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A Reassessment of Copper (II) Binding in the Full-Length Prion Protein

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Short title: Copper (II) binding in the full length prion protein

Synopsis

It has been shown previously that the unfolded N-terminal domain of the prion protein can bind up to six Cu^{2+} ions *in vitro*. This domain contains four tandem repeats of the octapeptide sequence PHGGGWGQ, which alongside the two histidine residues at positions 96 and 111, contribute to its Cu^{2+} binding properties. At maximum metal-ion occupancy each Cu^{2+} is coordinated by a single imidazole and deprotonated backbone amide groups. However two recent studies of peptides representing the octapeptide repeat region of the protein, have shown that at low Cu^{2+} availability an alternative mode of coordination occurs where the metal ion is bound by multiple histidine imidazoles. Both modes of binding are readily populated at pH 7.4, while mild acidification to pH 5.5 selects in favour of the low occupancy, multiple imidazole binding mode. We have used NMR to resolve how Cu^{2+} binds to the full-length prion protein under mildly acidic conditions where multiple histidine coordination is dominant. We show that at pH 5.5 the protein binds two Cu^{2+} ions and that all six histidines of the unfolded N-terminal domain and the N terminal amine act as ligands. These two sites are of sufficient affinity to be maintained in the presence of millimolar concentrations of competing exogenous histidine. A previously unknown interaction between the N-terminal domain and a site on the C-terminal domain becomes apparent when the protein is loaded with Cu^{2+} . Furthermore, the data reveal that sub-stoichiometric quantities of Cu^{2+} will cause self-association of the prion protein *in vitro* suggesting that Cu^{2+} may play a role in controlling oligomerisation *in vivo*.

Keywords: Prion, PrP, Copper (II), Metal binding, NMR, Fluorescence

Introduction

Prion diseases are a group of fatal neurodegenerative disorders that includes Creutzfeldt-Jakob disease in humans, scrapie in sheep and bovine spongiform encephalopathy in cattle. In humans such diseases can arise sporadically, be inherited, or occur through infection with prion-contaminated material [1, 2]. The protein only hypothesis states that the particles that cause infection, termed prions, are composed principally or entirely of a misfolded isoform of a host-encoded protein, termed the prion protein (PrP)[3-5], with prion propagation occurring by conformational conversion of the normal isoform, PrP^C, to the disease related isoform PrP^{Sc}. Mammalian PrP^C is a glycolipid anchored cell surface glycoprotein found at high levels in the central nervous system, lymphatic tissue, activated lymphocytes and at neuromuscular junctions [6]. The physiological function of PrP remains unclear; ablation of the PrP gene, *Prn-P*, in mice results in viable animals without gross developmental or behavioral abnormalities [7], although more detailed studies revealed some phenotypic effects, including altered synaptic physiology [8]. Biophysical and biochemical studies have demonstrated that PrP specifically binds Cu²⁺ and this has led to the proposal that PrP is a metalloprotein *in vivo* [9-21].

PrP^C comprises a flexibly disordered N-terminal domain (residues 23-125) [22] and a globular, mainly α -helical, C-terminal domain (residues 126-231)[23]. Residues 60-91 of the protein comprise four tandem repeats of the sequence PHGGGWGQ. It is this region, known as the octapeptide repeats, which was first discovered to bind Cu²⁺[10, 11] and a considerable body of work has since been devoted to identifying the number of binding sites, their affinities and the nature of coordination, in both the octapeptide repeats and the full length prion protein, PrP²³⁻²³¹ ‡[9, 12-21]. Until recently, the accepted model of Cu²⁺ binding to the full length prion protein, at pH 7.4, is that it binds six Cu²⁺ ions at separate sites, one in each of the four octapeptide repeats and two more at sites involving H96 and H111, and each with coordination from a single histidine imidazole and amide nitrogens from nearby glycines [19].

Two recent studies have shown that the model described above provides an incomplete picture of Cu²⁺ binding in the prion protein, by demonstrating that a peptide representing

the four octapeptide repeats displays multiple modes of Cu^{2+} binding at pH 7.4 [24, 25]. The peptide can bind a single Cu^{2+} ion through multiple histidine coordination but, in the presence of more Cu^{2+} , binding of up to four Cu^{2+} ions occurs, with all of these coordinated in identical sites - each involving deprotonated backbone amide nitrogen atoms and a single histidine. Perhaps counter-intuitively, these four Cu^{2+} binding sites replace a single site of higher affinity. Peptides representing the octapeptide repeats cannot, however, be considered a complete model for Cu^{2+} binding to the prion protein. Cu^{2+} binding sites outside the octapeptide repeats have also been reported, involving histidines 96 and 111 [12, 19, 26, 27]. It is thus clear that for a full understanding of Cu^{2+} binding to the prion protein, binding to the full-length protein needs to be investigated, and in particular the involvement of multiple histidine Cu^{2+} coordination. Given the low availability of Cu^{2+} in the extracellular milieu (examined in the discussion section), this higher affinity binding mode is more likely to be substantially populated *in vivo*.

Fortunately, although the full-length prion protein is poorly soluble above pH 6 [22], it can be studied at lower pH values where multiple histidine Cu^{2+} coordination should be favoured. In aqueous solution H^+ ions will compete for the basic coordinating groups that form a metal binding site, and thus the pK_a of the coordinating groups and the pH of the solution are both determinants of the stability of a metal complex. The pK_a of the histidine imidazole group is around 6, which is substantially lower than that of a backbone amide nitrogen, and thus coordination by multiple histidines will be maintained at lower pH compared with coordination modes involving deprotonated backbone amide groups. We find that binding of two Cu^{2+} ions is maintained in the full-length prion protein at pH 5.5, and have investigated the nature of the coordination in this complex. Here we report the locations of these binding sites and the identity of the coordinating groups. We demonstrate that these binding sites are of sufficient affinity to be maintained in the presence of substantial concentrations of exogenous histidine, a substance known to bind copper *in vivo* [28]. Finally, we show how Cu^{2+} binding is altered in a truncated form of PrP, comprising residues 91 to 231 - PrP⁹¹⁻²³¹, which has no octapeptide repeat regions, but which can still transmit infection.

Experimental

Expression, purification and metal stripping of PrP⁹¹⁻²³¹

Human PrP⁹¹⁻²³¹, with methionine at position 129, was expressed and purified as described previously [29, 30]. The PrP²³⁻²³¹ and PrP⁹¹⁻²³¹ constructs used here both have the additional three residue sequence SFR present at the N-terminus which forms part of the thrombin cleavage site. To ensure the protein was free of metal ions, the protein was repeatedly buffer exchanged with 8 M urea, 20 mM EDTA, 25 mM TRIS/acetate, pH 8.0 and then refolded in 5 mM MES, pH 5.5 in an Amicon ultrafiltration unit (3 kDa molecular weight cut off). The protein concentration for refolding was less than 1 mg/ml. All buffers used during and after the metal stripping step were prepared using AnalR grade water and, wherever possible, Aristar grade reagents, and were treated with Chelex resin (BioRAD).

Expression, purification and metal stripping of full length prion protein, PrP²³⁻²³¹

Full length human prion protein, with methionine at position 129, was expressed with a N-terminal his-tag containing a thrombin cleavage site, using standard molecular biology protocols. Expression of uniformly ¹⁵N, ¹³C labeled protein and the initial purification by Ni-NTA chromatography was carried out using the same procedures described previously for PrP⁹¹⁻²³¹ [29, 30]. Oxidation of the disulfide bond was checked by HPLC [30] and was complete after 24 hours, without the aid of a catalyst. The protein was diluted to less than 1 mg/ml in 6 M guanidine HCl, 50 mM TRIS/HCl, pH 8.0 and then refolded by dialysis against 150 mM acetate/TRIS, 0.02% w/v NaN₃, pH 3.5. In preparation for the thrombin cleavage step, the pH was gradually increased by successive dialysis steps against 10 mM TRIS/acetate, 0.02 % w/v NaN₃ at pH 4.5, pH 5.5, pH 6.5 and pH 7.0. The fusion protein was cleaved by adding 0.6 units of thrombin (Novagen) per milligram of protein and CaCl₂, to a concentration of 2.5 mM. The reaction was incubated at 37 °C for 24 hours. Cleavage was halted by the addition of the protease inhibitor cocktail Protease Complete (Roche). For the final purification step a higher pH was required and this had to be carried out under denaturing conditions for the protein to be soluble. Urea was thus added to the reaction mixture to give a final concentration of 8 M and the pH

was adjusted to 7.5. This material was then loaded onto an SP-Sepharose Fast Flow column (Amersham), pre-equilibrated with 8 M urea, 150 mM NaCl, 50 mM TRIS/HCl pH 7.5. The column was washed extensively with the equilibration buffer. A salt gradient from 150 mM to 600 mM NaCl in 8 M urea, 50 mM TRIS/HCl, pH 7.5 was run over 8 column volumes and the protein was eluted at approximately 300-400 mM NaCl. To remove any metal ions, the purified protein was repeatedly buffer exchanged against 8 M urea, 25 mM EDTA, 20 mM TRIS/acetate, pH 8.0 and then refolded in 150 mM acetate/TRIS, pH 3.5, in an Amicon ultra-filtration unit (3 kDa molecular weight cut off). The protein concentration for refolding was less than 1 mg/ml. The buffer was exchanged to 20 mM acetate/TRIS pH 4.5 and then finally 10 mM MES pH 5.5. All buffers used during and after the metal stripping step were prepared using AnalR grade water and, wherever possible, Aristar grade reagents, and treated with Chelex resin (BioRAD).

NMR sample preparation

For backbone and sidechain assignment experiments, metal-free full length prion protein in 20 mM MES, pH 5.5 was concentrated to approximately 1 mM protein. NaN_3 (to a final concentration of 1 mM), EDTA free Protease Complete (Roche) and 10% D_2O were added, and the pH readjusted to 5.5 by addition of MES. The final MES concentration was approximately 30 mM. The sample was spun for 5 minutes at 13,000 rpm in a microcentrifuge, to remove any precipitated material. For titrations with Cu^{2+} the samples were prepared likewise, but only concentrated to approximately 0.5 mM or 0.1 mM protein and no NaN_3 was added. An absorbance measurement at 280 nm was taken on an aliquot of the sample diluted into 6 M guanidine HCl, 50 mM Tris/HCl, pH 8.0. The protein concentration was determined based on a calculated extinction coefficient [31]. ICP-MS analysis showed that no significant quantities of transition metals were present in the NMR sample (transition metals above 1 μM were: Cd 5.5 μM , Cu 4.7 μM , Zn 3.6 μM , Fe 1.8 μM). For titration with Cu^{2+} a sample of 1.2 mM PrP⁹¹⁻²³¹ was prepared in 5 mM MES, pH 5.5, with 10% D_2O . Measurement of the protein concentration was carried out by the same method. Nitric acid washed NMR tubes were used for metal titration samples.

NMR resonance assignment experiments for the full length prion protein

- see supplementary online materials and methods

Cu²⁺ titrations monitored by NMR

The PrP⁹¹⁻²³¹ sample was titrated with 4 aliquots of 4 mM CuSO₄, 10 mM glycine, 5 mM MES pH 5.5 to give mole fractions of Cu²⁺ relative to protein of 0.25, 0.50, 0.75 and 1.00. NMR spectra were recorded at 303 K on a Bruker Avance DRX-600 spectrometer. At each point in the titration a ¹H¹⁵N HSQC and aromatic and aliphatic ¹H ¹³C HSQCs were recorded. The full length prion protein sample was titrated with 13 aliquots of 4 mM CuSO₄, 10 mM glycine, 5 mM MES pH 5.5, to give mole fractions of Cu²⁺ relative to protein of 0.05, 0.10, 0.20 and through to 2.20, in steps of 0.20. NMR spectra were recorded at 303K on a Bruker Avance AV-800 spectrometer. Again at each point in the titration a ¹H¹⁵N HSQC and aromatic and aliphatic ¹H¹³C HSQCs were recorded.

Determination of dissociation constants

Samples of 3.5 μM full length prion protein in 5 mM MES, pH 5.5 were prepared containing either 10 mM histidine or 100 mM histidine. CuSO₄ was added as a glycine complex in 5 mM MES at pH 5.5, to give the appropriate final Cu²⁺ concentrations. Fluorescence measurements were made at 30 °C with an excitation wavelength of 291 nm and an emission wavelength of 345 nm, using a Jasco FP-750 spectrofluorimeter. The contribution of collisional quenching effects was evaluated by titrating samples of 20 μM N-acetyltryptophanamide (matching the concentration of tryptophan residues in the prion protein sample) with Cu²⁺ under the same conditions. The prion protein data was then corrected for the measured collisional quenching effect.

Analysis of the data and least squares curve fitting was carried out using the program Grafit (Erithacus Software). To obtain the apparent dissociation constant for the second Cu²⁺ binding event, the fluorescence data recorded in the presence of 10 mM histidine were fitted to a discontinuous function describing two phases of metal binding (equation 1), where F₀ is the initial fluorescence and F_M is the fluorescence at a given metal concentration, A₁ and A₂ are the amplitudes of the fluorescence changes associated with

each event, M is the total metal concentration, P is the total protein concentration and K_{d2} is the apparent dissociation constant of the second binding event. A linear function describes the first event where the apparent K_d value is much less than the concentration of sites, whilst the second event, for which the apparent K_d value is similar to the concentration of sites, is described by the quadratically-derived ligand binding equation.

$$M \leq P \quad F_M = F_0 + M \left(\frac{A_1}{P} \right)$$

$$M > P \quad F_M = F_0 + A_1 + A_2 \left(\frac{M + K_{d2} - \sqrt{(M + K_{d2})^2 - 4(M - P)P}}{2P} \right)$$

Equation 1

The data acquired in the presence of 100 mM histidine were fitted to equation 2 which describes metal binding to a single site, with a dissociation constant of K_{d1} . The apparent K_d value of the second site, under these conditions, will be too large to make a significant contribution to the observed fluorescence quenching and hence the contribution of this site was ignored in the fitting procedure.

$$F_M = F_0 + A_1 \left(\frac{P + M + K_{d1} - \sqrt{(P + M + K_{d1})^2 - 4MP}}{2P} \right)$$

Equation 2

The true K_d values of first and second Cu^{2+} binding events at pH 5.5 were calculated using equation 3, where $K_{d(\text{app})}$, is the measured apparent dissociation constant, $K_{d(\text{His})}^1$ and $K_{d(\text{His})}^2$ are the step wise dissociation constants for formation of a $\text{Cu}(\text{histidine})_2$ complex at pH 5.5, (1.2 μM and 160 μM respectively [30]) and the concentration of histidine is $[\text{His}]$.

$$K_d = \frac{K_{d(app)}K_{d(His)}^1K_{d(His)}^2}{[His]^2}$$

Equation 3

Results

NMR-detected effects of Cu²⁺ addition

Observation of NMR signals as a protein is titrated with Cu²⁺ provides a site specific probe for the identification of nuclei close to the metal ion in a Cu²⁺ protein complex. Proximity to the paramagnetic Cu²⁺ ion causes acceleration of the T₂ relaxation of nuclei, and hence broadens NMR signals, leading to a loss of peak height [32]. Nuclei close to the metal binding site can thus be identified by a reduction in the height of their NMR signals as Cu²⁺ is titrated into the protein. Backbone and sidechain signals were monitored on addition of Cu²⁺ bis-glycinate to the full length prion protein, PrP²³⁻²³¹, using ¹H, ¹⁵N and ¹H, ¹³C heteronuclear single quantum coherence (HSQC) NMR spectra. The presence of glycine allows the Cu²⁺ to be added as a pH buffered solution, by preventing the precipitation of Cu²⁺ as copper hydroxide, which would otherwise occur at pH 5.5 [33]. The overlap in chemical shift of the signals from the octapeptide repeat region meant that these residues were represented by a single set of resonances, each of which was a combination of four or five signals, one from each repeating unit and from a pseudo octapeptide repeat sequence at positions 52-59.

The effects of titration with Cu²⁺ on the height of the protein's NMR signals fall into four broad categories, of which representative examples are shown in Fig. 1A. Certain signals are almost completely unaffected; others are initially unaffected but are attenuated after *ca.* 2 molar equivalents of Cu²⁺ have been added; a third group show substantial attenuation after addition of sub-stoichiometric quantities of Cu²⁺; the final group behave similarly to these but begin to recover after *ca.* 1.4 molar equivalents of copper have been added.

The full length prion protein binds two Cu²⁺ ions at pH 5.5

Numerous backbone [N,H^N][§] signals, for example those of T191 (Fig. 1A), show very limited reduction in height throughout the titration, indicating that these residues are remote from any metal binding site. In contrast, there are a groups of signals from residues such as S231 (Fig. 1A) for which, initially, the backbone [N,H^N] signal peak heights are largely unaffected by Cu²⁺ addition but which rapidly lose height after *ca.* two

molar equivalents of Cu^{2+} have been added. This behaviour occurs at multiple sites in the vicinity of residues that have a functional group with a propensity for metal binding (e.g. H140, D144, D178, E207, S231). Similarly, in terms of chemical shift, some $[\text{N},\text{H}^{\text{N}}]$ signals exhibit changes only after *ca.* two molar equivalents of Cu^{2+} have been added, including those of S143, D144, Y145 and S231 (Fig. 1B). The affected residues are positioned at several distinct sites on the surface of the protein, so the effects can not be due to a single specific Cu^{2+} binding site. Instead they are the result of several different transient interactions with Cu^{2+} ions, at separate locations on the protein's surface. The most noteworthy feature of these observations is that the NMR signals from residues involved in these Cu^{2+} binding sites are completely unaffected by the first two mole equivalents of Cu^{2+} added to the sample. The only explanation for this is that the protein completely sequesters two mole equivalents of Cu^{2+} in binding sites of substantially higher affinity. This shows that higher affinity Cu^{2+} binding in the full-length prion protein saturates at an apparent Cu:PrP stoichiometry of 1.8 ± 0.2 , which is compatible with a stoichiometry of 2:1.

Identification of the Cu^{2+} coordinating groups in the $\text{PrP}(\text{Cu}^{2+})_1$ and $\text{PrP}(\text{Cu}^{2+})_2$ complexes

The behaviour of the third category of NMR signals, of which M112 is typical (Fig. 1A), reveals the identity of the coordinating groups that form the two higher affinity Cu^{2+} binding sites. This group of $[\text{N},\text{H}^{\text{N}}]$ signals show a substantial loss of peak height on addition of the first aliquots of Cu^{2+} to the sample, with complete loss of the signal occurring prior to the addition of two molar equivalents of Cu^{2+} . The largest reductions in peak heights are in signals from the six histidines residues of the N-terminal domain and from residues nearby in the sequence. This can be seen in Fig. 2A, which shows the reductions in peak heights at a Cu:PrP stoichiometry of 1.8:1. At this stoichiometry, any reductions in peak heights due to the weaker binding sites, described above, can be eliminated. The $^1\text{H}^{15}\text{N}$ HSQC data thus point to all six histidines of the N-terminal domain being involved in the formation of the higher affinity complexes. Importantly, no large changes in behaviour are observed for any of the NMR signals on passing a Cu:PrP stoichiometry of 1:1, suggesting the first and second binding sites are not distinct from

each other - a point which is discussed in more detail below. The behaviour of signals such as S231 [N,H^N] (Fig. 1A), which are largely protected from attenuation by Cu²⁺ binding up to a Cu:PrP stoichiometry approaching 2:1, allows us to rule out the possibility that the effects we attribute to formation of a Cu²⁺ protein complex are instead due to non-specific interactions with free Cu²⁺ ions.

To confirm that all six histidines of the N-terminal domain are the coordinating residues, and to determine if any additional coordinating groups are involved, the behavior of [C,H] NMR signals on titration with Cu²⁺ was also measured. Signals completely attenuated by the addition of sub-stoichiometric amounts of Cu²⁺ included the [C β ,H β], [C δ 2,H δ 2] and [C ϵ 1,H ϵ 1] signals of H96, H111 and all of the octapeptide repeat histidines, but not those of other histidine residues such as H140 (Fig. 3). Attenuation of signals in this way is expected for nuclei that are close to Cu²⁺ in a protein complex, where the exchange rate of Cu²⁺ between protein molecules is fast on a NMR timescale (i.e. where the dissociation rate exceeds the chemical shift change induced by binding in Hz). Thus all six histidines (i.e. four from the octapeptide repeats plus H96 and H111) must coordinate Cu²⁺ in the PrP(Cu²⁺)₁ complex, and exchange on a 10 ms or faster timescale. The only other signals reduced in the same fashion were the [C α ,H α] and [C β ,H β] signals from the N-terminal residue, S20, which is the first of the three residues added to the N-terminus of the full length prion protein sequence to form a thrombin cleavage site. The N-terminal amine is thus also a coordinating group. The hydroxyl group of S20 may be an additional ligand, though the affinity of this group for Cu²⁺ is predicted to be orders of magnitude weaker than that of the amine [34, 35].

In summary, the data indicate that at least seven functional groups - the six histidines of the N-terminal domain and the N-terminal amine - contribute to coordinating a single Cu²⁺ ion in the PrP(Cu²⁺)₁ complex. Cu²⁺ is generally either 3, 4 or 5 coordinate in proteins [36] and hence these seven coordinating groups can not all be simultaneously involved in a single Cu²⁺ complex. The higher affinity Cu²⁺ binding in the full length prion protein must instead be derived from a rapidly exchanging ensemble of different coordination geometries, all of comparable stability, involving different combinations of

the seven coordinating groups.

On increasing the Cu:PrP stoichiometry beyond 1:1, no new protein signals become substantially broadened, until the second binding site is saturated. Therefore, the second binding site must be formed from a subset of the coordinating groups which take part in the ensemble of binding modes which collectively form the highest affinity site.

Self-association of the PrP(Cu²⁺)₁ complex

In the fourth group of NMR signals another distinct behaviour is observed - peak height is initially reduced by the addition of Cu²⁺ but the signals recover towards their initial height as a Cu:PrP stoichiometry of 2:1 is approached (e.g. those of residue K27 in Fig. 1A). This group of [N,H^N] signals includes those from residues in several stretches of the N-terminal domain, namely: 24-48, 100-108, 125-126. When the experiment was repeated with the protein concentration reduced from 440 μM to around 100 μM (K27 Fig. 1A), signals in this group were not dramatically affected by addition of Cu²⁺, indicating that their behaviour at the higher protein concentration must be caused by an intermolecular process. Thus at sub-saturating levels of Cu²⁺ and 440 μM protein concentration, a self-associated form of the prion protein must be at least transiently present. Concentration dependent effects are confined to the disordered N-terminal domain and thus show that it is this section of the protein which self-associates. The most probable cause of this self-association is that metal ions are readily coordinated by histidine sidechains from more than one protein molecule, since the concentration of the protein and the effective concentration of histidine residues in the same chain are likely to be similar. This self-associated protein species causes an acceleration of the relaxation of the nuclei in residues such as K27, either by placing them in closer proximity to the Cu²⁺ ion or through localized increases in the correlation time. At a Cu:PrP stoichiometry of 1.8:1, where these signals have fully recovered, we can infer that no significant amount of the self-associated species remains. At this point in the titration the effects on signals that arise solely from proximity to Cu²⁺ in the monomer can therefore be clearly distinguished and are illustrated in Fig. 2A.

Chemical shift changes in the PrP(Cu²⁺)₁ and PrP(Cu²⁺)₂ complexes

Large chemical shift changes were not observed on formation of either PrP(Cu²⁺)₁ or PrP(Cu²⁺)₂ complexes, indicating that the electronic environment for the paramagnetic ion must be close to isotropic in both cases [37]. However, smaller chemical shift changes of less than 0.1 ppm in the ¹⁵N dimension and less than 0.02 ppm in the ¹H dimension are present, confirming the rapid exchange of Cu²⁺ between prion protein molecules. The distribution of ¹⁵N chemical shift changes for the PrP(Cu²⁺)₂ complex is plotted in Fig. 2B. Certain signals change in chemical shift up to a Cu:PrP stoichiometry of *ca.* 2:1 and then stop. This saturable behaviour shows that these chemical shift changes must be associated with the formation of the PrP(Cu²⁺)₁ and PrP(Cu²⁺)₂ complexes. The largest shift changes in this category are seen in signals from residues in the region between H96 and H111 (for example N100, K101 and K110 in Fig. 1B), plus some from residues in the first helix (namely H155 and R156) and at the N terminus (namely K24, R25 and K27). Similar but less substantial changes are seen in signals from other residues close to the N-terminus and a few residues nearby H155 and R156 in the C-terminal domain. The locations of these chemical shift changes do not correlate with the changes in peak height, for example the height of signals like K101 and N100 are not substantially affected by Cu²⁺ binding, so these changes are unlikely to be a direct result of proximity to Cu²⁺. The shifts observed in the region between H96 and H111 (Fig. 2B) are most likely reporting a conformational redistribution associated with the formation of a partially ordered loop between these residues. However the lack of substantial ¹H chemical shift dispersion in the PrP(Cu²⁺)₁ and the PrP(Cu²⁺)₂ complexes indicates Cu²⁺ binding does not bring about a highly ordered, sidechain-immobilized structure in the N-terminal domain of the full length prion protein. Instead, Cu²⁺ binding at this second site uses the excess metal coordinating residues present in the ensemble of PrP(Cu²⁺)₁ complexes to produce a poorly ordered structure.

Interaction of the Cu²⁺ binding site with the C-terminal domain

Although the C-terminal domain does not contribute directly to Cu²⁺ coordination at the higher affinity site, the heights of certain [N,H^N] NMR signals from the C-terminal domain are substantially reduced in the PrP(Cu²⁺)₂ complex. The largest of these

reductions map to a region of the protein surface formed by residues close to the C-terminal end of helix1, (H155 and N159) and the nearby loop between the first beta strand and helix 1 (S135 and R136), (Fig. 4A). The reductions can be explained if this area of the C-terminal domain surface comes in close proximity to the section of the N-terminal domain that coordinates Cu^{2+} . An alternative explanation, where these reductions in peak height were due to direct weak Cu^{2+} binding at a site on the C-terminal domain can be eliminated – In this scenario the reductions in signal height would be expected to become more dramatic after the higher affinity Cu^{2+} binding was saturated, in the same way as is seen at the S231 $[\text{N},\text{H}^{\text{N}}]$ signal (Fig. 1A). This does not happen and instead the changes stop at the 2:1 point of the titration showing that they must be related to the saturable higher affinity binding.

Chemical shift changes are also observed for the same section of helix 1 (Fig. 2B), arising either from the interaction between domains forming in a Cu^{2+} dependent fashion, or from Cu^{2+} binding to a region of the N-terminus which is already interacting with the C-terminal domain. Strikingly, the changes observed for full length prion protein, PrP^{23-231} , bound to two Cu^{2+} ions are very similar to the changes we observe for the truncated construct PrP^{91-231} bound to a single Cu^{2+} in the same conditions (Fig. 4B, experiments on this construct are discussed fully in the final results section). Since signals for residues 115 to 134 are not greatly affected by Cu^{2+} binding, the sequence responsible for interacting with the C-terminal domain must lie within residues 91-114.

Measurement of Cu^{2+} binding affinity

There are two highly specific Cu^{2+} binding events in the full-length prion protein that are apparent from the NMR observations. These events can be monitored at much lower protein concentration by measuring the quenching of the intrinsic tryptophan fluorescence, revealing that the two sites have significantly different affinities. In these experiments, the apparent affinities of the two metal binding events are reduced to measurable values by the inclusion of a competitive chelator which competes for free metal ions. With knowledge of the affinity of the competition reagent for the metal, a true dissociation constant for the protein can be calculated from the measured apparent

dissociation constant. The affinities of both sites were measured using competition from the amino acid histidine, which forms a Cu^{2+} bis-histidinate complex. The affinity of the second of the two Cu^{2+} binding sites was measured in the presence of 10 mM histidine. Under these conditions, fluorescence quenching was linear to a stoichiometry of 1:1, indicating that the first binding event was at the strong binding limit (Throughout, we use “strong binding” to describe a regime where the dissociation constant is much less than the concentration of binding sites and “weak binding” for where it is similar to, or greater than, the concentration of sites. Weak binding is a condition necessary for measuring a dissociation constant in a titration experiment.). In contrast the second binding event had a measurable apparent dissociation constant of 6 μM (Fig. 5A), showing that the affinities of the two binding events are not equal, as might be predicted from the similarity of the coordinating groups in each. To reduce the apparent affinity of the first site to a measurable value it was necessary to use competition with 100 mM histidine. Under these conditions an apparent dissociation constant of 4 μM was measured, with no initial strong binding phase (Fig. 5B). In combination these fluorescence data also show that the first and second binding events are not cooperative, since the data shown in Figures 1A and 1B are not compatible with a single dissociation constant process.

The absolute affinity of histidine for Cu^{2+} can be used to calculate the affinity at pH 5.5 [30] and using this information we can make estimates of the true dissociation constants for the two binding events in the full-length prion protein at this pH. The interpretation of these competition experiments is ambiguous, however, because it is not known if the protein is interacting with a Cu^{2+} ion or a Cu^{2+} mono-histidinate complex. If the second binding event in the protein involves a Cu^{2+} mono-histidinate complex, $\log_{10} (K_d/\text{M})$ for the Cu^{2+} (histidine):protein complex would be -7.0 ± 0.4 , whilst if the protein is binding a free Cu^{2+} ion then $\log_{10} (K_d/\text{M}) = -10.8 \pm 0.4$ (The K_d values calculated here are K_d values at pH 5.5. To calculate intrinsic K_d values it would be necessary to take into account the protonation state of the metal binding site of the protein.). For the first binding event, the apparent dissociation constant translates to a true K_d value of $\log_{10} (K_d/\text{M}) = -8.2 \pm 0.5$ for binding of a Cu^{2+} mono-histidinate complex, or to $\log_{10} (K_d/\text{M}) =$

-13.1 ± 0.5 for binding of a free Cu^{2+} ion. Despite the ambiguities, it can be concluded that the affinities of the two Cu^{2+} binding events at pH 5.5 are both in the sub-micromolar range and are at least an order of magnitude apart.

Identification of Cu^{2+} coordinating groups in PrP^{91-231}

The integrated nature with which all of the histidine sidechains of the N-terminal domain are involved in Cu^{2+} coordination in the full-length prion protein, is somewhat surprising given that a truncated form of the protein without the octapeptide repeat regions (PrP^{91-231}) is also capable of sub-micromolar affinity Cu^{2+} binding [12]. In PrP^{91-231} , the Cu^{2+} binding site was proposed to involve H96 and H111 on the basis of broadening of NMR signals from backbone NH moieties and X-ray absorption data [12, 38]. To investigate the relationship between the two Cu^{2+} binding modes, we have carried out further characterization of the $\text{PrP}^{91-231}(\text{Cu}^{2+})_1$ complex under equivalent conditions to our investigation of the full-length protein.

The identity of the ligands was determined by following the titration of Cu^{2+} bis-glycinate into a sample of ^{15}N , ^{13}C labeled PrP^{91-231} using heteronuclear NMR methods, as above. After addition of 0.25 molar equivalents of Cu^{2+} to PrP^{91-231} , a specific group of [C,H] signals were completely attenuated, whilst the majority of signals, such as V122, were unaffected by Cu^{2+} addition (Fig. 6). The attenuated signals included the [C β ,H β], [C δ 2,H δ 2] and [C ϵ 1,H ϵ 1] resonances of H96 and H111. In addition, the [C α ,H α] and [C β ,H β] signals of the N-terminal serine, S88, also became unobservable. As in the case of our PrP^{23-231} construct, S88 was one of three residues inserted on the N-terminal side of the PrP^{91-231} sequence to form a thrombin cleavage site. The total quenching of these signals at sub-stoichiometric Cu^{2+} concentrations again shows that exchange of Cu^{2+} between PrP^{91-231} molecules occurs rapidly within the timescale of the NMR experiment. None of the sidechain signals for other potential ligands (namely Q91, T95, S97, Q98, N100, S103, T107, N108, M109 and M112) or signals for backbone amides exhibited this complete quenching at the first addition of Cu^{2+} , thus demonstrating that at pH 5.5 PrP^{91-231} coordinates Cu^{2+} through the two histidine imidazoles of the N-terminal region and the N-terminal amine.

In previous work the affinity of Cu^{2+} binding in the PrP^{91-231} construct was over-estimated. The affinities reported for the interaction of the truncated prion protein constructs PrP^{91-231} (and PrP^{52-98}) with Cu^{2+} [12] were calculated from apparent dissociation constants measured in the presence of glycine at pH 8, but without taking into account the extent of protonation of the glycine amino group at this pH, which reduces the stability of the glycinate complex and weakens the competition for Cu^{2+} . The stepwise dissociation constants for the Cu^{2+} bis-glycinate complex at pH 8 are 0.3 and 3 μM , 40 times larger than the absolute dissociation constants for the formation of this complex from fully deprotonated glycine, which were used in the original calculations. When this is considered, the dissociation constant for free Cu^{2+} binding to PrP^{91-231} is in the picomolar range, with a $\log(K_d/\text{M})$ of -10.1. The equivalent value for PrP^{52-98} is -10.5.

Discussion

A model of Cu^{2+} binding to the full-length prion protein

A model of Cu^{2+} binding to the full length prion protein is illustrated in Fig. 7A. The $\text{PrP}(\text{Cu}^{2+})_1$ complex is an ensemble of species in which Cu^{2+} coordination is shared between the six histidines of the N-terminal domain and the N-terminal amine, with rapid exchange of ligands at the metal centre. Some fraction of the $\text{PrP}(\text{Cu}^{2+})_1$ species exists as an oligomer, which is likely to simply comprise Cu^{2+} ions coordinated by histidine residues more than one protein molecule. The $\text{PrP}(\text{Cu}^{2+})_2$ complex coordinates Cu^{2+} through total occupancy of the ligands used in the $\text{PrP}(\text{Cu}^{2+})_1$ ensemble of complexes. With the present approach it can not be determined which ligands coordinate each Cu^{2+} ion, and it is possible that $\text{PrP}(\text{Cu}^{2+})_2$ is also a rapidly exchanging ensemble of species with different coordination modes. Although the formation of two distinct sites would be a simpler model, this can not account for the broadening of NMR signals from all seven coordinating groups by the first addition of Cu^{2+} and the observation that no further signals are broadened after the 1:1 point of the titration. In the wild type protein, lacking the three residue addition in the construct used here, coordination by the S20 amine would almost certainly be replaced by the K23 backbone amine, since the basicity of the backbone amines and the effective molarity of the two amino acids are virtually the same. The backbone amine group in lysine has a pK_a of 9.18 and the dissociation constant for formation of a 1:1 Cu^{2+} complex is $10^{-7.6}$ M, whilst for serine these values are 9.60 and $10^{-7.9}$ M respectively [30]. The pK_a of the sidechain amine in lysine is 10.72, indicating it is far less likely to be involved in metal binding.

Here we have shown that at pH 5.5 the full length prion protein is capable of binding two Cu^{2+} ions with coordination from multiple histidine imidazole groups (Fig. 7A). At first sight, this may appear to contradict some other studies that have reported that the protein can bind five or six Cu^{2+} ions at pH 7.4 with a coordination involving deprotonated backbone amides [19] (Fig. 7B), but the crucial difference between the experiments is the pH. The mode of Cu^{2+} coordination that occurs at neutral pH, with maximum Cu^{2+} occupancy, has been extensively studied. The octapeptide repeats can bind four Cu^{2+} ions, with one ion in each repeat unit, and with coordination of each ion by a histidine

imidazole N δ 1, two deprotonated backbone amides from adjacent glycine residues, and a carbonyl oxygen [16]. Cu²⁺ ions can also bind at two additional and independent sites, involving the residues histidine 96 and 111, again with coordination from the histidine imidazole and nearby backbone amides [19, 26]. This brings the total number of sites to six.

However recent studies of peptides, which comprise the four repeats of the octapeptide sequence, have shown that if the peptide binds only a single Cu²⁺ ion, then coordination involves multiple histidine imidazole groups [24, 25]. When Cu²⁺ binding in the peptide is saturated, this form of binding is replaced by the four identical sites involving backbone amides. The latter binding sites are very sensitive to reduction in pH because each involves two deprotonated backbone amide groups and the pK_a of this group is well above 7.4. In contrast, multiple histidine coordination would be expected to be maintained at lower pH values because the pK_a of the histidine group is around 6, and this is indeed what we observe in the full-length protein at pH 5.5.

The peptide studies of Valensin *et al.* and Chattopadhyay *et al.*, and our own unpublished data, suggest that the multiple histidine Cu²⁺ coordination mode we observe in the full-length protein at pH 5.5 will be similar to the binding mode which occurs at neutral pH at low Cu²⁺ occupancy, even though at maximum Cu²⁺ occupancy coordination will be very different (Fig. 7B.). Consistent with this proposal EPR investigations also report that, in the presence of one or two molar equivalents of Cu²⁺ at pH 7.4, Cu²⁺ coordination is different to that which occurs in the Cu²⁺ saturated species [19]. It is further supported by an X-ray absorption study which found evidence that single and multiple imidazole Cu²⁺ coordination might both be accessible in the full length protein at neutral pH [39].

The affinity of the prion protein for Cu²⁺ and the availability of Cu²⁺ are the key determinants of whether the prion protein binds Cu²⁺ *in vivo*. The high capacity, backbone amide coordinated form of Cu²⁺ binding, which is accessible at pH 7.4, produces a substantial circular dichroism signal, and this has been used to determine that the affinity of these sites is in the low micromolar range [14]. *In vivo*, virtually all

extracellular Cu^{2+} is sequestered in complexes with affinities significantly higher than micromolar, for example with amino acids such as histidine or with proteins such as serum albumin, which have affinities in the low nanomolar and low picomolar range, respectively [28, 40]. A micromolar affinity therefore suggests that the cellular prion protein does not bind Cu^{2+} *in vivo*. However, the preference of the prion protein for multiple histidine Cu^{2+} coordination at low Cu^{2+} concentrations and at lower pH values, shows that this is likely to be the highest affinity form of binding in the protein. The affinity measurements carried out here indicate that this form of coordination has an affinity at least in the nanomolar range and potentially higher. While not conclusively showing that the prion protein is an authentic cuproprotein, it is clear that the multiple histidine mode of Cu^{2+} binding is the most likely to be of biological relevance. In addition, the capability for Cu^{2+} binding at pH 5.5 indicates that Cu^{2+} binding could be maintained during the cycling of the prion protein through acidic endosomal compartments [41], which have been postulated to be the site of PrP^{Sc} formation [42].

It should be emphasized that the Cu^{2+} dependent oligomerisation we observe is a distinct process from the oligomerisation involved in formation of either PrP^{Sc} or PrP fibrils. We observe association of the disordered N-terminal domain with no evidence for any structural changes in the globular C-terminal domain. In contrast formation of PrP^{Sc} or PrP fibrils from monomeric PrP^{C} involves a substantial conformational change within residues 90-231, giving an increase in β -sheet type structure [43,44] and does not require residues 23-89 [45], which contain the octapeptide repeats. Nevertheless, our work suggests that metal binding may play a role in controlling self-association of PrP *in vivo* which could influence the rate of formation of other PrP oligomers such as PrP^{Sc} . Although the Cu^{2+} dependent self-association is relatively weak at pH 5.5, requiring protein concentrations in the order of 0.5 mM, at pH 7.4 self-association is likely to be stronger. At this pH we have found that Cu^{2+} binding to a peptide comprising the four tandem octapeptide repeats causes self-association with peptide concentrations in the sub-micromolar range (Wells, M. A. and Jackson, G. S., unpublished data). Also, *in vivo*, effective concentrations of the prion protein may be vastly elevated above the true concentration because the protein is anchored to the cell membrane and is further

localized in cholesterol rich lipid rafts [46], which may be sufficient to allow Cu^{2+} binding to trigger self-association.

The involvement of H96 and H111 in Cu^{2+} binding in the full-length protein shows that metal ion occupancy must play a role in determining the conformation of this section of the protein in the PrP^{C} isoform, as has also been shown for PrP^{Sc} [47]. Given the known importance of this region of the protein for prion propagation, the role of metal ions in the conversion process should be considered. The formation of a single Cu^{2+} binding site by histidine residues from throughout the unstructured N-terminal domain, also has possible implications for understanding the mechanism by which a unique group of pathogenic mutations, the octapeptide repeat insertion mutations, cause disease. Most of the pathogenic mutations seen in inherited prion disease occur within residues 89-231 [1], which form the relatively protease resistant core of PrP^{Sc} and are sufficient to support prion replication and the development of pathology [45, 48]. Insertional mutations comprising addition of integral numbers of the octapeptide repeat sequence to the four repeats of the wild type protein are the exception [1]. The mechanism by which the octapeptide repeat region influences the formation of PrP^{Sc} is unclear, especially since this section of the protein is unfolded and highly mobile in PrP^{C} , and is rapidly digested by proteinase K in PrP^{Sc} [49]. However the Cu^{2+} mediated interaction demonstrated here between the octapeptide repeats and H96 and H111, illustrates that the insertions would cause alterations in metal coordination which have the potential to modulate PrP^{Sc} formation by causing conformational changes within residues 91-231.

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Abbreviations

HSQC, heteronuclear single quantum coherence; K_d , dissociation constant; PrP, prion protein; PrP^C, cellular isoform; PrP^{Sc}, pathogenic (scrapie) isoform; Prion protein constructs will be referred to as PrP^{x-y} with the residues they are composed of given as a superscript, e.g. PrP²³⁻²³¹ for full length prion protein; PrP(Cu²⁺)₁ and PrP(Cu²⁺)₂, complexes of the full-length prion protein with one and two Cu²⁺ ions respectively; 2D ¹H¹⁵N HSQC and 2D ¹H¹³C HSQC crosspeaks will be described by giving the two coupled nuclei which produce the crosspeak in brackets, e.g. [Cβ,Hβ].

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Tables and Figures

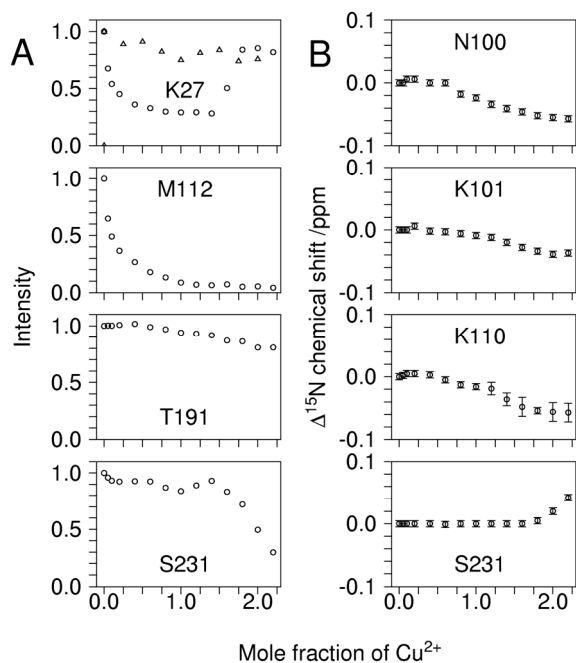


Figure 1. Titration of full length human prion protein with Cu²⁺ bis-glycinate monitored by ¹H ¹⁵N HSQC. (A) NMR signal heights for residues K27, M112, T191 and S231. (B) ¹⁵N chemical shift changes for residues N100, K101, K110 and S231. All data is from a titration of 440 μM protein, except the data shown by triangles in the plot for K27, which is from a titration of 110 μM protein.

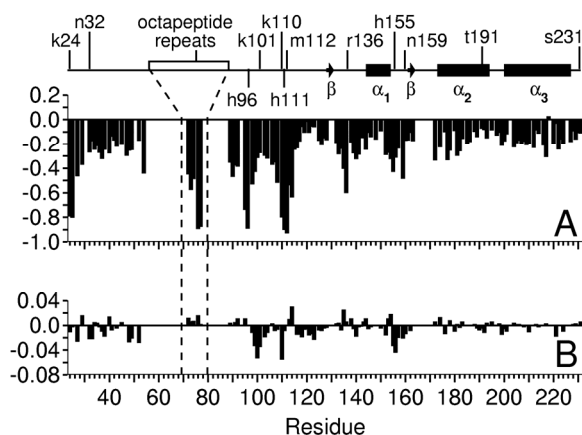


Figure 2. Changes in the backbone $[N,H^N]$ NMR signals induced by Cu^{2+} binding to full length human prion protein plotted against residue number. The degenerate octapeptide repeat signals are plotted in the order (GG)WGQ(P)HG and no data is presented for the bracketed glycines residues due to signal overlap. Signal height changes are shown, as a fraction of the starting value, for the addition of 1.8 mole equivalents of Cu^{2+} bis-glycinate (A). The large reductions at residues 24-25, 95-96, 110-112 and the octapeptide repeat HG sequence arise from paramagnetic quenching due to the proximity of these residues to Cu^{2+} in the $PrP(Cu^{2+})_2$ complex. (B) The ^{15}N chemical shift changes after addition of 1.8 mole equivalents of Cu^{2+} bis-glycinate in ppm.

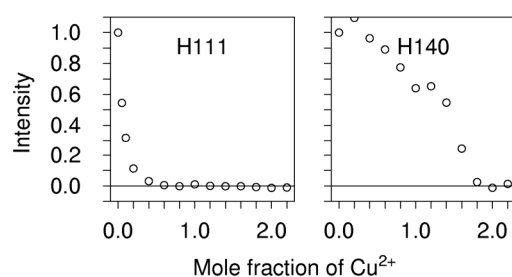


Figure 3. Reductions in the heights of histidine sidechain $[C\delta,H\delta]$ NMR signals induced by proximity to Cu^{2+} in PrP^{23-231} . Plots show the signal height as a fraction of its starting value during titration with Cu^{2+} for H111 (A), part of the higher affinity binding site, and H140 (B), which only binds Cu^{2+} after higher affinity binding is saturated.

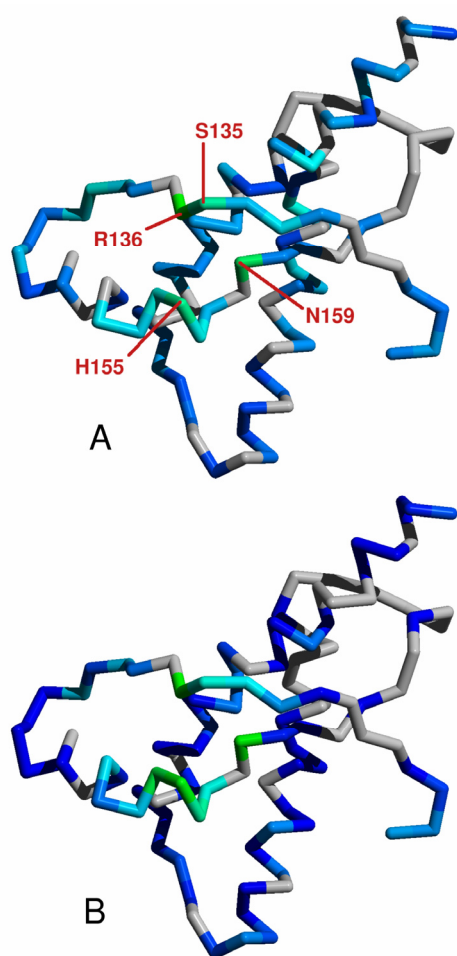


Figure 4. The Cu^{2+} -bound N-terminal domain contacts the C-terminal domain. The reductions in the heights of backbone $[\text{N},\text{H}^{\text{N}}]$ signals in the C-terminal domain of PrP, induced by proximity to the Cu^{2+} bound to the N-terminal domain, are illustrated on the backbone structure by a color scale, ranging from dark blue to cyan (0-30% peak height reduction) and cyan to green (30-60% reduction). Data is shown for the full length prion protein loaded with two Cu^{2+} ions (A) and the truncated PrP^{91-231} construct, loaded with one Cu^{2+} ion (B). Residues for which there are no data are coloured grey. Signals from the area of the C-terminal domain that contacts the unfolded N-terminal domain see the largest reductions (coloured green) because they are closer to the Cu^{2+} ion bound to the unfolded domain. The unfolded domain of the protein and the Cu^{2+} ion bound to it are omitted for clarity.

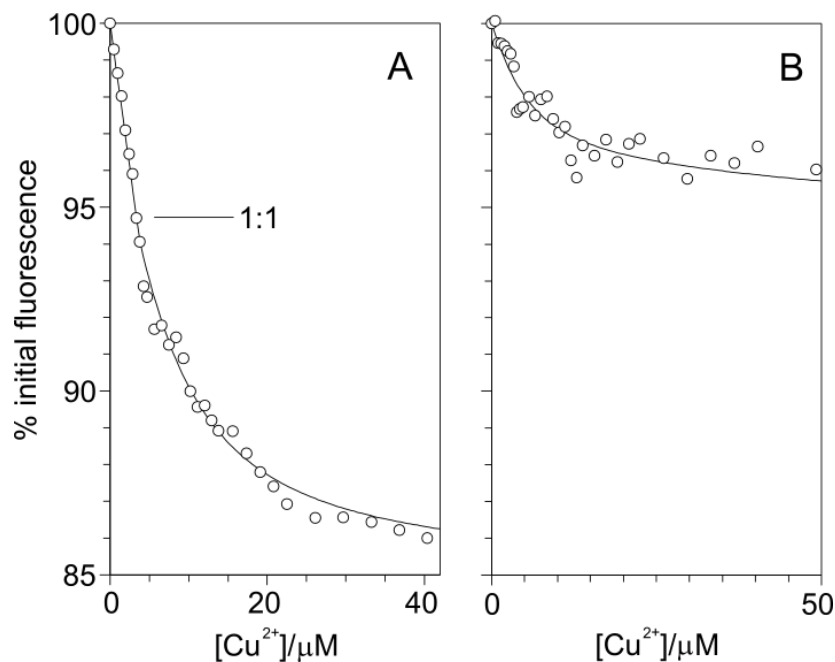


Figure 5. Cu²⁺ binding to full length human prion protein monitored by tryptophan fluorescence quenching. The graphs show the tryptophan fluorescence, as a percentage of the starting fluorescence, as Cu²⁺ bis-glycinate is titrated into samples of 3.5 μM prion protein at pH 5.5 in the presence of two different competition systems, (A) 10 mM histidine and (B) 100 mM histidine. The point at which one mole equivalent of Cu²⁺ has been added to the protein, and the highest affinity site is therefore saturated, is labeled 1:1 in (A). The lines of best fit to the binding equations (described in the materials and methods section) are shown.

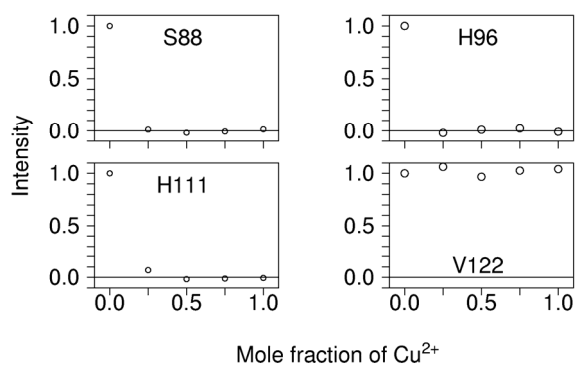


Figure 6. Effects of titration with Cu^{2+} bis-glycinate on the heights of sidechain [C β ,H β] NMR signals in PrP⁹¹⁻²³¹. Reductions induced by proximity to Cu^{2+} show the coordinating groups for the Cu^{2+} binding site in this truncated construct. The plots show the signal height as a fraction of its starting value during titration with Cu^{2+} .

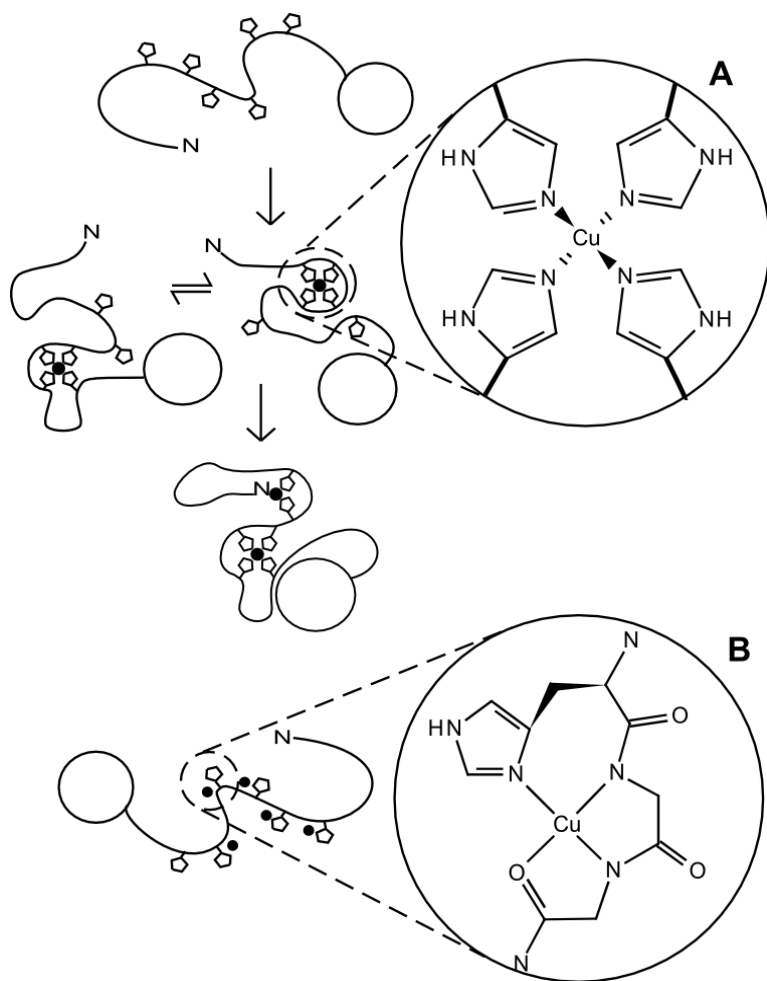


Figure 7. An illustration of the two alternative modes of Cu²⁺ coordination in the full length prion protein with details showing the coordination environment of the Cu²⁺ ion in each case. Binding of Cu²⁺ by the full length prion protein at pH 5.5 involves coordination by an exchanging combination of histidine imidazoles (A). The figure shows apo-PrP (top), the PrP(Cu²⁺)₁ ensemble (middle) and PrP(Cu²⁺)₂ (bottom), which may also be an ensemble of different coordinations involving the same ligands. Note that the assignment of particular combinations of ligands to any one complex or metal centre is arbitrary. The histidine Nε coordination shown is based on an infra-red study of multiple histidine coordination in the octapeptide repeats [13]. At pH 7.4, with saturating concentrations of copper, the full-length prion protein can bind at least five Cu²⁺ ions, each coordinated by a single imidazole and nearby backbone amides (B).