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Evaluation of Copper^{2+} Affinities for the Prion Protein

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ABSTRACT: The prion protein (PrP) is a cell-surface Cu^{2+} binding glycoprotein which can bind six copper ions. The role of Cu^{2+} in PrP function, misfolding, and prion disease has generated much interest; however, the field has been hampered by a lack of consensus with regard to the affinity of Cu^{2+} for PrP^C. Here we build on our understanding of the appearance of visible CD spectra for full-length PrP and fragments to determine the affinity of Cu^{2+} for four different binding modes, with dissociation constants ranging between 13 and 66 nM at pH 7.4.

The normal cellular mammalian prion protein (PrP^C) is typically 209 residues in length and has two structurally distinct domains: a mainly α-helical C-terminal domain (residues 126−231) and an unstructured N-terminal domain (residues 23−125) (*1*) which exhibits a high degree of main-chain flexibility (*2*). It is this region that binds a number of Cu^{2+} ions (*3*−*8*). Binding of Cu^{2+} has been linked to both the normal function of the prion protein and also the disease state (for reviews, see refs (*9*−*11*)). A misfolded oligomeric isoform of the prion protein, PrP^{Sc}, has been identified as the infectious particle responsible for prion diseases in humans and cattle (*12*).

Recently, we have used visible circular dichroism (CD) and electron paramagnetic resonance (EPR) to characterize coordination of Cu^{2+} to full-length PrP^C (*3*). These studies show that fragments from the unstructured region of PrP^C represent a good model for binding of Cu^{2+} to full-length PrP^C. It is clear multiple Cu^{2+} ions load sequentially onto PrP^C, with Cu^{2+} initially binding to full-length PrP^C in the amyloidogenic region, between the octarepeats and the structured domain, at His^{95} and His^{110}. Only subsequent Cu^{2+} ions bind to the octarepeat region, eventually loading onto each individual histidine residue (*3*).

Here we determine the affinity of Cu^{2+} for full-length PrP, as well as analogues and fragments, using visible CD and glycine as a competing ligand. At present, there are large discrepancies reported for the affinity of Cu^{2+} for PrP. In the past, Cu^{2+} affinities have often been underestimated; our own previous studies failed to account for both stepwise affinities (K_{a1} and K_{a2}) of competing ligands (glycine and histidine). Additionally, others have underestimated affinities by not taking into consideration the presence of competing buffers or chelators, which have a small but significant affinity for Cu^{2+} ions, as highlighted for parallel studies on amyloid-β peptide of Alzheimer's disease (*13*, *14*).

At least four different modes of coordination of Cu^{2+} to the unstructured N-terminal domain of PrP^C are well-documented (see the Supporting Information) (*3*, *11*). They include complexes centered at His^{95} as well as His^{110}. In addition, Cu^{2+} binds to multiple or individual histidine residues from the octarepeat region. The visible CD spectra for these various binding modes are well-characterized (*3*, *5*, *6*, *15*−*17*). From Figure 1, we can see striking differences in their appearance.

Building on our understanding of the appearance of visible CD spectra, we have used the competitive effects of glycine to determine the affinity of Cu^{2+} for a range of PrP constructs, including full-length mPrP(23−231), and various fragments and recombinant analogues, shown in Figure 1 (see the Supporting Information for experimental details). Free glycine will bind to Cu^{2+} ions in a $Cu(Gly)_2$ complex, coordinating via the amino and carboxylate groups. Glycine is nonchiral, and the $Cu(Gly)_2$ complex does not give rise to any visible CD signals; thus, as more glycine competes for the Cu^{2+} ions, a reduction in the intensity of the Cu−PrP signal is observed. The stepwise apparent affinities for glycine at pH 7.4 are known: $K_{a1} = 7.4 \times 10^5\ M^{-1}$, and $K_{a2} = 7.4 \times 10^4\ M^{-1}$ (*35*). Using both affinities and the concentration of glycine that is required to remove half of 1 molar equiv of Cu^{2+} ions from PrP, the apparent dissociation constant for the Cu−PrP complex at pH 7.4 may be calculated (see also the Supporting Information) (*13*).

$$[Cu^{2+}{}_{\text{free}}] = [1/(1 + K_{a1}[Gly] + K_{a1}K_{a2}[Gly]^2)]([Cu^{2+}{}_{\text{total}}] - [Cu^{2+}{}_{\text{bound to PrP}}]) \quad (1)$$

We show in Figure 1 the apparent dissociation constants at pH 7.4 for each of the PrP constructs in the presence of 1 molar equiv of Cu^{2+} ions. We have also determined similar values using an alternative approach (*18*) (see the table in the Supporting Information).

It has been established that Cu^{2+} binds to PrP in a further binding mode not represented by visible CD spectra in Figure 1 (*18*, *19*). In this case, a single Cu^{2+} ion binds to all four histidine residues from the octarepeat region of PrP. This complex will not generate visible CD spectra, as it lacks amide main-chain coordination (*17*). The affinity was therefore measured using a fluorescence quenching experiment. Figure 2a shows the quenching of the four tryptophan fluorescence signals by Cu^{2+} ions in the Cu−PrP(57−90) complex. A similar experiment performed on the single octarepeat, PrP(57−67), is shown in Figure 2b. Here Cu^{2+} coordinates using the amide main chain and therefore does give rise to a visible CD spectrum shown in Figure 1a. As with the CD experiments, the affinity is determined using the competitive effects of glycine. In the fluorescence experiments, addition of glycine causes the tryptophan fluorescence signal to return to its maximal intensity as Cu^{2+} ions are removed from PrP. Similar Cu^{2+} affinities for the PrP single octarepeat are calculated using this second independent method, validating the fluorescence approach. Visible CD indicates a K_d

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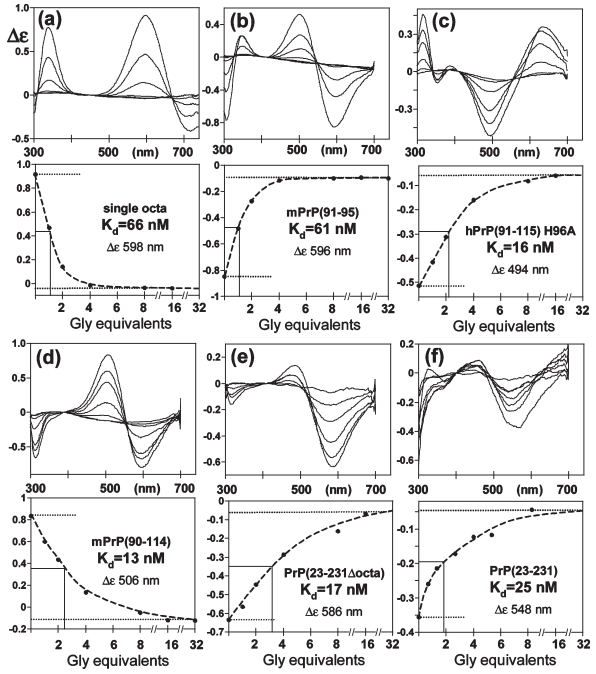

FIGURE 1: Visible CD spectra (top row and third row) show glycine competition studies for Cu^{2+} bound to various PrP proteins and/or fragments at pH 7.4 and 25 °C. Cu^{2+}−PrP signals at specific wavelengths corresponding to CD bands are shown (second row and bottom row) with molar ellipticity ($\Delta\varepsilon$) plotted vs molar equivalents of glycine. For each plot, the half-height of the disappearing Cu^{2+}−PrP band is shown. Apparent K_d values at pH 7.4 are also indicated for each titration. Protein concentrations ranged between 50 and 200 μM with 1.0 molar equiv of $CuCl_2$ in each case: (a) single octarepeat mPrP(57−67), (b) mPrP(91−95), (c) hPrP(95−115) H96A, (d) mPrP(90−114), (e) mPrP(23−231Δocta), and (f) mPrP-(23−231).

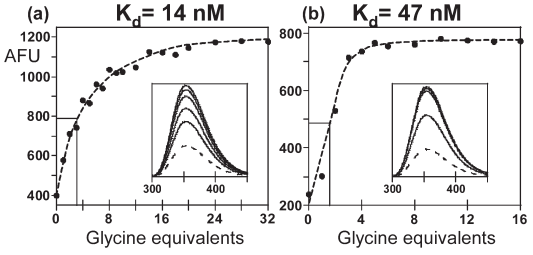

FIGURE 2: Tryptophan fluorescence quenching by Cu^{2+} ions, showing the competitive effects of glycine. Fluorescence signal at 353 nm plotted vs increasing equivalents of glycine for (a) mPrP(57−90) and (b) the single octarepeat, mPrP(57−67). For each plot, the half-height of the returning fluorescence signal is shown and K_d values are calculated. Insets show fluorescence spectra recorded with 50 μM PrP fragments and 50 μM $CuCl_2$ at pH 7.4 and 22 °C. The dashed line represents the Cu^{2+} loaded PrP fragment (with tryptophan signal quenched), while the solid lines represent incremental glycine additions.

at pH 7.4 of 66 nM (log K_a = 7.2 M^{-1}) for the single octarepeat, while the fluorescence quenching method indicates a similar K_d of 47 nM (log K_a = 7.3 M^{-1}).

The affinities for fragments of PrP calculated agree well with what is known about the sequential loading of Cu^{2+} ions, binding first at the amyloidogenic region and then loading onto individual single octarepeats (*3*). In particular, the amyloidogenic region binds copper more tightly at 13 nM [represented by the mPrP(90−114) fragment], compared to the single-His binding mode within the octarepeat region which has an affinity of 66 nM. The relative affinities also agree with previous competition studies which showed that Cu^{2+} binds to the hPrP(91−115) fragment preferentially over the octarepeat fragment (*20*).

As would be expected, the recombinant construct PrP-(23−231Δocta), which lacks the octarepeat region, produces a visible CD spectrum similar to that of mPrP(90−114) and also a very similar affinity, with K_d values of 17 nM (log K_a = 7.8 M^{-1}) and 13 nM (log K_a = 7.9 M^{-1}), respectively. Furthermore, the affinity of Cu^{2+} for full-length PrP^C is also similar, 25 nM (log K_a = 7.6 M^{-1}). These three values are within the accuracy of these measurements, based on repeat experiments performed. The visible CD signal for mPrP(23−231) with 1 molar equiv of Cu^{2+} present has an intensity similar to that of PrP(23−231Δocta), suggesting that the CD silent multiple-octarepeat His complex has an affinity slightly lower than the affinities of complexes centered at His^{95} or His^{110} in full-length $mPrP^C$. The fluorescence measurements of the single Cu^{2+} ion binding to multiple His residues within PrP(58−91) indicates an affinity comparable to that of PrP(90−114), 14 nM.

The affinity of Cu^{2+} for PrP is hotly debated in the literature with reported affinities varying significantly (*7*, *18*, *19*, *21*−*23*). For example, two early studies put the affinity in the micromolar range for full-length PrP (*4*, *21*). These studies were of PrP^C loaded with five Cu^{2+} ions or did not take into account the competitive effects of glycine and so underestimated the affinity for PrP^C. Subsequent studies suggested Cu^{2+} affinities a number of orders of magnitude higher, with a femtomolar dissociation constant for large PrP fragments (*7*) and full-length protein (*22*). More recently, however, the same laboratories have determined affinities more in line with those reported here (*18*, *23*). The variation for recent studies identified is typically within 2 orders of magnitude for each mode, as summarized in the table in the Supporting Information. Recent studies highlight affinities for the fifth site (His^{95} and His^{110}) between 100 (*18*) and 0.03 nM (*23*), while for the multiple-histidine complex, K_d values of 3 nM (*18*), 0.1 nM (*19*), and 0.06 nM (*23*) are reported. Further additions of Cu^{2+} cause this multiple-histidine octarepeat site to be outcompeted by a lower-affinity binding mode involving single histidines within the octarepeats (*18*, *19*).

We would argue our approach has some advantages over indirect methods such as fluorescence quenching and isothermal titration calorimetry. The application of visible CD is a straightforward robust method that enables us to directly measure the amount of Cu^{2+} ions bound to PrP^C from their highly characteristic Cu^{2+} visible CD absorption bands and takes advantage of the nonchiral, CD-silent, nature of the competing ligand. Use of competing ligands also identifies clearly the proportion of Cu^{2+} that will bind to the competing ligands and helps mimic to an extent the metal binding behavior in vivo.

Cu^{2+} ions within cerebrospinal fluid (CSF) and extracellular brain interstitial fluid typically reach concentrations of 250 nM (*24*), 1 order of magnitude more concentrated than the K_d of Cu^{2+} for PrP^C. The locality of PrP^C tethered at a presynaptic membrane surface is very significant (*25*), since local concentrations of free Cu^{2+} ions may reach as much as 100 μM at the synaptic cleft during neurotransmission (*26*). In these instances, extracellular Cu^{2+}-chelating proteins such as metallothionein-III and serum albumin may become saturated with Cu^{2+} ions. With PrP^C affinities for Cu^{2+} shown to be in the range of 13−66 nM, and with potentially up to six binding sites, PrP^C is therefore well placed to bind these fluxes of free Cu^{2+} ions. Hence, the function of PrP^C may be to scavenge Cu^{2+} ions released during neuronal depolarization and so protect the cells

from toxic effects of redox-active free copper ions which are capable of generating hydroxyl radicals via Fenton's/Haber Weiss reactions. This hypothesis is supported by reports that show heightened sensitivity of PrP knockout mice to copper-induced oxidative stress, compared to wild-type mice (*27*). In vitro PrP^C chelation of Cu^{2+} acts as a sacrificial quencher for free radicals, through oxidation of exposed methionine residues in the vicinity of the Cu^{2+} binding site (*28*).

A role for Cu^{2+} as a cofactor in prion disease should not be ruled out; indeed, metal ions may confer the strain of prion disease (*29*). Furthermore, some forms of familiar prion disease are linked with extra copies of the copper binding octarepeats (*30*), while binding of Cu^{2+} to the amyloidogenic region of PrP^C is linked to the neurotoxic peptide PrP(106−126) (*31*). Coordination of Cu^{2+} promotes conformational changes in the unstructured domain and self-association of PrP^C (*6*, *15*), although in vitro Cu^{2+} binding seems to inhibit amyloid fibril growth (*32*). There is evidence in Alzheimer's disease that diffusible oligomers of Aβ may concentrate Cu^{2+} ions at the neuronal cell surface, resulting in copper-catalyzed reactive oxygen species, which may be the cause of Aβ cytotoxicity (*14*, *33*, *34*). It is interesting to speculate that a similar process could account for PrP^{Sc} toxicity.

SUPPORTING INFORMATION AVAILABLE

A summary of the Cu^{2+} binding modes of the prion protein and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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