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Contribution of Individual Histidines to Prion Protein Copper Binding

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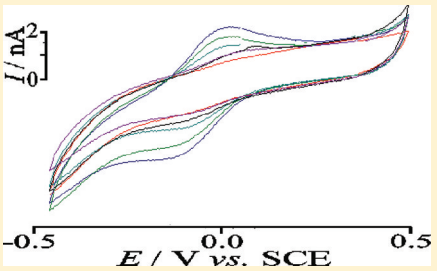
ABSTRACT: The prion protein is well-established as a copper binding protein. The N-terminus of the protein contains an octameric repeat region with each of the four repeats containing a histidine. The N-terminus has two additional histidines distal to the repeat region that has been commonly known as the fifth site. While binding of copper by the protein has been extensively studied, the contribution of each histidine to copper binding in the full-length protein has not. Here we used a battery of mutants of the recombinant mouse prion protein to assess copper binding with both isothermal titration calorimetry and cyclic voltammetry. The findings indicate that there is extensive cooperativity between different binding sites in the protein. The two highest-affinity binding events occur at the fifth site and at the octameric repeat region. However, the first binding is that to the octameric repeat region. Subsequent binding events after the two initial binding events have lower affinities within the octameric repeat region.



The cellular prion protein (PrP^c) is a normal cellular glycoprotein strongly expressed in neurons[1] and to some degree in other cell types.[2,3] The prion protein (PrP) is associated with a number of diseases grouped together under the term prion diseases or transmissible spongiform encephalopathies.[4] In these diseases, protein becomes misfolded, contains increased β-sheet content, and forms aggregates. An abnormal isoform of PrP is associated with disease transmission, and propagation of the diseases is associated with the ability of the disease specific isoform to induce conformational change and aggregation of host PrP by a nucleation mechanism.[5] The mechanism of the disease process is not completely understood, but a potentially improved understanding of this may be realized via a fuller investigation of the cell biology and biochemistry of PrP^c. One of the most widely investigated properties of PrP^c is its potential to bind metals such as copper.[6]

The octarepeat region in the human protein is composed of a sequence of eight amino acids (PHGGGWGQ) repeated four times, each containing a histidine that is generally thought to be the primary residue responsible for the copper coordination.[7,8] Detailed studies involving electron paramagnetic resonance imaging (EPR) and X-ray crystallography on recombinant peptide fragments have demonstrated that a single copper is coordinated by each octarepeat segment in a pentacoordinate complex involving residues HGGGW.[7,8] This equatorial coordination involves the histidine imidazole, deprotonated amides from the two adjacent glycines, and a deprotonated carboxyl from the last glycine. A water molecule may also be involved by allowing an oxygen to coordinate axially forming a bridge to the NH group of the indole on the last tryptophan. Recent work has demonstrated that the coordination of copper was dependent on the degree of copper occupancy on the protein.[9] Three distinct coordination modes were proposed, clearly divisible at different relative copper concentrations. These consisted of a multiple-His coordination mode at low copper occupancy, moving through a transitional coordination to the maximal occupancy at a physiological pH of 7.4. Work on the octarepeat region has also revealed evidence of dipolar copper−copper centers suggesting a copper−copper distance of 3.5−6 Å, short enough for van der Waals interactions.[9] These interactions may be responsible for driving a hydrophobic collapse and consequent N-terminal structural organization at full copper occupancy.

A large body of literature that demonstrates that copper is able to bind outside of the octarepeat region of PrP also exists.[10−14] Work by Jones et al.[10,15] highlighted these copper binding regions as His96 and His111 in the human protein. They also identified the minimal sequences necessary for copper binding to this region of PrP are amino acids 92−96 and 107−111. Recently, the same group utilized NMR and visible circular dichroism (Vis-CD) to fully elucidate the coordination of copper to this so-called fifth site.[16] Interestingly, they found that the coordination of copper changed dramatically depending on chain length and pH. They concluded that this striking effect was caused by a change in the relative affinity of His95 and His110 for copper. Although His110 seems to display the highest affinity for copper, the affinity of His95 increases dramatically upon addition of the 11-amino acid hydrophobic segment. They also discovered a multicoordination mode that was strongly influenced by pH.

While some aspects of binding of copper to PrP are much more fully understood, the true importance of the fifth site and the octameric repeat in a relative sense with respect to copper binding has not been clearly defined. In addition, the contributions of each histidine to the copper binding are unknown. There have also been suggestions of both positive cooperativity and negative cooperativity between the various

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copper binding sites. For these reasons, we examined the contribution of all six histidines of the N-terminus of PrP both individually and in different combinations to assess the contribution of each site to copper binding. Our findings indicate that the fifth site and octameric repeat both contribute a high-affinity site in the wild-type protein but that these two high-affinity sites are a result of cooperativity between all sites. The remaining sites also exhibit some cooperativity with each other. Our findings provide little evidence of negative cooperativity.

METHODS

Recombinant Protein Production. Recombinant mouse PrP protein was produced as described previously.[17,18] All proteins used for this study were not tagged. Briefly, the polymerase chain reaction-amplified product was cloned into expression vector pET-23 (Novagen) and transformed into *Escherichia coli* ad494(DE3) or BL21(DE3). Expressed proteins were solubilized by sonication in 8 M urea and recovered by immobilized metal ion affinity chromatography (IMAC). Columns were charged with copper. The eluted material was treated with 0.5 mM EDTA to ensure the protein was free of metal ions that had potentially leached from the IMAC column. Additionally, all subsequent treatments were performed with doubly deionized water treated with Chelex resin (Sigma) to remove residual metal ions. The denatured protein was refolded by a 10-fold dilution of the urea in deionized water followed by concentration by ultrafiltration and two rounds of dialysis to remove residual urea, imidazole, and EDTA. Starting stock solution protein concentrations were measured using theoretical extinction coefficients at 280 nm (http://us.expasy.org/tools/protparam.html) and confirmed by a Bradford assay (Sigma). Protein purity was checked using polyacrylamide gel electrophoresis under denaturing conditions with gels stained with Coomassie brilliant blue.

Isothermal Titration Calorimetry (ITC) Measurements. ITC experiments were conducted on a Microcal VP-ITC instrument. A series of injections of metal were made into an isolated chamber containing the protein at a constant temperature of 25 °C. Heat changes within the cell were monitored during each injection of metal and recorded as the total heat change per second over time. Protein samples were prepared by adding a small amount of concentrated and pH-adjusted MOPS buffer, yielding final concentrations of 10 mM buffer and either 10, 12, 15, or 20 μM protein. Different concentrations were used to ensure that results were repeatable over a variety of conditions. The choice of buffers was based on initial trials, which revealed that these buffers offered minimal background noise.

The use of free copper in the titrations results in protein aggregation and nonspecific binding.[19] ITC binding experiments involving tight binding models in which the affinity for the ligand is likely to exceed 10^9 M^{-1} require the use of a weaker chelator to accurately determine dissociation constants.[20,21] In addition, no free copper exists in vivo, so a copper chelate was used. Copper solutions were prepared by adding copper sulfate to 10 mM buffer (as above) along with glycine at a molar ratio of 1:4. Glycine chelates two molecules of copper, forming $Cu(gly)_2$ and $Cu(gly)$ complexes. At pH 7, the $Cu(gly)_2$ complex dominates.[22] The excess glycine thus ensures that there is never any free copper in the reaction cell, preventing the complications mentioned above. The use of buffer in only one of the solutions would result in significant background noise; therefore, both protein and metal solutions were buffered. The concentration of copper used was dependent on the concentration of protein in the reaction cell. The final ratio of protein to copper in the reaction cell after each ITC experiment was 1:10. During each experiment, 30 × 4 μL doses of copper were injected into the chamber with protein, which was stirred constantly at 300 rpm. Each injection was followed by a 2 min period to ensure equilibration of the solution. All experiments were repeated three times and where possible using different concentrations of separately prepared protein.

ITC Data Anlaysis. Initial data analysis was conducted using the sequential fitting model in Microcal Origin 5. Regression was conducted until the minimal χ^2 value was achieved while variation in the fractional K and H values remained within ±10%. To compare the resultant apparent affinity values between mutants, we accounted for the effect of the glycine chelate with eq 1.

$$K_n = \frac{K_d(\text{app})K_d(\text{che})}{[\text{che}]^2} \quad (1)$$

where K_n is the adjusted value at any given site, K_d(app) is the association constant from the ITC regression fitting, K_d(che) is the literature association constant for the chelator, and [che] is the concentration of the chelator. In the case of glycine, the constant is used for the bis complex with Cu(II)-bound protein.[23]

Cyclic Voltammatry. Cyclic volatammetry was conducted with adsorbed protein as previously described.[18] Voltammetric measurements were taken with a μ-Autolab III potentiostat system (Eco Chemie, Utrecht, The Netherlands) in a conventional three-electrode electrochemical cell. Experiments were performed in staircase voltammetry mode (step potential of 0.6 mV) with a platinum gauze counter and saturated calomel reference electrode (SCE, REF401, Radiometer). The working electrode was a 3 mm diameter boron-doped diamond electrode (Diafilm, Windsor Scientific). Electrodes were polished on fresh micro cloths (Buehler) with 1 μm alumina (Buehler) as a polishing aid. After the final polish on a clean micro cloth, electrodes were rinsed with demineralized water. Aqueous solutions were thoroughly deaerated with argon (BOC) before data were recorded. All measurements were taken at 22 ± 2 °C. Voltammetric measurements were conducted in an aqueous buffer solution [5 mM MES/Tris (pH 7)] thoroughly deaerated with argon. The working electrode was polished and the background current recorded in the absence of protein. Next, the working electrode was immersed in a protein solution (containing 20 μM recombinant mPrP in buffer) and after 60 s removed and rinsed with water. A longer incubation did not significantly increase the amount of immobilized protein. The resulting protein-modified electrode was reimmersed in the pure buffer solution in the measurement cell, and cyclic voltammograms were recorded starting from open circuit conditions. Adhesion of protein to the boron-doped diamond electrode surfaces was excellent, and stable signals were obtained for many potential cycles.

RESULTS

The mouse prion protein (PrP) has six histidine residues in the N-terminus. Mutation of the PrP gene to replace all six histidines with alanines resulted in recombinant protein that does not bind copper.[18] To assess the contribution of each

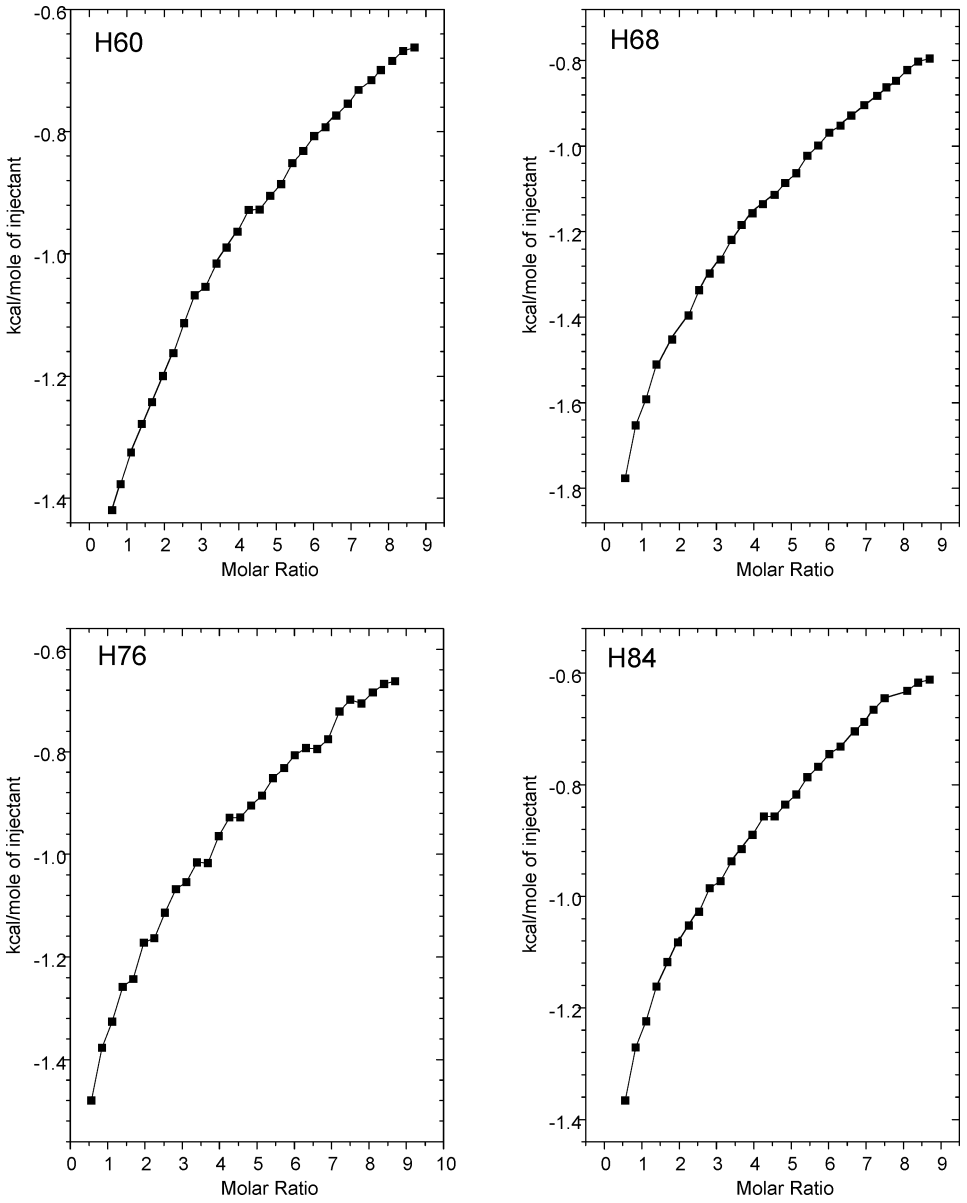

Figure 1. ITC analysis of PrP with one N-terminal histidine in the octameric repeat region. Shown are example isotherms for mutant PrPs retaining only one of the four histidines of the octameric repeat region.

histidine to copper binding, we generated a battery of mutations with either one, two, three, four, or five histidines. To simplify the presentation of the results, we have named the mutant PrPs by the histidine residues that are not mutated. The six histidines are present at positions 60, 68, 76, 84, 95, and 110 in the mouse sequence. Therefore, for example, a mutant protein with all histidines replaced with alanines except those at positions 60 and 110 is named H60+110, while a protein with a histidine replaced with alanine only at position 76 is named H60+68+84+95+110.

Copper Binding to PrP with a Single N-Terminal Histidine. ITC was used to assess the binding of copper to mutant PrP with a single histidine present in the octameric repeat region. Examples of ITC isotherms for H60, H68, H76, and H84 are present in Figure 1. The affinity for copper at each individual histidine on its own was identical with a value of log affinity of 6.2, and in each case, the error between experiments (n = 4) was less than 5% of the value. This implies that each individual octameric repeat can coordinate copper at a concentration of ~1 μM.

The so-called "fifth site" for copper binding by PrP consists of H95 and H110. Mutant PrP with only one of these two histidines present exhibited behavior quite different from that of the single-octameric repeat histidines. H110 bound copper with an affinity similar to those of the histidines within the octameric repeat region (log K_a = 6.3). In comparison, H95 showed no copper binding at all. The lack of binding of copper under the conditions used probably reflects the inability of the site to outcompete glycine for copper occupancy.

Copper Binding to PrP with Two N-Terminal Histidines. While the interpretation of binding of copper to a single histidine is straightforward, introduction of a second histidine introduces the possibility of cooperation in copper binding between the sites present. First, copper binding to the fifth site (H95+110) was examined using ITC. In this case, two copper binding sites were observed. While the affinity of one site matches that seen for H110, the second site shows a

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considerably higher affinity (Table 1). This implies there is positive cooperativity in copper binding to the fifth site.

Table 1. Changes in Fifth Site Affinity[a]

	second histidine			
first histidine	none	H84	H95	H110
H60	6.2	6.2	6.2	10.6
	–	6.2	nd[b]	6.2
H84	6.2	–	7.8	7.8
	–		6.8	6.3
H95	nd[b]	–	–	10.6
	–			6.1

[a]Log affinity values determined from ITC experiments for mutant PrP with one or two histidines. The standard error in each case was between 1 and 5% of the value. [b]No site detected.

Copper binding was also analyzed for mutant proteins with one histidine present from the fifth site and one present from the octameric repeat (either H60 or H84). While H95 on its own did not bind copper, H60+95 bound a single copper ion with an affinity similar to that of H60 alone, implying that H95 and H60 did not show cooperative binding. In contrast, H84+95 bound two copper ions both with affinities higher than that of H84 alone, suggesting positive cooperation between histidines of the octameric repeat region and the fifth site. H60+110 and H84+110 both bound two copper ions with one site in both cases having a higher affinity. The highest affinity was seen with H60+110, suggesting that cooperativity in copper binding was greater the more distal the histidine from the fifth site.

A similar analysis was conducted with the histidines within the octameric repeat. In this case, six mutant proteins were analyzed constituting all possible combinations of the four histidines (H60+68, H60+76, H60+84, H68+76, H68+84, and H76+84). ITC isotherms are shown in Figure 2. All six mutants were fitted to a two-site sequential model, with χ^2 values all at least 62% lower than those of other possible fits. All errors in affinity values were <5 and <8% for the calculated enthalpy of binding. Comparing the affinities of the six mutants with the complete octarepeat, we again noticed striking differences (Table 2). The values fell into two clear groups. These two groups depended on the proximity of the two histidines to each other. In the three mutants in which the two histidines were not adjacent (i.e., separated by at least one repeat with alanine replacing a histidine), there was no difference in the affinity for the two sites when compared to the affinity for the same sites when present alone. In contrast, mutant proteins with two adjacent histidines (H60+68, H68+76, and H76+84) showed two distinct sites, one with an affinity equivalent to that of a single-histidine mutant and the second site with an affinity at least 1 order of magnitude higher than that for the single-histidine mutants. These data suggest positive cooperativity between adjacent octameric repeats for copper binding.

Copper Binding to PrP with Three N-Terminal Histidines. The data presented above clearly demonstrate cooperativity exists in the binding of copper to PrP. This cooperativity is seen between adjacent octameric repeats or H110 of the fifth site and any other histidine regardless of its distance from the fifth site on the protein. We also examined the effect of a complete fifth site on the binding of copper to an octameric repeat proximal or distal to that site (H60+95+110 and H84+95+110). In this case, only two sites were observed for both mutants. The log K_a values for H60+95+110 were 10.2 and 7.14, while the values for H84+95+110 were 9.6 and 7.18. These data confirm greater cooperativity between the fifth site and the most distal histidine of the octameric repeat. While in both cases the affinity values are greater than that when H95 is absent, the contribution of this histidine is clearly less important than that of H110, and H95 is unable to maintain the binding of an additional copper ion. Some example isotherms are shown in Figure 3.

We also examine binding of copper to mutants with three histidines present in the octameric repeat region in the absence of fifth site histidines (H60+68+76, H60+68+84, H60+76+85, and H68+76+84). Example isotherms of all four mutants are shown in Figure 4, and the affinity values obtained are listed in Table 3. All four mutants bound three copper ions. All mutants exhibited similarly stepped affinities, with the lowest-affinity site being similar to that seen with single-histidine mutants. The highest affinity was seen with mutants in which all three histidines were in consecutive octameric repeats (H60+68+76 and H68+76+84). These results contrast to those seen with the fifth site in that no combination of three histidines that includes the fifth site was able to bind three copper atoms, but in both cases, the presence of the fifth site resulted in a much higher affinity for copper.

Copper Binding to PrP with Four or Six N-Terminal Histidines. We have previously studied binding of copper to PrP without the fifth site histidines.[18] When all four histidines are present in the octameric repeat region (H60+68+76+84), the highest affinity seen via ITC is 2 orders of magnitude higher than the affinity of the protein with only three histidines present. The log K_a values obtained from ITC experiments were 10.5, 7.0, 7.0, and 6.0. Only one binding site shows an affinity similar to the affinity of mutant PrP with only one histidine present. This implies that cooperation between the histidines of the octameric repeat region increases the highest affinity values seen by more than 4 orders of magnitude. In wild-type PrP with all six histidines present, only five binding events are observed. The values obtained are similar to those for the mutant without the fifth site histidines except for the addition a site with a log K_a of 10.1. Upon comparing all data obtained with all mutants, we can conclude that the two highest-affinity binding events are due to the octameric repeat and the fifth site in cooperation. The binding of additional copper atoms has a lower affinity but is restricted to the octameric repeats. It is also likely that the initial binding of copper is not to a single site but involves coordinated binding of copper by both the octameric repeat region and the fifth site.

Relation between the Number of Histidines in the N-Terminus and Electrochemistry. We previously used cyclic voltammetry (CV) to investigate the redox cycling of adsorbed Cu−PrP complexes,[18] and there has been considerable recent interest in voltammetry measurements with PrP also in solution.[24] In our study, we showed that Cu−PrP complexes, when adsorbed onto a boron-doped diamond electrode, were able to fully switch in a chemically reversible manner between oxidized and reduced forms. It was tentatively assumed that the protein adsorption process (presumably via electrostatic interaction) only insignificantly affected the structure, binding to copper, and redox reactivity and that the surface-bound protein could provide information about the type and reactivity of copper sites. On the basis of our previous findings,[18] the redox activity observed in measurements was postulated to be

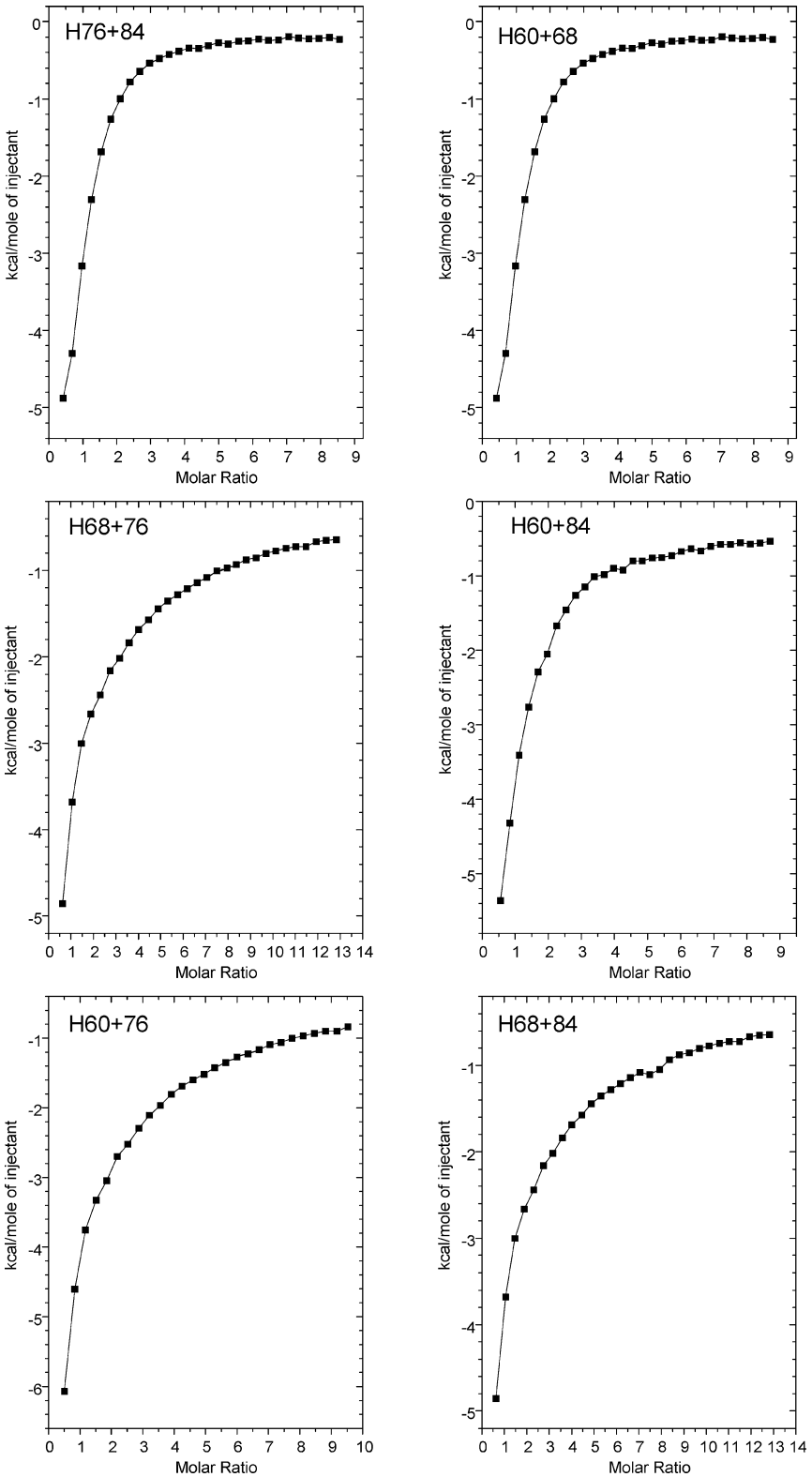

Figure 2. ITC analysis of PrP with three N-terminal histidines. Shown are example isotherms for mutant PrPs retaining two of the four histidines of the octameric repeat region.

primarily related to copper binding at the octameric repeat region. Here, we used CV for adsorbed protein to investigate the contribution of the octameric repeats to the redox cycling of the Cu−PrP complex. Examples of voltammograms obtained are shown in Figure 5, and the midpoint potentials and integrated oxidation peak currents are listed in Table 4. For PrP without the fifth site, deletion of one histidine from the octameric repeat region did not disrupt the redox-switchable nature or the midpoint potential but did reduce the peak current of the signal by ∼50%. When we assume that the coverage of the electrode surface with protein is similar, this suggests that a detected loss of copper is associated with the

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Table 2. Changes in Octarepeat Affinity[a]

	second histidine			
first histidine	none	H68	H76	H84
H60	6.2	7.7	6.4	6.2
	–	6.2	6.2	6.2
H68	6.2	–	7.8	6.4
	–		6.2	6.2
H76	6.2	–	–	7.8
	–			6.2

[a]Log affinity values determined from ITC experiments for mutant PrPs with two histidines. The standard error in each case was between 1 and 5% of the value.

change in the copper binding sites in the protein. Deletion of two or three histidines effectively removes the current response.

DISCUSSION

The use of a single histidine to alanine mutation or multiple histidine to alanine mutations within each region of the PrP N-terminus represents a new approach to the detailed study of copper binding at each individual site. Previous studies using truncation peptides[12,25−27] were at risk of missing important aspects of the copper binding chemistry by not allowing for protein folding and inter-region interactions. There have indeed been some studies involving peptides encompassing

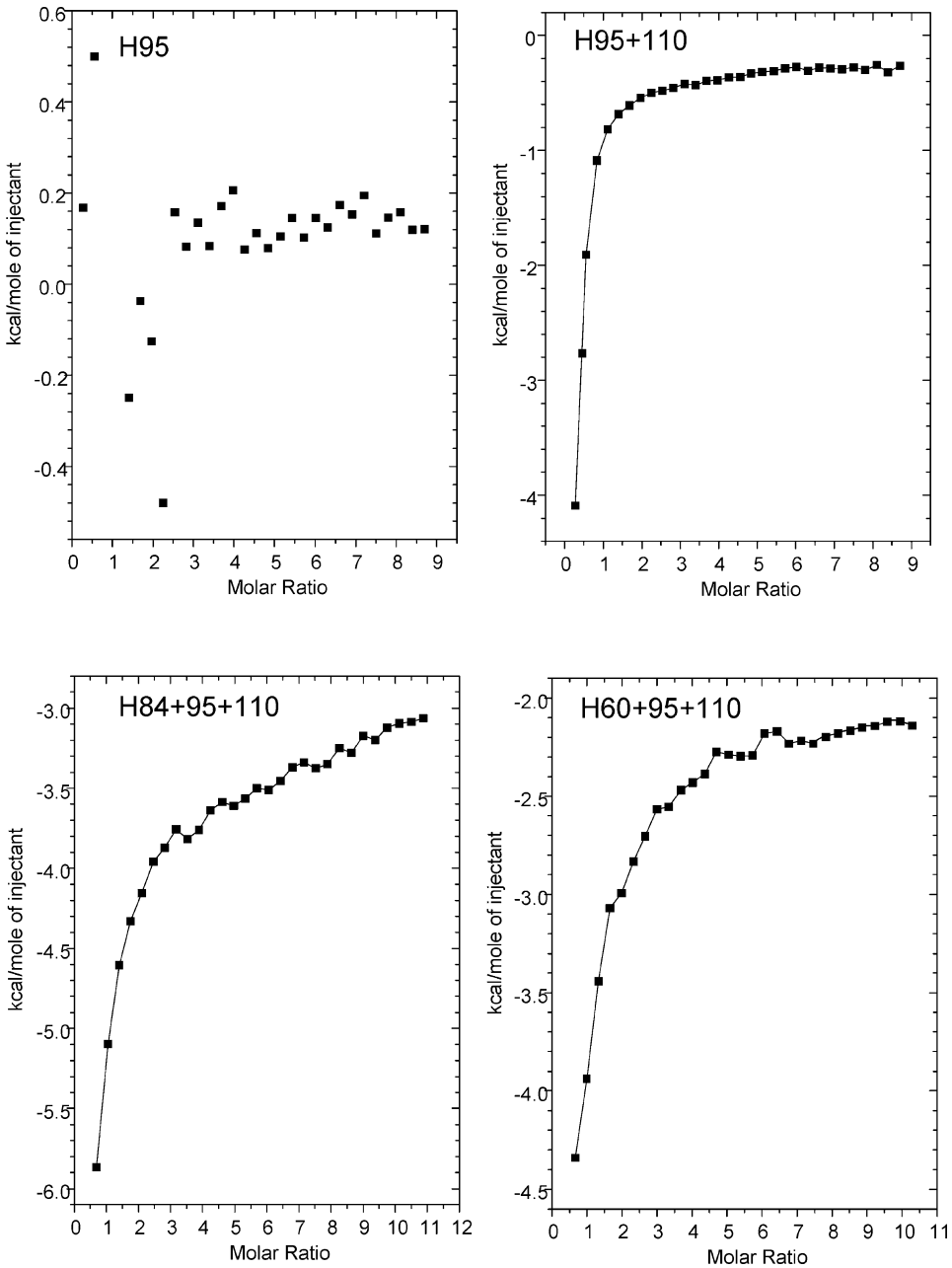

Figure 3. ITC analysis of PrP with histidines from the fifth site. Shown are example isotherms with at least one histidine in the fifth site. PrP with only H95 shows no binding. However, PrP with a complete fifth site (H95+110) has two binding sites. PrP with the complete fifth site and one histidine from the octameric repeat region still binds only two copper ions.

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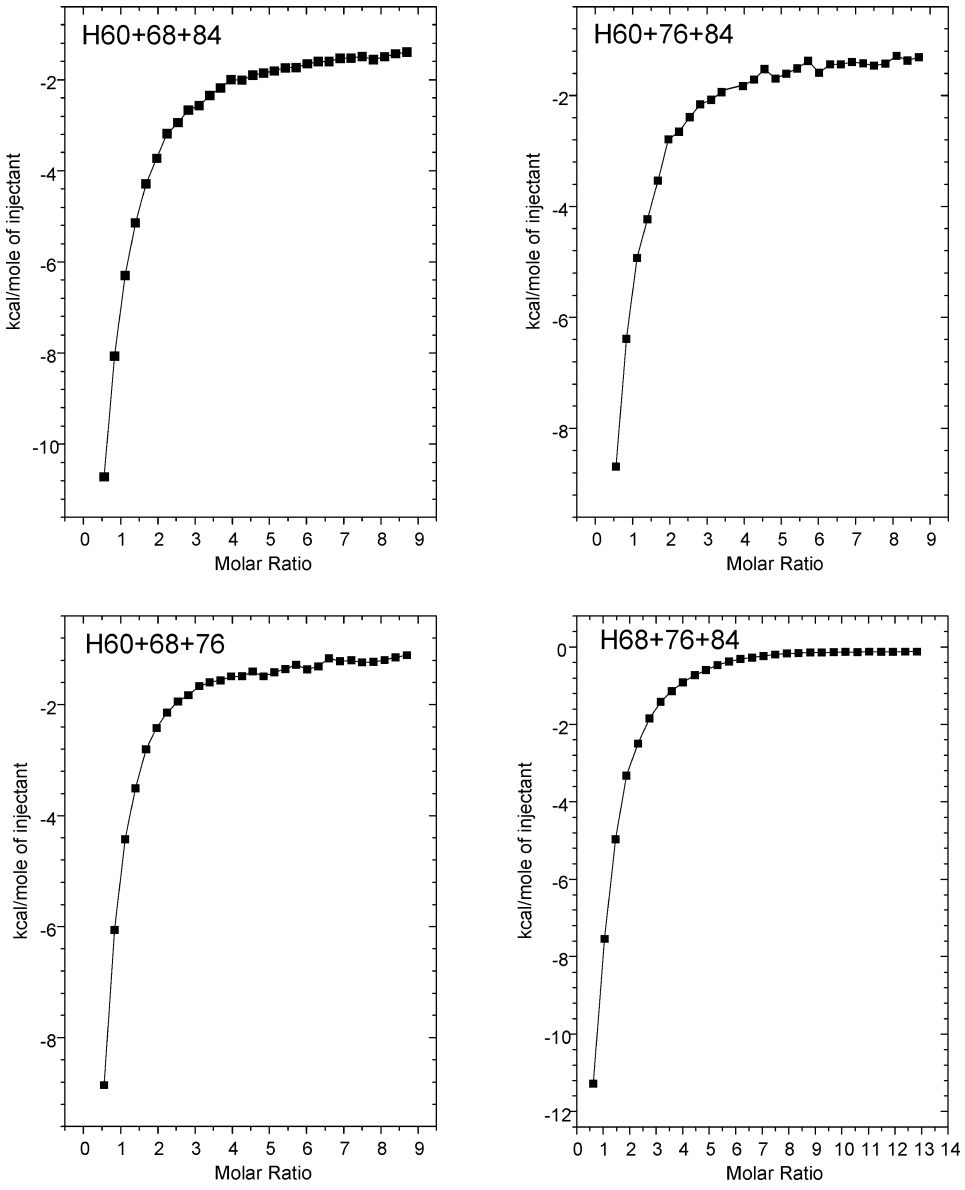

Figure 4. ITC analysis of PrP with three N-terminal histidines. Shown are example isotherms for mutant PrPs retaining three of the four histidines of the octameric repeat region.

both the octarepeat and the fifth site.[28] However, for the sake of completeness, a study of the full-length protein is a useful and necessary contribution to understanding the complex relationships between the prion protein and copper.

In this study, we have used the well-characterized chelator glycine to deliver the metal ion to the proteins being analyzed. This is necessary because of the solubility issues of copper salts at physiological pH and the sensitivity range of ITC ($K_d = 10^2-10^9$).

Table 3. Affinities Determined from ITC Experiments with Mutant PrPs with Three Octameric Repeat Histidines[a]

mutant	site 1	site 2	site 3
H60+68+76	8.2	7.1	6.2
H60+68+84	7.3	6.9	6.1
H60+76+84	7.4	7.0	6.1
H68+76+84	8.3	7.1	6.4

[a]Values are mean log K_a values. The standard error in each case was between 1 and 5% of the value.

There is evidence that when using this system for binding analyses, a ternary copper−glycine−macromolecule species is formed[29] and hence should be considered. We feel, on the basis of our previous observations,[17,18] that this effect can largely be igonored for the purposes of this study. We are comparing relative differences between protein states, and as such, experimental conditions remain the same for each analysis.

The data from the mutants with the fifth site histidines replaced allow us to make a detailed assessment of the binding of copper(II) within the octarepeat region. When one histidine is replaced within the region, a dramatic effect on the stability of copper binding at a low copper occupancy is apparent. When compared to that in the intact octarepeat region, the high-affinity event in the 0.1 nM range is no longer detectable and is replaced with an event in the 10−100 nM range. This represents a significant reduction in the stability of copper(II) binding during the first equivalent of the ion. Previous structural work by Chattopadhyay et al.[9] revealed a complex, multistage coordination of copper(II) within the octarepeat

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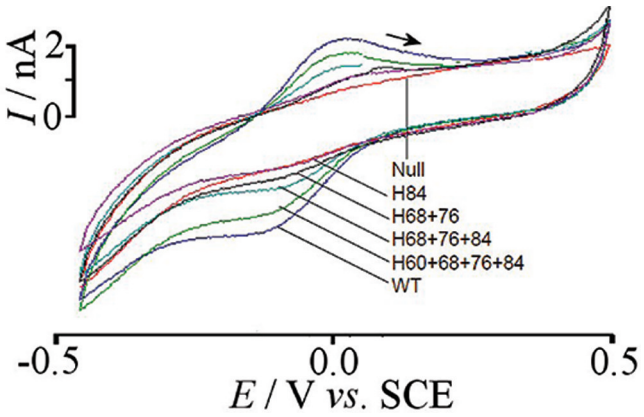

Figure 5. Cyclic voltammagram comparing various mPrP mutants with wild-type protein at a scan rate of 10 mV/s. All reactions were conducted on a 3 mm boron-doped diamond working electrode immersed in 5 mM MES/Tris buffer at pH 7 and room temperature. The mutant labels refer to the number of histidine residues remaining within the octarepeat. Null refers to the protein in which all histidines in the copper binding N-terminus were replaced with alanine.

Table 4. Comparison of the Integrated Peak Charges and Midpoint Potentials for PrP Mutants As Obtained from Cyclic Voltammetry[a]

protein	midpoint potential (V vs SCE)	integrated oxidation peak charge (nC)
wild-type PrP	0.03 ± 0.02	255 ± 11
H60+68+76+84	0.03 ± 0.02	201 ± 5
H68+74+84	0.03 ± 0.02	95 ± 5
H68+76	0.08 ± 0.02	18 ± 1
H84	0.07 ± 0.02	15 ± 1
H95+110	0.10 ± 0.03	13 ± 1
PrP null histidine	not available	not detected

[a]All reactions were conducted at a scan rate of 10 mV/s on a 3 mm boron-doped diamond working electrode immersed in 5 mM MES/Tris buffer at pH 7 and room temperature. Midpoint potential $E_{mid} = {}^1/_2(E_p^{ox} + E_p^{red})$.

region that was dependent on copper occupancy. They showed that, at a copper occupancy of one or below, all four octarepeat histidines were responsible for the coordination of one metal ion by forming a 4N binding arrangement. The group predicted that this "component 3" binding mode would have significantly higher stability than the binding modes at higher copper occupancies. The effect of this remarkable feature of the octarepeat region is highlighted by the thermodynamic data. With one histidine and therefore one octarepeat region unavailable for copper(II) coordination, the highly stable 4N arrangement is replaced by a 3N or 3N1O coordination, resulting in a reduction in the binding stability of at least 2.5 orders of magnitude. As the ability of the octarepeat region to bind copper(II) in this multihistidine mode is utterly dependent on dramatic protein folding around the ion,[8] the location of histidines within the octarepeat region would be expected to be critical to the stability of the copper(II) site. This is exemplified by the thermodynamic data. In mutants with the remaining histidine residues located sequentially, the first binding event has a significantly higher stability than that of the mutants in which the sequence of the remaining histidines is broken. Where the missing histidine is within the sequence of histidines, the reduction in the stability of the first copper(II) site is reduced by 3.5 orders of magnitude.

Further evidence of the component 3 binding mode involving multiple-histidine coordination is apparent in the mutants with two octarepeat histidines replaced. When the two remaining histidines are in sequence, the affinity of the first binding event (~50 nM) is almost identical to that seen in the mutants with just one histidine removed. This provides strong evidence that the initial event involves both remaining histidine residues in a likely 2N2O coordination with the oxygen ligands coming from water molecules present in solution. This coordination and stability are strongly suggestive of a primary contribution from the two remaining histidine imidazoles. In contrast, when the remaining histidines are separated by a mutated octarepeat, the stability for the first copper decreases into the 500 nM nanomolar range. As the affinity is higher than that expected for a single octarepeat (see below), there must be some inter-octarepeat cooperativity. This can be explained by findings that show an intermediate binding mode within the intact octarepeat.[9] This "component 2" mode involves not only an imidazole nitrogen but also an exocyclic nitrogen from one other histidine residue and two oxygens from water molecules. Only existing for an instant in the complete region when copper occupancy is increasing, this mode of copper(II) coordination is usually extremely difficult to resolve.[7] This mutant with the two remaining histidines separated may then provide a model for the study of this intermediate binding mode. Further evidence of this type of coordination comes from the fact that increasing the distance between histidine residues further produces two identical copper(II) binding events, with a stability almost identical to those of two individual octarepeats.

The single octarepeats display a copper binding affinity that is identical irrespective of where they are located. This provides good evidence that it is the inter-octarepeat interactions that are responsible for the high degree of stability for copper(II) at low occupancies. The affinity of each octarepeat for copper(II) very closely matches that previously reported.[30] This single copper to single octarepeat binding mode is described in the literature as "component 1"[9] and consists of a primary coordinating imidazole ligand with contributions from deprotonated amides from adjacent glycines and a carboxyl from the last glycine in the HGGGW sequence. This would naturally form an equatorial arrangement.

Copper binding within the fifth site region has been the cause of much debate within the prion field. Via removal of the effect of the octarepeat region through the mutation of all four histidines to alanine, a detailed assessment of the contribution of the two fifth site histidines can be made. The most striking observation from the thermodynamic data presented here is that binding within the fifth site appears to be more than the sum of its contributing parts. Via removal of the histidine at position 110, it is clear that the histidine at position 95 is unable to coordinate copper(II) independently. By reversing this process and looking at the site at position 110, we find only very low nanomolar binding is apparent. Upon comparison to the intact fifth site region, or mutants with one histidine present from the fifth site and the octameric repeat, two atoms of copper(II) are coordinated with one in the 0.1 nM range and one in the 1 μM range, a striking example of intraregion cooperation on PrP. Previous work confirms the importance of both fifth site histidines with position 110 providing the primary coordinating ligand.[16] The observation that the copper binding at either site is so dramatically different from that when both sites are present is very difficult to explain but is likely to

involve structural effects from either the mutation of histidine to alanine or copper−copper interactions. This effect will be exaggerated greatly by the presence of multiple proline residues in the fifth site region. Of interest, and in support of using the full-length protein for binding studies, it was shown that the relative affinity of each histidine for copper was dependent on peptide chain length.[9]

Our previous work has already suggested that there is an interaction between the fifth site and the octameric repeat region with regard to the coordination of copper.[18] To investigate this phenomenon, mutants with the fifth site present and one site within the octarepeat present either close or distal to the fifth site were utilized. It is clear from the data that the presence of the fifth site has a significant effect on copper binding within the octarepeat. The affinity of copper binding within a single octarepeat region increased by 1 order of magnitude when copper was present within the fifth site. A similar but slightly different picture emerges when only H110 is present with one of the two octameric repeat histidines. In this case, the affinity of binding of copper to the octameric repeat is unaltered (i.e., no effect of cooperativity for this site). However, the affinity for H110 increases above that of H110 alone. This suggests the cooperativity with H110 increases the affinity at this site principally. Also, the degree of cooperativity (i.e., increased affinity) is seen with the distal octarepeat histidine (H60). This suggests that this cooperativity is dependent on the flexible nature of the N-terminus domain allowing the more distal histidine to move into the proximity of H110. In contrast, H95, which shows no copper binding under our conditions, was influenced by a proximal histidine from the octameric repat region (H84). This mimics the cooperativity seen between H95 and H110 in the fifth site only mutant. These data clearly indicate both the importance of copper binding to the fifth site H110 and the dependence of this high-affinity binding on interactions with the octameric repeat.

Looking at our data as a whole, we find the most striking outcome is the extent of cooperativity in terms of copper binding within the N-terminus. The majority of mutant PrPs with two or more histidines show affinity values greater than those with a single histidine. The fifth site or the intact octameric repeat region can bind copper in the 0.1 nM range. When combined, the two highest affinity values seen in our analysis clearly can be distributed one to each site. However, as the highest-affinity site observed is present in the presence and absence of the fifth site, then the first high-affinity copper binding site is found within the octameric repeat with the second binding event occurring within the fifth site. Additional binding events are then coordinated as indicated above by an altered coordination associated with individual histidines within the octameric repeat region.

One of the most heated topics of debate concerning the prion protein's function is whether it is able to act in an antioxidant role. Much recent evidence has been contradictory, with studies confirming antioxidant activity[31−35] and other studies showing this not to be so.[36,37] The need to develop a technique that can be built to contribute to this argument is therefore desirable. We previously developed an electrochemical technique to study the binding of metal to surface-bound PrP, which may aid in developing a solution to this question.[18] By adsorbing PrP to a diamond electrode and subjecting it to cyclic voltammetry, we have shown that the adsorbed protein is capable of exchanging electrons when bound to Cu(II) or Cu(I). This, however, assumes that (i) the bound protein remains structurally intact and (ii) the coverage with protein is similar for different mutants. It is shown here that this electrochemistry appears to be dependent on the copper bound to the octarepeat region. Similar recently published data[24] demonstrated that the amount of copper ions bound to the octarepeat region (using peptides) governed the extent of redox cycling by the bound metal centers. This same publication also showed that PrP can generate hydrogen peroxide from this process, which is the known product of superoxide dismutation, and further supports our findings.[24] The Cu(II)−Cu(I) redox switching process is fascinating and may lend extra support to the finding that the octarepeat region protects the fifth site from oxidative damage.[38]

We showed previously that the measured PrP antioxidant activity is related to the number of octameric repeats present in the protein that are able to bind copper.[37] While deletion of each individual octameric repeats led to a stepwise loss of copper binding, loss of antioxidant activity was lost stepwise in that there was no difference between the activity of mutants with two or three copper ions bound and those with only one copper ion showed no activity. These previous observations match quite closely with what was observed in this study with cyclic voltammetry in terms of the loss of peak amplitude.

The redox cycling for the wild-type protein appears to be unaffected by continuous oxidation and reduction. Previous work has demonstrated this property.[39] The midpoint potential of 0.03 V versus SCE would mean in vivo conditions would favor a redox-switchable state. Apart from other potential mechanistic roles, this redox cycability could aid biological charge transport.

Many blue copper proteins display some sort of redox activity.[40] A fully reversible redox property, however, is usually reserved for type I blue copper sites as these tend to form a distorted tetrahedral environment with a strong Cu−S bond to the thiolate of cysteine residue, two Cu−N imidazole bonds, and a long Cu−S bond to a methionine. The highly covalent Cu(II)−S(Cys) bond provides a strong electronic coupling into protein pathways to facilitate rapid long-range electron transfer. This ensures that the rapid transition between the two redox states of the copper does not cause the metal center to be lost. PrP binds copper(II) in the classic type II format with a square planar geometry.[27] Proteins in this class are not normally able to accommodate copper(I), so once reduced, the copper tends to fall off the protein. This clearly does not happen to copper bound to PrP. Type I proteins that need to cycle electrons in a stable way normally have structural elements that prevent this from happening. Superoxide dismutase (SOD), for example, has its copper center buried deep within the protein structure.[41] This pattern of protein evolution is consistent across the type II proteins, and those that are able to undergo stable redox cycling all have some significant catalytic role involving redox chemistry at their copper centers.[42] Additionally, the pattern and midpoint potentials of these catalytic proteins are all very similar. Using Cu/Zn SOD as an example, the range and potential closely match those of PrP previously published.[41] With the enormous body of evidence supporting the importance of oxidative stress in prion disease[43] and the evidence supporting PrP as an antioxidant, this new evidence further supports the idea that PrP contributes to both the normal defense against oxidative stress and its cause in prion disease.[44,45]

In summary, we have assessed the contribution of the six N-terminal histidine residues to the binding of copper to PrP. The

protein can bind five atoms of copper, four of which bind to the octameric repeat region and one of which binds to the fifth site distal to this region. Two of the events represent high-affinity binding. The first of these occurs within the octameric repeat region and involves cooperation between the histidine centered binding sites but is independent of binding of copper to the fifth site. The fifth site is also the site of the second high-affinity binding event but requires cooperation with the octameric repeat region and most likely from H60. This suggests that flexibility in the N-terminus is necessary for the high-affinity binding at the fifth site. Both high-affinity binding events contribute to the switchable Cu(II)−Cu(I) redox activity of the copper−PrP complex.

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