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Copper Binding Extrinsic to the Octarepeat Region in the Prion Protein

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Abstract

Current research suggests that the function of the prion protein (PrP) is linked to its ability to bind copper. PrP is implicated in copper regulation, copper buffering and copper-dependent signaling. Moreover, in the development of prion disease, copper may modulate the rate of protein misfolding. PrP possesses a number of copper sites, each with distinct chemical characteristics. Most studies thus far have concentrated on elucidating chemical features of the octarepeat region (residues 60-91, hamster sequence), which can take up to four equivalents of copper, depending on the ratio of Cu^{2+} to protein. However, other sites have been proposed, including those at histidines 96 and 111, which are adjacent to the octarepeats, and also at histidines within PrP's folded C-terminal domain. Here, we review the literature of these copper sites extrinsic to the octarepeat region and add new findings and insights from recent experiments.

Introduction – Copper and the Prion Protein

Misfolding of the prion protein (PrP) is responsible for a unique class of neurodegenerative diseases referred to as the Transmissible Spongiform Encephalopathies (TSEs) [1,2]. Although there are parallels to other protein folding diseases (i.e., protein aggregation and formation of amyloid) [3], prion diseases are unique in that they can be transmitted via prions, infectious particles that consist only of misfolded PrP. As a result, prions are source of great interest not only as a new paradigm for disease contagions, but also as a public health concern.

Similar to proteins involved in other aggregation/amyloid diseases, the prion protein in healthy tissue exists in a properly folded cellular form termed PrP^{C} , which is expressed throughout the body. The precise function of PrP^{C} is not yet known. Structural information, both by NMR [4,5] and x-ray crystallography [6], shows that the C-terminal domain of PrP^{C} adopts a predominantly α -helical conformation. Interestingly, the N-terminal half of the protein is flexible and unstructured [4].

In 1997, Brown *et al.* reported that the prion protein exhibits a strong affinity for copper, with binding localized primarily in the N-terminal half of the protein [7]. This study and other investigations identified the octarepeat region as the major site of copper coordination [8-11], although these earliest reports also suggested that copper could be taken up by other portions of the protein as well. Since then, most attempts to assign a native function to PrP have focused

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Dedication: This work is dedicated to our dear colleague Tony Fink. Tony was an inspiration in the manner in which he conducted his science and, more importantly, in the way he lived his life. Tony exuded a love of work and family. He always had a few minutes, a smile and a friendly word for those of us who enjoyed seeing him on a daily basis.

on copper [12]. Studies show that PrP may protect cells against apoptosis [13] and oxidative stress [14], but in both cases, this function is lost if the protein does not include the octarepeat region. Likewise, the ability of PrP to stimulate nerve growth is also dependent on the copper binding octarepeats [14]. Other putative functions include SOD activity [15], copper dependent cell signaling [16] and protection from Cu^{2+} oxidative effects by sequestration [17]. Studies of neurons in culture reveal that Cu^{2+} (and Zn^{2+}) cause PrP to undergo endocytosis [18]. This cellular trafficking is lost when PrP is expressed with mutations of the octarepeat region; both reduction of the number of repeats from the normal four to two, and expansion to nine octarepeats blocks copper induced endocytosis [19]. PrP expression is stimulated by copper [20,21], suggesting that PrP is involved with copper homeostasis.

In addition to native function, a number of studies have attempted to ascertain the influence of copper, and the copper-binding region of PrP, on prion disease. Such studies are motivated, in part, by the elegant work of Uversky, Li and Fink on the interplay between metal ions and α -synuclein [22]. At this juncture, there is no consensus as to whether copper impedes or promotes disease. Scrapie infected animals display a significant delay in disease onset when dosed with copper [23], and a similar effect was observed in neuroblastoma cell cultures. Baskakov and co-workers showed that copper inhibits the *in vitro* formation of fibrils in full-length recombinant protein (the N-terminally truncated PrP 90-231 shows a similar, but less pronounced effect) [24]. Conversely, reduction of total brain copper in animals by treatment with chelators can delay disease onset [25]. Copper may also promote the conversion of PrP^{C} to a protease resistant form [26].

Our understanding of the number, location, structure, affinity and cooperativity of the copper sites in PrP continues to evolve in the literature. Fig. (1) highlights important structural features, as well as documented and potential copper binding sites [27-31]. The octarepeat region, with its four histidine containing octapeptide segments is most often the focus of study, however, as noted above, other sites have also been identified as potential copper binding regions. Below, we review the literature with an emphasis on these non-octarepeat copper binding sites and add insights from some new experiments in our lab.

The octarepeat region can take up to 4 equivalents of copper [17,32,33]. Early studies indicated that the affinity for copper was only micromolar [9,34,35], but with very strong positive cooperativity [7,35,36]. Work from our lab showed that the octarepeat region binds copper in a series of different binding modes that depend on the precise concentration of available copper [37]. We have determined the copper affinity for each of these binding modes and found that while the K_d for the fully copper loaded octarepeat region (four equivalents of copper, component 1) is indeed in the micromolar range, the first equivalent of copper is bound in a multi-histidine binding mode (component 3) with sub-nanomolar affinity, Fig. (2). By tracking the amounts of each of binding mode as a function of copper, we showed that the cooperativity was actually strongly negative [38]. Our findings were recently supported by a combination of equilibrium dialysis and spectroscopic experiments [39]. In addition to the octarepeat region, copper uptake has also been attributed to His96 and His111. These sites lie to the C-terminal side of the octarepeats, but are still in the flexible N-terminal portion of the protein. A number of reports have also discussed copper binding within the structured C-terminal half of the protein. These copper binding sites extrinsic to the octarepeat region will be considered in depth in the following sections.

Copper Binding Outside the Octarepeat Region

The octarepeat region of the prion protein can bind up to four equivalents of copper, one for each of the four octarepeats, whereas the full length protein has been reported to have a copper capacity ranging from 5.3 to 12 equivalents [17,40]. This discrepancy has prompted an

examination of residues 90-231 to locate non-octarepeat copper sites. Copper binding in this region of PrP is of particular interest because this part of the protein has the most relevance to disease. First, all known pathogenic point mutations are found to the C-terminal side of residue 90 [41]. This segment also corresponds to the protease resistant core of the pathogenic form of the protein; after treatment with proteinase K, pathogenic PrP (PrP^{Sc}) retains residues beyond position 90 and this treated material remains infective [42]. Animal models that express an N-terminally truncated form (PrP(90-231)) are susceptible to prion disease (unlike PrP knockout animals), although with altered pathology and rate of progression [43]. On the other hand, animals that express only the structured C-terminal half of the protein, PrP(122-231), suffer from ataxia [44]. Interestingly, peptides derived from the sequence between the octarepeats and the structured portion of PrP (residues 106-126) are neurotoxic [45], but transgenic animals that express PrP with that same portion deleted, do not survive past the embryonic state [46]. Deletion of this segment in recombinant PrP prevents the protein from forming amyloid *in vitro* [47]. In humans, a number of pathogenic mutations are found just to the C-terminal side of the octarepeats, including P102L and P105L [41]. We consider copper binding by residues within 90-231 as divided into two groups: the so-called 5th site involving His96 and/or His111, and sites within the folded C-terminal domain, PrP(125-231).

Copper Binding Sites Between the C-Terminal Domain and the Octarepeat Region

From the early experiments on copper uptake by PrP, it was realized that His96 was also a likely candidate for a copper site. His96 is adjacent to the octarepeats and, like the octarepeats, is in a glycine rich environment. Using mass spectrometry, Whittal *et al.* found that while a peptide encompassing only the octarepeat region would bind four equivalents of Cu²⁺, PrP(57-98) (extended to include His 96) would bind five [9]. Likewise Burns *et al.* found the same result using EPR to examine similar peptide segments of the protein [17]. Using Circular Dichroism to detect copper binding in peptides extended to include His111, Jones *et al.* showed that both His96 and His111 bind copper [48]. These experiments also highlighted the relevance of these sites: the 5th site could successfully compete with the octarepeat region for copper. In follow up work, this same group determined that His96 and His111 are each part of two independent copper binding sites [49], with coordination features consistent with those previously identified by Burns *et al.* [17]. Others used analysis of metal catalyzed oxidation [50] and NMR (although at pH 5.5) [51] to confirm that both H96 and H111 bound copper *in vitro*.

The relative copper affinity of His96 and His111, and their relation to the nearby octarepeat region remains to be settled. Some disparity is due to the variety of experimental methods, but new experiments show that the construct length also affects affinity and selectivity. Using peptides approximating PrP(90-115), several groups find that His96 has a greater affinity for copper than His111 [17]. Recently, Klewpatinond and Viles found that site preference depended on fragment length; His96 is the preferred first binding site for PrP(90-115), while His111 is the first site occupied in PrP(90-126) [52]. There is also no agreement in the literature as to the site of highest affinity in the protein as a whole. Reported affinities for the octarepeat region [38] and the 5th site [12] both range from sub-nanomolar to micromolar. Using metal catalyzed oxidation mass spectrometry of PrP(57-98), Srikanth finds that His96 is the histidine most often involved in binding [53], but does not assign preference. Using separate peptides (PrP(57-91) and PrP(91-115)) Wells *et al.* found that the highest affinity binding mode for the octarepeat region was 3 nM, while the 5th site was 100 nM [39]. Conversely, Klewpatinond found that when full length protein is titrated with copper, the first identifiable circular dichroism signal is from the 5th site, although they also note that the strongest octarepeat binding mode is not CD active [54]. Our lab recently reported titrations studies of full-length recombinant PrP with copper, with binding modes reflected through EPR spectra. Least squares

fitting of those EPR spectra (with a basis spectra derived from peptide models) shows that the first few equivalents of copper are almost equally distributed between the octarepeats (component 3) and the 5th site (labeled non-octarepeat binding), Fig. (3). This would indicate that the strongest binding mode of the octarepeats has approximately the same affinity for copper as the 5th site. However, high amounts of Zn²⁺, which binds exclusively to the octarepeat region, can shift copper to non-octarepeat binding sites [55].

Recent experiments in our lab address the order of histidine occupation in the fifth site, the effect of construct length on binding affinity, and the relative affinity compared to the octarepeat region [56]. To determine if there was a preference for copper coordination to either His 96 or His 111, metal catalyzed oxidation was employed, a valuable approach for mapping copper binding sites in peptides and proteins [53,57]. This technique uses ascorbate to reduce the bound metal and sodium persulfate to generate sulfate radical. This radical then reacts by oxidizing amino acid sidechains near the metal binding site. Ascorbate is used at high concentrations to both reduce the copper centers and to scavenge unreacted sulfate radical, thus avoiding oxidation of amino acids away from the binding site. With these conditions, only amino acid sidechains within ~10Å are oxidized [57].

Solutions of the peptide mouse PrP(90-114) were first allowed to equilibrate with a series of different copper concentrations for 30 minutes to ensure full binding. These solutions were then allowed to react with ascorbate and persulfate for 10 seconds before the reaction was quenched with EDTA. The resulting solutions were then analyzed via reverse phase HPLC. As shown in Fig. (4), the addition of any amount of copper resulted in two new peaks in the chromatogram. Mass spectrometry of these new product peaks is consistent with one oxidation each (+ 16 amu), with signal integration showing that each possesses approximately the same area.

Ion trap mass spectrometry (MS₂ and MS₃) was used to identify the oxidation sites for each product. Fragmentation of peak 1 (Fig. (4)) showed that the oxidation was on the segment GTHN, which includes His96. MS₂ of peak 2 reveals that the oxidation is located in the final nine C-terminal residues. MS₃ of the smallest fragment displays that the oxidation is located directly on His 111. These results, coupled with consistently equal distribution of reacted species with addition of increasing amounts of copper, show that within the construct MoPrP (90-114) copper binds to each histidine site with equal affinity and there is no preference between the two.

The copper affinities of this peptide and the truncated protein PrP(90-231) were determined by EPR competition, a method we have previously applied to the octarepeat region [38]. Competition of PrP(91-115) with oxidized glutathione gave a K_d value of 2.5 (±0.25) nM, while the same experiment yielded an affinity of 0.13 (±0.01) nM for PrP(90-231). This ten-fold increase in K_d may result from the change in the local binding environment, particularly on the pK_a of residues near the binding site. Several groups report that the copper binding mode of the 5th site is strongly dependent on pH [52,58,59]. Fig. (5) shows the EPR spectrum from a pH titration of PrP(90-115) and PrP(90-231). As observed in the perpendicular region of the EPR spectra, between 3200 G and 3400 G, these two constructs respond differently to pH. We find that the spectrum of the protein at pH 7.5 most closely matches the PrP(90-115) peptide spectrum at pH 8.2. To determine whether pH also influences copper affinity, competition experiments were repeated at a pH of 8.2 on the peptide PrP(91-115). The K_d determined at pH 8.2 was 0.08 (±0.008) nM, approximately matching the K_d for the fifth site histidines in the protein at pH 7.4. Thus, the peptide becomes a good model for the protein, both in coordination details and affinity, only if the pH is raised by 0.8 units. One explanation for this effect could be that an interaction between the N-terminal domain and the folded C-terminal domain lowers the pK_a values of the functional groups involved in copper binding. A study

using chemical crosslinking, MS and structural calculations [60] suggests that PrP(90-125) forms a loop with G90 close to E152, at a distance of approximately 5 Å. This sort of structure would place His96 and His111 in close proximity to the more acidic C-terminal domain.

Copper Binding in the C-Terminal Domain PrP(126-231)

Several studies suggest that there are copper binding sites within the folded C-terminal domain of PrP. Conventional EPR detected copper bound by PrP(121-231) [40] and pulsed EPR (ESEEM and ENDOR) implicated the involvement of one of the three histidines in the C-terminal domain [61]. These findings were followed by experiments using a series of His→Ser mutants of the C-terminal histidines to identify specific sites [62]. Unfortunately, only PrP(121-231, H140S) remained folded after the addition of copper, and this mutant showed no change in copper binding. Although aggregation prevented the H177S mutant from being tested, they found that the mutation of a nearby acidic residue, D178K, did not change the copper binding. The authors felt that this ruled out H177 as a likely copper site. An EPR spectrum of the H187S mutant had an altered signal, but the authors urged caution because of aggregation in the sample [62].

Because of the remaining uncertainty, several recent studies have the putative copper site at H187. Brown et al studied the peptide PrP(180-193) using EPR, CD, UV-visible spectrometry and ESI mass spectrometry [63]. They found that the peptide bound copper and had similar EPR parameters to those reported in the earlier literature. More recently, two groups reported QM/MM (combined quantum mechanical/molecular mechanical) calculations for the potential C-terminal PrP copper sites [64,65]. Both of these studies found that a site anchored by H187 best matches the experimental EPR parameters. Interestingly, H187 is the site of a pathogenic mutation that causes a GSS-like disease in humans [66].

Using mutagenesis combined with EPR characterization, our lab pursued investigations of possible copper sites in the C-terminal domain of PrP. We developed constructs where histidines involved in the 5th site were mutated to tyrosine (PrP(23-231, H96Y, H111Y)). These proteins were purified using our normal protocol [55] (i.e. using the octarepeat region as a natural histidine affinity tag), but gave a simplified copper EPR spectrum enabling identification of additional copper binding sites. Three recombinant proteins were produced, each with a distinct C-terminal His→Tyr mutation (H140Y, H177Y, H187Y), and all folded to the expected α -helical form characteristic of PrP^C. None of the C-terminal His→Tyr mutations produced significant changes reflected in their EPR spectra. As an example, Fig. (6) shows the overlaid spectra of PrP(23-231, H96Y, H111Y) and PrP(23-231, H96Y, H111Y, H187Y), each with a considerable excess of copper (8 equivalents). Integration of the respective EPR absorption spectra finds that the amount of bound copper varies by less than 5%. The features of the two spectra in Fig. (6) overlap well, indicating preservation of the coordination environment of bound Cu²⁺. Similar spectral comparisons were found for the other two mutant proteins, PrP(23-231, H96Y, H111Y, H140Y) and PrP(23-231, H96Y, H111Y, H177Y), with spectral absorption integrations approximately matching the wild type C-terminal domain. Previously reported ESEEM experiments suggested the involvement of C-terminal histidine residues, but these experiments were performed on PrP(23-231), in which the N-terminal domain histidines are also present [61].

Conclusion

Although copper binding by the prion protein has been studied for over a decade, details of this phenomenon remain unresolved. However there does seem to be a coalescence of opinion on copper binding by the so-called 5th site, with consensus that both His96 and His111 each bind an equivalent of copper. As demonstrated here, the binding mode is very sensitive to pH

and, when using peptides, to fragment length as well. We also showed that copper uptake at these sites in the protein exhibits a ten fold higher affinity than the corresponding peptide models encompassing residues 96 and 111. In the full-length recombinant protein, the affinity is approximately nanomolar, placing it equal to the strongest binding mode of the octarepeat region. This strong affinity and inclusion of these sites in the protease-resistant, pathogenic core of PrP^{Sc}, encourages further study of their role of copper in prion disease [67]. Both *in vivo* and *in vitro* studies have explored the importance of the octarepeat region to putative function, but few have extended these studies to investigate the physiological relevance of the 5th site.

Issues of solubility or copper promoted aggregation have hampered previous studies of copper binding in the folded C-terminal domain of the prion protein. Identification has therefore had to rely on imperfect samples, indirect mutations or computational studies. These studies consistently identify H187 as the site of C-terminal copper binding. Here, we report that we have been able to express and properly fold these mutants, yet we do not observe noticeable differences in copper binding relative to wild type. We do detect additional C-terminal copper association at very high copper levels, however, this is unlikely to be physiologically relevant. Taken together, we conclude that relevant, high-affinity copper coordination related to PrP^C function is restricted to the N-terminal domain of PrP.

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Fig. (2). Model Representing Copper Binding Sites in the Prion Protein. Model on the left shows the N-terminal region at full copper occupancy, as well as indicating the locations of histidines located in the globular C-terminal domain. Far right shows octarepeat region binding in Component 3 (low copper occupancy, high affinity, multi-histidine mode).

Fig. (3).

Copper binding modes of PrP 23-231 in the presence and absence (solid lines) of zinc. A solution of recombinant PrP(23-231) was titrated with copper with either 0 (dashed lines) or 300 μ M (solid lines) zinc. EPR spectra of this titration were decomposed into a basis reflecting the different copper binding modes and plotted against total copper bound. Adapted from Walter *et al.* 2007.

Fig. (4).
Chromatograph of the metal catalyzed oxidation of mouse PrP(90-115). Separation was performed on a C18 analytical column. Adapted from Stevens (2008).

Fig. (5). EPR spectra recorded at a series of pH values of copper loaded peptide (left side) and protein (right side). Both constructs are sensitive to pH in a seemingly independent fashion, but a comparison exists between the spectra of the protein with the spectra of the peptide at a higher pH (~ 0.8 pH units). Adapted from Stevens (2008).

Fig. (6).
EPR spectra of PrP(23-231, H96Y, H111Y) and PrP(23-231, H96Y, H111Y, H187Y). Spectra are nearly equivalent, consistent with the lack of a copper binding site at His187.