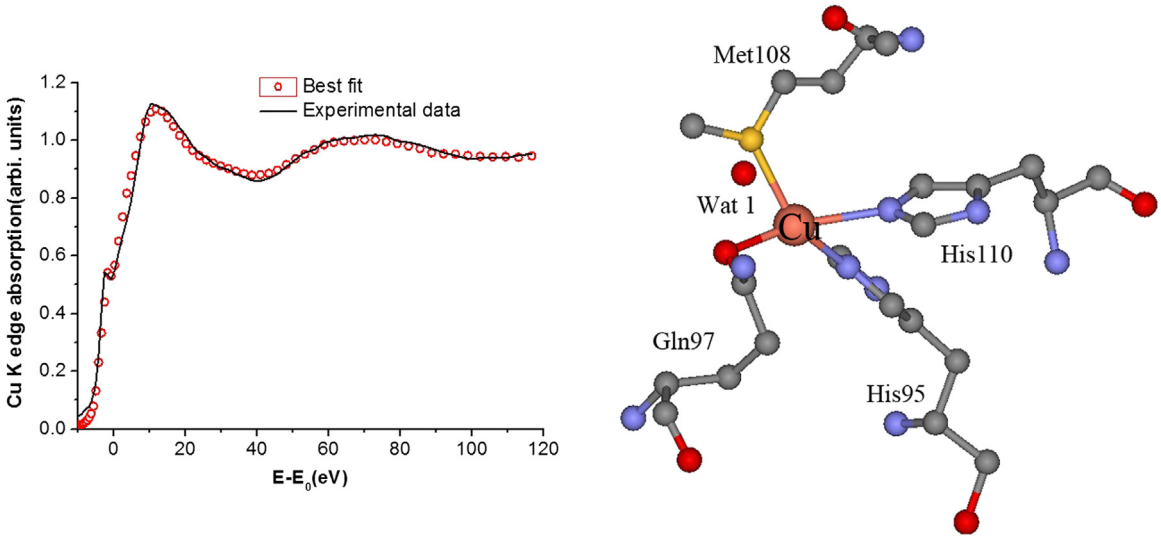


**Fig. 2.** (a) Best fit of the Cu K-edge XANES spectrum (red dots) compared with experimental spectrum (black line). (b) The coordination of the copper ion (pink ball). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Table 1**
The distance between Cu ion and first coordination atoms.

| Atom pairs | Cu–N(His95) | Cu–N(His110) | Cu–O(Glu97) | Cu–S(Met108) | Cu–O(Wat1) |
|---|---|---|---|---|---|
| Distance (Å) | $2.05 \pm 0.02$ | $2.25 \pm 0.02$ | $1.91 \pm 0.03$ | $2.27 \pm 0.03$ | $3.15 \pm 0.06$ |

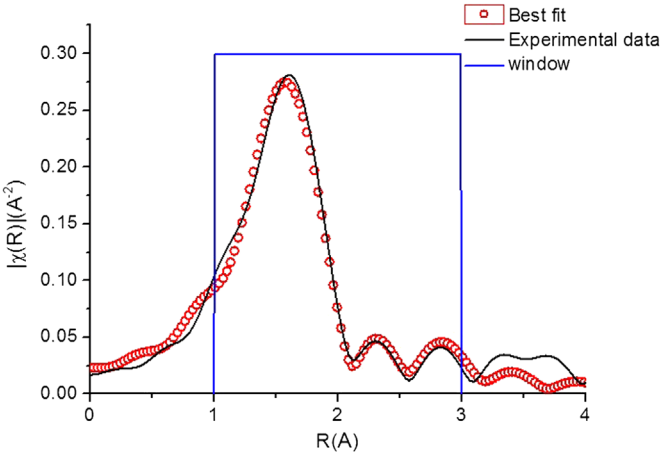


**Fig. 3.** Best fit of the Cu K-edge EXAFS spectrum (red dots) compared with experimental spectrum (black line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

much less in RaPrP after binding copper. It may provide valuable hints for understanding the inability of $RaPrP^{C}$ to form $PrP^{Sc}$, and give a structural proof of the detailed molecular mechanism of the conformation conversion for prion proteins.

## 4. Conclusion

In this work, we investigated the structure of the copper binding site at $RaPrP^{90-228}$ by XANES spectroscopy combined with *ab initio* full multiple scattering calculations. An approximate model and the 3D structure of the Cu ion binding site in the $RaPrP^{C}$ loop region are presented. This is the first structural model of the metal ion binding site in the PrP fifth copper-binding site of TSE-resistant mammalian species. Results may provide a structural basis for the TSE-resistant mechanism at the atomic level. The work is also a new effective way to explore geometrical structures of metal ion binding sites in system where it is not possible to determine the structure with crystallographic or NMR methods.

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