

RESEARCH COMMUNICATION

Normal prion protein has an activity like that of superoxide dismutase

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We show here that mouse prion protein (PrP^C) either as recombinant protein or immunoprecipitated from brain tissue has superoxide dismutase (SOD) activity. SOD activity was also associated with recombinant chicken PrP^C confirming the evolutionary conserved phenotype suggested by sequence similarity. Acquisition of copper by PrP^C during protein folding endowed SOD activity on the protein but the addition of copper following

refolding did not. PrP^C dependent SOD activity was abolished by deletion of the octapeptide-repeat region involved in copper binding. These results describe an enzymic function for PrP^C consistent with its cellular distribution and suggest it has a direct role in cellular resistance to oxidative stress.

Key words: copper, oxidative stress, re-folding.

INTRODUCTION

The prion protein (PrP^C) is a copper binding [1] glycoprotein expressed by neurons [2], glia [3,4] and other cells [5]. In prion diseases such as bovine spongiform encephalopathy (BSE) and Creutzfeldt–Jakob's disease, PrP^C is converted into a proteinase-resistant form (PrP^{Sc}). The main features of prion diseases are neurodegeneration, gliosis and accumulation of PrP^{Sc} in central nervous tissue [6]. PrP^{Sc} is believed to be the agent of transmission and its formation from host PrP^C is closely associated with the cause of the neurodegeneration which eventually leads to death of the affected individual. Conversion of host PrP^C into PrP^{Sc} is, therefore, closely associated with the main features of the disease. Understanding the differences between these two isoforms of this protein is essential to understanding how prion diseases occur. However, despite broad investigation of the conversion process leading to PrP^{Sc} formation, little is known about the biological activity of the normal cellular isoform of this protein.

PrP^C is highly conserved throughout evolution but its cellular function remains undefined. Evidence suggests that PrP^C is important for synaptic activity [7,8] but its expression in many cell types is indicative of a more cosmopolitan function. Recent work has shown that PrP^C binds four atoms of copper under certain conditions [1,9,10]. Copper is probably bound to an octapeptide-repeat region in which the binding of copper is coordinated by four histidine residues [11]. Additionally, cell culture experiments suggest that the presence of copper causes an increase in the turnover rate of PrP^C [12]. There is evidence that PrP^C expression aids cellular resistance to copper toxicity and oxidative stress [13,14].

Research using mice that lack PrP^C expression (Prnp^{0/0} mice) [15] also provides evidence for a link between PrP^C copper binding and resistance to oxidative stress [1,14]. PrP^C is normally highly expressed in the synapses of wild-type mouse neurons [16]; however, synapstosomal fractions of Prnp^{0/0} mice are deficient in copper [1]. Furthermore, exogenous copper decreases the amplitude and frequency of spontaneous inhibitory currents

recorded from Purkinje cells from Prnp^{0/0} mice but not wild-type mice [1]. In culture, neurons from Prnp^{0/0} mice are more sensitive to both oxidative stress and copper toxicity than wild-type neurons [13,14,17]. Neurons from Prnp^{0/0} mice show decreased activity of Cu/Zn superoxide dismutase (SOD) [14], probably because of the decreased incorporation of copper into the enzyme [18]. Cytosolic extracts from the brains of adult Prnp^{0/0} mice also show a reduction in SOD activity [1,14]. This and other evidence suggest that expression of the PrP^C somehow affects copper distribution and utilization by cells [19]. For these reasons it would be interesting to identify a link between PrP^C copper and resistance to oxidative stress at the molecular level.

We undertook experiments using recombinant PrP^C refolded to incorporate copper to try to identify this link. Recombinant chicken and mouse PrP^C, as well as PrP^C immunoprecipitated from brain tissue, has SOD activity. PrP^C-dependent SOD activity was abolished by deletion of the octapeptide-repeat region involved in copper binding or by the addition of the copper chelator diethyldithiocarbamate (DDC). These results suggest that PrP^C has an enzymic function, dependent on copper incorporation, consistent with its cellular distribution and indicating that it has a direct role in cellular resistance to oxidative stress.

MATERIALS AND METHODS

Unless described otherwise all reagents were from Sigma. The anti-hexahistidine antibody was from Boehringer.

Production of recombinant PrP^C

Production of mouse recombinant PrP^C (Mo-rPrP^C) and chicken recombinant PrP^C (Ch-rPrP^C) was as described previously [20]. rPrP^C was recovered from urea solubilized bacteria by immobilized-metal-chelate affinity chromatography (IMAC) and the eluted material refolded by several successive rounds of dilution in 5 mM CuCl₂ followed by ultrafiltration and dialysis. The protein was finally concentrated to approx. 1–2 mg/ml and its identity confirmed by N-terminal sequencing and Western

Abbreviations used: SOD, superoxide dismutase; PrP^C, cellular prion protein; PrP^{Sc}, proteinase-resistant form of PrP; Mo-rPrP^C, mouse recombinant PrP^C; Ch-rPrP^C, chicken recombinant PrP^C; TXRF, total reflection X-ray fluorescence spectroscopy; NBT, Nitro Blue Tetrazolium; DDC, diethyldithiocarbamate.

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blot analysis. The conformation of the final preparation of the Mo-rPrP^C was also examined by CD spectroscopy and the sensitivity to proteinase K was measured. The secondary structure of the protein was similar to that described by Hornemann et al. [21] and the protein remained sensitive to added protease (results not shown). Trypsin digestion of Mo-rPrP^C was performed by incubation of the protein with trypsin bound to agarose beads. Following a 20 min incubation with trypsin at 37 °C, the beads were pelleted and the cleaved protein dialysed to remove the hexahistidine-containing protein fragment.

Site-directed mutagenesis

Specific deletion of the octapeptide repeats present in the mouse prion sequence was accomplished using site-directed mutagenesis (QuickChange, Stratagene) using a splint oligonucleotide to remove residues 51–90. The mutation was confirmed by DNA sequence analysis across the deletion site and the encoded protein purified and refolded as described above.

Total reflection X-ray fluorescence spectroscopy (TXRF)

The metal ion content of protein samples was determined using TXRF as described previously [22,23]. Elemental determinations were carried out using an EXTRA II energy dispersive X-ray fluorescence spectrometer (Rich, Seifert and Co., Ahrensburg, Germany) fitted with multiple total-reflection optics. Determinations were performed by operating the X-ray tube at 50 keV and 10 mA with a count time of 300 s. Background readings for the quartz reflectors were recorded (count time 50 s; ISIS Processor Unit, Rich, Seifert and Co.); acceptable reflectors were identified as those reflectors which when scanned gave less than 1.2 c.p.s. for the elements of interest. All sample handling was performed in a positive-pressure filtered air cabinet using Gilson Microman (5–25 µl) positive-displacement pipettes. A 10 µl aliquot of each protein sample together with a 10 µl volume of internal standard (10 p.p.m. Cobalt) was placed directly on to the surface of a quartz reflector and dried in air by placing the reflector in a positive-pressure filtered air cabinet.

SOD assays

Two SOD assays were used in these experiments. The first employing superoxide production from xanthine oxidase and detection of a coloured formazan product formed from Nitro Blue Tetrazolium (NBT), was as described previously [24]. The second assay, based on production of superoxide by KO₂ dissolved in solution, was as described by Marklund [25].

Animals

The Prnp^{0/0} mice used in this study were as originally described by Büeler et al. [15]. The wild-type mice used were descendants of an F1-generation mouse produced by interbreeding the original parental strains [C57BL/6J and 129/Sv(ev) mice] used to generate Prnp^{0/0} mice.

Immunoprecipitation

Immunoprecipitation of PrP^C was carried out using a monoclonal antibody produced against a peptide based on the mouse sequence corresponding to amino acid residues 142–160. The method was as described previously [18]. Extracts were equilibrated according to the protein content, using the bicinchoninic acid method (Sigma) at 200 µg/ml, reacted with 5 µl of antibody, and Protein A–Sepharose was added to the extracts at 10 µg/ml.

Cell culture

Cerebellar cells from wild-type mice were as described previously [17]. PrP^C were added directly from stock solutions at the concentrations indicated. Exposure to xanthine oxidase (Sigma) and 500 µM xanthine (Sigma) was for 24 h one day after plating. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) based survival assay was as described previously [17].

RESULTS AND DISCUSSION

PrP^C has been identified as a copper binding protein [1]. A copper-dependent molecular activity of PrP^C is unknown. To investigate the role of copper in PrP^C activity we expressed full-length mouse PrP^C as a hexahistidine-tagged fusion protein in *Escherichia coli*. Copper binding is a property of the N-terminal octapeptide repeats [1,9,10,26]. A deletion precisely removing the repeat region was also expressed to provide a specificity control. The sequence and structure similarity between all *prp* genes is high [27,28]. Thus any activity observed to be dependant on copper binding should also be a property of an evolutionarily distant PrP. Accordingly, a full-length form of chicken PrP^C was similarly prepared. PrP proteins, produced at high levels, required solubilization in urea before purification, effectively stripping them of any bound ligand. All PrP^C forms were refolded in the presence of 5 mM Cu²⁺ following elution from the immobilized-metal-chelate affinity chromatography column. This resulted in soluble rPrP^C preparations. Residual free copper was removed by dialysis before analysis of the final preparations by SDS/PAGE (Figure 1). To ascertain whether any of the refolded rPrP^C preparations had retained copper, purified protein was analysed for copper content by TXRF. Both full-length Ch-rPrP^C and Mo-rPrP^C, refolded in the presence of copper, retained equivalent amounts of copper (Table 1). Deletion of the octapeptide-repeat region from MoPrP^C reduced the level of copper present in the purified preparations consistent with binding of the ion to the octapeptide repeats as described previously [1,9,10]. Residual copper associated with rPrP^C preparations lacking the repeat region (Table 1) is probably due to copper associated with the histidine tag which is still present during analysis although it is possible that copper binds at PrP sites outside of the repeats. To verify this, samples of Mo-rPrP^C (both full length and lacking

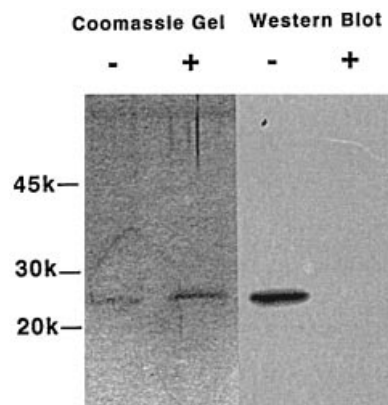


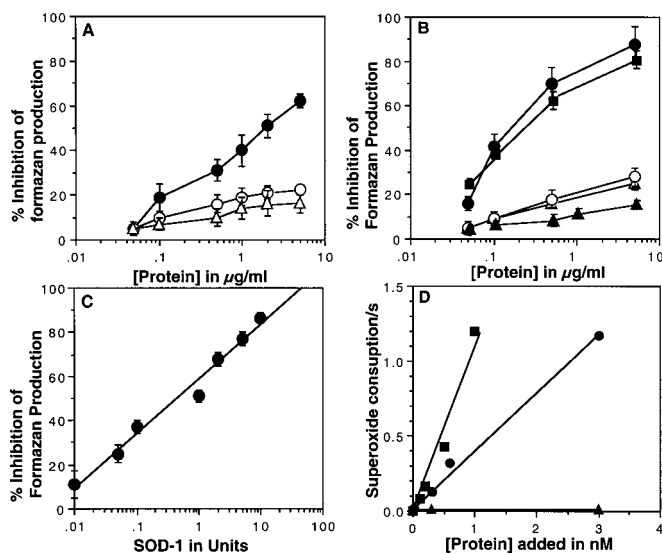
Figure 1 Analysis of Mo-rPrP^C following trypsin digestion

Coomassie Blue staining of trypsin digested (+) and undigested (–) Mo-rPrP^C after electrophoresis on a PAGE gel (left panel). Western blotted protein digested with trypsin could not be recognised by an antibody specific for hexahistidine (right panel). k, kDa.

Table 1 Bivalent cation content of isolated PrPs determined by TXRF analysis

Protein (200 μg) was reacted with 5 μl of PrP-specific antibody and 10 mg of Protein A–Sepharose. $\Delta 51$ –91 indicates a Mo-rPrP with a deletion of amino acid residues 51–90. Trypsin indicates that the samples were treated with trypsin attached to beads after refolding with Cu^{2+} . Immunoprecipitants were derived from extracts of adult mouse brain (wild-type or *Prnp*^{0/0}). TXRF values are expressed as μg of metal ion/mg of protein or μg of metal ion/10 mg of Protein A–Sepharose. The molar ratio indicates the approximate number of copper atoms per molecule of PrP to the nearest whole integer. Data are the means \pm S.E.M. for 6–25 measurements. n.d., not detectable; n.a., not applicable. Additionally for all the samples tested other bivalent cations, including Ni^{2+} and Fe^{2+} , were detected at less than 0.03 μg of metal ion/mg of protein.

PrP	TXRF values			Molar ratio of Cu^{2+} /PrP
	Cu^{2+}	Zn^{2+}	Mn^{2+}	
Chicken				
Wild-type untreated	0.01 \pm 0.01	0.03 \pm 0.01	0.03 \pm 0.01	0
Wild-type exposed to Cu^{2+}	2.01 \pm 0.01	n.d.	0.01 \pm 0.01	< 1
Wild-type refolded with Cu^{2+}	51.8 \pm 1.1	0.03 \pm 0.01	0.03 \pm 0.01	10
Mouse				
Wild-type untreated	0.01 \pm 0.01	0.06 \pm 0.01	0.01 \pm 0.01	0
Wild-type exposed to Cu^{2+}	2.3 \pm 0.51	n.d.	0.01 \pm 0.01	< 1
Wild-type refolded with Cu^{2+}	52.0 \pm 1.2	n.d.	0.05 \pm 0.01	10
Wild-type reported with Cu^{2+} /trypsin	25.2 \pm 2.2	n.d.	0.01 \pm 0.01	4–5
$\Delta 51$ –90 untreated	0.01 \pm 0.01	0.01 \pm 0.01	0.01 \pm 0.01	0
$\Delta 51$ –90 refolded with Cu^{2+}	32.0 \pm 3.0	0.01 \pm 0.01	0.01 \pm 0.01	6
$\Delta 51$ –90 with Cu^{2+} /trypsin	3.2 \pm 2.0	n.d.	n.d.	< 1
Wild-type immunoprecipitant	50 \pm 6	0.05 \pm 0.01	0.05 \pm 0.01	n.a.
<i>Prnp</i> ^{0/0} immunoprecipitant	0.05 \pm 0.05	0.05 \pm 0.01	0.05 \pm 0.01	n.a.

**Figure 2** PrP SOD activity

SOD activity was assessed for purified (A) Ch-rPrP^C or (B) Mo-rPrP^C using an NBT-based assay. The samples tested were either refolded without Cu^{2+} (△), refolded with 5 mM Cu^{2+} added as CuSO_4 (○), or refolded in the presence of Cu^{2+} (●). Samples of Mo-rPrP^C and Ch-rPrP^C refolded in the presence of copper (●) showed significant levels of activity. Additionally a mutant Mo-rPrP^C lacking amino acids 51–90 lacked SOD activity even after refolding in the presence of 5 mM Cu^{2+} (▲). Mo-rPrP^C refolded with copper and digested with trypsin (■) was also assayed for activity. The activity of trypsin-digested protein was not significantly different from undigested protein (Student's *t* test, $P < 0.05$). (C) Bovine Cu/Zn SOD with known activity was assayed in parallel to determine the relative activity level of the rPrP^C samples. (D) A second KO_2 based SOD assay was used to verify the observation that Ch-PrP has SOD activity. The activity of Ch-rPrP^C refolded without Cu^{2+} (▲) and refolded with Cu^{2+} (●) was compared with that of bovine Cu/Zn SOD (■). Cu^{2+} -refolded Ch-rPrP^C also showed strong SOD activity in this assay. The data are means \pm S.E.M. for a minimum of three experiments.

the octapeptide-repeat region) were treated with trypsin (0.5 unit/ml, 20 min at 37 °C), which is known to cleave off the very end of the C-terminus of PrP^C [29]. Western blot analysis using an anti-hexahistidine antibody (Boehringer) confirmed that this treatment removed the histidine tag without degrading the protein (Figure 1). TXRF analysis of the resulting C-terminal cleaved material following dialysis confirmed that this treatment removed residual copper binding with the histidine tag (see Table 1). Trypsin cleavage of the full-length Mo-rPrP^C resulting in protein with four to five atoms of copper binding to one PrP^C molecule and cleavage of the deletion mutant resulted in less than one atom copper binding to one PrP^C molecule. The residual copper binding to the trypsin digested deletion mutant is possibly a result of incomplete digestion. However, the result confirms that four atoms of copper can bind to the octapeptide-repeat region of PrP^C.

Addition of 5 mM Cu^{2+} to a preparation of full-length Mo-rPrP^C or Ch-rPrP^C refolded in the absence of copper and then dialysed to remove free ions showed very low copper retention (Table 1) suggesting that Cu^{2+} acquisition occurred during protein folding. To determine whether native protein also retained copper, PrP^C was immunoprecipitated from brain extracts of either wild-type or *prp*-gene-ablated (*Prnp*^{0/0}) mice [15]. Material immunoprecipitated from wild-type mouse brain contained significantly more copper (Table 1). Thus, evolutionarily diverse PrP^C of both recombinant and native origin contains copper, suggesting that this may be an important physiological ligand required for normal prion function.

PrP^C expression aids cellular resistance to oxidative stress produced by exposure to superoxide radicals [14]. It is possible that PrP^C directly protects against superoxide damage by an antioxidant activity such as that possessed by SOD. Accordingly, rPrP^C preparations were tested for SOD activity using a formazan formation assay [24]. Ch-rPrP^C refolded in the absence of copper had little effect on the rate of formazan produced (Figure 2A),

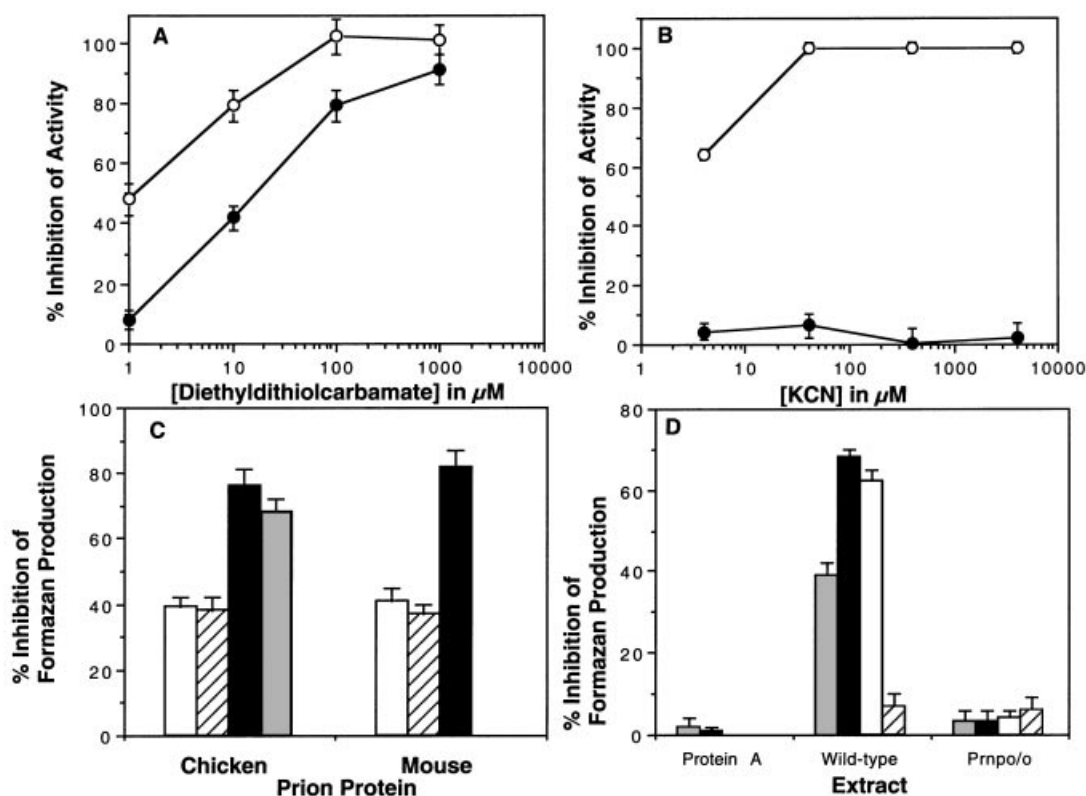


Figure 3 Inhibition of PrP SOD activity

Effect of (A) DDC and (B) KCN on the activity of Ch-rPrP^C SOD (1 $\mu\text{g}/\text{ml}$; ●) and Cu/Zn SOD (0.1 unit/ml; ○). DDC inhibited the activity of Cu²⁺ refolded Ch-rPrP^C in a dose-dependent manner whilst the addition of KCN had no effect. Both agents inhibited Cu/Zn SOD. (C) The SOD activity of Mo-rPrP^C and Ch-rPrP^C was tested after refolding under different conditions using the NBT-based assay. The highest activity was seen when rPrP^C was refolded in the presence of 5 mM Cu²⁺ (black bars). Protein refolded in the presence of 50 μM Cu²⁺ had significantly less activity (Student's *t* test, $P < 0.05$; open bars). Protein refolded in the presence of both 50 μM Cu²⁺ and 50 μM Zn²⁺ (hatched bars) had activity that was not significantly different from that of protein refolded with Cu²⁺ only. The addition of 5 mM Zn²⁺ during refolding of Ch-rPrP^C with 5 mM Cu²⁺ did not alter its activity (grey bar). (D) PrP was immunoprecipitated from protein extracts of wild-type and Prnp^{0/0} (which express no PrP) mouse brain using an anti-mouse PrP monoclonal antibody. The SOD activity of the immunoprecipitants was assayed using the NBT-based assay. Aliquots of 5 μl (grey bars) and 10 μl (black bars) of either a 10 $\mu\text{g}/\text{ml}$ Protein A-Sepharose solution or the precipitates from wild-type and Prnp^{0/0} mouse brain (in PBS) were used. DDC (1 mM) fully inhibited the activity of the wild-type immunoprecipitant (hatched bars) whilst treatment with 40 mM KCN did not (open bars). The data are means \pm S.E.M. for a minimum of three experiments.

whilst Ch-rPrP^C refolded in the presence of copper showed a dose-dependent inhibition of formazan production. A similar inhibition was seen with Mo-rPrP^C refolded with copper (Figure 2B). These results show that PrP^C has SOD activity amounting to between 15% (Ch-rPrP^C) and 30% (Mo-rPrP^C) of that observed for the equivalent molar amount (protein) of bovine Cu/Zn SOD (Figure 2C). Ch-rPrP^C was also subjected to a second, independent SOD activity assay (Figure 2D) based on the production of superoxide by KO₂ [25]. This second assay indicated that Ch-rPrP^C stimulated superoxide decay with a rate constant for the reaction of $4 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ (PrP). SODs can also contain Zn, Mn, Fe or Ni but the possibility that the observed activity is due to a contaminating known SOD can be ruled out from the TXRF analysis which revealed an absence of these elements (Table 1).

A series of control experiments support the idea that this activity is enzymic and not due to copper-mediated catalysis of superoxide dismutation by the copper associated with PrP^C. Firstly, addition of 5 mM Cu²⁺ to Ch-rPrP^C or Mo-rPrP^C refolded without copper, resulted in no SOD-like activity (Figure 2A and 2B). Secondly, EDTA (100 μM) added to Mo-rPrP^C refolded with copper does not inhibit the SOD-like activity [inhibition of formazan production = $71 \pm 3\%$ with EDTA, $69 \pm 3\%$ without

EDTA, 0.5 $\mu\text{g}/\text{ml}$ PrP^C, $n = 4$, using the NBT-based assay]. Thirdly, a peptide based only on the octapeptide-repeat region of mouse PrP [10] (amino acid residues 59–91; PHGGGWGQ \times 4) to which 5 mM CuCl₂ was added showed no SOD activity (160 $\mu\text{g}/\text{ml}$ peptide inhibited formazan production in the NBT based assay by $11 \pm 2\%$ without the addition of copper and $12 \pm 3\%$ with the addition of copper, $n = 4$). Fourthly, a mutant Mo-rPrP^C lacking the octapeptide-repeat region, prepared and refolded in the presence of copper as for the wild-type Mo-rPrP^C, retained approx. 60% of the copper associated with the wild-type (Table 1) but lacked any SOD activity (Figure 2B). Finally, trypsin digested Mo-rPrP^C, which lacks the hexahistidine tag, and the copper that bound to it (see Table 1) possessed identical activity to Mo-rPrP^C with the hexahistidine tag, indicating that copper binding to the hexahistidine tag was not involved in the activity measured by the reaction (Figure 2B). Thus the presence of the octapeptide-repeat region in full-length PrP^C, and the copper which binds to it, are necessary for the measured SOD activity.

That SOD activity depended upon the presence of the N-terminal repeats is consistent with their mapping as the primary site for PrP^C copper binding [1,9–11,26]. But the fact that residual copper associated with the deleted forms of PrP^C or added to full-

length PrP^C refolded without copper, suggest that the presence of the ion is insufficient in its own right for catalysis. The amount of copper binding to the deleted form without trypsin digestion is equivalent to 60% of the amount of copper that bound to the full-length form. This amount did not produce substantial SOD activity (Figure 2B).

The importance of copper to the observed dismutation was assayed using the xanthine oxidase/NBT method [24]. The copper chelator DDC, which inhibits Cu/Zn SOD [30], also inhibited the dismutase activity of refolded Ch-rPrP^C (Figure 3A). By contrast KCN, also a potent inhibitor of Cu/Zn SOD (K_i of 62 μ M at pH 7.8) [31], did not inhibit the SOD activity of Ch-rPrP^C (Figure 3B).

Alterations in the method used for refolding the protein were investigated. A lower concentration of Cu²⁺ (50 μ M) added to both Ch-rPrP^C and Mo-rPrP^C during refolding still led to rPrP^C with SOD activity but at a level lower than that observed when 5 mM Cu²⁺ was used. Zn is incorporated into Cu/Zn SOD. However, Ch-rPrP^C and Mo-rPrP^C refolded in the presence of both Cu²⁺ and Zn²⁺ had no more activity than protein refolded with only Cu²⁺ (Figure 3C).

Immunoprecipitated PrP^C from mouse brain was also assayed for SOD activity. Immunoprecipitated material from wild-type mouse brain, but not from Prnp^{0/0} mouse brain, showed SOD activity. This activity was inhibited by DDC at 10 μ M but not by KCN at 10 mM (Figure 3D). These results confirm that native PrP^C also possesses SOD activity.

Previous studies [1,14,18] showed that the brains of Prnp^{0/0} mice have reduced activity of cytosolic Cu/Zn SOD. This reduction in activity was not due to the absence of PrP SOD activity as the difference between wild-type and Prnp^{0/0} mice could be abolished by KCN. As we have demonstrated in the present paper the SOD activity of PrP is not sensitive to KCN. Furthermore, PrP is a cell-membrane-bound protein and preparations of the brain extracts for assay of cytosolic Cu/Zn SOD require clearance of the membrane fractions before assay. Therefore PrP is unlikely to contribute to measurements of cytosolic SOD. PrP^C is located primarily in synapses [16] for which no SOD has been described previously. Therefore it is possible that PrP^C is the main or only SOD-like protein in this region. Superoxide is known to have negative effects on synaptic activity which might explain the high synaptic expression of PrP^C [16].

We have provided the first evidence for an enzymic activity associated with rPrP^C. Activity was dependent on the presence of Cu²⁺ incorporated into the molecule specifically at the octapeptide-repeat region during refolding. Change in binding of copper by PrP^C may provide a switch for the expression of SOD activity. Discovery of a function for PrP^C in oxidative metabolism also has important consequences for prion diseases. Hydrogen peroxide is the product of superoxide dismutation. It is an intriguing possibility that increased extracellular accumulation of non-degradable PrP^C in the form of PrP^{Sc} may alter the extracellular balance of reactive oxygen species and by products. This in turn may be causal to the pathology of prion diseases.

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