Copper and the structural biology of the prion protein

John H. Viles1, Mark Klewpatinond and Rebecca C. Nadal
School of Biological and Chemical Sciences, Queen Mary, University of London, Mile End Road, London E1 4NS, U.K.

Abstract
PrP (prion-related protein) is a cell-surface Cu2+ -binding glycoprotein which, when misfolded, is responsible for a number of transmissible spongiform encephalopathies. The co-ordination geometry, stoichiometry and affinity of Cu2+ for PrP are the subject of much debate. In the present paper, we review the recent progress we have made in these areas. As many as six Cu2+ ions bind to PrP with submicromolar affinity. Initially, two Cu2+ ions bind to full-length PrP in the amyloidogenic region, between the octarepeats and the structured domain, at His95 and His110. Only subsequent Cu2+ ions bind to single histidine residues within the octarepeat region. Competitive chelators have been used to determine the affinity of the first molar equivalent of Cu2+ bound to full-length PrP; this approach places the affinity in the nanomolar range. The affinity and number of Cu2+-binding sites support the suggestion that PrP could act as an antioxidant by binding potentially harmful Cu2+ ions and sacrificially quenching of free radicals generated as a result of copper redox cycling. Finally, the effect of Cu2+ on the prion structure and misassembly into oligomers and fibres is discussed.

Introduction
Metal imbalances and oxidative stress have been linked with a number of neurodegenerative diseases such as Alzheimer’s, Parkinson’s and Huntington’s disease. These diseases all involve protein misfolding and misassembly. Metal ions can influence the misfolding, aggregation, oligomerization and fibril formation of these disease-associated proteins. Furthermore, redox-active metal ions can contribute to the oxidative stress often associated with these diseases [1]. Of particular interest are the transmissible spongiform encephalopathies or prion diseases. These fatal neurodegenerative diseases include scrapie in sheep, BSE (bovine spongiform encephalopathy) in cattle and CJD (Creutzfeldt–Jakob disease) in humans. Prions, meaning proteinaceous infectious particles, are wholly novel infectious agents and are distinct from bacteria and viruses in that the infectious pathogen contains no nucleic acid. PrP (prion-related protein) has the ability to infect a host and that infectivity is conferred by its conformation. The concept of an infectious protein presents implications for function and disease.

PrP (prion-related protein) is a cell-surface Cu2+ -binding glycoprotein which, when misfolded, is responsible for a number of transmissible spongiform encephalopathies. The co-ordination geometry, stoichiometry and affinity of Cu2+ for PrP are the subject of much debate. In the present paper, we review the recent progress we have made in these areas. As many as six Cu2+ ions bind to PrP with submicromolar affinity. Initially, two Cu2+ ions bind to full-length PrP in the amyloidogenic region, between the octarepeats and the structured domain, at His95 and His110. Only subsequent Cu2+ ions bind to single histidine residues within the octarepeat region. Competitive chelators have been used to determine the affinity of the first molar equivalent of Cu2+ bound to full-length PrP; this approach places the affinity in the nanomolar range. The affinity and number of Cu2+-binding sites support the suggestion that PrP could act as an antioxidant by binding potentially harmful Cu2+ ions and sacrificially quenching of free radicals generated as a result of copper redox cycling. Finally, the effect of Cu2+ on the prion structure and misassembly into oligomers and fibres is discussed.

Key words: copper, misfolding, prion protein, visible circular dichroism.
Abbreviations used: CSF, cerebrospinal fluid; PrP, prion-related protein; PrPc, cellular isoform of PrP; PrPSc, scrapie isoform of PrP; ROI, reactive oxygen species.
1 To whom correspondence should be addressed (email j.viles@qmul.ac.uk).

The misfolding prion protein structure
The mammalian PrPc is a cell-surface glycoprotein, composed of a single polypeptide chain, typically 209 amino acids long, attached to the cell surface by a GPI (glycosylphosphatidylinositol) anchor. There are now a number of solution structures for mammalian PrPs in their Cu2+-free form. Structurally, PrPc contains two distinct regions, the C-terminal domain, residues 121–231, is largely α-helical [11,12], as shown in Figure 1. In contrast, in the absence of Cu2+, the N-terminal domain, residues 23–120, is unstructured [13] and has a high degree of main-chain flexibility [14]. It is this natively unfolded domain which includes octarepeat sequences that binds a number of Cu2+ ions [4,15,16].

It is believed that the fundamental event in prion diseases is the conversion or misfolding of PrPc (helical form) to a β-sheet-rich conformation, known as the scrapie isoform, PrPSc, which is accompanied by the formation of neurotoxic oligomers and amyloid fibrils. The primary sequences of the two isoforms are identical and only differ in their conformation, and consequently their biophysical properties. Additional weight to the prion hypothesis came when...
synthetic replicating infectious prions were generated from misfolded recombinant PrP [17].

Studying insoluble amyloid fibrils presents problems for structural biologists, and the molecular structure of PrPSc has yet to be determined; however, recent biophysical studies suggest a model containing an extended cross-β-structure [18,19]. Understanding what triggers the conversion of PrPc into PrPSc is critical to our understanding of the mechanism of prion disease and could assist in the rational drug design of inhibitors of the misfolding process. Intermediates of PrPc are therefore an area of intense research [20]. Crucially, the Cu2+-bound forms of PrP are physiologically relevant to those studying the misfolding and fibrillization of PrP [21].

Overview of Cu2+ binding to fragments of PrPc and their associated visible CD

Utilizing peptide fragments, specifically centred on two regions within the unstructured N-terminal domain of PrPc, a number of studies have examined Cu2+ binding to PrPc. These studies include the octarepeat region, residues 57–90, and the amyloidogenic region located between the octarepeats and the structured C-terminal domain. Using both 1H-NMR and visible CD spectroscopy, we have shown that Cu2+ binding outside the octarepeats is centred at His95 and His110 in the mouse sequence (His96 and His111 in the human sequence). It is clear from EPR the co-ordination geometry at each of these histidine residues is square-planar/tetragonal [15,22]. Through the use of Ni2+ ions, as a diamagnetic mimic in 1H-NMR studies, the co-ordination of the metal complex was shown to involve the histidine imidazole nitrogen δN and the main-chain amides that precede it (see complexes I and II in Figure 2) [15]. Binding in this amyloidogenic region of PrP has a reported affinity in the range 1–100 nM [23,24]. In addition, mammalian PrPs contain a repeating
motif of eight amino acids, typically consisting of four octarepeats between residues 57 and 90, with each repeat containing a histidine residue. This octarepeat region, which is highly conserved across mammalian species, is natively unstructured. It is here that up to four Cu$^{2+}$ ions bind with identical coordination geometry [4,25]. A Cu$^{2+}$-bound crystal structure of HGGGW, the minimal binding motif within the octarepeats, has been published [26], revealing that Cu$^{2+}$ co-ordinates with three nitrogen atoms and one oxygen atom as equatorial ligands, plus an axial water molecule to form a square-pyramidal geometry, as shown in Figure 2 (complex III). Affinity for this binding mode has been placed at a $K_d$ of 1–10 μM at physiological pH [24,25,27]. Recent studies have shown that, at substoichiometric levels, a single Cu$^{2+}$ ion will bind to multiple histidine residues from the octarepeats with a higher affinity, a $K_d$ in the 0.1–3 nM range [24,27] (complex IV, also shown in Figure 2).

Visible CD has been shown to be a very powerful probe for studying Cu$^{2+}$ interactions with PrP. Using a number of fragments and analogues for the unstructured N-terminal domain, we have characterized the various Cu$^{2+}$-binding modes described above. As also shown in Figure 2, we have summarized the striking differences in the visible CD spectra for these four different binding modes at physiological pH. For example, Cu$^{2+}$ binding at His$^{95}$ produces a positive CD band at 500 nm with a negative band at 580 nm, whereas the almost reverse CD spectrum is observed for Cu$^{2+}$ binding centred at His$^{110}$, exhibiting a negative CD band at lower wavelengths, 480 nm, and a positive band at higher wavelengths at 580 nm [15,22,28]. In marked contrast, Cu$^{2+}$ binding to individual histidine residues in the octarepeats gives a positive CD band at 580 nm, but with a negative band at 690 nm [4,25]. The multiple-histidine-binding mode, observed at substoichiometric levels of Cu$^{2+}$ is CD-silent in the visible region [16,28].

We were intrigued by the almost mirror image appearance of Cu$^{2+}$ binding centred at His$^{95}$ and His$^{110}$. EPR and 1H-NMR studies of pentapeptides, PrP-(91–95) and PrP-(106–110), which represent the minimum motif for this Cu$^{2+}$-binding region, suggest that the co-ordination geometry for the two binding sites is very similar. However, the visible CD spectra of the two sites are very different, producing almost mirror image spectra. We have used a series of analogues of the pentapeptides containing His$^{95}$ and His$^{110}$ to rationalize these differences in the visible CD spectra. In addition, using simple histidine-containing tripeptides, we have formulated a set of empirical rules that can predict the appearance of Cu$^{2+}$ visible CD spectra involving histidine and amide main-chain co-ordination [28]. In summary, we predict that Cu$^{2+}$ (or Ni$^{2+}$) binding to histidine-containing proteins or peptides to form 4N complexes, will result in a CD spectrum with a positive CD band at shorter wavelengths and negative at longer wavelengths for GXH peptides (where X can be any primary amino acid). In contrast, a ZXH sequence (where Z can be any amino acid, except glycine or proline) will result in a negative CD signal at shorter wavelength and a positive CD signal at longer wavelengths [28].

**Cu$^{2+}$ binding to full-length PrP$^c$**

Recently, we have built on our understanding of the appearance of visible CD and EPR spectra for various PrP fragments to characterize Cu$^{2+}$ co-ordination to full-length PrP [16]. Figure 3(a) shows visible CD spectra for full-length PrP$^c$ with increasing additions of Cu$^{2+}$ ions. It is clear that...
multiple Cu\(^{2+}\) ions load sequentially on to PrP\(^c\). Figure 3(b) shows difference spectra of Cu\(^{2+}\)-loaded full-length PrP\(^c\), with spectra of the first two molar equivalent of Cu\(^{2+}\) ions subtracted. The difference spectra shown in Figure 3(b) have a striking resemblance to visible CD spectra of Cu\(^{2+}\) bound to the octarepeat fragments of PrP, PrP-(57–90), shown in Figure 3(d). Figures 3(c) and 3(e) show visible CD spectra of recombinant PrP constructs in which His\(^{95}\) and His\(^{110}\) are replaced by alanine residues, or the octarepeat region has been removed. Again the spectra show striking resemblance to Cu\(^{2+}\) binding to the analogous small fragments of PrP-(57–90), the octarepeat fragment (Figure 3d), and PrP-(90–114), a peptide fragment from the amyloidogenic region (Figure 3f).

These studies show that fragments from the unstructured region of PrP represent a good model for Cu\(^{2+}\) binding to full-length PrP. It is clear that, at physiological pH, Cu\(^{2+}\) initially binds to full-length PrP in the amyloidogenic region, between the octarepeats and the structured domain, at His\(^{95}\) and His\(^{110}\). Only subsequent Cu\(^{2+}\) ions bind to the octarepeat region, eventually loading on to each individual histidine residue [16].

**A nanomolar affinity of Cu\(^{2+}\) for PrP\(^c\)**

The precise affinity of Cu\(^{2+}\) for PrP\(^c\) is hotly debated [24,27,29–31]. The affinity quoted for Cu\(^{2+}\) binding to full-length PrP differs quite significantly. For example, two early studies put the affinity in the micromolar range for full-length PrP [3,29]. However, subsequent studies suggest a number of orders of magnitude higher, with a femtomolar dissociation constant for the full-length protein [30,31]. Studies of PrP\(^c\) fragments have Cu\(^{2+}\) affinities with quite a reasonable consensus in the nano- to micro-molar range [23–25,27].

Our competitive metal-capture studies place the affinity of the first equivalent of Cu\(^{2+}\) for full-length PrP at approx. 10\(^8\) M\(^{-1}\) [16]. This agrees with the affinities reported for PrP fragments [24,27] and the earliest reports of Cu\(^{2+}\) affinity for full-length PrP [3,29].

Cu\(^{2+}\) concentrations in CSF (cerebrospinal fluid) and extracellular brain interstitial fluid are typically 0.1 \(\mu\)M [32]. Concentrations of serum albumin are found at much lower levels in the CSF than in blood plasma (typically at 3 \(\mu\)M), but much of the Cu\(^{2+}\) is still found to be bound to serum albumin, with a picomolar affinity for Cu\(^{2+}\) ions [33]. PrP\(^c\) is found concentrated at the synaptic cleft [34]; here, fluxes of Cu\(^{2+}\) ions may be as high as 0.1 mM during neuronal depolarization [35]. Thus PrP\(^c\), with a dissociation constant for Cu\(^{2+}\) of \(\sim\)10 nM, is well placed to bind fluxes of Cu\(^{2+}\) released at the synapse, whereas albumin is not capable of coping with these localized spikes of Cu\(^{2+}\) ions with its single Cu\(^{2+}\)-binding site.

**PrP\(^c\) is a sacrificial scavenger of Cu\(^{2+}\)-generated hydroxyl radicals**

Free Cu\(^{2+}\) ions are potentially highly toxic to cells, generating hydroxyl radicals via Fenton’s chemistry. The localization of PrP\(^c\) at presynaptic membranes [34] means that it is well placed to bind fluxes of Cu\(^{2+}\) ions released during neurotransmission [35]. We have demonstrated, in a cell-free system, that PrP\(^c\) behaves as a sacrificial antioxidant, resulting in oxidative chemical modification of PrP\(^c\), localized at methionine and histidine residues [36]. Methionine residues within proteins can act as an ROS (reactive oxygen species) sink, effectively scavenging ROS before they damage other cellular components [37]. In human PrP\(^c\), Met\(^{109}\) and Met\(^{112}\) are solvent-exposed, close to His\(^{96}\) and His\(^{111}\), which represents a locus for Cu\(^{2+}\) binding. The unusually rapid turnover of PrP, every 3–6 h [38], together with its ability to bind as many as six Cu\(^{2+}\) ions, supports the hypothesis that PrP\(^c\) could act as a sacrificial quencher of free radicals generated by redox-active Cu\(^{2+}\), resulting in the oxidation of solvent-exposed methionine residues close to the Cu\(^{2+}\)-binding sites [36]. PrP\(^c\) function may well be to scavenge Cu\(^{2+}\) ions released during neuronal depolarization and so protect cells from the toxic effects of Cu\(^{2+}\). This idea is supported by the observation that PrP-null mice are more sensitive to Cu\(^{2+}\)-induced oxidative stress than the wild-type mice [39,40].

**Cu\(^{2+}\)-induced misfolding and self-association: a link to prion disease?**

It is clear that misfolding of PrP\(^c\) is central to prion disease in familial and acquired prion diseases. This does not preclude the possibility that perturbed Cu\(^{2+}\) homoeostasis within the brain could influence this misfolding process. Indeed, different protease-resistance profiles of PrP observed with and without the presence of Cu\(^{2+}\) ions correspond to similar profiles seen in different strains of prion disease [10]. Cu\(^{2+}\) seems to promote self-association of PrP\(^c\) and protease resistance, but results from in vitro studies suggest that Cu\(^{2+}\) inhibits fibril growth [21].

The unstructured region between the octarepeat region and the structured C-terminal domain, PrP-(90–126), is considered to be essential for amyloid formation and infectivity in prion disease [41]. Residues in this region are thought to play a pivotal role in the misfolding of PrP\(^c\) into the \(\beta\)-sheet-rich conformation. The presence of Cu\(^{2+}\) binding within this region (residues 90–126) should have profound implications for the Cu\(^{2+}\)-loaded structure of PrP\(^c\). We have shown that Cu\(^{2+}\) binds preferentially to His\(^{110}\) and His\(^{95}\) before the octarepeat region of PrP\(^c\) [23]. Furthermore, our UV CD studies indicate a loss of irregular structure within this region upon Cu\(^{2+}\) co-ordination, with an increase in extended \(\beta\)-sheet-like conformation [23]. The influence Cu\(^{2+}\) ions have on PrP\(^c\) secondary structure is still not well understood and is a key area of interest in our laboratory.

---

**Thanks to the BBSRC (Biotechnology and Biological Sciences Research Council) for financial support, D. Brown for continued collaborations, and A. Garnett, C. Jones, D. O’Sullivan and N. Younan for their inspired research.**
References


13 Ablation of the metal ion-induced endocytosis of the prion protein by disease-associated mutation of the octarepeat region. Curr. Biol. 11, 519–523


25 Klevapinat, M. and Viles, J.H. (2007) Fragment length influences affinity for Cu2+ and Ni2+ binding to His96 or His111 of the prion protein and spectroscopic evidence for a multiple histidine binding only at low pH. Biochim. J. 404, 393–402


Received 29 August 2008
doi:10.1042/BST0361288