MANGANESE BINDING TO THE PRION PROTEIN

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Short Title: Mn and PrP

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There is considerable evidence that the prion protein binds copper. However, there have also been suggestions that PrP binds manganese. We used isothermal titration calorimetry to identify the manganese binding sites in wild-type mouse PrP. The protein showed two manganese binding sites with affinities that would bind manganese at concentrations of 63 µM and 200 µM at pH 5.5. This indicates that PrP binds manganese with similar affinity to other known manganese binding proteins. Further study indicated that the main manganese binding site is associated with H95 in the so called “5th site” normally associated with copper binding. Additionally, it was shown that occupancy by copper does not prevent manganese binding. Under these conditions, manganese binding results in an altered conformation of PrP, displacement of copper and altered redox chemistry of the metal-protein complex. Cyclic voltammetric measurements suggest complex redox chemistry involving Mn bound to PrP while Cu bound PrP is able to undergo fully reversible electron cycling. Additionally, Mn binding to PrP converts it to a form able to catalyse aggregation of metal free PrP. These results further support the notion that manganese binding could cause conformation change in PrP and trigger changes in the protein similar to those associated with prion disease.

The prion protein (PrP⁰) is a metal binding glycoprotein expressed on the plasma membrane of a variety of cell types¹. In particular, PrP⁰ is highly expressed at neuronal synapses². The function of the protein is still debated, but data has suggested a number of possible different roles including, an antioxidant, a copper transport protein and involvement in cell adhesion or cell signalling³-⁶. While the protein’s role in normal cell activity is still being defined, it is widely agreed that conversion of PrP⁰ to an abnormal isoform (PrPSc) plays a central role in the disease process of a family of disorders termed prion diseases or transmissible spongiform encephalopathies¹. These diseases include sporadic forms such as scrapie and Creutzfeldt-Jakob disease (CJD), inherited forms such as Fatal Familiar Insomnia and transmitted forms such as bovine spongiform encephalopathy and variant CJD.

The majority of data clearly points to PrP⁰ being a cuproprotein⁷-¹³. In addition, various cellular activities have been suggested resulting from the interaction of copper and PrP⁰ ³,⁴,¹⁴,¹⁵. As well as possible beneficial effects of copper binding, other data suggests that inappropriate interactions between copper and PrP result in the protein’s aggregation and increased proteinase K resistance¹⁶-¹⁷. Other studies have shown that copper can enhance the infectivity of prions and copper chelators extend the incubation period of the disease¹⁸-¹⁹. This picture is further complicated by the fact that copper can also prevent prion fibrils
and increased copper diet can delay the onset of prion disease symptoms\textsuperscript{21}. Other metals have been suggested to bind to the PrP\textsuperscript{C}. These include manganese, zinc and nickel \textsuperscript{8,11,22-25}. The binding of manganese to PrP potentially results in the conversion of the protein to an abnormal isoform with properties reminiscent of PrP\textsuperscript{Sc} \textsuperscript{8,26}. In particular, manganese bound PrP shows greater protease resistance\textsuperscript{27}, increased beta-sheet content, the ability to aggregate\textsuperscript{28} and the ability to seed polymerisation of further prion protein\textsuperscript{26}. These effects are observed on recombinant protein and protein expressed in cells\textsuperscript{29}. It has been suggested that substitution of copper with manganese could bring about changes in PrP, initiating prion disease\textsuperscript{8}. Despite geochemical evidence that high environmental manganese coincides with some clustering of prion disease cases\textsuperscript{30} and the cellular and biochemical effect of manganese on PrP, there is currently no evidence that exposure to increased levels of manganese is anything other than a potential risk factor for prion diseases.

Despite the interest in manganese binding to PrP, a thorough analysis of the interaction of the metal with the protein has not appeared. In this paper we examine the affinity of manganese PrP and demonstrate that manganese can bind to PrP even while copper is still bound and induce a conformational change. The use of cyclic voltammetry indicated that two copper redox active centres are displaced in manganese charged PrP, and that the metal-protein complex has an estimated reversible potential of +0.08 V. We have shown that the main manganese binding centre of the protein is associated with a histidine a position 95 in the mouse PrP sequence with an affinity equivalent to other known manganese binding proteins.

**Experimental Procedures**

Unless stated all reagents were purchased from Sigma.

**Protein Purification** Recombinant mouse prion proteins were prepared as previously described\textsuperscript{13}. Briefly, bacterial expression was used to generate recombinant protein. Bacteria were transformed with a plasmid (pET) containing the open reading frame of wild-type mouse PrP (amino acid residues 23-231) or mutants of this construct. Protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and inclusion bodies isolated from the bacteria with standard techniques. The inclusion bodies were solubilised in a buffer containing 8M urea. Recombinant PrP was purified using immobilised metal affinity chromatography (IMAC). The column was charged with copper and the protein bound to the column eluted with 300 mM imidazole in 8M urea. All proteins were generated tag-free. 0.5 mM EDTA was added to the protein to chelate any metals present. All subsequent steps used double deionised water treated with Chelex resin, (Sigma) to remove residual metal ions. The denatured protein was refolded by a ten fold dilution of the urea in deionised water, concentration by ultrafiltration and two rounds of dialysis to remove residual urea, imidazole and EDTA. Protein concentrations were measured using theoretical extinction coefficients at 280 nm (http://us.expasy.org/tools/protparam.html) and confirmed by BCA assay (Sigma). Protein purity was checked using polyacrylamide gel electrophoresis under denaturing conditions stained with Coomassie brilliant blue.

For some experiments recombinant proteins were charged with metal (either MnSO\textsubscript{4} or CuSO\textsubscript{4}) during the refolding process. This results in saturation of the available metal binding sites with the metal. The method was as previously described\textsuperscript{8} and the metal occupancy confirmed with mass spectroscopy. For some experiments PrP was treated with Diethylpyrocarbonate (DEPC) to block potential metal binding sites. As PrP contains seven histidine
molecules, DEPC was incubated with PrP at a ten molar excess (in 50 mM Na$_2$PO$_4$ pH 6.8) to ensure all histidine molecules were modified. Modification of histidine was monitored as increased absorbance at 245 nm. Once the reaction was complete excess DEPC was removed by dialysis.

The mutant proteins used in this study were generated as previously described$^{13,31}$. The mutant proteins used in this study include a deletion of N-terminal residues 23-89 (PrP90-231), deletion of residues 23-112 (PrP113-231), deletion of the octameric repeat region 51-89 and three mutants with alanine substituted for histidine at amino acid residues, 95, 110 or both (H95A, H110A and H95A-H110A). In addition two peptides were used for these experiments.

The first was a 32 amino acid residue peptide based on the octameric repeat sequence (residues 51-89) as previously described$^8$ termed OctaRepeat (PHGGGWGQPHGGSGWQPHGGSGWQ PHGGGWGQ) and a peptide with the sequence equivalent to amino acid residues 90-120 of the mouse PrP sequence (GGGTHNQWNKPSKPKTNLKHVAGAA AAGAVV). Both peptides were synthesised with modified N- and C-terminal residues to ensure no non-specific metal interactions with terminal groups as previously described$^{32}$.

Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) was carried out as previously described$^{13}$. All measurements were made on a Microcal VP-Isothermal Titration Calorimeter instrument. Briefly, a time course of injections of metal to PrP was made in an enclosed reaction cell maintained at a constant temperature. The instrument measured the heat generated or absorbed as a ligand-macromolecule reaction occurs. A binding isotherm was fitted to the data, expressed in terms of heat change per mole of metal against metal to PrP molar ratio. From the binding isotherm values for the reaction stoichiometry and association constants ($K_a$) were obtained.

All solutions were filtered through a 0.22 µm filter and degassed prior to use. Typically an initial injection of 2 µl metal solution was followed by a further 29 injections of 4 µl of metal solution into the protein in the sample cell stirred at 300 rpm. Injections were separated by 120 seconds to allow equilibration and sample temperature was maintained at 25 °C. All experiments were repeated at least three times. Data were analyzed with the Origin 5.0 software package from MicroCal. A baseline correction was applied to each experiment by subtraction of data from a series of injections of metal solution into a buffer blank correlating to the heat of dilution of the metal solution. After subtraction of the blank data a nonlinear least squares method was used to minimize $\chi^2$ values and obtain best fit parameters for the association constants. In all cases best-fit parameters were obtained from the sequential binding sites model, whereby the user defines the number of binding sites to be fitted in a sequential manner.

Circular Dichroism Spectroscopy

For analysis of protein samples by Circular Dichroism Spectroscopy (CD) the samples were diluted to 0.1 mg/ml. The protein concentration was determined for the samples by first assessing the concentration of a metal free PrP sample using a scan of the UV spectra with a Carey Spectrophotometer (Varian) using an extinction co-efficient of 63495 M$^{-1}$ cm$^{-1}$. As metals can alter the UV absorbance of protein, a Bradford assay was used to assure that the metal binding PrP samples were at the same concentration as apo-PrP. CD spectra for apo-PrP and metal charged PrP were obtained using a Applied Photophysics Chirascan spectropolarimeter as previously described$^{31}$. Protein concentration and pathlength were used to equate the measured spectra and plotted in the form of molar ellipticity between 185-260 nm.
Cyclic Voltammetry. Voltammetric measurements were conducted with a µ-Autolab III potentiostat system (Eco Chemie, The Netherlands) in a conventional three-electrode electrochemical cell. Experiments were performed in staircase voltammetry mode with platinum gauze counter and saturated calomel reference electrode (SCE, REF401, Radiometer). The working electrodes used were 5 mm diameter edge plane pyrolytic graphite (Pyrocarbon, Le Carbon, UK) or 3 mm diameter boron-doped diamond (Windsor Scientific, UK). Electrodes were polished on fresh cloths (Buehler, UK) with alumina (1 micron, Buehler, UK) as polishing aid. After the final polish on a clean cloth, electrodes were rinsed with demineralised water. Aqueous solutions were thoroughly de-aerated with nitrogen (BOC, UK) prior to recording data. All measurements were undertaken at 25 +/- 2 °C. For voltammetric measurements an aqueous buffer solution (5 mM Mes at pH 7) was thoroughly de-aerated with nitrogen. The working electrode was polished and the background current recorded in the absence of protein. Next, the working electrode was immersed into the protein solution (containing 20 µM WT-PrP in MilliQ filtered water pH 7) and after 60 seconds removed and rinsed with MilliQ filtered water pH 7. The resulting protein-modified electrode was re-immersed into the pure buffer solution in the measurement cell and cyclic voltammograms were recorded. Protein adhesion to the edge plane pyrolytic graphite and to the boron-doped diamond electrode surfaces was excellent and stable signals were obtained for many potential cycles. Redox potentials were also determined with reference to the saturated calomel electrode (SCE, reference electrode).

Aggregation Studies. Recombinant PrP was generated as described and either refolded to bind manganese or refolded to bind copper. Some copper refolded protein was exposed to manganese as described for ITC experiments. The basis for the experiment was the ability of the metal charged protein to cause aggregation of monomeric, metal free PrP as determined by a change in absorbance at 325 nm. Metal free WT PrP was prepared at 1 mg/ml in 1 mM MES pH 6.5. 1 ml of this solution was placed into a cuvette and 0.1 mg/ml of seeding PrP (with metals) was added in 50 microlitres of 1 mM MES pH6.5). All measurements were performed using a Cary 100Bio UV-Visible spectrophotometer (Varian) at 325 nm using a quartz cuvette of 5 mm path length. Polymerisation was observed as an increase in solution turbidity. The resultant scattering of UV light at 325 nm resulted in an increased absorbance measurement. Measurements were taken at 1-10 minute intervals until no further change in absorbance was measured in all samples.

RESULTS

Manganese Affinity for PrP and pH dependence. We used isothermal titration calorimetry (ITC) to study Mn binding to mouse PrP. Wild-type mouse PrP (WT-PrP) was generated by expression in bacteria and isolated using an IMAC technique. This protein consisted of the full length mouse PrP lacking the signal and GPI signal peptides (PrP23-231). The purified protein was tag free and dialysed after treatment with EDTA to remove any trace metals contamination. Manganese was titrated into PrP at a variety of pHs to determine the affinity within the pH range of 4.5-8. ITC experiments showed strong isotherms for the binding of manganese (Figure 1). Using a data fitting program, two sequential bindings sites were determined and the affinity for each site calculated. Two sites were identified at all pHs tested. Figure 1 shows that the highest affinity of Mn for WT-PrP was observed at pH5.5. At this pH the calculated dissociation constants for the two sites would be 63 µM and 200 µM. These values lie with the expected range for manganese affinity. At pH7.5 the dissociation constant values rise to 630 µM and ~10 mM. At this pH it is unlikely that
the second site would be occupied. Further experiments were carried out at pH 5.5. Previous studies have suggested that manganese binds to PrP in association with histidine residues. Proteins can be specifically modified by reacting DEPC with the imidazole groups of histidines, blocking the ability to bind metals. In order to assess whether histidines were important to manganese binding, PrP was modified with DEPC and ITC experiments repeated at pH 5.5. As shown in figure 1B, no manganese binding to PrP was observed. This suggests that histidine residues play a central role in manganese binding to PrP and further support the notion that manganese is a ligand for PrP.

Identification of Mn binding sites. In order to assess the location of the two manganese binding sites, mutant forms of PrP were also used in ITC experiments. Table 1 shows the results of the ITC experiments and includes the affinity values calculated. Only one of the mutants showed no binding of manganese. The mutant PrP113-231 lacks the N-terminus of the protein. This indicates that both sites lie within the N-terminus. Deletion of the N-terminus up until residue 90 (ie PrP90-231) resulted in the loss of the low affinity binding site suggesting that the high affinity site lies between residues 90 and 112. Analysis of a mutant lacking only the octameric repeat region confirmed that the low affinity binding site lies within the octameric repeat. In order to confirm this we used two synthetic peptides corresponding to these two regions. The peptide 90-120 bound manganese with high affinity, an order of magnitude higher than for the wild type protein PrP23-231. In comparison the affinity for the octarepeat peptide was very low, confirming work from previous studies.

As studies with DEPC suggested that manganese binds to histidine in the protein and two histidines form the so-called “5th site” for copper binding, we examined mutants lacking either or both of these histidines. The double mutant with alanine substituted at H95 and H110 lacked the high affinity site. This confirms that this site is the high affinity manganese binding site in PrP. Substitution of H95 alone resulted in loss of the high affinity site while substitution of H110 did not (Table 1). This implies that H95 is central to the high affinity manganese binding site of PrP.

Metal Binding to Manganese Saturated PrP
In order to assess whether copper and manganese bind to equivalent sites in the protein we carried out experiments with manganese saturated PrP. Following purification of PrP in the presence of 8 M urea, the protein was refolded to its native conformation in the presence of manganese. This results in occupation of all available manganese binding sites. Protein purified in this way was subjected to ITC experiments with manganese. Manganese binding to manganese saturated PrP resulted in one binding event (Figure 2) with very low affinity (Table 2). Under the same conditions manganese saturated H95A-H110A also showed binding at one site with same affinity. Therefore this low affinity binding site (if physiologically relevant) is not at the 5th site but was associated with the octameric repeats.

We also assessed copper binding to manganese saturated PrP with ITC. Studies of copper binding to PrP at four sites at pH 5.5 indicate 4 binding sites with a range of affinities as shown in Table 2. However, when saturated with manganese, only two sites could be detected with ITC. This implies that manganese bound to PrP blocked copper binding at two sites at pH 5.5. The H95A mutant showed the same number of sites as the wild-type protein, but the H110A and H95A-H110A mutant showed the loss of the high affinity copper binding site. This implies that in manganese saturated PrP copper can bind at H110 and somewhere in the octameric repeat region. Despite this result the PrP90-231 mutant showed no binding of copper or manganese when first loaded with manganese during refolding. This would suggest that metal
binding at the 5th site is different in the absence of the amino acid residues 23-89 which include the octameric repeat region.

**Manganese Binding to Copper Saturated PrP** It has been suggested previously that when manganese binds in place of copper, it can alter the conformation of PrP. However, it has not been determined whether manganese can bind to PrP when it already binds copper. Manganese was titrated into PrP refolded in the presence of copper. The copper sites in PrP were filled with copper, but ITC experiments suggest that WT-PrP was still able to bind two atoms of manganese (Figure 3, Table 2). This result suggests that copper binding to PrP does not prevent manganese binding. In comparison the H95A mutant lacked the high affinity manganese site while H110A did not. This implies that for copper saturated PrP, manganese is able to bind at the fifth site via H95. Interestingly, PrP90-231 saturated with copper showed no binding of manganese. This suggests that in the absence of the N-terminus, co-ordination of metal binding to the 5th site is altered.

We used CD spectroscopy to look at changes in the secondary structure of PrP on binding of manganese to copper saturated PrP. Figure 4 shows typical CD spectra from WT-PrP. Freshly prepared copper and manganese saturated WT-PrP produced similar CD spectra characterised by a deep minimum between 210 and 225 nm indicative of alpha helical content. In contrast WT-PrP saturated with copper, with subsequent manganese binding resulted in an altered spectrum with reduced helical content. This suggests that manganese binding to PrP results in an altered protein conformation.

**Electrochemical Analysis if Manganese bound to PrP** Voltammetric experiments were conducted in aqueous solution containing 5 mM Mes buffer at pH 7. We have previously shown that PrP adsorbs onto edge plane pyrolytic graphite or boron-doped diamond electrode and results in immediately obvious and highly reproducible reversible current responses. Experiments could not be conducted at pH5.5 as the copper centres would not remain strongly bound to the protein at this pH. Due to considerably lower capacitive back ground current using a boron-doped diamond electrode, this form of electrode was chosen for further examination. WT-PrP that had been saturated with either copper or manganese were examined as well as copper refolded protein that had been exposed to manganese or manganese refolded protein that had been exposed to copper. There was no obvious difference between the latter two conditions so only one trace is shown. Figure 5 shows the voltammograms obtained with a scan rate of 1 mVs⁻¹. Figure 5(ii) shows the back ground as a dotted line and a clear difference can be observed that can be attributed to reduction and oxidation by the manganese or copper centres. The midpoint potential for the copper and manganese saturated proteins were also determined. This is defined by equation 1.

**Equation 1.**

\[ E_{\text{mid}} = \frac{E_{\text{pa}} + E_{\text{pc}}}{2} \]

The redox potential for PrP binding copper was -0.01 V versus SCE, while the redox potential for PrP binding Mn was +0.08 V vs SCE.

The peak current ratio on protein containing copper only was 1, suggesting a chemically fully reversible reaction for this condition. As the start current and voltage was approximately 0, this reaction represents the oxidation of Cu⁺ to Cu²⁺ and its subsequent reduction back to Cu⁺. Where protein with only manganese was tested, the oxidation peak was much larger than the corresponding reduction, suggesting a partially irreversible oxidation of manganese. Subsequent cycles demonstrated an oxidation of equal intensity along with a lesser reduction equivalent to the initial cycle. This is likely to represent an oxidation...
of manganese from Mn$^{2+}$ to Mn$^{3+}$ and Mn$^{4+}$. In addition, the effect of electron transfer kinetics are apparent by the wide separation between peaks. Protein that had been exposed to both manganese and copper showed a significantly smaller oxidation/reduction corresponding to the copper centres, suggesting that manganese had displaced some of the bound copper and thus reduced the copper redox signal.

The copper bound protein contains 5 copper centres, 4 localised relatively closely together in the octa-repeat unit and 1 within the 5th site. The oxidation signal produced for the wild-type copper refolded protein corresponded to an integrated peak charge of 270nC. For the copper/manganese refolded protein, this was reduced to 110nC for the copper signal and 90nC for the manganese signal. The manganese only protein showed an integrated peak charge of 125nC. Experiments with pure mPrP protein in the absence of copper did not show this signal. As this charge is directly related to the amount of metal available for electron transfer on the protein, it would appear that three atoms of copper are displaced by manganese in the copper/manganese binding protein.

**Seeded Aggregation.** One of the characteristics of PrP$^{Sc}$ is the ability of the protein to catalyse aggregation of further PrP. In this regards “seeds” of PrP can be used to nucleate further aggregation. We have shown previously that recombinant PrP binding manganese can act as a seed to catalyse aggregation of metal free recombinant PrP$^{Sc}$. As we have shown that Mn can bind to copper saturated WT PrP and alter its conformation we wished to determine Mn binding PrP generated this way can also cause aggregation. Mn saturated, and copper saturated PrP were prepared and dialysed free of unbound metals. The seeding material was added to fresh prepared, metal free PrP As shown in figure 6, Mn saturated PrP was able to stimulate aggregation as indicated by increase absorbance in a turbidity assay. Cu saturated PrP was not able to stimulate aggregation. Cu saturated PrP was then titrated with MnSO$_4$ as for ITC experiments. Excess manganese was removed by dialysis. Mn binding PrP generated this way was able to stimulate PrP aggregation. This result suggests that binding of Mn can convert PrP to an aggregation promoting protein even when Cu is previously bound to the protein.

**Discussion**

The prion protein is now well recognised as a copper binding protein. It has also been suggested to bind manganese. Previous studies have shown that PrP isolated from brains or from cultured cells has manganese bound. Until this report there has been no estimate of the affinity of free manganese for full length PrP. Our results show that mouse PrP binds Mn at two sites. The two sites are detectible across a range of pHs. The optimum pH for manganese binding was pH5.5. This suggests that Mn bind to PrP in a more acid compartment of the cell such as endosomes. At this pH both sites would be occupied when the protein would be exposed to micromolar concentrations of Mn. Unlike other metals such as copper, affinity values for Mn binding to know Mn binding proteins are quite low and in the micromolar range. Divalent metal transporter-1 (DMT-1) is the only known transporter for Mn$^{2+}$. The affinity of Mn for this protein has not been determined but kinetic studies of Mn uptake suggest that its $K_m$ is 3 mM, suggesting a similarly low affinity. Therefore our values for the affinity of Mn and PrP are in the range expected for a manganese binding protein. However, the concentration of manganese in mammalian brain is lower than this value, in the nanomolar range. Based on this value it would be assumed that PrP would not bind manganese in vivo. If this was true then very few of the known manganese binding proteins would bind manganese as they have similar affinities for
the metal. Therefore, it is likely that manganese binding to proteins is regulated in a manner that is currently not understood. This metal has no known specific transport protein in the blood and has been suggested to enter cells through DMT-1, a protein known to transport a variety of divalent cations across the plasma membrane. Given the deficits in understanding of manganese metabolism, it is likely the understanding of how manganese binding proteins are able to associate manganese will have to wait until more has been discovered of this system.

Previous assessment of Mn affinity for a recombinant PrP was for human 91-231. In this case a dissociation constant 202 µM was determined. We also determined the affinity for a similar protein, mouse PrP 90-231. However, affinity we measured for this protein suggests a dissociation constant of 63 µM. This affinity was equivalent to the high affinity site we measured in the full length protein. Our previous work suggested that full length PrP bound four atoms of manganese per molecule. These new findings suggest that only two atoms bind per molecule. The difference between the two sets of data relates to the methods used to associate manganese with PrP. In the former experiments the metal was incorporated during refolding and appeared to associate with the octameric repeat region. In the latter findings metal was incorporated after PrP was refolded. The current study used a variety of mutants and peptides to determine the metal binding sites and also the amount of metal bound was confirmed with cyclic voltammetry. Therefore we feel that this data is a truer reflection of metal binding as it would occur in vivo.

Studies with recombinant PrP have shown previously that binding of manganese results in a gradual change in protein conformation. Initially, PrP binding Mn shows the same conformation of PrP binding copper (see Figure 4), but over time showed increased beta-sheet content and proteinase K resistance. We have shown here that this change in structure can occur even when PrP still binds copper, implying that substitution of copper with manganese is not necessary. The change in structure of PrP on binding Mn has been recently verified using optical Raman spectroscopy. Other studies have shown protein conversion in mammalian cells and yeast cells when high levels of manganese are applied exogenously. Protein conversion has also been observed in protein extracts from brains when manganese is added.

Studies of manganese binding to peptides based on the sequence of PrP have produced variable results. In particular some studies suggest that Mn binds to peptides related to the octameric repeat region and some suggest it does not. Regardless of this, any measured affinity at this site was quite low. Our findings confirm this as the octameric peptide showed binding of only one atom per molecule. This low affinity site is present in measurements with full length protein. Manganese remains bound at the low affinity site despite extensive dialysis. Our CV data also confirm that PrP binds two atoms, but also show irreversible oxidation of the manganese centres on the protein. This would suggest a change in the valence state of the metal. In this case there could be a change in the dissociation constant for bound manganese explaining why it doesn’t simply fall off the protein after extensive dialysis.

Research using peptides covering or overlapping the region 90-120 have been more useful in confirming manganese binding to PrP. 1H NMR studies with a peptide related to amino acid residues 106-126 of the human PrP sequences suggested co-ordination of Mn by Gly-126, Leu-125, Gly-124, and His-111 of this sequence. Our studies with the 90-120 also showed manganese binding with a similar affinity to that reported for the 106-126 fragment. However, our studies suggested that mutating the histidine of this sequence (H-110 in mouse) had little effect on Mn binding and that His-95 played a much greater role in co-ordinating Mn binding. We
also observed strong binding to a peptide 90-120, but only at one site. The differences between these results cannot be attributed pH as the experiments of Gaggelli et al. were performed at pH 5.7 close to that used on our experiment (pH 5.5). Therefore, as the 106-126 peptide lacks H-95 it is possible that the full length protein, Mn is inhibited from binding at H-110 due to structural constraints or electrostatic properties of the surround amino acid residues.

Our studies with the peptide 90-120 showed a higher affinity than for the full length wild-type protein. One possible reason for this is the greater potential flexibility of the shorter peptides allows the formation of stronger bonds or abrogates repulsive forces present in the full length protein.

It is interesting to note that copper and manganese have bind to different histidines within the 5th site in the full length protein. Manganese binds at H95 while copper binds at H110. When studying the truncated mutant, PrP90-231, this preference is probably lost as occupancy of the 5th site by one metal excludes binding of the other at this site. This implies a change in co-ordination of both metals within the 5th site in the truncated protein. In this alternative binding mode manganese could either bind to both histidines or associate with the protein as suggested by Gaggelli et al.

The affinity of copper PrP is much higher than for manganese. We previously suggested that manganese could occupy copper binding sites and compete equivalently for these sites. The current research suggests that manganese will occupy different sites to copper (as determined by ITC), but can cause the displacement of copper (from CV data). This opens the possibility that binding of manganese to PrP can occur under any conditions and does not require the availability of copper depleted protein. There is strong evidence that PrP can be isolated with manganese bound from both brain and also from cell exposed to high non-toxic levels of manganese. The consequence of such interactions is a change in conformation of the protein as observed here and reported previously. Initially, manganese binding does not result in an altered conformation, but over time, the protein is more susceptible to oxidative damage than metal free or copper binding protein which then possible results in changes in the protein’s structure. The conformation change in Cu charged PrP on exposure to manganese did not show this time dependence. This suggests a more specific effect which may include the copper bound to the protein as well. Interactions between PrP and copper under certain conditions have also been shown to cause changes in PrP such as increased PK resistance.

The importance of manganese to research into prion disease touches many aspects of the field. Most importantly, Mn is bound to PrP isolated from the brains of patients with CJD. PrP with manganese bound is able to initiate seeded polymerisation of metal free prion protein. This ability is retained by the protein even when the metal is extracted from the protein. The current manuscript has expanded on these findings by showing that PrP with manganese bound is able to seed PrP polymerisation even if the protein already has copper bound. This suggests that in vivo binding of manganese to PrP does not require any alteration in its copper binding capacity. In the presence or hydrogen peroxide, binding of manganese or copper at the 5th metal binding site results in cleavage of the protein within the octameric repeat domain. PrP with manganese bound also has other qualities of the PrP Sc. It becomes protease resistant and it is neurotoxic. In summary, we have identified the manganese binding sites in the prion protein and shown that the high affinity site is associated with His-95 in the so called 5th metal binding site. We have show that the affinity for this site is high for a manganese binding protein and clearly implies that PrP could bind manganese in vivo. We also show...
that manganese bound to PrP becomes oxidised and is able to displace copper that may already be bound to the protein. As manganese binding to PrP is detrimental and causes a conformation change in the protein, its ability to bind to PrP when copper is still bound suggests that manganese binding could potential play a role in prion disease progression in vivo.
REFERENCES


Footnotes

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Figure Legends
**Figure 1** ITC analysis of Mn binding to PrP  
A WT-PrP was placed in the ITC calorimeter and MnSO₄ titrated into the solution at pH 5.5. Shown is a typical trace indicating the raw energy change per injection (upper panel) and the net energy change for the titrations in relation to the moles of Mn injected. Mn binding produce a strong isotherm as indicated by the curve.  
B WT PrP was modified with DEPC. Experiments were repeated with the same conditions as for A. In this case there was little evidence for metal binding and no isotherm was obtained.  
C ITC experiments with unmodified WT PrP were carried out at a variety of pHs to determine the pH sensitivity of the Mn binding sites. Experiments were repeated three times. The highest affinity values were seen at pH 5.5. As the values are on a log scale, no error is shown. However standard error values were less than 5% of the mean values. ■ = high affinity site, △ = low affinity site.

**Figure 2** Metal binding to Mn saturated WT PrP  
Examples of ITC isotherms from different experiments.  
A MnSO₄ was titrated into WT-PrP refolded in the presence of Mn. The resulting isotherm indicated one Mn binding site.  
B CuSO₄ was titrated into Mn saturated WT-PrP. The isotherm indicated two Cu binding sites.  
C CuSO₄ was titrated into Mn saturated PrP-H95A. Two Cu binding sites.  
D CuSO₄ was titrated into Mn saturated PrP-H110A. One low affinity Cu site.

**Figure 3** Mn binding to Cu saturated Mutant PrP  
Examples of ITC isotherms from different experiments.  
A MnSO₄ was titrated into WT-PrP refolded in the presence of Cu. The resulting isotherm indicated two Mn binding sites.  
B MnSO₄ was titrated into Cu saturated PrP90-231. No isotherm produced and no Mn binding sites indicated.  
C MnSO₄ was titrated into Cu saturated PrP-H95A. One low affinity Mn binding site.  
D MnSO₄ was titrated into Cu saturated PrP-H110A. Two Mn binding sites.

**Figure 4** CD Analysis  
Analysis of WT-PrP prepared with different metal content using CD. Samples are taken from ITC experiments where metals were titrated into the protein. The samples were dialysed before analysis. Spectra are shown for copper saturated (thick line) and manganese saturated (thin line) protein. In comparison WT PrP refolded to bind copper and then exposed to manganese in the ITC experiment showed a different spectrum (medium line).

**Figure 5** CV Analysis  
Cyclic voltammogram comparing wildtype PrP binding either (I) Copper, (II) Copper and manganese or (III) manganese. The protein was adsorbed onto a 3 mm boron doped diamond electrode by incubation in 20 µM protein solution pH 7 for 60 seconds. The scan rate used was 1 mV/s in an oxygen excluded buffer of 5 mM Mes, pH 7 25°C. The dashed line on scan (II) represents the background signal where protein without metal is adsorbed to the electrode.

**Figure 6** Seeded Aggregation  
Fresh, metal-free WT-PrP at 1 mg/ml was placed in a cuvette in a spectrophotometer. PrP seeds were added to the solution to a final concentration of 0.1 mg/ml. Change in absorbance
was then assessed over 60 min at 325 nm. Seed samples were Mn saturated PrP (squares), Cu saturated PrP (circles) or Cu saturated PrP exposed to Mn (triangles). An increase in absorbance is indicative of aggregation. The data shown is an example experiments that were repeated four times.
Table 1  Affinity of Mn for PrP mutants

<table>
<thead>
<tr>
<th>PrP Mutant</th>
<th>Site 1</th>
<th>Site 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT PrP</td>
<td>4.2</td>
<td>3.7</td>
</tr>
<tr>
<td>PrP90-231</td>
<td>4.2</td>
<td>n.d.</td>
</tr>
<tr>
<td>90-120</td>
<td>5.3</td>
<td>n.d.</td>
</tr>
<tr>
<td>PrPΔ51-89</td>
<td>4.2</td>
<td>n.d.</td>
</tr>
<tr>
<td>PrP113-231</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>H95A</td>
<td>n.d.</td>
<td>3.9</td>
</tr>
<tr>
<td>H110A</td>
<td>4.2</td>
<td>3.6</td>
</tr>
<tr>
<td>H95A-H110A</td>
<td>n.d.</td>
<td>3.6</td>
</tr>
<tr>
<td>OctaPeptide</td>
<td>n.d.</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Shown are the log of the affinity values (M⁻¹) determined from ITC experiments in which MnSO₄ was titrated into a solution PrP protein at pH5.5 buffered with 5 mM MES. n.d. = not detected. Site 1 is the high affinity site associated with the “5th Site” while the 2nd Site is that associated with the octameric repeat region. Experiments were repeated three times.
Table 2: Affinity of Copper and Manganese for PrP when metal bindings its are occupied.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Copper</th>
<th>Manganese</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo- WT PrP</td>
<td>10.5, 9.5, 8.2, 7.4</td>
<td>See Table 1</td>
</tr>
</tbody>
</table>

**Cu Saturated**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Copper</th>
<th>Manganese</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT PrP</td>
<td>-</td>
<td>4.2, 2.3</td>
</tr>
<tr>
<td>PrP90-231</td>
<td>-</td>
<td>n.d.</td>
</tr>
<tr>
<td>H95A</td>
<td>-</td>
<td>2.2</td>
</tr>
<tr>
<td>H110A</td>
<td>-</td>
<td>4.2, 2.1</td>
</tr>
</tbody>
</table>

**Mn Saturated**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Copper</th>
<th>Manganese</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT PrP</td>
<td>7.5, 6.3</td>
<td>2.2</td>
</tr>
<tr>
<td>PrP90-231</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>H95A</td>
<td>7.5, 6.4</td>
<td>-</td>
</tr>
<tr>
<td>H110A</td>
<td>6.4</td>
<td>-</td>
</tr>
<tr>
<td>H95A-H110A</td>
<td>6.2</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Shown are the log of the affinity values (M⁻¹) for each site detected as determined from ITC experiments in which MnSO₄ or CuSO₄ (glycine chelate) was titrated into a solution PrP protein at pH5.5 buffered with 5 mM MES. Proteins used were either refolded in the presence of MnSO₄ (Mn saturated) CuSO₄ (Cu saturated) or no metal (apo WT PrP). n.d. = not detected. – indicates no experiment performed.
Brazier et al.
Figure 2
Figure 3

A, B, C, D: Graphs showing changes in microcalories per second and molar ratio with time (min) for different conditions.

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Figure 4
Figure 5
Brazier et al.
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Figure 6
Manganese binding to the prion protein
Marcus W. Brazier, Paul Davies, Esmie Player, Frank Marken, John H. Viles and David R. Brown

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