Comparative Analysis of the Human and Chicken Prion Protein Copper Binding Regions at pH 6.5*§

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Recent experimental evidence supports the hypothesis that prion proteins (PrPs) are involved in the Cu(II) metabolism. Moreover, the copper binding region has been implicated in transmissible spongiform encephalopathies, which are caused by the infectious isoform of prion proteins (PrPSc). In contrast to mammalian PrP, avian prion proteins have a considerably different N-terminal copper binding region and, most interestingly, are not able to undergo the conversion process into an infectious isoform. Therefore, we applied x-ray absorption spectroscopy to analyze in detail the Cu(II) geometry of selected synthetic human PrP Cu(II) octapeptide complexes in comparison with the corresponding chicken PrP hexapeptide complexes at pH 6.5, which mimics the conditions in the endocytic compartments of neuronal cells. Our results revealed that structure and coordination of the human PrP copper binding sites are highly conserved in the pH 6.5–7.4 range, indicating that the reported pH dependence of copper binding to PrP becomes significant at lower pH values. Furthermore, the different chicken PrP hexarepeat motifs display homologous Cu(II) coordination at sub-stoichiometric copper concentrations. Regarding the fully cation-saturated prion proteins, however, a reduced copper coordination capability is supposed for the chicken prion protein based on the observation that chicken PrP is not able to form an intra-repeat Cu(II) binding site. These results provide new insights into the prion protein structure-function relationship and the conversion process of PrP.

A group of lethal neurodegenerative disorders known as transmissible spongiform encephalopathies are related to prion proteins (PrPs),1 which are synaptic glycosyl-phosphatidylinositol-anchored surface glycoproteins (1, 2) that are expressed mainly in the central nervous system (3, 4). Prion diseases are apparently caused by conversion of the monomeric 28-kDa cellular prion protein (PrPC) into a pathogenic conformational isoform, usually denoted as the scrapie form, PrPSc (5, 6). These abnormally folded proteins accumulate into highly protease-resistant aggregates (7) that act as a template for the transition of native PrP (8). Despite numerous efforts to elucidate the physiological role of the cellular prion protein, its function remains enigmatic. However, the binding of copper to mammalian PrPSc in vivo and in vitro suggests an involvement in copper homeostasis and metabolism (9–11) comprising contributions to endocytic transport mechanisms (12) as well as neuroprotective pathways (13, 14). Furthermore, the close association of Cu(II) with prion diseases (15–17) emphasizes the need for detailed structural characterization of both PrP isoforms.

The overall structure of recombinant mammalian prion proteins, consisting of a globular C-terminal domain (residues 121 to 231) and a disordered N-terminal tail (residues 23 to 120), are established mainly by NMR investigations (18–21) as well as by two x-ray diffraction studies (22, 23). A total of five high affinity copper binding motifs of PrPC were identified, one at the beginning of the C-terminal domain centered at residues His-96 and His-111 (24, 25) and four within the His-containing sequence P[PHGGWGQ] that is repeated four times between residues 60 and 91 (9, 10, 26–31). This N-terminal octapeptide repeat region, which is highly conserved in mammals (32, 33), cooperatively binds up to four Cu(II) ions with dissociation constants in the nanomolar to micromolar range, reflecting a significant pH-dependence (9, 26, 31, 34).

Despite the large number of studies, detailed structural models of Cu(II) binding are still discussed. Recently, Morante et al. (35) suggested that the coordination shell of Cu(II) varies depending on the occupancy of the available metal sites. This x-ray absorption spectroscopy analysis of bovine octapeptide complexes linked the two common copper binding models, which differ mainly in the number of coordinated imidazole side chains (11,
Consequently, a conversion of the inter-repeat binding mode of Cu(II) into an intra-repeat site geometry is proposed once the copper concentration increases (35). Despite the low sequence identity of ~44% (32), the essential features of mammalian prion proteins are also conserved in chicken PrP (36). Homologous Cu(II) binding capability was identified for the tandem hexapeptide (PHPNGY) repeats located between residues 53 and 94 (31, 34, 37). To date, the particular interest in analyzing and understanding the exact Cu(II) coordination geometry of avian PrP is driven by the fact that prion diseases have, up to now, not been observed for birds (38, 39).

In this study, we present a comparative x-ray absorption spectroscopy analysis of Cu(II) coordination to the corresponding repeat sequences of both chicken and human PrP at pH 6.5. According to the putative involvement of PrP in copper homeostasis, this slightly acidic pH value mimics conditions close to the endocytic compartments of neuronal cells, where Cu(II) might be released (9, 16, 40). The results we obtained revealed that the structure and the coordination of the copper binding sites of human PrP are highly conserved between pH 6.5 and pH 7.4, indicating that the reported pH dependence of Cu(II) binding becomes significant at lower pH values. Although chicken PrP has a considerably different copper binding region, the hexarepeat motifs display a homologous Cu(II) coordination at sub-stoichiometric copper concentrations. In contrast, a reduced copper binding ability is proposed for chicken PrP in the fully cation-saturated state, supporting the hypothesis that prion diseases have, up to now, not been observed for birds (38, 39).

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**MATERIALS AND METHODS**

**Peptide Synthesis and Purification**—Synthetic peptides representing various fragments of the repeat region of human and chicken prion proteins were synthesized with a Biotronik ECOSYN P solid phase automated peptide synthesizer (Eppendorf, Hamburg, Germany) using the Fmoc (9-fluorenyl-methoxycarbonyl) method. After elution from the resin and deprotection, the crude peptides were further purified by reverse phase high performance liquid chromatography and assayed for mass and purity (>98%) by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) analysis, amino acid analyses, and analytical high performance liquid chromatography. To mimic the peptides within the full-length PrP, all peptides were blocked by N-acetyl and amide groups at the N terminus and the C terminus, respectively.

**Preparation of Cu(II) Complexes of Synthetic Peptides**—For the x-ray absorption spectroscopy measurements, peptide-Cu(II) complexes were prepared by adding an appropriate amount of CuCl₂ stock aqueous solution to the synthetic peptides (7.4 mM) in 10 mM MES buffer, pH 6.5. The copper concentration was kept sub-stoichiometric to ensure that all Cu(II) ions were complexed with the ligand. The final solutions were centrifuged, transferred to polyamide foil-covered sample holders of 1-mm thickness, and placed into liquid nitrogen immediately before the measurement. The number of Cu(II) ions that were coordinated to the peptides was analyzed by linear flight MALDI-TOF mass spectrometry using a sinapic acid matrix and a Bruker Autospec mass spectrometer. Subsequent to the incubation with Cu(II), the corresponding samples were reacted with a 5-fold molar excess of diethyl pyrocarbonate at room temperature for 30 min to flag the histidine residues that were not bound to Cu(II) ions (41). The peptide concentrations were determined using extinction coefficients at 280 nm, which were calculated using 5690 M⁻¹ cm⁻¹ multiplied by the number of Tyr residues and 1280 M⁻¹ cm⁻¹ multiplied by the number of Tyr residues, respectively (42).

**RESULTS**

We have applied x-ray absorption spectroscopy, in particular EXAFS techniques, to analyze in detail the Cu(II) binding geometry and coordination for mammalian and chicken PrP. Our intention was to obtain three-dimensional information to unravel the functional differences of aChPrP-(24–249) and aHuPrP-(23–231) that are potentially associated with the N-terminal copper binding region. Because of the extremely low solubility of the full-length prion proteins at pH 6.5, in particular in complex with Cu(II), we have analyzed synthetic peptides resembling 1–4 tandem copies of the avian hexarepeat and the human octarepeat, respectively (Table 1). To mimic these sequences within the full-length proteins, all peptides were blocked at the N terminus by N-acetyl and at the C terminus by amide groups. The copper concentration was kept slightly sub-stoichiometric because of a limitation of x-ray absorption spectroscopy, which requires full saturation of the observable Cu(II) by the ligand to avoid heterogenic spectra caused by free metal ions.

The obtained x-ray absorption near-end structure pattern showed a high similarity for all ChPrP and HuPrP peptide Cu(II) complexes (Fig. 1). The almost identical edge energies of 8990 eV resemble the electronic transitions characteristic of Cu(II) ions (45), indicating an identical Cu(II) oxidation state at pH 6.5. The shapes of the copper K-edges of human and chicken complexes appeared to be almost superimposable. However, a

| Peptide | Peptide sequence | Cu(II) sites | Cu(II)/PrP | Cu(II)/repeat |
|---------|-----------------|-------------|------------|--------------|
| ChPrP-(53–58) | Ac-PHNPGY-NH₃ | 1 | 0.5 | 0.5 |
| ChPrP-(53–64) | Ac-PHPNGY₁-NH₃ | 2 | 1.5 | 0.75 |
| ChPrP-(53–70) | Ac-PHPNGY₁-NH₃ | 3 | 1.5 | 0.5 |
| ChPrP-(53–76) | Ac-PHPNGY₂-NH₃ | 4 | 2.0 | 0.5 |
| ChPrP-(60–67) | Ac-PHPGGWGQ₂-NH₃ | 1 | 0.5 | 0.5 |
| ChPrP-(60–83) | Ac-PHPGGWGQ₂-NH₃ | 3 | 1.5 | 0.5 |
| ChPrP-(60–91) | Ac-PHPGGWGQ₂-NH₃ | 4 | 2.0 | 0.5 |
Cu(II) ChPrP-(24–249). Displayed traces correspond to the following complexes: a, Cu(II) HuPrP-(60–67); b, Cu(II) HuPrP-(60–83); c, Cu(II) HuPrP-(60–91); d, Cu(II) ChPrP-(53–58); e, Cu(II) ChPrP-(53–64); f, Cu(II) ChPrP-(53–70); g, Cu(II) ChPrP-(53–76).

A visual analysis of the extracted experimental k³-weighted EXAFS spectra and their corresponding Fourier transforms confirmed the existence of three groups of Cu(II) complexes (Figs. 2 and 3). The different shapes of the dominating low frequency signal at 4.5 Å⁻¹ in the EXAFS spectra, the different amplitudes of the outer shell peaks at 3.0 and 4.2 Å in the Fourier transforms, and the characteristic unsteady side of the EXAFS peak at 6.5 Å⁻¹ (Figs. 2A and 3A) strongly indicate the presence of histidine ligands in the primary coordination sphere of the copper ions (24, 45, 46).

Cu(II) Site Geometry in the Human Octarepeat Complexes at pH 6.5—As observed for the x-ray absorption near-end structure pattern, the EXAFS spectrum of Cu(II) HuPrP-(60–67) also differs from the spectra obtained for Cu(II) HuPrP-(60–83) and Cu(II) HuPrP-(60–91). This difference is evidenced by small peaks observed in the low frequency region of the corresponding difference spectra, which contain signals caused by the nearest neighbor interactions (Fig. 4, traces a-b and a-c). However, the spectra of the human Cu(II) peptide complexes containing three and four octarepeats are almost superimposable, suggesting a comparable Cu(II) coordination for the octarepeat peptides.

For an initial calculation, the experimental EXAFS spectrum of the Cu(II) HuPrP-(60–67) complex was fitted on the basis of the already known coordination of Cu(II) in complex with mammalian PrP octarepeats at pH 7.4 (11, 35). As a result, the initial models were composed of one to four imidazole groups as well as an appropriate moiety of oxygen/nitrogen ligands to match the typical coordination numbers of Cu(II) ranging from four to six. Based on maximum agreement, constrained refinement indicated that the first coordination shell of Cu(II) in complex with HuPrP-(60–67) at pH 6.5 is formed by one nitrogen atom assigned to an imidazole group and three other light single-bonded nitrogen or oxygen atoms (Fig. 2 and Table II). The consistent distance values of 1.97 ± 0.01 Å are as expected for a Cu(II)-nitrogen coordination (47). Further refinement of the oxygen/nitrogen magnitude is prevented by the high similarity of the nitrogen and oxygen backscattering amplitude as...
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Table II

| Cu(II) complexes | Ligands | N | r  | 2σ(λ0) | ΔE_F | F² | N_HIS |
|------------------|---------|---|----|---------|------|----|-------|
| ChPrP-(53–58)    | Cu-N/O  | 4 | 1.98(1) | 0.005(2) | -8(1) | 34.4 | 2.0(3) |
| ChPrP-(53–64)    | Cu-N/O  | 4 | 1.97(1) | 0.008(2) | -8(1) | 21.0 | 2.0(2) |
| ChPrP-(53–70)    | Cu-N/O  | 4 | 1.97(1) | 0.007(1) | -8(1) | 30.9 | 2.3(4) |
| ChPrP-(53–76)    | Cu-N/O  | 4 | 1.98(1) | 0.007(3) | -8(2) | 24.4 | 2.1(2) |
| HuPrP-(60–67)    | Cu-N/O  | 4 | 1.97(1) | 0.008(2) | -9(1) | 28.1 | 1.3(3) |
| HuPrP-(60–83)    | Cu-N/O  | 4 | 1.98(1) | 0.006(1) | -9(2) | 32.1 | 2.1(2) |
| HuPrP-(60–91)    | Cu-N/O  | 4 | 1.96(2) | 0.005(3) | -8(1) | 25.9 | 1.8(2) |

* The R-factor reflects the goodness of the fit.
* Constrained to have the same value during refinement for similar atom types.

The optimal R-factor of 0.28 increased significantly if the coordination number was modified further, suggesting a four-atom copper coordination for the single octarepeat peptide.

Utilizing the parameters calculated for the monorepeat, a comparable but slightly different tetragonal Cu(II) coordination was proposed by fitting the EXAFS signals of the Cu(II) HuPrP-(60–83) and Cu(II) HuPrP-(60–91) complexes independently. The data obtained from these complexes, which contain a higher number of octarepeat copies, support the coordination of two imidazole groups to a single Cu(II) ion. Thereby, the amount of oxygen/nitrogen ligands is reduced without affecting the coordination number (Fig. 2). The refined distance values are summarized in Table II and are in the expected range. The marginal variation of the corresponding R-factors is caused by minor differences in the signal-to-noise ratios of the detected spectra.

In addition, the assumption of an additional Cu(II) coordination by a remote oxygen ligand (e.g., from a water molecule) did not result in a significant decrease of the associated R-factors for all human octarepeat Cu(II) complexes (see supplemental data in the on-line version of this article). The exceptionally high Debye-Waller factors obtained for these scenarios indicate that such coordination is unlikely. However, it cannot be fully rejected.

**Cu(II) Site Geometry in the Chicken Hexarepeat Complexes at pH 6.5**—In contrast to the human Cu(II)-complexes, the detailed analysis of the EXAFS difference spectra of Cu(II) ChPrP-(53–58), Cu(II) ChPrP-(53–64), Cu(II) ChPrP-(53–70), and Cu(II) ChPrP-(53–76) suggested an almost identical Cu(II) coordination sphere among the chicken repeat complexes at pH 6.5 (Fig. 4, traces d-e, d-f, and d-g). On the other hand, comparison with spectra of the human mono-octarepeat and tetra-octapeptide Cu(II) complexes showed significant differences in the low frequency region, where the imidazole groups mainly contribute (Fig. 4, traces a-d, c-g). The increased amplitude of the outer shell peaks observed in the corresponding Fourier transform spectra are, most probably, connected with changes in the magnitude and orientation of coordinated histidine side chains (46).

The EXAFS data of the Cu(II) ChPrP-(53–58) complex were initially fitted using the geometric parameters previously determined for the human mono-octarepeat as a starting model. The best results were again obtained considering a tetragonal assembly of ligands at a distance of 1.98 ± 0.01 Å. For this configuration, the R-factor improved significantly after replacing one light oxygen/nitrogen atom from the first shell by contributions of an additional imidazole group. Therefore, the postulated coordination sphere contains two imidazole groups and two light oxygen/nitrogen atoms (Fig. 3 and Table II). Considering the experimental standard deviations, this model is appropriate for all Cu(II) chicken hexarepeat complexes. Consequently, almost identical Cu(II) binding geometry and coordination were expected for all chicken hexarepeats. Again, the coordination of an additional remote axial oxygen ligand appears to be rather unlikely, although it cannot be fully excluded for chicken complexes at pH 6.5 as well (see supplemental data in the on-line version of this article).

**DISCUSSION**

Our results suggest that the structure of Cu(II) binding to the selected human octarepeat peptides differs only in the amount of involved histidine residues, depending on the number of repeat copies present within a single peptide. This is indicated by the EXAFS data and by the qualitative analysis of the corresponding X-ray absorption near-edge structure pattern. Moreover, the essential mode of copper binding to the octarepeats of human PrP® at pH 6.5 was found to be highly conserved regarding the already established models proposed for a pH of 7.4 (27, 34, 35). This suggestion is initially supported by the fundamental agreement of the simulated solution structure of the Cu(II)-HuPrP-(60–67) complex (Fig. 5A) containing the mono-octarepeat sequence with the crystallographic model of the Cu(II)-HGGGW complex determined at physiological pH conditions (11). Both structures consistently indicate an intra-repeat Cu(II) motif that involves the equatorial coordination of a single His residue as well as three nitrogen/oxygen ligands provided by the peptide backbone. Taking into account the sterical constraints caused by the planarity of the peptide chain, the nitrogen/oxygen-atoms observed by EXAFS most likely correspond to the ligands determined in the crystallographic structure (11). Consequently, the coordination pattern of the Cu(II) ion was apparently not affected by the slightly acidified conditions, indicating that the reported pH dependence of copper binding to PrP® (28, 30) becomes significant at pH values below 6.5.

On the other hand, a conversion of the intra-repeat to an inter-repeat Cu(II) site was supposed within the Cu(II) complexes of human peptides containing three and four octarepeats. Considering the sub-stoichiometric copper concentration, this observation is again consistent with a model of cooperative Cu(II)-binding proposed at pH 7.4 (35). The comparable inter-repeat site geometry is featured by the direct engagement of two His residues in equatorial positions, whereas a tetragonal coordination sphere is most likely retained (Fig. 5B). According to previous investigations, which support the similarity of Cu(II) binding to peptides containing four octarepeats as well as to PrP®C, the observed inter-repeat...
coordination geometry is presumably also applicable to the full-length prion protein (30, 33, 35).

Furthermore, we have to point out that the majority of studies report a penta-coordination of Cu(II) in the intra- as well as in the inter-repeat geometry, considering the presence of an additional axially bound solvent water molecule at a distance of ∼2.4 Å (11, 35). Because of the relatively low contributions of an outer shell ligand to the EXAFS signal, we cannot exclude a penta-coordination geometry of the Cu(II) ions via solvent. In fact, the presence of an additional remote water ligand does not alter the proposed structural models significantly. However, based on the present refinement data, the coordination of further remote oxygen ligands appears to be rather unlikely.

Initially, these restraints are in agreement with our reported results. A Cu(II) binding geometry close to the intra-repeat motif of the human mono-octarepeat was not supported by our investigations. On the contrary, all Cu(II) complexes of the chicken hexarepeats consistently showed an almost identical tetragonal Cu(II) coordination apparently involving two histidine residues to bind one copper ion. Based on the similar x-ray absorption near-end structure spectra, we suggest that this Cu(II) coordination is almost comparable with the inter-repeat binding site of the human peptides, considering the modification that the required amide nitrogen atoms are most probably provided by the adjacent asparagine residues. The coordination of other ligands, including the phenolate oxygen atom of tyrosine residues to bind one copper ion. Based on the similar x-ray absorption near-end structure spectra, we suggest that this Cu(II) coordination is almost comparable with the inter-repeat binding site of the human peptides, considering the modification that the required amide nitrogen atoms are most probably provided by the adjacent asparagine residues. The coordination of other ligands, including the phenolate oxygen atom of tyrosine, is unfavorable because of steric constraints. The proposed model for Cu(II) binding (Fig. 5C), which can be both intramolecular and intermolecular in the full-length chicken PrP, is also supported by circular dichroism experiments (34).

In conclusion, considering the pH of 6.5 as well as the sub-stoichiometric copper concentrations, we propose almost identical coordination spheres for Cu(II) ions bound to human and chicken prion peptides containing more than two repeat copies, which is consistent with the model of Cu(II) sites proposed for human PrP at a pH of 7.4 (35). This remarkable result is most likely depending on the available copper concentration. With regard to human PrP, a cooperative Cu(II) binding process is observed at pH 7.4 (11, 28, 34), which is reflected by the formation of a high number of intra-repeat sites once the copper content is increased up to full site occupancy (35). This characteristic feature is potentially preserved together with the overall ligand geometry in human PrP at pH 6.5. However, intra-repeat Cu(II) binding modes are assumed to be prevented in chicken PrP because of the additional proline residue within the hexarepeat sequence. Consequently, the copper coordination is supposed to differ significantly between the fully cation-loaded human and chicken PrPs.

The analysis of the prion copper binding sites provides a structural basis for a putative pH-dependent molecular switch that governs the uptake and release of Cu(II) within the proposed copper metabolism (9–11). Our results evolved at a pH of 6.5, which is close to conditions in the endocytic compartments of neuronal cells where the Cu(II) is potentially released, closing the gap between the detailed structural models determined at pH 7.4 and the studies performed at pH 6.0 (48). In addition, the reduced ability of copper binding suggested for the chicken PrP at high Cu(II) concentrations in the environments of membrane rafts is highly indicative of different physiological functions of human and chicken prion proteins. Furthermore, the absence of prion diseases in chicken, which are shown to be closely associated with changes of the copper content within the neuronal cells (15–17), appears to be related to the differences in the Cu(II) coordination between human and avian prion proteins.

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