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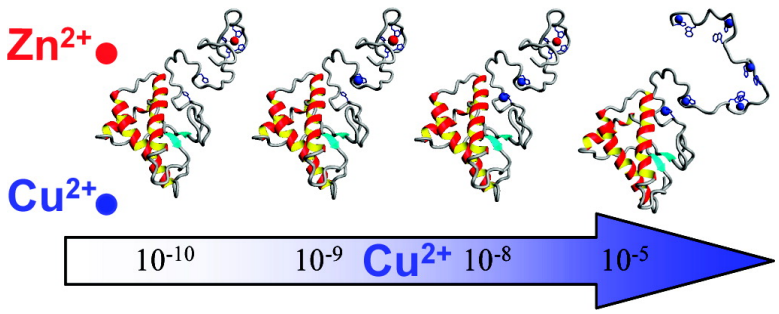

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The Prion Protein is a Combined Zinc and Copper Binding Protein: Zn^{2+} Alters the Distribution of Cu^{2+} Coordination Modes

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The transmissible spongiform encephalopathies (TSE) are a unique class of neurodegenerative diseases where the transmissible agent (the prion) consists of misfolded protein,[1] designated PrP^{SC}. The normal cellular form (PrP^{C}) is expressed throughout the body, but mainly in the brain. The physiological role of PrP^{C} is not known, but growing evidence points to a function related to copper binding.[2] PrP has been shown to bind copper in vivo, and although PrP knockout mice flourish, they show increased signs of oxidative stress. Cellular studies show that copper induces PrP endocytosis.[3,4]

PrP binds copper in the highly conserved, unstructured N-terminal half of the protein. The octarepeat region, PrP(60−91), consists of four tandem repeats of PHGGGWGQ and binds 4 equiv of copper at full occupancy.[5] Adjacent to the octarepeats are two additional histidines (H96 and H111) that may also bind copper (the so-called "non-octarepeat" copper binding sites).[6] We recently showed that, when the octarepeat region is titrated with Cu^{2+}, the copper binding mode depends on the precise molar ratio of copper to protein.[7] The first equivalent of copper coordinates in a multi-histidine mode involving 3−4 imidazole side chains, and we identify this mode by its CW electron paramagnetic resonance (EPR) spectrum as component 3. With additional copper, component 3 decreases and is replaced by component 2 (2 histidines per copper) and then 4 equiv of component 1 (one copper per octarepeat), at saturation.[8]

In addition to copper, other metals have been associated with PrP,[9] with zinc (Zn^{2+}) having the next highest affinity.[10] Zinc is also the only metal other than copper that induces PrP endocytosis.[3,4] Zinc, like copper, also inhibits fibril formation (synthetic prions)[11] and promotes intermolecular interactions.[12] It has been suggested that in vivo PrP may actually bind zinc rather than copper given the abundance of available zinc in the brain, with peak levels up to 300 μM in the synaptic cleft of glutaminergic neurons.[13] While peak copper levels in the synaptic cleft may be as high as 100−250 μM,[14] the basal level in cerebral spinal fluid (CSF) is micromolar, with most exchangeable copper bound by amino acids and peptides. In this work, we show that even large excesses of zinc are unable to displace copper from either the octarepeat region or the full-length protein. However, EPR reveals that physiologically relevant levels of zinc significantly alter the distribution of copper among the available binding modes. Diethyl pyrocarbonate (DEPC) modification[10] and mass spectrometry are used to identify the octarepeat region as the zinc binding site and to confirm that the PrP−Zn^{2+} dissociation constant is ~200 μM, reflecting an affinity significantly lower than that for copper.

First we employ a direct competition between copper and zinc for binding sites in the octarepeat region, with the copper binding monitored by X-band EPR. Zn^{2+} is diamagnetic and therefore has no EPR signal; likewise, unbound Cu^{2+} at pH 7.4 exists as antiferromagnetically coupled hydroxides and is EPR silent. Component analysis of the copper−PrP EPR spectrum, developed by our lab, is used to determine both the concentration and the binding mode.[8]

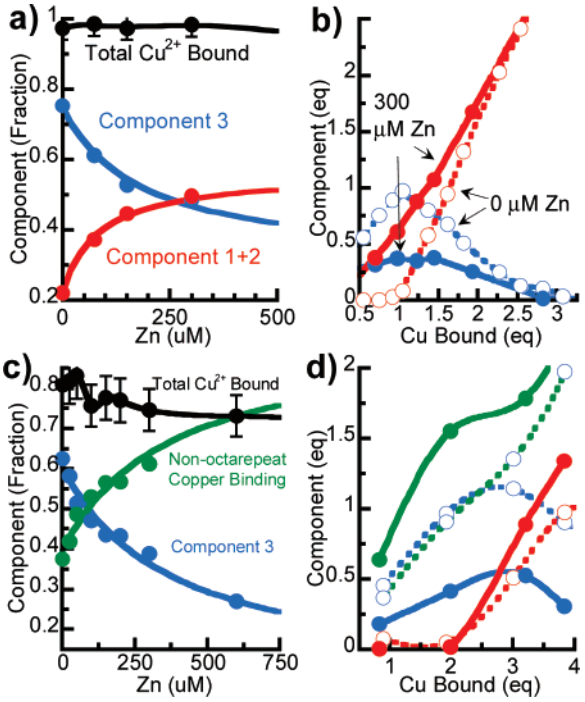

Figure 1. Effect of zinc on the distribution of the components of the copper EPR spectrum. (a) PrP(23−28,57−91) with 1 equiv of copper titrated with zinc; (b) PrP(23−28,57−91) with and without 300 μM Zn titrated with copper; (c) recombinant PrP(23−231) with 2 equiv of copper titrated with zinc; (d) PrP(23−231) with and without 300 μM Zn titrated with copper. Total copper bound (black), component 3 (blue), 1 + 2 (red), and non-octarepeat (green). Copper and zinc (solid), copper only (dotted).

Figure 1a shows the analysis of the EPR spectra from a zinc titration of PrP(23−28, 57−91) with copper held constant at 1 equiv. At physiologically relevant zinc levels (<1 mM), the total amount of bound copper is unchanged. However, a decomposition of the spectra into component spectra shows that the copper binding mode shifts dramatically with zinc concentration, from mostly component 3 in the absence of zinc to a majority of component 1 (component 2 remains a minor component throughout and is summed with component 1 for simplicity). When these spectral changes are fit to saturation curves (solid lines), they indicate a Zn^{2+} affinity on order of 10^{-4} M (Supporting Information). Alternatively, Figure 1b shows a copper titration in the absence of zinc or with zinc held at 300 μM, to match the anticipated maximal synaptic concentration. The influence of zinc is the greatest with <2 equiv of bound copper; with >3 equiv of bound copper, zinc has little effect. When these techniques were applied to peptides encompassing the non-octarepeat copper binding sites, PrP(90−114), no change in the EPR spectrum was evident. Additionally, Ca^{2+} and Cd^{2+} were substituted for zinc; Ca^{2+} showed no effect, while Cd^{2+}

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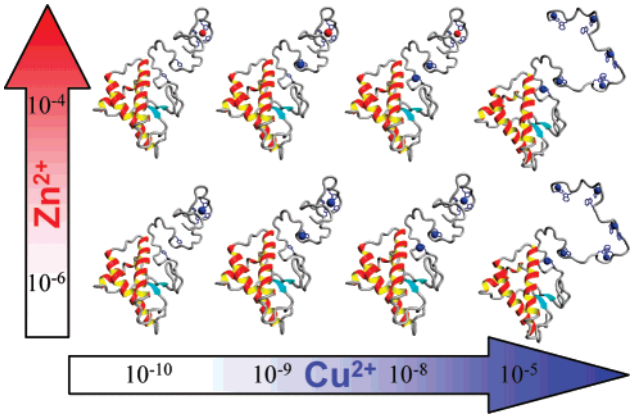

Figure 2. Models representing metal binding in the N-terminal domain of PrP. Top row (high zinc): zinc (red) is bound by the octarepeat region (left), while non-octarepeat sites (H96 and H111) are available for copper binding (blue, middle). Copper at high concentration will displace zinc from octarepeats to form up to 4 equiv of component 1 (right). Bottom row (low zinc): copper (blue) is bound by the octarepeats in component 3 when copper is low (left), with increasing copper loads the non-octarepeat sites (middle). High copper (right column) results in component 1 copper binding by the octarepeats. Approximate molar metal concentrations are shown in the arrows. Octarepeat structures based on data from Chattopadhyay et al.[7]

showed an effect qualitatively similar to Zn, but with weaker (~10×) affinity.

Similar experiments were also performed with full-length recombinant protein from Syrian hamster, SHaPrP(23−231). SHaPrP-(23−231) with 2 equiv of copper was titrated with Zn^{2+}. (Note that the extra copper equivalent relative to the peptide experiment is added to partially populate the non-octarepeat sites.) The spectra showed only component 3 and non-octarepeat binding, with the amounts of each as a function of zinc shown in Figure 1c. While the zinc binding affinity derived from the fit saturation curves is slightly less than for the octarepeat peptide (300 μM vs 200 μM; see Supporting Information), the change in copper distribution is even more significant. As with the octarepeat peptide, the presence of zinc shifts copper away from the multi-histidine component 3 binding mode. Figure 1d shows that the binding mode, to which the copper is diverted, depends on how many equivalents of copper are bound. With less than two copper equivalents, it is the high copper affinity non-octarepeat binding sites that show increased copper. At higher copper levels, when the non-octarepeat sites are saturated, the distribution change occurs in the octarepeat region, favoring component 1.

To test zinc binding affinity in the absence of copper, we used DEPC modification and mass spectrometry. Peptides spanning the octarepeat region, PrP(60−91), and the non-octarepeat copper binding sites, PrP(90−114), were allowed to react with zinc. Then DEPC was added and allowed to react for 1 min. The reaction was then quenched with imidazole. The reaction products were separated and quantified by reverse-phase HPLC and identified by ESI-MS (see Supporting Information). DEPC modifies the imidazole side chain of histidines to give a characteristic change in mass; however, histidines that are involved in metal binding will be protected from such modification. When PrP(60−91), $(PHGGGWGQ)_4$, is titrated with $ZnCl_2$, the amount of unmodified peptide goes up and the amount of peptide with four modifications goes down, giving a saturable binding curve with a K_d of 200 μM (Supporting Information). Similar experiments with peptides spanning the non-octarepeat copper binding region PrP(90−114) and peptides with 1−3 octarepeats show no change in DEPC modification distribution with the addition of zinc. Together, these show that zinc is bound exclusively by the octarepeat region, with four octarepeats necessary to bind 1 equiv of Zn^{2+}.

These findings show that Zn^{2+} binding to the PrP octarepeat domain is possible with reported synaptic zinc concentrations. As summarized in Figure 2, when copper levels are low, PrP can simultaneously bind both copper and zinc. At higher copper levels, it accommodates the zinc by shifting to binding modes (components 1 and 2) that minimize the ratio of histidines to copper. However, when no rearrangement can accommodate both zinc and the available copper, it is the zinc that is displaced, not the copper. This is true even at millimolar zinc concentrations.

Our results indicate that zinc may effect changes in two different ways: either directly by being bound by PrP or indirectly by changing the copper binding mode. A shift in binding mode may explain how both copper and zinc are able to stimulate PrP endocytosis. Unlike copper, zinc is redox inactive; however, zinc can change the overall Cu^{2+} redox properties of the protein by shifting copper coordination from the redox accessible component 3 binding mode to redox inactive component 1 and non-octarepeat binding modes. If so, proposed reductase properties based on component 3 binding may not be relevant in vivo. Finally, there are implications for experimental design; in vivo studies on either metal must consider the basal levels of the other. In vitro results for each metal individually may not extrapolate to the combination.

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Supporting Information Available: Experimental parameters and details for peptide and protein production, DEPC/MS footprinting, and EPR. HPLC chromatograms and spectra for ESI-MS, and EPR. This material is available free of charge via the Internet at http://pubs.acs.org.

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