

Consequences of manganese replacement of copper for prion protein function and proteinase resistance

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The prion protein (PrP) binds copper and has anti-oxidant activity enhancing the survival of neurones in culture. The ability of the PrP to bind other cations was tested and it was found that only manganese could substitute for copper. Although initially manganese-loaded PrP exhibited similar structure and activity to copper-loaded PrP, after aging, manganese-loaded PrP became proteinase resistant and lost function. It was also found that manganese could be incorporated into PrP expressed by astrocytes and that this PrP was partially proteinase resistant. These results show that it is possible to generate proteinase-resistant PrP from cells and suggest a possible mechanism for the formation of the scrapie isoform of the PrP as generated in sporadic prion disease.

Keywords: copper/manganese/oxidative stress/prion/proteinase K

Introduction

The majority of cases of naturally occurring prion diseases (transmissible spongiform encephalopathies) arise sporadically with no known cause (Prusiner, 1991). These include the majority of human cases of Creutzfeldt–Jakob disease (CJD), scrapie of sheep, chronic wasting disease of deer and others. Other human diseases are associated with point mutations in the prion protein (PrP) gene (Hsiao and Prusiner, 1990; Prusiner and Hsiao, 1994; Ghetti *et al.*, 1996). Bovine spongiform encephalopathy may have been spread among cattle by the feeding of infected offal but the original disease may also have arisen sporadically.

Prion diseases are characterized by the conversion of the normal cellular form of the prion protein (PrP^c) to an altered isoform (PrP^{Sc}). Theoretically, transmission of disease can be induced by introduction of PrP^{Sc} into the brain, which catalyses conversion of the host's normal PrP^c to the abnormal isoform (Cohen and Prusiner, 1998). It is characteristic of all prion diseases that PrP^{Sc} accumulates in the central nervous system in infected individuals and it probably has a causal role in inducing neurodegeneration and gliosis, the pathological hallmarks of these diseases (DeArmond *et al.*, 1992).

PrP^{Sc} unlike PrP^c is resistant to digestion with proteinase K (PK) and aggregates to form fibrils (Bolton *et al.*, 1982; Prusiner, 1982). It is high in β -sheet content. *In vitro* fibril formation from PrP^c using PrP^{Sc} has been demonstrated (Kocisko *et al.*, 1994; Bessen *et al.*, 1997). However, the mechanism by which PrP^{Sc} is formed *in vivo* from PrP^c is unknown. The seeding theory (Cohen and Prusiner, 1998) is one popular theory, which suggests that a number of 'seeds' of PrP^{Sc} are formed slowly from host protein. Once such nuclei of PrP^{Sc} are formed large amounts of PrP^{Sc} can form more rapidly. There is some evidence to suggest that caveolae-like domains in cell membranes are the possible point in which PrP^{Sc} and PrP^c may interact (Vey *et al.*, 1996). There is also a strong argument that another protein, 'factor X', may be necessary for the formation of PrP^{Sc} from PrP^c (Kaneko *et al.*, 1997).

The cellular isoform of the PrP is a 30–35 kDa glycoprotein that is anchored to the cell membrane via a GPI anchor. PrP^c binds copper (Brown *et al.*, 1997a, 1999; Stöckel *et al.*, 1998) and its expression appears to aid resistance to oxidative stress and copper toxicity at least *in vitro* (Brown *et al.*, 1996a, 1997b, 1998). PrP^c has been shown to influence copper uptake into neurones (Brown, 1999a). Copper taken up in association with PrP^c can be utilized at the synapse (Brown *et al.*, 1997a; Brown, 1999a) or can be incorporated in Cu/Zn superoxide dismutase (Brown and Besinger, 1998). More importantly, PrP^c with copper bound to it can act as a superoxide dismutase (Brown *et al.*, 1999), possibly explaining why cells deficient in PrP^c expression are sensitive to stress from cell culturing conditions (Brown *et al.*, 1997b; Kuwahara *et al.*, 1999).

Although it has been established that PrP^c is a copper-binding protein it is unknown whether the protein can bind other divalent cations. In this report we found that recombinant PrP^c will bind manganese and nickel. Of these, manganese appeared to alter PrP^c to a proteinase-resistant form that forms fibrils. Furthermore, PrP^c expression influences uptake of manganese into cells. Therefore, we speculate that incorporation of manganese into PrP^c may be one way in which PrP^{Sc} can be formed *in vivo*.

Results

Binding of copper to PrP

We have shown previously that full-length PrP binds up to four copper atoms to the octameric repeat region (Brown *et al.*, 1999). PrP refolded with copper was demonstrated to have an antioxidant activity like that of superoxide dismutase. It has been suggested from studies with peptides based on the octameric repeat region of PrP (Viles *et al.*, 1999) that the four histidines in the repeat region coordinate four copper atoms, each one associated with two histidines. Alternatively, each histidine could bind one copper atom

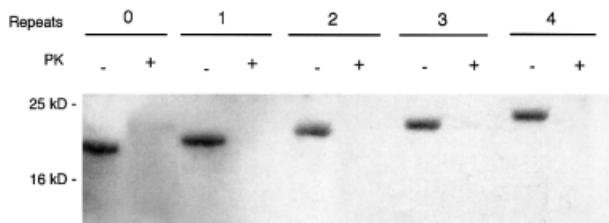


Fig. 1. Western blot of mutant PrPs. Protein from different deletion mutants of recombinant mouse PrP following refolding with copper was electrophoresed and blotted using the Western technique. Parallel samples were treated with 2 $\mu\text{g}/\text{ml}$ proteinase K (PK+). PrP was detected using a polyclonal antibody specific for PrP (see Materials and methods). Wild-type PrP (four octameric repeats) showed a band at 25 kDa. The deletion mutants, with three, two, one and no (0) octameric repeats showed bands of decreasing size.

Table I. Copper binding to recombinant PrP with different numbers of octameric repeats

No. of repeats	$\mu\text{g Cu}/\text{mg protein}$	Atoms/molecule
0	0.2 ± 0.2	0.1 ± 0.1
1	2.6 ± 0.4	1.0 ± 0.2
2	6.2 ± 0.6	2.4 ± 0.3
3	8.3 ± 0.5	3.2 ± 0.2
4	11.2 ± 0.7	4.4 ± 0.3

Values of $\mu\text{g metal ion}/\text{mg}$ of protein were obtained from TXRF analysis of different PrP proteins with 0–4 octameric repeats. Atoms/molecule indicates the molar ratio of copper atoms to PrP molecule. No. of repeats indicates the number of octameric repeats in the PrP molecule studies. All PrP proteins were refolded with CuCl_2 . Values were determined for a minimum of six measurements; the mean and SEM are shown.

each. To test this possibility we prepared mutants of mouse PrP with none, one, two or three repeats. The mutants and full-length PrP were expressed in bacteria, extracted from inclusion bodies and refolded in urea in the presence of 5 mM CuSO_4 . The resulting proteins could be detected following Western blotting with a specific PrP antibody and were PK sensitive (Figure 1). The copper not bound to PrP was removed by dialysis. The histidine tag was removed as previously described (Brown *et al.*, 1999). The resulting protein was analysed by total X-ray reflection fluorescence (TXRF) spectroscopy (Table I). The mutant PrP with no octameric repeats bound a residual level of copper. In comparison, the other mutants bound the equivalent of one, two or three atoms of copper depending on how many octameric repeats were left in the protein. These results suggest that the four histidines in the repeat region can bind one copper atom each.

The activity of the different octameric repeat mutants was assessed using an assay for superoxide dismutase based on the production of oxygen radicals by xanthine oxidase and the formation of formazan from nitro-blue tetrazolium (NBT).

The mutants containing one or no octameric repeats showed no activity in the assay (Figure 2). The minimal requirement for superoxide dismutase activity was two octameric repeats. A mutant with three octameric repeats binding three copper atoms had no more activity than (not significant by Student's *t*-test, $p > 0.05$) a mutant with two. The activity of wild-type PrP (binding four atoms of copper to the octameric repeats) was the highest, and was

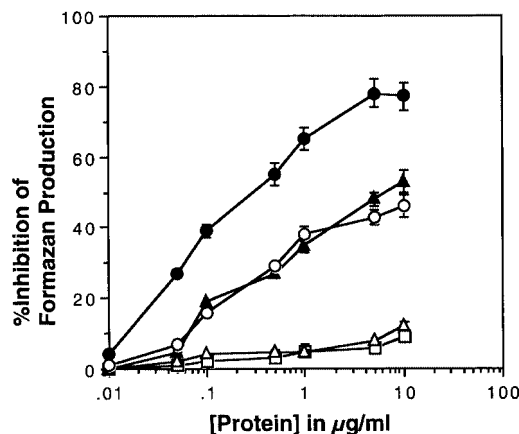


Fig. 2. Activity of octameric repeat mutants of PrP. The superoxide dismutase activity of PrP refolded with copper was assessed using a standard NBT/xanthine oxidase-based assay. Increasing concentrations of PrP with four (●), three (○), two (▲), one (△) and no (□) octameric repeats were added to individual assays. Values indicate the ability of the added protein to inhibit the formation of formazan. Inhibition of formazan in the reaction is indicative of superoxide dismutase activity. Shown are the mean and SEM for four assays.

significantly greater than that of PrP with only two or three repeats (Figure 2).

Refolding of PrP with other divalent cations

Recombinant full-length and deleted PrP was refolded as described with other divalent cations including calcium, magnesium, manganese, nickel, iron and zinc at concentrations of 5 mM. Following refolding and dialysis to remove excess cations the protein was examined with TXRF analysis to determine which cations were retained. Values shown in Table II indicate the number of atoms per molecule of PrP. The deletion mutant did not retain any of the divalent cations, indicating that any retention by the full-length protein was a result of binding to the octarepeat region. Of the cations tested only nickel, zinc and manganese were retained by PrP. Of these, zinc was poorly retained. In order to determine whether manganese and nickel could replace copper when in competition, full-length PrP was refolded with either nickel and copper or manganese and copper in equimolar concentrations (5 mM). In these cases (Table II) copper was preferentially bound in place of nickel but manganese was bound equivalently with copper (i.e. two atoms each per molecule). When the concentration of manganese was decreased during refolding (5 mM copper, 0.5 mM manganese) the amount of manganese binding was reduced to one-tenth that of copper. These results suggest that manganese can substitute equivalently for copper in the holo-form of PrP.

Analysis of activity of manganese- and nickel-loaded PrP

Given that only manganese and nickel substituted for copper in refolded PrP, samples refolded only with these cations were used for assaying superoxide dismutase activity of these proteins. As indicated above, copper-loaded PrP has substantial activity in this assay. PrP loaded with nickel showed no activity in the same assay (Figure 3A). However, manganese-loaded PrP showed some activity, plateauing at ~50% of that seen for the

Table II. Binding of divalent cations to full-length recombinant PrP

Refolding cation	Atoms/molecule						
	Cu	Mn	Zn	Mg	Ni	Ca	Fe
Copper	4.4 ± 0.3	nd	nd	nd	nd	nd	nd
Manganese	nd	4.2 ± 0.4	nd	nd	nd	nd	nd
1:1 copper:manganese	2.1 ± 0.1	2.2 ± 0.2	nd	nd	nd	nd	nd
1:10 copper:manganese	0.5 ± 0.2	3.9 ± 0.3	nd	nd	nd	nd	nd
Nickel	nd	nd	nd	nd	4.6 ± 0.3	nd	nd
1:1 copper:nickel	3.4 ± 0.4	nd	nd	nd	0.6 ± 0.4	nd	nd
Zinc	nd	nd	1.8 ± 0.2	nd	nd	nd	nd
Magnesium	nd	nd	nd	0.3 ± 0.2	nd	nd	nd
Iron	nd	nd	nd	nd	nd	nd	0.8 ± 0.4
Calcium	nd	nd	nd	nd	nd	0.03 ± 0.03	nd

The cation content of wild-type recombinant PrP determined after refolding with different cations (refolding cation) was determined using TXRF analysis. The values are expressed as the number of atoms of the cation bound to each molecule of PrP (atoms/molecule). nd indicates that levels were below detection with this method. Values were determined for a minimum of six measurements; the mean and SEM are shown.

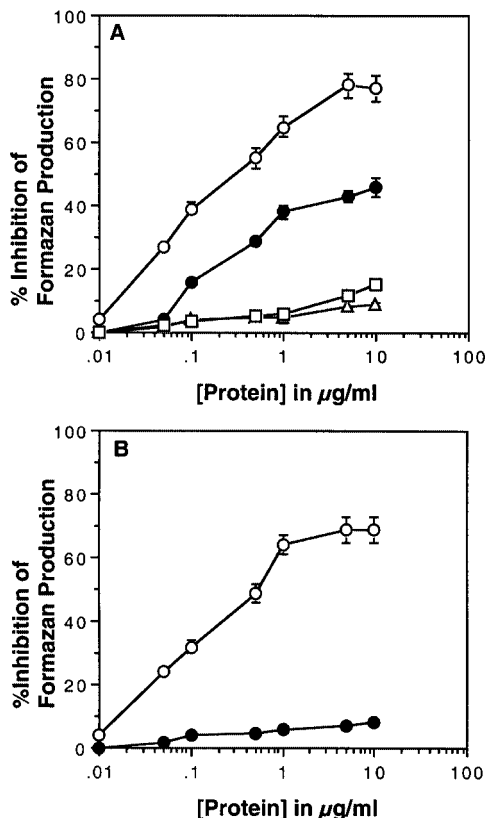


Fig. 3. Activity of full-length PrP refolded with different cations. (A) The superoxide dismutase activity of PrP refolded with either copper (○), nickel (△), manganese (●) or zinc (□) from chloride salts was assessed using a standard NBT/xanthine oxidase-based assay. Increasing concentrations of PrP were added. (B) The superoxide dismutase activity of aged PrP refolded with either copper or manganese was assessed using the NBT/xanthine oxidase assay. After 2 weeks of aging, PrP refolded with copper (○) retained activity but that refolded with manganese did not (●). Shown are the mean and SEM for four assays.

same mass of copper-loaded PrP. The samples for the studies were used directly after completion of the refolding and dialysis procedure.

Copper- and manganese-loaded PrP that had been left to age for 2 weeks at 4°C were assayed again for superoxide dismutase activity using the same assay. In this case, copper-loaded PrP showed the same activity as

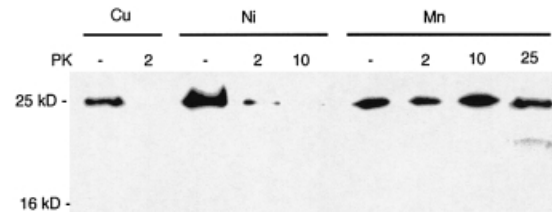


Fig. 4. PK resistance of recombinant PrP. Protein from wild-type recombinant mouse PrP following refolding with either copper, nickel or manganese was aged for 2 weeks and then electrophoresed and blotted using the Western technique. Parallel samples were treated with increasing concentrations of PK (µg/ml). PrP was detected using a polyclonal antibody as described in Materials and methods. The experiment was repeated four times.

fresh material. However, the manganese-loaded PrP showed almost no activity, suggesting that some change had occurred in the protein during aging (Figure 3B).

Analysis of protease resistance

Recombinant PrP refolded with either copper, manganese or nickel was treated with increasing concentrations of PK. The digested protein was electrophoresed on a polyacrylamide gel, blotted and PrP detected with a specific antibody. All freshly refolded PrP was PK sensitive to low concentrations (0.2 µg/ml) (data not shown). However, when PrP that had been aged for 2 weeks was treated with PK, manganese-refolded PrP showed increased PK resistance up to 25 µg/ml (Figure 4). Copper-refolded PrP could be completely degraded with 2 µg/ml PK and nickel-refolded PrP could be mostly degraded with 2 µg/ml PK and completely degraded with 10 µg/ml PK. This suggests that the binding of manganese in place of copper may alter its PK sensitivity, possibly due to changes in its secondary structure.

CD analysis

Previously we have shown that there is a shift in circular dichroism (CD) spectra when PrP is refolded in the presence of copper. The CD spectra for PrP freshly refolded with copper or manganese were identical (Figure 5). Also, the CD spectrum of copper-refolded PrP that was aged for 2 weeks was no different to that of freshly prepared PrP. However, when manganese-refolded PrP was aged for 2 weeks, the CD spectrum showed strong changes at

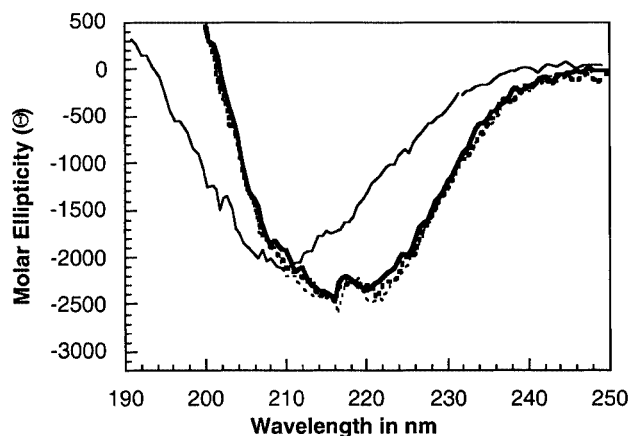


Fig. 5. Far UV CD spectra of recombinant PrP were determined for PrP refolded with either copper or manganese. Freshly prepared samples were compared with those for samples that had been aged for 2 weeks. Samples of copper-refolded protein showed an identical spectrum when prepared fresh (thick dotted line) or aged (thin dotted line). Fresh PrP refolded with manganese (thick line) showed the same spectrum. However, PrP refolded with manganese and aged showed a different spectrum (thin line) indicative of increased β -sheet content and decreased α -helical content.

~210 nm, consistent with greatly increased β -sheet content. This suggests that manganese-refolded PrP underwent a spontaneous change in secondary structure when manganese was bound.

Analysis of the turbidity of manganese- and copper-refolded aged protein indicated that there was no change in measured turbidity ($n = 4$; data not shown). Furthermore, analysis of the manganese content of aged manganese-refolded PrP by TXRF analysis indicated that this material retained the same amount of manganese (3.9 ± 0.4 atoms per molecule; $n = 4$). These results indicate that the change in activity and structure of aged manganese-refolded PrP is not due to loss of manganese content or aggregation.

Cell culture studies

Experiments were carried out to determine whether cells grown in a manganese-rich medium would retain levels of manganese that might then be incorporated into PrP. Manganese is very toxic to neurones, therefore astrocytes were used. Monolayer cultures of pure murine astrocytes were prepared. The astrocytes were grown for 7 days in serum-free medium. The astrocytes were exposed to either copper or manganese or both in varying concentrations for 2 days. The concentrations used were either equivalent to or slightly higher than the levels of these cations found in serum (Rukgauer *et al.*, 1997). The cells were then thoroughly washed before harvesting. The cation content of the cells was assessed using TXRF analysis (Figure 6). According to this analysis the serum-free medium used contained $0.1 \mu\text{M}$ copper and $0.2 \mu\text{M}$ manganese ($n = 3$). Increasing the manganese or copper in the medium increases the amount of either metal in the astrocytes. Addition of a high concentration of manganese in the presence of added copper decreased the copper content of the cells (Figure 6A). However, added copper had only a minor effect on the manganese content of cells (Figure 6B). This suggests that manganese can enter cells through competition with copper at more sites than copper can

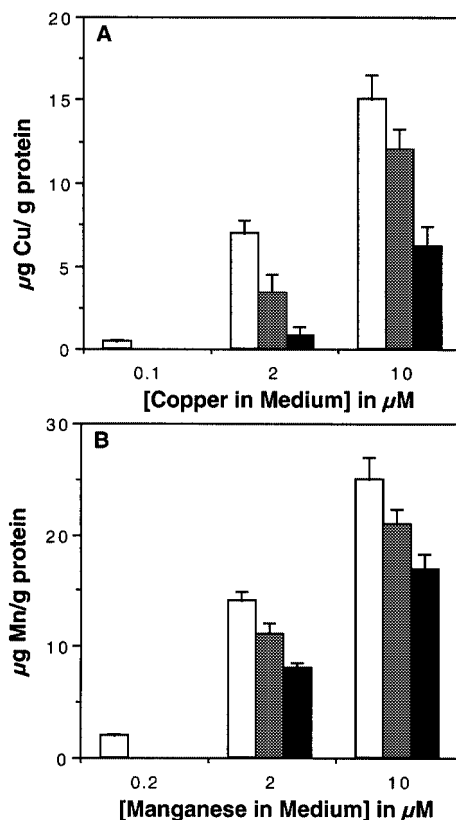


Fig. 6. Manganese uptake by cells. TXRF analysis was used to determine the cation concentration in extracts of cells. (A) Astrocytes were treated with various concentrations of copper (chloride) alone (open bars) or with addition of either $2 \mu\text{M}$ (grey bars) or $5 \mu\text{M}$ (black bars) manganese (chloride). Determination of copper content is shown. (B) Astrocytes were treated with various concentrations of manganese alone (open bars) or with addition of either $2 \mu\text{M}$ (grey bars) or $5 \mu\text{M}$ (black bars) copper. Determination of manganese content is shown. The mean and SEM of six measurements are shown. Values are based on the protein content of the starting material.

Table III. Cation content of immunoprecipitated PrP from astrocytes

Treatment	Cu	Mn
Medium	5.2 ± 0.8	0.8 ± 0.2
$2 \mu\text{M}$ Cu	15.1 ± 2.1	0.01 ± 0.01
$10 \mu\text{M}$ Cu	22.0 ± 2.2	nd
$2 \mu\text{M}$ Mn	3.2 ± 2.2	4.3 ± 1.2
$10 \mu\text{M}$ Mn	1.2 ± 0.5	11.2 ± 1.2

Values for the cation content of immunoprecipitated protein are in μg cation/mg protein. Shown are the mean and SEM for three determinations each. Treatment indicates the conditions that the cells were grown in (see Materials and methods; Figure 6).

compete with manganese. These experiments show that astrocytes can retain high levels of copper or manganese.

Astrocytes grown in the presence of manganese or copper were collected and a protein extract made by homogenization in phosphate-buffered saline (PBS) containing 0.1% NP-40. PrP was immunoprecipitated from the extracts using affinity chromatography based on a specific antibody to PrP. Analysis of the affinity-purified protein by TXRF analysis indicated that native PrP isolated from cells grown in the presence of manganese could incorporate manganese (Table III). Other immunoprecipitated PrP was digested with PK and electrophoresed on a

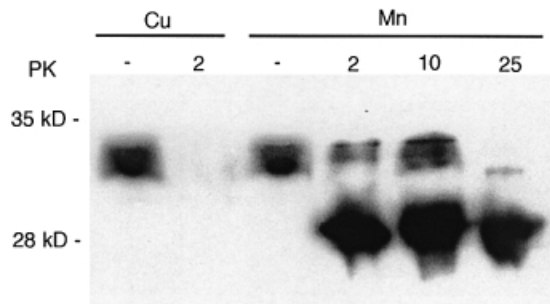


Fig. 7. PK resistance of PrP expressed by cells. Extracts were made from astrocytes grown in the presence of high manganese or copper concentrations. The PrP content was immunoprecipitated. Samples of the immunoprecipitants were treated with PK and studied using Western blotting. Samples from cells grown with added copper were sensitive to PK but the extract from cells grown with manganese contained PK-resistant PrP. Undigested lanes (PK-) were loaded with one-tenth of the amount loaded for PK-digested samples. PrP was detected on the blot using a specific polyclonal antiserum to PrP. Values for PK are in $\mu\text{g/ml}$.

polyacrylamide gel. After Western blotting, PrP was detected with a specific antibody. It was found that PrP from manganese-treated cells was more resistant to PK treatment than that from cells grown in the presence of copper (Figure 7). It should be noted that PrP from astrocytes has only glycosylated forms of PrP, of which the double glycosylated form predominates (Brown, 1999b). The bands seen after PK treatment were more reminiscent of the bands seen after PK treatment of PrP^{Sc} than of those for the recombinant protein seen in Figure 4. These results suggest that cells can incorporate manganese into PrP and that some of the PrP is PK resistant.

Discussion

There is increasing evidence that PrP^C has a function in the regulation of cellular resistance to oxidative stress and that it is involved in or dependent upon copper metabolism in the brain. PrP^C expression regulates uptake of copper into neurones and its utilization at the synapse (Brown, 1999a). Copper taken up in association with PrP^C can be utilized intracellularly by enzymes such as Cu/Zn superoxide dismutase (Brown and Besinger, 1998) or can be utilized by PrP^C itself for superoxide dismutase activity. Therefore, not only does PrP^C have a molecular function but also its expression influences copper utilization by cells.

In the present work we demonstrate that the minimum number of copper ions required for superoxide dismutase activity is two and that the molecule shows increased activity when a further two atoms of copper bind. As binding of copper to PrP induces a more ordered structure (Miura *et al.*, 1996, 1999; Wong *et al.*, 1999), it is likely that the increased activity on binding of two further atoms results from increased order in the molecule. Increased redox potential resulting from increased binding of copper is insufficient to explain this as binding of three copper atoms did not produce a corresponding increase in activity.

Previous experiments with peptides related to the octameric repeat region suggested that binding of each copper atom required coordination between two histidine residues (Viles *et al.*, 1999). However, we found that full-length PrP with two octameric repeats and thus two histidines

was able to bind two copper atoms. Viles *et al.* (1999) found that peptides with three repeats did bind three copper atoms. Similarly, PrP with three repeats bound three copper atoms. The binding of this third copper atom did not add activity to the protein as described above. In studies using equilibrium dialysis (Brown *et al.*, 1997a) based on an N-terminal fragment of PrP it was found that there was cooperativity in the binding atoms. This implies that once one atom of copper is bound further atoms can bind more easily. Therefore, it is likely that the binding of one copper atom changes the conformation of PrP to allow the binding of other copper ions. In a short peptide it is unlikely that binding of one copper ion would induce sufficient changes in the molecule to allow binding of the second atom of copper. Therefore, although coordination of copper between two histidines might facilitate copper binding to peptides and might even be involved in binding of copper to the whole molecule, it is likely that the copper ions bind principally to one histidine each.

It is known that copper-binding proteins can often bind other divalent cations such as zinc under certain circumstances. However, recombinant PrP did not show high retention of zinc. PrP did bind nickel and manganese. This is in contrast to the work of Stöckel *et al.* (1998) who did not find that recombinant PrP binds manganese. However, the same study also indicated that PrP binds only two atoms of copper. We and others (Viles *et al.*, 1999) have provided evidence that PrP can bind four copper atoms. Therefore, it is possible that the methods used by Stöckel *et al.* (1998) to incorporate copper or manganese into PrP were not adequate to saturate the molecule. Refolding the protein in the presence of cations possibly represents the optimal method for cation incorporation. Furthermore, we have shown that manganese is incorporated into native PrP from cells cultured with manganese in the medium. Therefore, it is possible that manganese can be incorporated into or binds to PrP^C *in vivo*.

It has previously been shown that copper bound to PrP can be taken up by cells (Brown, 1999a). It is possible that PrP^C could influence uptake of other cations. Indeed, incorporation of manganese into PrP^C from cells that take up manganese as we have demonstrated here would suggest that PrP^C may also influence manganese metabolism. Manganese is used as an electron acceptor in manganese superoxide dismutase, which is found in mitochondria, and it is interesting that recombinant PrP showed some superoxide dismutase activity on binding manganese.

Studies on replacement of cations in superoxide dismutase have indicated that replacement can occur. In copper-containing proteins the copper can be replaced by cobalt with subsequent changes in activity and structure (O'Neill *et al.*, 1982; Salvato *et al.*, 1989). Similarly, studies of manganese-containing superoxide dismutase indicate that the manganese can be replaced by iron and the enzyme retains activity. Manganese superoxide dismutase is a potent enzyme but the lower redox potential for manganese probably means that it cannot be as active as the copper-containing enzyme. However, more significant for this study is the finding that replacement of copper with other cations can lead to changes in secondary structure. It appears that PrP is less stable on binding manganese and quickly converts to a misfolded form.

The observation that on binding manganese PrP^C

becomes proteinase resistant is probably the most important finding in the present report. Proteinase resistance, along with fibril formation, is one of the most important characteristics distinguishing PrP^{Sc} from PrP^C. Although there are now many reports of the generation of proteinase-resistant recombinant protein, this is the first report of the formation of proteinase-resistant protein from native PrP^C from cells without the interaction of those cells with PrP^{Sc}. Cells expressing PrP carrying mutations associated with inherited prion disease have been known to generate proteinase-resistant PrP but this does not occur with cells expressing the native protein. This therefore represents *de novo* formation of PK-resistant PrP.

When the mechanism of infection in prion disease is understood there will still remain sporadic prion disease, which occurs without any known infection event. The cause of sporadic prion disease is a mystery, and attempts to explain human CJD by suggesting that it comes from eating infected sheep have been disreputed. Therefore, an environmental cause for sporadic disease remains a possibility. Recent investigations of scrapie, CJD and chronic wasting disease clusters in Iceland, Slovakia and Colorado, respectively, have indicated that the soil in these regions is low in copper and higher in manganese (Purdy, 2000). The possibility that imbalances in environmental cations entering the food chain may induce conditions favouring the formation of proteinase-resistant PrP is a controversial but intriguing possibility. This idea is supported by the findings presented in this paper.

The finding that incorporation of manganese into PrP makes it proteinase resistant and abolishes its function is a long way from explaining sporadic prion disease. However, a current favourite among hypotheses concerning conversion of PrP^C to PrP^{Sc} requires the formation of 'seeds' or 'nuclei' of proteinase-resistant PrP (Cohen and Prusiner, 1998). The formation of sufficient proteinase-resistant PrP to create such a seed is the limiting step in this model of PrP^{Sc} formation. Incorporation of manganese in place of copper in the holo-form of PrP^C may represent one possible way in which the substance of a 'seed' could form.

In summary, the work presented here confirms that copper is the preferred cation incorporated into PrP^C at the octameric repeat region. This copper can be replaced by manganese but the consequence was found to be loss of function and increased proteinase resistance. Each atom of copper incorporated into PrP is coordinated by the histidine in a single octameric repeat. Although PrP can bind the same number of manganese atoms as copper atoms the resulting protein is aberrant. These results show for the first time a mechanism by which proteinase-resistant native PrP^C can be expressed by cells.

Materials and methods

Protein purification and refolding

The production of recombinant mouse PrP has been described previously (Wong *et al.*, 1999). Briefly, amplified product was cloned in the expression vector pET-23 (Novagen) and transformed in *Escherichia coli* AD494(DE3). The expressed proteins were recovered from urea-solubilized bacterial lysate after sonication by immobilized nickel-based affinity chromatography (Invitrogen). The eluted material was refolded by several successive rounds of dilution in either deionized water, 5 mM CuCl₂ or 5 mM CuSO₄ followed by ultrafiltration and dialysis to remove

unbound copper. The final protein was typically >95% pure and was concentrated to ~1–2 mg/ml. Its identity was confirmed by N-terminal sequencing and Western blotting using the polyclonal antibody to mouse PrP (142–160). Protein concentration was determined using the Bio-Rad protein assay reagent or as previously described (Brown *et al.*, 1999). Refolding with other divalent cations was carried out similar to that described above. The same concentration (5 mM) of the chloride salt of each cation was used in place of CuCl₂.

Some samples were stored for 2 weeks at 4°C. These samples are referred to as 'aged'. Aggregation of protein was assessed using spectrophotometric determination of turbidity ($n = 4$) per sample. The method used was as previously described for amyloid proteins (Evans *et al.*, 1995). Samples showing increased turbidity were not used for further study.

Site-directed mutagenesis

Specific deletions of one, two, three or four of the octameric repeats present in the mouse prion sequence were accomplished by site-directed mutagenesis (QuickChange; Stratagene) using paired splint oligonucleotides to remove residues 51–68 (one repeat plus the incomplete repeat), 51–76 (two repeats), 51–82 (three repeats) or 51–90 (four repeats). The mutation was confirmed by DNA sequence analysis across the deletion site and the encoded protein purified and refolded as above.

TXRF analysis

Metal ion content of protein samples was determined using TXRF as described previously (Brown *et al.*, 1999). Briefly, elemental determinations were carried out using an EXTRA II Energy Dispersive X-ray fluorescence spectrometer by operating the X-ray tube at 50 keV and 10 mA with a count time of 200 s. A 10 ml aliquot of each protein sample together with an equal volume of internal standard (10 p.p.m. cobalt) was placed directly onto the surface of a quartz reflector and dried in air by placing the reflector in a positive pressure filtered air cabinet. Background readings for the quartz reflectors were also recorded.

Superoxide dismutase assay

The assay was performed as described previously (Brown *et al.*, 1999) employing superoxide production from xanthine oxidase and detection of a coloured formazan product formed from NBT. Inhibition of formazan production by added protein is equivalent to the superoxide dismutase activity. Formazan production was measured spectrophotometrically at 560 nm on a U1100 spectrophotometer (Hitachi).

CD analysis

Samples were dissolved at a concentration of 10 µM in 1 mM MES pH 5.5, as determined using the Bio-Rad protein assay. CD spectra were recorded in a 0.1 cm path length quartz cell at room temperature under constant nitrogen flush using the Jasco J720 spectropolarimeter. At least four spectra were accumulated, and the appropriate blanks were subtracted. The values were expressed as molar ellipticity (θ).

PK analysis

Purified recombinant proteins or immunoprecipitated native protein were assessed for proteinase sensitivity by treatment with 2–25 µg/ml PK (Boehringer Mannheim) at 37°C for 1 h (Herrmann and Caughey, 1998). The reaction was stopped by adding Perfabloc (Boehringer Mannheim) to 2 mM and the products of digestion were analysed by Western blotting using a rabbit polyclonal antibody against mouse PrP (142–160) as previously described (Brown *et al.*, 1999).

Cell culture

Pure cultures of astrocytes from wild-type mice were prepared as previously described (Brown *et al.*, 1996b). Astrocytes were cultured in serum-free medium for experiments involving treatment with divalent cations. Astrocytes were kept in serum-free culture for 7 days with changes of medium every third day. Copper or manganese was added to cultures in the form of a chloride salt and as a histidine chelate. Treatment with cations was for 2 days; after this time the cells were scraped off the flasks and collected for TXRF analysis of cellular metal content for immunoprecipitation of the PrP. Immunoprecipitated PrP from astrocytes was used for TXRF analysis of manganese and copper content of or PK resistance of the PrP. The cells were collected and digested in PBS containing 0.1% NP-40. Immunoprecipitation was as previously described (Brown *et al.*, 1999).

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