Deconvoluting the Cu$^{2+}$ Binding Modes of Full-length Prion Protein$^{*\dagger}$

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The prion protein (PrP) is a cell-surface Cu$^{2+}$-binding glycoprotein that when misfolded is responsible for a number of transmissible spongiform encephalopathies. Full-length PrP (23–231) and constructs in which the octarepeat region has been removed, or His$^{95}$ and His$^{110}$ is replaced by alanine residues, (23–231) and constructs in which the octarepeat region has been transmissible spongiform encephalopathies. Full-length PrP-protein that when misfolded is responsible for a number of prion diseases in humans, including Creutzfeldt Jakob Disease (1–3). Normal cellular prion protein (PrPC) is typically 209 residues long, devoid of nucleic acids, which is responsible for bovine spongiform encephalopathy in cattle and a number of prion diseases devoid of nucleic acids, which is responsible for bovine spongiform encephalopathy in cattle and a number of prion diseases

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 single histidine residues within the octarepeat region. Ni$^{2+}$ ions are used to further probe metal binding and, like Cu$^{2+}$, Ni$^{2+}$ will bind individually to His$^{95}$ and His$^{110}$, involving preceding main chain amides. Competitive chelators are used to determine the affinity of the first mole equivalent of Cu$^{2+}$ bound to full-length PrP; this approach places the affinity in the nanomolar range. The affinity and number of Cu$^{2+}$ binding sites support the suggestion that PrP could act as a sacrificial quencher of free radicals generated by copper redox cycling.

The normal cellular prion protein, PrP$^\text{C}$, is a cell surface glycoprotein that binds Cu$^{2+}$ ions. A misfolded form of the prion protein, PrP$^\text{Sc}$, is a proteinaceous infectious particle, devoid of nucleic acids, which is responsible for bovine spongiform encephalopathy in cattle and a number of prion diseases in humans, including Creutzfeldt Jakob Disease (1–3). Normal cellular prion protein (PrP$^\text{C}$) is typically 209 residues long, attached to the cell surface by a glycosylphosphatidylinositol anchor. The C-terminal domain between residues 126 and 231 is mainly $\alpha$-helical (4). In contrast, in the absence of copper ions, the N-terminal domain between residues 23 and 125 is unstructured (5) and exhibits a high degree of main chain flexibility (6). It is this natively unfolded domain that includes octarepeat sequences that bind a number of Cu$^{2+}$ ions (7–9).

Metal imbalance is a feature of prion disease (10) and when isolated from diseased brain, PrP$^\text{Sc}$ has been found to be occupied with cup (11). Metal binding to the prion protein is altered in human prion disease (12), with levels of cellular copper affected by scrapie infection (13) and the ability for disease progression in infected mice to be slowed with the use of copper-specific chelation therapy (14). Copper-catalyzed redox damage of PrP (15) is linked to prion disease (16, 17), although, the copper binding octarepeat region is not required for prion infectivity and propagation (18). Cu$^{2+}$ binding has also been noted outside the octarepeats, in the so called 5th site (8, 19–23). This region is an amyloidogenic, neurotoxic segment of PrP that is essential for prion replication (18, 24–29). Interestingly, the presence, or absence, of Cu$^{2+}$ ions may also confer different strains of prion disease (11, 30).

PrP$^\text{C}$ is concentrated at presynaptic membranes in the central nervous system, where Cu$^{2+}$ is also highly localized (32). The ability of PrP$^\text{C}$ to bind Cu$^{2+}$ in vivo and in vitro infers PrP may have a physiological role in copper homeostasis (7, 9). Copper has been shown to promote the endocytosis of PrP$^\text{C}$ (33, 34), but PrP expression levels do not seem to affect copper delivery (35, 36). Copper-induced cleavage of the PrP$^\text{C}$ main chain has also been reported (37, 38) and PrP$^\text{C}$ can act as an antioxidant by sacrificially quenching hydroxyl radicals produced via Cu$^{2+}$/Cu$^{+}$ Fenton’s cycling (15). PrP knock-out mice show heightened sensitivity to Cu$^{2+}$-induced oxidative stress (12, 39).

Despite a whole arsenal of spectroscopic studies directed at Cu$^{2+}$-bound fragments of PrP, there have been surprisingly few structural studies of Cu$^{2+}$-bound to full-length PrP. Studies on full-length PrP$^\text{C}$ have been restricted by the solubility of PrP$^\text{C}$ at physiological pH required for Cu$^{2+}$ binding. Structural details of Cu$^{2+}$ binding to full-length PrP and large recombinant fragments have included $^1$H NMR studies of PrP (90–231) (20) and full-length PrP at low pH (40), EPR studies (22), and mass spectrometry (30).

There have been numerous Cu$^{2+}$ binding studies of fragments of PrP centered on two regions within the unstructured N-terminal domain of PrP. These studies include the octarepeat region, residues 58–91, and a second Cu$^{2+}$ binding region between the octarepeats and the C-terminal structured domain, between residues 90 and 126. Mammalian PrPs contain a repeating motif of 8 amino acids, typically, four octarepeats between residues 60 and 91 with each repeat containing a histidine residue. It is this unstructured, highly conserved...
region that binds four Cu\(^{2+}\) ions with identical coordination geometry (9, 41). A crystal structure of the copper-bound octarepeat motif (HGGGW) has been reported (42) that shows Cu\(^{2+}\) coordinates with three nitrogen atoms and one oxygen atom as equatorial ligands, and an axial water molecule to form a square-pyramidal geometry (complex II), shown in Fig. 1. Affinity for this binding mode has been placed at a \(K_d\) of 1–10 \(\mu M\) at physiological pH (41, 43, 44). Recent studies have shown that at substoichiometric levels, Cu\(^{2+}\) will bind to multiple His residues with a higher affinity, \(K_d\) in the 0.1–3 nM range (43, 44) (complex I), also shown in Fig. 1. In addition, we have shown by \(^1\)H NMR and visible CD spectroscopy that Cu\(^{2+}\) also binds to PrP outside the octarepeats region between residues 90 and 126, centered at His\(^{95}\) and His\(^{110}\) (His\(^{96}\) and His\(^{111}\) in the human sequence). These complexes form a square planar-tetragonal complex (8, 23). \(^1\)H NMR of the diamagnetic Ni\(^{2+}\) analogue indicates coordination of the metal complex involves the His imidazole nitrogen \(\delta N\) and the main chain amides that precede the His, also shown in Fig. 1. Binding here is in the 1–100 nm range (19, 43). 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 various Cu\(^{2+}\) binding modes to PrP. Also in Fig. 1 we have summarized the striking difference in the visible CD spectra for these four different binding modes at physiological pH. In particular, Cu\(^{2+}\) binding to individual histidine residues in the octarepeats, which gives a positive CD band at 580 and a negative band at 690 nm (9, 41). In contrast, Cu\(^{2+}\) binding centered at His\(^{95}\) (His\(^{96}\) in humans) gives a positive band at 500 nm with a negative band at 580, whereas the almost reverse CD signal is observed for Cu\(^{2+}\) binding at His\(^{110}\) (His\(^{111}\) in human sequence), with a negative CD band at 480 nm and a positive band near 580 nm (8, 23, 45, 46).

In this study we have used our understanding of the appearance of visible CD spectra binding to PrP fragments to characterize Cu\(^{2+}\) binding to full-length PrP. We aim to investigate whether Cu\(^{2+}\) binds to full-length PrP in a similar fashion to the fragments, by differentiating these various Cu\(^{2+}\) binding modes and identifying whether the Cu\(^{2+}\) coordination is sequential, or simultaneous, to these various binding sites. In addition to mPrP-(23–231), we have used constructs of full-length PrP outside the octarepeats region between residues 90 and 126, designated mPrP-(23–125) and mPrP-(23–231)H95A, H110A. In addition, a construct with the four octarepeat sections removed containing residues 23–56, followed by residues 90–231, designated mPrP-(23–231)Δocta. Finally, a truncated version of PrP was obtained with only the natively unstructured domain, residues 23–125, designated mPrP-(23–125).

**Peptide Synthesis and Purification**—Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry was used to synthesize various fragments of PrP, which were N-terminal acetylated and C-terminal amidated to mimic this region of PrP within the full-length protein (A.B.C., Imperial College, London). The peptides were removed from the resin and de-protected before purification by reverse-phase high pressure liquid chromatography. The samples were characterized using mass spectrometry and \(^1\)H NMR spectroscopy. Peptide fragments of both mouse and human PrP are shown in Table 1.

**Titrations**—UHQ water (10\(^{-18}\) \(\Omega\) \(^{-1}\) cm\(^{-1}\) resistivity) was used throughout. Small aliquots of fresh aqueous solutions were used to add metal ions (Cu\(^{2+}\) as CuCl\(_2\)-2H\(_2\)O, Ni\(^{2+}\) as NiCl\(_2\)-6H\(_2\)O; typically 50 m\(\mu\)m solutions). Stock solutions of competing Cu\(^{2+}\) chelators, Gln, l-His, and N-(2-hydroxyethyl)iminodiacetic acid (HIMDA) (Sigma) were made up in water. Gly and l-His were added as small aliquots, whereas HIMDA was added in equivalent volume and concentrations to avoid any solubility problems of the HIMDA complex at pH 7.4. Protein and peptide concentrations were determined using an extinction coefficient at 280 nm, which is the sum of the extinc-

| Table 1: PrP peptide fragments |
|-------------------------------|
| All peptides were acetylated and amidated at the N terminus and C terminus, respectively. Note, His\(^{96}\) and His\(^{110}\) in the mouse sequence are His\(^{95}\) and His\(^{111}\) in the human PrP numbering. |

| Peptide | Acceptor Peptide | Sequence |
|---------|-----------------|----------|
| hPrP(58-91) | GQHHSQSSQH | SEQGGSFQHGSQG |
| mPrP(57-90) | GQHHSQSSQH | SEQGGSFQHGSQG |
| hPrP(90-120) | GQGSSHQSKQ | HAGAAGAVVGLG |
| mPrP(91-115) | GQGSSHQSKQ | HAGAAGAVVGLG |
| hPrP(90-120) | H95A | |
| mPrP(90-120H111A) | GQGSSHQSKQ | HAGAAGAVVGLG |
| hPrP(90-120H196A) | GQGSSHQSKQ | HAGAAGAVVGLG |
| hPrP(91-115H111A) | GQGSSHQSKQ | HAGAAGAVVGLG |
| hPrP(91-115H196A) | GQGSSHQSKQ | HAGAAGAVVGLG |
| hPrP(92-96) | GQGSSHQSKQ | HAGAAGAVVGLG |
| mPrP(91-95) | GQGSSHQSKQ | HAGAAGAVVGLG |
| hPrP(107-111) | GQGSSHQSKQ | HAGAAGAVVGLG |
| mPrP(106-110) | GQGSSHQSKQ | HAGAAGAVVGLG |

**Experimental Procedures**

Expression and Purification of Recombinant Mouse Prion PrP-(23–231) Protein—The coding region of the full-length mouse PrP-(23–231) was cloned into a pET-23 vector to produce a tag-free protein as previously described (50). Expression and purification is identical to previously described procedures (19) (Sequence 1). The positions of the histidines involved in Cu\(^{2+}\) binding are highlighted in bold. Three further constructs of recombinant mouse PrP were used, full-length mPrP-(23–231) with both His\(^{95}\) and His\(^{110}\) replaced by alanine, designated mPrP-(23–231)Δocta. Finally, a truncated version of PrP was obtained with only the natively unstructured domain, residues 23–125, designated mPrP-(23–125).
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ion coefficients for the total number of aromatic residues and disulfide bonds; 5690 m$^{-1}$ cm$^{-1}$ multiplied by the number of Trp residues in the peptide, plus 1280 m$^{-1}$ cm$^{-1}$ multiplied by the number of Tyr residues, plus 120 m$^{-1}$ cm$^{-1}$ multiplied by the number of disulfide bonds (51). This gave an extinction coefficient of 62,280 m$^{-1}$ cm$^{-1}$ at 280 nm for full-length mPrP-(23–231). Protein concentrations were also determined using a Bradford assay and were in close agreement (within 95% of each other). In the case of pentapeptides that lacked aromatic residues, the weight was used to approximate the concentration and 20% water content was assumed. Peptide concentrations were typically 100 μM for visible CD experiments and 50 μM for EPR. Titrations were carried out in the absence of buffers, except for the 0.005% sodium azide in protein samples and 10 mM sodium acetate buffer. Samples were diluted for individual titrations; sodium acetate concentration was typically 1 mM. The pH was measured before and after acquiring each spectrum, adjusting the pH when necessary using small aliquots of 100 mM NaOH or HCl.

**Circular Dichroism (CD)**—CD spectra were recorded on an Applied Photophysics Chirascan instrument at 25 °C. A cell with a 1-cm path length was used for spectra recorded between 300 and 800 nm, with sampling points every 2 nm. Typically, four scans were recorded, and baseline spectra were subtracted from each spectrum. Data were processed using Applied Photophysics Chirascan Viewer, Microsoft Excel, and the KaleidaGraph spreadsheet/graph package. The direct CD measurements ($\theta$, in millidegrees) were converted to molar ellipticity, $\Delta \varepsilon (m^{-1} cm^{-1})$, using the relationship $\Delta \varepsilon = \theta/33,000 \cdot c \cdot l$, where $c$ is the concentration and $l$ is the path length.

**Absorption Spectroscopy (UV-visible)**—UV-visible electronic absorption spectra were obtained with a Hitachi U-3010 double beam spectrophotometer, using a 1-cm path length.

**Electron Paramagnetic Resonance Spectroscopy (EPR)**—X-band EPR spectra were acquired on a Bruker Elexsys E500 spectrometer operating at a microwave frequency of 9.33 GHz. The spectra were acquired over a sweep width of 2500 gauss, a modulation frequency of 10 gauss, and a temperature of ~20 K. At least three scans were acquired per sample.

**RESULTS**

Copper$^{2+}$ Binding to Full-length mPrP-(23–231) and the Natively Unfolded Domain, PrP-(23–125), Monitored by Visible CD—Visible CD has been used extensively to study Cu$^{2+}$ binding to fragments from PrP (summarized in Fig. 1). Here, we use the same approach to study full-length PrP. Fig. 2 shows a Cu$^{2+}$ titration of full-length PrP-(23–231) at pH 7.4 monitored by visible CD. Panel a presents the titration up to 1.8 mol eq of Cu$^{2+}$, increasing in 0.3 mol eq with each aliquot. Initial Cu$^{2+}$ additions caused the appearance of a negative CD band at ~576 nm, accompanied by a weak positive CD band at 500 nm. After ~1.8 mol eq the intensity of the two bands plateaued. By 2 mol eq of Cu$^{2+}$, the band at 576 nm turns more positive as more Cu$^{2+}$ is added, as shown by the binding curve inset. The Cu$^{2+}$ titration is shown in two separate panels for clarity; panel b shows the titration from 1.8 mol eq of Cu$^{2+}$ up to 9 mol eq in 0.9 mol eq steps. In Fig. 2b we observe the negative CD at 576 nm adopting a more positive signal, and a negative band also emerging at 692 nm only after more than 1.8 mol eq of Cu$^{2+}$ are added. The Cu$^{2+}$ titration reveals a number of distinct sequential binding modes. It is notable that even at 0.3 mol eq of Cu$^{2+}$ the CD band at 576 nm is apparent, the signal increases linearly in intensity between 0 and 0.9 mol eq of Cu$^{2+}$ added.

Fig. 2c is the visible CD difference spectra that results when the spectrum for 1.8 mol eq of Cu$^{2+}$ is subtracted from all subsequent spectra of full-length mPrP-(23–231). The new difference spectrum that emerges is very comparable with that observed for the visible CD for Cu$^{2+}$ binding to peptides of the octarepeat region (9, 41). This is evident by comparing the visible CD spectra for Cu$^{2+}$ binding to mPrP-(57–90), a peptide of
PrP incorporating the four octarepeats, shown in Fig. 2d. In both sets of spectra two positive CD bands are observed around 340 and 590 nm, accompanied by a negative band at 680 nm. The wavelength position, sign, and relative intensity of CD bands are strikingly similar. There is a notable difference in intensity of the CD bands for Cu$^{2+}$ binding to full-length PrP and the smaller mouse octarepeats fragment. To summarize, the data in Fig. 2 suggest there are at least two modes of Cu$^{2+}$ binding; the first $\sim$2 mol eq of Cu$^{2+}$ produce a negative CD band at 580 nm and a weak positive CD band at 500 nm. This is followed by Cu$^{2+}$ binding that produces CD signals indicative of binding to single histidines within the octarepeats of PrP.

We were interested in whether the structured domain, residues 125–231, influences Cu$^{2+}$ binding to full-length PrP. A similar Cu$^{2+}$ titration was performed on the recombinant fragment mPrP-(23–125), which consists of just the unstructured, flexible N terminus of PrP, shown in Fig. 3. The visible CD spectra have a very similar appearance to the full-length protein, as we observe an initial negative signal at $\sim$580 nm up to 1.8 mol eq of Cu$^{2+}$ with $\Delta\varepsilon \approx 0.35$ M$^{-1}$ cm$^{-1}$, before producing positive CD bands at 340 and 590 nm and a negative band at 680 nm as the titration continues. As with full-length PrP, the appearance of the octarepeats signal after 2 mol eq is more apparent in the difference spectrum, shown in Fig. 3c. The binding curve at 576 nm shows the first 2 eq of Cu$^{2+}$ binding, followed by 4 Cu$^{2+}$ ions binding to the octarepeats. Saturation of the binding sites is suggested above 6 eq of Cu$^{2+}$. There is a close similarity between the visible CD spectra for PrP-(23–231) and PrP-(23–125) (although not identical), which suggests the Cu$^{2+}$ binding modes are very similar and Cu$^{2+}$ binding to PrP is largely unaffected by the structured domain of PrP. The weak positive band at 500 nm seen for PrP-(23–231) is not apparent in the PrP-(23–125) spectra. The band could be masked by another negative signal at 500 nm, which is suggested in spectra shown in Fig. 4a.

Cu$^{2+}$ Binding to Analogues of Full-length mPrP-(23–231) Monitored by Visible CD—In an attempt to further deconvolute the binding modes of full-length PrP, an analogue of mPrP-(23–231) was produced, where the two His residues in the so called “5th site” were mutated into Ala residues, to remove His$^{95}$ and His$^{110}$ as potential ligands for Cu$^{2+}$ binding. This allowed us to isolate the effects of Cu$^{2+}$ binding to the rest of the protein, in particular, the octarepeats. Fig. 4a shows the resultant visible CD spectra from a Cu$^{2+}$ titration at pH 7.4. Interestingly, despite the replacement of His$^{95}$ and His$^{110}$ to Ala, Cu$^{2+}$ additions do not initially result in the characteristic visible CD spectra for Cu$^{2+}$ ions bound to single His residues within the octarepeat region. In particular, Cu$^{2+}$ additions cause a single negative band at 500 nm. However, after in excess of 1 mol eq of Cu$^{2+}$ has been added, the spectra become very similar to that for the octarepeats peptide fragment PrP-(58–91). Fig. 4b shows a difference spectra of the wild-type mPrP-(23–231)H95A/H110A after 1.8 Cu$^{2+}$ mol eq have been subtracted, whereas Fig. 4c shows the spectra of the four octarepeat peptide, Cu-mPrP-(57–90), for a direct comparison. In particular, two positive CD bands are produced at 340 and 590 nm, with a negative band at 680 nm apparent. It is also notable that the initial weak negative band, apparent at 500 nm, is not directly observed for full-length PrP and mPrP-(23–231)Δ4octa, and could be a contaminant.

A second construct of mPrP was produced, with the octarepeat region deleted, between residues 57 and 90, whereas His$^{95}$ and His$^{110}$ remain present and is designated as mPrP-(23–231)Δ4octa. Fig. 5a shows visible CD spectra for a Cu$^{2+}$ titration of mPrP-(23–231)Δ4octa at pH 7.4. Very distinct CD bands are apparent, a positive band at 500 nm and negative band of similar intensity at 580 nm, both bands exhibit similar binding curves (Fig. 5c). The binding curves do not exhibit a linear behavior, but saturate by 2 mol eq of Cu$^{2+}$. At first appearance, the CD spectra in Fig. 5a seem to suggest a single Cu$^{2+}$ binding mode. However, closer inspection of the histidine ligand-to-metal-charge-transfer bands at 314 and 344 nm indicates a sequential loading of two binding sites, with Cu$^{2+}$-loading causing the saturation of the band at 314 nm before the site associated with 344 nm. Significantly, this is consistent with a 2:1 stoichiometry. Fig. 5d shows the Cu$^{2+}$ titration for the pep-

FIGURE 2. Visible CD of Cu$^{2+}$ binding to full-length mPrP-(23–231). Cu$^{2+}$ titration at pH 7.4 for mPrP-(23–231): a, up to 1.8 mol eq of Cu$^{2+}$ in 0.3 mol eq additions; b, 1.8 mol eq of Cu$^{2+}$ and above in 0.9 mol eq additions. Binding curves for Cu$^{2+}$ at 576 and 692 nm are shown in the inset for the two panels, respectively. Difference spectra resulting from subtraction of 1.8 mol eq of Cu$^{2+}$ from subsequent spectra are presented in c. Spectra for Cu$^{2+}$ bound to the four octarepeats fragment, mPrP-(57–90), is shown for comparison in d. Protein concentrations of 100 μM.

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tide fragment, mPrP-(90–114), containing His\(^{95}\) and His\(^{110}\). The position and intensity of the CD bands are strikingly similar to those observed for Cu\(^{2+}\)/H\(^{110}\) binding to mPrP(23–231)/H\(^{90}\) tetra, with binding curves for the bands at 500 and 580 nm. In addition, the sequential loading of 2 Cu\(^{2+}\)/H\(^{110}\) ions at 314 nm followed by 344 nm is also apparent.

We have studied the binding of Cu\(^{2+}\)/H\(^{110}\) to His\(^{95}\) and His\(^{110}\) (complexes III and IV) from fragments of the unstructured region of PrP that lack the octarepeats, in particular human PrP-(90–126). Visible CD spectra of these two binding sites (for both Cu\(^{2+}\) and Ni\(^{2+}\)) have been studied extensively by \(^{1}\)H NMR and visible CD (8, 23). Interestingly, the visible CD signals of these two binding sites produce almost mirror-image spectra (45). Simulations of the appearance of visible CD spectra of Cu\(^{2+}\) binding based on pentapeptides, mPrP-(91–95) and mPrP-(106–110), are also shown in supplementary Fig. S1. Loading of Cu\(^{2+}\) best simulates mPrP-(90–114) and the recombinant full-length construct by loading Cu\(^{2+}\) at His\(^{95}\) and His\(^{110}\), but preferentially to His\(^{95}\) first (see supplementary Fig. S1). For a more detailed description of this type of approach see Refs. 8 and 23.

For the PrP(23–231)/H\(^{90}\) tetra construct, 2 Cu\(^{2+}\) ions bind both at His\(^{95}\) and His\(^{110}\). The visible CD signal is dominated by the signal from Cu\(^{2+}\)/H\(^{110}\) binding to His\(^{95}\), but is significantly reduced by the overlap of His\(^{110}\), which gives a weaker almost mirror-image spectrum.

We are now in a position to more fully interpret the wild-type full-length PrP visible CD spectra. It is notable that we observed very clear binding of Cu\(^{2+}\)/H\(^{110}\) to His\(^{95}\) and His\(^{110}\) for the mPrP(23–231) analogue. In full-length PrP the octarepeat signal is clearly apparent once the protein has been loaded with 2 mol eq of Cu\(^{2+}\). Fig. 6 shows a number of mouse PrP constructs all with 2 mol eq of Cu\(^{2+}\) present at pH 7.4. These included mPrP-(23–231), mPrP-(23–231)/Δ4octa, mPrP-(90–114), and finally, the addition of spectra for the equivalent two pentapeptides, mPrP-(91–95) and mPrP-(106–110). The position of the visible CD bands is very similar for all four constructs. The intensity of mPrP-(23–231) has been attenuated by approximately one-third, relative to the other spectra.

To summarize, two Cu\(^{2+}\) ions bind to His\(^{95}\) and His\(^{110}\), as it does for small peptide fragments of this region, in full-length PrP (complexes III and IV). Subsequent Cu\(^{2+}\) ions bind to individual His residues within the octarepeats (complex II). Coordination of Cu\(^{2+}\) to multiple histidines in the octarepeats (complex I) (43, 44), is not ruled out. However, this complex is CD silent and is therefore clear complex I does not make a signifi-
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$^{2+}$ Binding Full-length mPrP-(23–231) and Analogues Detected by Visible CD—$^{2+}$ is often a good model for $^{2+}$ binding to square planar complexes involving amide main chain coordination. We have previously shown that $^{2+}$ can mimic $^{2+}$ binding to His$^{95}$ and His$^{110}$ in PrP fragments, but will not bind to the octarepeat peptide fragments of PrP because of the presence of a proline preceding the histidine residues, which blocks the coordination of amides preceding the histidine residue (8, 47). Thus, $^{2+}$ can be used to further differentiate binding to His$^{95}$ and His$^{110}$, and the octarepeat region of PrP. The $^{2+}$ visible CD spectra are shown in Fig. 7a for full-length mPrP-(23–231) at pH 9. Two visible CD bands are observed: one positive band at 410 nm and a negative band at 498 nm of similar intensity. From the $^{2+}$ binding curves shown as inserts it is clear these two bands increase in intensity to the same extent with each $^{2+}$ addition, and saturate after 2 mol eq of $^{2+}$ are added. In Fig. 7b, it is clear that the mPrP-(23–231)$^{40}$ analogue also gives rise to almost identical visible CD spectra. Clearly, the presence or absence of the octarepeats does not affect $^{2+}$ binding to full-length PrP, as predicted from studies with peptide fragments. The Ni-mPrP-(23–231) visible CD spectra are compared with $^{2+}$ binding to a shorter peptide fragment, mPrP-(90–114), which lies between the octarepeats and the structured domain. It is clear from Fig. 7c that $^{2+}$ binding to full-length PrP produces visible CD spectra that have striking similarity to $^{2+}$ binding to mPrP-(90–114), as shown in Fig. 7c.

Previous studies by $^1$H NMR and visible CD on the hPrP-(90–126) and hPrP-(91–115) fragments have shown that $^{2+}$ forms two independent diamagnetic complexes centered at His$^{96}$ and His$^{111}$ (His$^{95}$ and His$^{110}$ in mouse sequence) (complexes III and IV in Fig. 1) (8, 23). Visible spectra of peptide analogues containing single histidine reveal almost mirror-image visible CD spectra (8, 23, 45), shown in Fig. 7c as an inset. Also shown as an inset in Fig. 7c is the resulting spectra of the addition of two $^{2+}$-bound pentapeptides, Ni$_2$mPrP-(91–95) and Ni$_2$mPrP-(106–110). These pentapeptides have striking resemblance to full-length PrP and the mPrP-(90–114) fragment. This approach has been described previously (8, 23). Simulations show His$^{95}$ and His$^{110}$ sites load simultaneously with similar affinities. A very different appearance in the set of spectra for the $^{2+}$ titration is generated when sequential loading is simulated (data not shown).

Finally, as expected, $^{2+}$ additions to the mPrP(23–231)H956A,H110A analogue (Fig. 7d) gives rise to weak visible CD signal compared with the other $^{2+}$-bound spectra, even after 4 mol eq of $^{2+}$ are added. This indicates the lack of $^{2+}$ coordination to single histidines of the octarepeats (complex II).

To summarize, as with $^{2+}$ binding to full-length PrP, $^{2+}$ binds to both His$^{95}$ and His$^{110}$. In the case of $^{2+}$, His$^{95}$ loads predominantly before His$^{110}$, but $^{2+}$ loads to His$^{95}$ and His$^{110}$ simultaneously. The presence of the octarepeat region has no direct effect on $^{2+}$ binding to full-length PrP.
EPR of Cu$^{2+}$ Binding to PrP—To complement the visible CD data, Fig. 8 is a comparison of continuous wave EPR spectra of Cu$^{2+}$ titrations at pH 7.4 for full-length PrP-(23–231) and various fragments of the protein. The EPR spectra are typical Cu$^{2+}$ (type II) axial spectra with $A_0^J$ and $g_\perp$ values indicative of 4N or 3N1O coordinating ligands. Analysis of this data has been greatly informed by studies from Millhauser and co-workers (44). In agreement with our visible CD data, Fig. 8a suggests Cu$^{2+}$ initially binds to His$^{95}$ and His$^{110}$ before binding to individual octarepeats (complex II). The EPR spectrum for the full-length protein bound to 1 mol eq of Cu$^{2+}$ is almost identical to that obtained for the shorter peptide, hPrP-(90–126), where the octarepeat region is absent. In contrast, the spectra of mPrP-(57–90), a peptide fragment containing the four octarepeats, gives a very different EPR spectrum when loaded with two equivalents of Cu$^{2+}$ (Fig. 8a), with a higher $g_\perp$ value.

We note that for the first equivalent of Cu$^{2+}$ binding to full-length PrP (Fig. 8a, inset), the axial complex formed is sufficiently planar to resolve the $g_\perp$ hyperfine coupling. The same complex in the hPrP-(90–126) fragment must have axial ligands slightly out of the plane, as $g_\perp$ hyperfine coupling is not resolved.

The Cu$^{2+}$ titration at pH 7.4 for the truncated protein, mPrP-(23–125), is presented in Fig. 8b. The first two equivalents of Cu$^{2+}$ produce EPR spectra very similar to that for hPrP-(90–126), complexes III and IV. Further addition of Cu$^{2+}$ produces a new set of signals typical of Cu$^{2+}$ binding to a single His in the octarepeats, complex II. The difference in $g_\parallel$ values for complex III/IV and complex II suggest a change from a 4N coordination to $g_\parallel$ values more typical of 3N1O coordination, as expected (52). Because we established the first 2 mol eq of Cu$^{2+}$ are binding to His$^{95}$ and His$^{110}$, difference spectra were obtained where the EPR spectra for the first 2 mol eq of Cu$^{2+}$ are subtracted from all subsequent spectra in the titration. This produced spectra that were directly comparable with those obtained for the isolated octarepeats peptide fragment, and are shown in Fig. 8c. Closer inspection of the hPrP-(58–91) spectra indicates the first Cu$^{2+}$ ion binding to the octarepeats produce spectra typical of a multiple His complex, complex I, described previously (44). After addition of further
mole equivalents of Cu\(^{2+}\), this binding is out-competed by the single histidine complex within the octarepeats; complex II.

**Cup**\(^{2+}\) **Affinity of mPrP-(23–231) Using Competing Chelators**—The affinity of Cu\(^{2+}\) for PrP was investigated using various competitive Cu\(^{2+}\) chelators and both EPR and visible CD. Fig. 9 presents continuous-wave EPR spectra for mPrP-(23–231) bound to 0.9 mol eq of Cu\(^{2+}\) at pH 7.4. The EPR spectrum gives rise to Type II axial spectra with \(g_\alpha\) and \(A_\alpha\) values of 2.25 and 17.2 mK, respectively. According to Peisach and Blumberg (52), these values indicate a coordination of Cu\(^{2+}\) typical for 4N or 3N1O ligands. Subsequent spectra show the effect of increasing additions of a competitive copper chelator, HIMDA. HIMDA binds to Cu\(^{2+}\) ions at pH 7.4 with an affinity of log \(K_a\) 10.6 m\(^{-1}\) and coordinates Cu\(^{2+}\) using oxygen ligands (53). This enables us to distinguish the nitrogen coordination mode of Cu\(^{2+}\)-PrP from oxygen coordination for the Cu\(^{2+}\)-HIMDA complex. A preference of Cu\(^{2+}\) for HIMDA over PrP may be readily detected as a change in \(A_\alpha\) and \(g_\alpha\) values in the EPR spectra. Upon the addition of just 0.2 mol eq of HIMDA, the hyperfine \(A_\|\) splitting reduces in magnitude and overall, the \(g_\|\) signals shift to higher field strengths. These changes are typical of ~4N coordination shifting to a more oxygen-rich coordinating environment. HIMDA-bound Cu\(^{2+}\) EPR confer \(g_\|\) and \(A_\|\) values of about 2.29 and 14.8 mK, respectively. As the binding curves show (inset), it only requires 1 mol eq of HIMDA to successfully remove all the Cu\(^{2+}\) bound to PrP and observe a spectrum almost identical to Cu\(^{2+}\)-HIMDA. Fig. 10b shows a direct comparison of the HIMDA:PrP:Cu mixture with Cu\(^{2+}\)-HIMDA on its own. This study suggests that Cu\(^{2+}\) is binding preferentially to HIMDA over mPrP-(23–231). Therefore, we can infer the affinity Cu\(^{2+}\) has for PrP in its tight binding mode must be less than HIMDA, and therefore must have a log \(K_a\) less than 10.6 m\(^{-1}\).

Complementary HIMDA competition studies were performed using visible CD. A HIMDA titration of mPrP-(23–231) bound to 0.9 mol eq of Cu\(^{2+}\) is shown in Fig. 9c. In agreement with the EPR spectra, we see that it takes just 1 mol eq of HIMDA to remove 1 mol eq of Cu\(^{2+}\) from PrP. In particular, the negative CD signal at 576 nm from Cu\(^{2+}\)-PrP disappears, with all the Cu\(^{2+}\) now binding to the non-chiral, CD silent, Cu\(^{2+}\)-HIMDA complex.

Further competition studies were carried out with full-length mPrP-(23–231) at pH 7.4, using free glycine and free l-histidine as competitors, in order that the affinity of Cu\(^{2+}\) for PrP could be further elucidated. Fig. 10a is a Gly titration of mPrP-(23–231) bound to 0.9 mol eq of Cu\(^{2+}\). Free glycine is non-chiral and coordinates to Cu\(^{2+}\) via its amino and carboxylate ligands with a \(K_a\) of log 5.9 m\(^{-1}\) (53). The Cu\(^{2+}\)-Gly complex does not give rise to any visible CD signals, so one would expect any CD bands to disappear if Cu\(^{2+}\) were binding preferentially to glycine over PrP. The Cu\(^{2+}\)-PrP complex has a negative CD band around 576 nm, and as more Gly is titrated in there is a decrease in the intensity of this signal. However, even after 12 mol eq of Gly are added, an appreciable Cu\(^{2+}\)-PrP visible CD signal still remains. The inability of Gly to pull off the PrP-bound Cu\(^{2+}\) indicates PrP has a higher affinity for Cu\(^{2+}\) than Gly, and therefore, must have a log \(K_a\) greater than 5.9 m\(^{-1}\). Fig. 10b shows a similar experiment, but for l-His instead of Gly. l-His, with a log \(K_a\) of 8.4 m\(^{-1}\) at pH 7.4 has a higher affinity for Cu\(^{2+}\) than Gly, 

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**Copper**\(^{2+}\) **Binding to Full-length Prion Protein**
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but has a lower affinity than HIMDA for Cu$^{2+}$ (53). As Fig. 10b indicates, it only takes 2 mol eq of i-His to bind most of the Cu$^{2+}$ from PrP, resulting in the characteristic visible CD spectrum for Cu$^{2+}$-bound i-His (Cu(His)$_2$) with a single positive band at 704 nm. The inset panel is the visible CD spectra for Cu$^{2+}$ bound to free i-His, for both the 2 His-1 Cu$^{2+}$ complex and the 1 His-1 Cu$^{2+}$ complex. Potentially, some Cu$^{2+}$ could be bound to PrPC in a multiple His complex, which lacks main chain amide coordination. We note that this type of complex is likely to be CD silent. However, i-His-Cu can be used to quantify directly the amount of Cu$^{2+}$ being detected and it is clear from Fig. 10b that 70% of the Cu$^{2+}$ added to the equilibrium mixture is bound to His after 2 mol eq of His has been added. At 4 mol eq of His, >90% of the added Cu$^{2+}$ is detected bound to the free i-His. A similar i-His competition study has been performed for the truncated PrP fragment, mPrP-(23–125). Supplementary Fig. S2 shows visible CD spectra for i-His competing with the N-terminal peptide mPrP-(23–125) for Cu$^{2+}$. Studies with mPrP-(23–125) shown in Fig. 3 indicate that the removal of the C-terminal domain has little impact on Cu$^{2+}$ binding. Like the studies with full-length mPrP-(23–231), 3 mol eq of i-His is all that is required to extract almost all the first mole equivalent of bound Cu$^{2+}$ from mPrP-(23–125).

Using two spectroscopic techniques we have shown that at pH 7.4 the first equivalent of Cu$^{2+}$ ions binds to full-length PrP with an affinity significantly greater than glycine, but comparable or slightly less than i-His. This puts the dissociation constant for the first mole equivalent of Cu$^{2+}$ with mPrP-(23–231) at ~10 nm, an affinity of 10$^8$ M$^{-1}$.

**DISCUSSION**

Cu$^{2+}$ binding to PrP has been linked to the normal function of PrP, as well as prion disease (54, 55). The affinity of Cu$^{2+}$ for PrP, coordination geometry, and effect on main chain conformation should give us further understanding of the role of Cu$^{2+}$ in normal function of PrP, as well as prion diseases. Visible CD has proved to be a powerful technique to discriminate between four different binding modes to PrP. Previous attempts to obtain Cu$^{2+}$-bound visible CD spectra for full-length PrP have been hampered by the solubility of Syrian hamster PrP at pH values above 6, a pH suitable for Cu$^{2+}$ binding studies (9). However, we have found that the mouse mPrP-(23–231) is sufficiently soluble even at pH 7.4 and above to carry out the visible CD experiments described. Thus, for the first time we present a Cu$^{2+}$ titration of full-length mammalian PrP, monitored by visible CD.

**Sequential Loading of Cu$^{2+}$ to His$^{95}$ and His$^{110}$ before the Octarepeats**—It is very clear from visible CD spectra of Cu-mPrP-(23–231) and the complementary spectra from the recombinant analogues (Δocta and H95A + H110A), as well as smaller peptide fragments, that there are a number of sequential modes of Cu$^{2+}$ coordination. The first 2 mol eq of Cu$^{2+}$ will form two isolated complexes at His$^{95}$ and His$^{110}$ that involve the preceding main chain amides (complexes III and IV in Fig. 1). Any formation of the CD-silent multiple octarepeats complex (complex I) must form after Cu$^{2+}$ loading at His$^{95}$ and His$^{110}$. Subsequent binding of Cu$^{2+}$ ions produces CD signals indicative of binding to single imidazole ring histidines and main chain amides within the octarepeats (complex II). These observations are supported by our complementary EPR data. Like Cu$^{2+}$, Ni$^{2+}$ binding to full-length PrP forms a complex centered at His$^{95}$ and His$^{110}$ with preceding amide coordination (complexes III and IV). The presence of the octarepeat region has no direct effect on Ni$^{2+}$ binding to full-length PrP.

It is often assumed that the main mode of Cu$^{2+}$ coordination is to the octarepeats followed by the so-called 5th site. This in part may be for historical reasons, as the octarepeats were identified as the Cu$^{2+}$ binding center before the 5th site (His$^{95}$ and His$^{110}$). It is notable that competition studies with Cu$^{2+}$ for two peptide fragments, hPrP-(58–91) and hPrP-(91–115), support our observation for full-length PrP, indicating that Cu$^{2+}$ would bind initially to His$^{95/110}$ (His$^{96/111}$ in the human sequence) followed by the octarepeats (19). This study on full-length PrP confirms studies on fragments that suggested 6 Cu$^{2+}$ ions bind to PrPC with appreciable affinity (8). An important observation is to note that Cu$^{2+}$ added to full-length PrP produces a significant visible CD signal even at substoichiometric amounts of Cu$^{2+}$. Coordination of Cu$^{2+}$ to multiple His has previously been observed (complex I) (43, 44). This type of complex with no main chain coordination from amides, will be CD silent in the visible region (43, 45). However, even after only 0.3 mol eq of Cu$^{2+}$ added, visible CD signal is apparent and increases almost linearly in intensity with Cu$^{2+}$ addition up to the first equivalent. It is clear that even at substoichiometric levels of Cu$^{2+}$ the initial complex formed must involve fixed main chain amides, centered at His$^{95}$ and His$^{110}$, at pH 7.4. At lower pH values, amide deprotonation is less favored and multiple His complexes will be formed.

There have been relatively few structural studies of Cu$^{2+}$ binding to full-length PrP due to its poor solubility at physiological pH. Crystallographic data of the flexible N-terminal tail has been difficult to obtain. Paramagnetic Cu$^{2+}$ ions cause profound broadening of 1H NMR signals and the interpretation of this effect can be complicated by the rapid exchange of Cu$^{2+}$ ions between different binding sites. However, recent studies of Cu$^{2+}$ bound to full-length PrP at pH 5.5, where the multiple His complexes are favored, have been reported (40). Cu$^{2+}$-EPR is powerful at indicating coordination geometry and ligand type, however, all Cu$^{2+}$ binding modes in PrP have been identified as Type II axial complexes and the differences between their EPR spectra can quite subtle. In particular, it is hard to discriminate by continuous wave-EPR, complex III at His$^{95}$ and complex IV at His$^{110}$ (45). Despite this, there has been significant progress in this area; Burns et al. (22) have shown Cu$^{2+}$ binding to full-length PrP at His$^{96}$ as well as the octarepeats, although binding at His$^{111}$ was thought to be of low affinity.

**Affinity of Cu$^{2+}$ for PrP**—The affinity of Cu$^{2+}$ for PrP is hotly debated (20, 43, 44, 48, 49). 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 (7, 48). However, subsequent studies suggest a number of orders of magnitude higher, with a femtomolar dissociation constant for the full-length protein (20, 49). Studies of fragments have Cu$^{2+}$ affinities with quite a reasonable consensus in the nanomolar to micromolar range. Key studies of Cu$^{2+}$ binding to the four octarepeats fragment suggests at substoi-
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The octarepeats polypeptide chain on either side of the octarepeats affecting the unstructured region of PrP. However, there has always been some concern whether this approach was valid. The close similarity between the visible CD spectra for mPrP-(23–231) and mPrP-(23–125) indicates that Cu$^{2+}$ binding to PrP is largely unaffected by the structured domain of PrP. In this study, Cu$^{2+}$ binding to smaller fragments within the unstructured domain of PrP have been invaluable for aiding in the characterization of the various binding modes to the full-length protein. In our work we show that in most key respects, the modes of Cu$^{2+}$ binding in the fragments are very similar to full-length PrP. The visible CD and EPR spectra for Cu$^{2+}$ and Ni$^{2+}$ binding indicate the same coordination geometries for full-length PrP and fragments of the protein.

Cu$^{2+}$ coordination geometry and affinity for His$^{96}$ and His$^{111}$ (complexes III and IV) are very similar. We have previously shown that fragment length and pH influence the subtle preference of Cu$^{2+}$ (and Ni$^{2+}$) over His$^{96}$ or His$^{111}$. Thus, for human fragments, Cu$^{2+}$ binds at His$^{111}$ preferentially over His$^{96}$, whereas the reverse is true for Ni$^{2+}$ (23). Here we show for the mouse sequence that the preference is reversed; Cu$^{2+}$ binds to His$^{95}$ preferentially over His$^{110}$, in both the mPrP-(91–115) fragment and full-length mPrP.C. Perhaps Met$^{109}$ in hPrP stabilizes the His$^{111}$ complex over Leu$^{108}$ in the mouse sequence.

There are some differences in the intensity of CD bands for full length PrP but not their position, relative to the complex in the octarepeats peptide fragment. The signal in the fragments is more intense than the same Cu$^{2+}$ CD band in full-length PrP. It is notable that CD intensities for Ni$^{2+}$ binding to full-length and fragments are very comparable. We have confidence that the bands observed are correctly assigned to binding to the octarepeats because the wavelength position, relative intensity, and sign of CD bands are identical. In addition, removal of the octarepeats in the mPrP(23–231)Δocta recombinant construct or the use of Ni$^{2+}$, which will not bind to the octarepeats, supports this assertion. The concentration of the protein is measured using Bradford assay and absorbance at 280 nm. We suggest that the difference in intensity is a subtle effect of the length of polypeptide chain. The most likely explanation is that there is a slight difference in strain on the complex formed by the length of chain outside the Cu$^{2+}$ binding region. The effect is not apparent for Ni$^{2+}$, which has a different ionic radius. The EPR supports this with the observation of g hyperfine splitting resolved in full-length PrP, but not fragments. This would imply full-length PrP has slightly less strain in the complex and is more planar, which would result in a reduced dichroism, as observed.

CONCLUSIONS

Cu$^{2+}$ and Prion Diseases—PrP$^{C}$ is unusual in that half of the protein is natively unstructured, facilitating the coordination of up to six Cu$^{2+}$ ions at physiological pH, with the first 2 eq of Cu$^{2+}$ binding to the His$^{96}$ and His$^{110}$ sites in the amyloidogenic region of PrP, followed by the octarepeats. This unstructured neurotoxic region (24), PrP-(90–126), has been shown to be vital for prion propagation (18). In vitro studies suggest Cu$^{2+}$ can confer the strain of prion disease (11). Copper binding, particularly at the octarepeats causes a profound ordering of the main chain, but does not directly cause β-strands to be formed (9, 41). However, coordination of Cu$^{2+}$ reduces solubil-
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ity and promotes aggregation of PrP, although in vitro Cu$^{2+}$ binding seems to inhibit amyloid fibril growth (59).

Copper$^{2+}$ and Prion Function—PrP$^C$ is tethered to the cell surface and is highly localized at presynaptic membranes (32). Release of free Cu$^{2+}$ ions during neurotransmission can cause fluxes of Cu$^{2+}$, consequently local concentration of free Cu$^{2+}$ may be as high as 0.1 mM at the synaptic cleft (31). Free Cu$^{2+}$ ions are potentially highly toxic to cells, generating hydroxyl radicals via Fenton chemistry. PrP$^C$ affinities of Cu$^{2+}$ of 1–100 nM are well placed to bind these Cu$^{2+}$ ions, with as many as six binding sites. The unusually rapid turnover of PrP, every 3–6 h (31), supports this hypothesis that PrP could act as a sacrificial quencher of generated free radicals, resulting in the oxidation of solvent-exposed Met (and Trp) residues close to the Cu$^{2+}$ binding sites (15). PrP$^C$ function may well be to scavenge Cu$^{2+}$ ions released during neuronal depolarization and so protect cells from the toxic effects of copper. This idea is supported by the observation that PrP knockouts are more sensitive to copper-induced oxidative stress than wild-type PrP$^C$ (12, 39).

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