Copper induces increased β-sheet content in the scrapie-susceptible ovine prion protein PrP^{VRQ} compared with the resistant allelic variant PrP^{ARR}

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Prion diseases are characterized by conformational change in the copper-binding protein PrP (prion protein). Polymorphisms in ovine PrP at amino acid residues 136, 154 and 171 are associated with variation in susceptibility to scrapie. PrP^{VRQ} [PrP(Val^{136}/Arg^{154}/Gln^{171})] or PrP^{ARQ} [PrP(Ala^{136}/Arg^{154}/Gln^{171})] animals show susceptibility to scrapie, whereas those that express Ala^{136}/Arg^{154}/Arg^{171} (PrP^{ARR}) show resistance. Results are presented here that show PrP^{VRQ} and PrP^{ARR} display different conformational responses to metal-ion interaction. At 37 °C copper induced different levels of β-sheet content in the allelic variants of ovine full-length prion protein (amino acid 25–232). PrP^{VRQ} showed a significant increase in β-sheet content when exposed to copper at 37 °C, whereas PrP^{ARR} remained relatively unchanged. The conversion of α-helical PrP^{VRQ} to β-sheet form was shown by CD spectroscopy and the decreased binding of C-terminal specific monoclonal anti-PrP antibodies. This conversion to an increased β-sheet form did not occur with truncated PrP^{VRQ} (amino acids 89–233), which demonstrates that additional metal-binding sites outside of the N-terminus may not overtly influence the overall structure of ovine PrP. Despite the difference in β-sheet content, both the scrapie-susceptible and -resistant allelic forms of ovine PrP acquired resistance to proteinase K digestion following exposure to copper at 37 °C, suggesting the potential for disease-associated PrP^{ARR} to accumulate *in vivo*. Our present study demonstrates that allelic variants of ovine PrP differ in their structure and response to the interaction with copper. These observations will contribute to a better understanding of the mechanism of susceptibility and resistance to prion disease.

Key words: conformational change, α-helix, copper, metal ion, prion protein (PrP), scrapie.

INTRODUCTION

Prion diseases, also referred to as transmissible spongiform encephalopathies, are chronic neurodegenerative disorders that include scrapie in sheep, bovine spongiform encephalopathy in cattle and Creutzfeldt–Jakob disease in humans. These conditions are characterized by the accumulation of PrP^{Sc} [scrapie (protease-resistant) prion protein isoform], an abnormal isomer of the host protein PrP^{C} (cellular PrP isoform). The protein-only hypothesis postulates that the transmissible prion agent comprises solely of proteinaceous material [1]. Consequently, it is proposed that PrP^{Sc} forms part, or all, of the infectious prion agent and that this abnormal isomer is responsible for the modification of the normal cellular form, PrP^{C}. PrP^{Sc} is derived from PrP^{C} by a posttranslational event [2], which does not appear to involve any covalent modification between the two isomers of PrP [3]. PrP^{Sc} is characterized by its partial resistance to proteolytic digestion and its ability to form highly insoluble aggregates [4,5]. Fouriertransform infrared spectroscopy has shown that PrP^{C} is predominantly α-helical (42 %) with little β-sheet (3 %), whereas PrP^{Sc} has considerably more β-sheet content (43 %) and a similar α-helical content (30 %) [6]. These observations indicate that during conversion of PrP^{C} to PrP^{Sc} a major refolding event occurs that results in a more extensive β-sheet conformation. This conformational change would appear to be fundamental to prion propagation, as PrP^{Sc} may be the only component of the infectious prion agent.

The conformational forms of PrP have been modelled with recombinant PrP. NMR studies of full-length [7–10] and truncated recombinant PrP [7,11] from several species predict a predominantly α-helical structure comprising a globular C-terminal domain and flexible N-terminal region. The globular domain contains three α-helices, with helix-2 and helix-3 joined by a single disulphide bond, and two short anti-parallel β-sheet regions flanking helix-1. The α-helical nature of helix-2 and helix-3 is influenced by the length of the N-terminus region and is maximal in full-length PrP. Recombinant PrP refolded under oxidizing conditions yields predominantly α-helical protein, whereas refolding under reducing conditions generates a form with a higher β-sheet content [12,13]. The β-sheet form of recombinant PrP displays characteristics similar to PrP^{Sc}, which include partial resistance to proteolytic digestion and the propensity to form insoluble amorphous aggregates [14]. The conversion pathway of PrP^{C} to PrP^{Sc} has been investigated with these different isoforms of recombinant PrP, either in solution [15–21] or associated with lipid membrane [22].

PrP has been shown to bind copper [23–29], and this metal ion may be involved in the normal function of the protein [30,31]. The principal copper-binding sites are located in the N-terminal octapeptide repeat regions of PrP, although a site more C-terminal to these has been proposed [32,33]. The N-terminal octapeptide repeats in different species comprise typically the amino acid sequence PHGGGWGQ. Each octapeptide binds a single copper(II) ion via a nitrogen from the histidine imidazole side chain and deprotonated amide nitrogens from the second and third glycine residues [26,34]. The octapeptide repeat region of PrP binds copper ions with a K_d in the micromolar range, and a second site, around residues 96–111 in human PrP, binds with higher affinity [24,25,29,32,35,36]. Binding of copper to the octapeptide repeat is pH dependent [33–35]. The binding of copper to PrP results in a conformational change in the normally unstructured N-terminal region of PrP [35]. The interaction of

Abbreviations used: PBS-T, PBS containing 0.1 % Tween 80; PK, proteinase K; PrP, prion protein; PrP^{C}, cellular PrP isoform; PrP^{Sc}, scrapie (protease resistant) PrP isoform; SEC, size exclusion chromatography.

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PrP with copper also promotes formation of more β-sheet structure within the protein and conveys acquisition of resistance to protease digestion [29,37]. In addition to copper, manganese has been reported to induce protease resistance in PrP [38].

The N-terminus region of PrP^C is well conserved amongst mammalian species, which is consistent with its proposed role in binding copper. In contrast, the C-terminal portion of the molecule shows more inter- and intra-species variation. In ovine PrP, polymorphisms at amino acid residues 136, 154 and 171 are associated with differences in susceptibility to natural scrapie [39,40]. Animals that express the allelic variant $Val^{136}/Arg^{154}/Gln^{171}$ (PrP^{VRQ}) or $Ala^{136}/Arg^{154}/Gln^{171}$ (PrP^{ARQ}) show susceptibility to scrapie, whereas those with $Ala^{136}/Arg^{154}/Arg^{171}$ (PrP^{ARR}) show resistance. The molecular mechanism that accounts for the variation in natural scrapie susceptibility is unknown. Clearly, critical polymorphic amino acid residues will influence the extent or stability of structural changes within ovine PrP, or its interaction with potential cofactors such as Protein X [41], as it converts from the normal to disease-associated form of PrP. It is already established that interactions at the N-terminal portion of PrP, such as metal-ion binding, influence conformational changes in the C-terminal portion of the molecule [42,43]. It is reasonable to assume therefore that different allelic variants of ovine PrP show different structural changes in response to metal-ion binding. In the present study we have investigated the effect of metal-ion interaction on the conformation of ovine PrP^{VRQ} and PrP^{ARR}. Our study shows that the interaction of copper with PrP at physiological temperature is capable of converting the α-helical forms of this protein to a more β-sheet form that displays proteinase K (PK) resistance. Significantly, copper induced a higher β-sheet content in the scrapie-susceptible genotype PrP^{VRQ} compared with that seen in the scrapie-resistant genotype PrP^{ARR}. These data have implications for the mechanism of resistance by PrP^{ARR} sheep to natural scrapie.

EXPERIMENTAL

Purification of recombinant PrP and truncated PrP peptides

Recombinant PrP was purified from BL21(DE3)pLysS bacteria transformed with the prokaryotic expression vector pET-23b (Novagen) that contained the open-reading frame encoding the sequence of full-length ovine PrP^{VRQ} or ovine PrP^{ARR} (residues 25–232), murine PrP (residues 23–231), or truncated ovine PrP^{VRQ} or ovine PrP^{ARR} (residues 89–233) in a method adapted from Hornemann et al. [44]. Briefly, transformed bacteria were grown at 37 °C in Luria–Bertani medium supplemented with 100 μg/ml ampicillin and 30 μg/ml of chloramphenicol, and induced overnight with 1 mM isopropyl β-D-thiogalactoside. Bacteria were harvested by centrifugation at 4000 ***g*** for 15 min at 4 °C, resuspended in 20 mM Tris/HCl, 50 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 10 μg/ml DNase and 1 mg/ml lysozyme, and incubated at 21 °C for 2 h prior to further lysis by sonication. Samples were centrifuged at 13 000 ***g*** for 20 min and resuspended in buffer consisting of 8 M urea/20 mM Tris/HCl (pH 8.0). The soluble fraction, collected after centrifugation at 13 000 ***g*** for 20 min at 21 °C, was applied to a nickel ion-charged Sepharose column (Pharmacia). PrP protein was eluted with 20 mM Tris/HCl/8 M urea (pH 4.5) and reduced with 100 μM dithiothreitol. PrP was further purified by application to a cation-exchange column (SP-Sepharose, Pharmacia) and eluted with 50 mM Hepes buffer (pH 8.0) containing 200 mM NaCl/8 M urea. Eluted PrP was oxidized using copper sulphate (5 times molar concentration of PrP) and refolded by dialysis into three changes of 50 mM sodium acetate buffer (pH 5.5) containing 100 mM EDTA, followed by extensive dialysis into the same buffer without EDTA. Oxidized and refolded recombinant PrP was stored at − 70 °C. Recombinant PrP proteins were verified by MS to confirm the correct protein sequence and the presence of a disulphide bond.

Generation of monoclonal antibodies

Monoclonal anti-PrP antibodies were prepared by conventional hybridoma technology. Briefly, 6-week-old $Prnp^{o/o}$ mice were immunized by subcutaneous injection with 50 μg of either refolded full-length murine recombinant PrP (residues 23–231), in the case of monoclonal antibodies 241, 242 and 249 [42] or a peptide of murine PrP (residues 161–231) in the case of monoclonal antibody 683 (A. M. Thackray and R. Bujdoso, unpublished work), emulsified in Complete Freund's adjuvant and boosted twice at monthly intervals with a similar amount of protein in Incomplete Freund's adjuvant. Mice were finally injected intravenously with antigen in PBS 3 days prior to the fusion. Spleens were removed and single cell suspensions were fused to the NS0 cell line and selected in hypoxanthine aminopterin thymidine medium. Supernatants were screened by ELISA using murine and ovine recombinant PrP as antigens, and positive cell lines were cloned by limiting dilution. Antibody was purified from hybridoma tissue culture supernatant by affinity chromatography on Protein G columns. The isotype of the monoclonal antibodies was determined by capture ELISA with anti-mouse Ig-isotype specific reagents (Sigma, catalogue no. ISO-2).

ELISA

Recombinant PrP protein at the desired concentration was coated on to 96-well flat-bottomed plates, and left overnight at 4 °C. Excess protein was removed and the wells blocked with PBS containing 5 % non-fat milk for 60 min at 37 °C. Plates were washed three times with PBS-T (PBS containing 0.1 % Tween 80). Appropriate dilutions of purified anti-PrP antibodies diluted in PBS were added and the plates incubated for 60 min at 21 °C, followed by three washes with PBS-T. Anti-mouse IgG–biotin conjugate (Sigma, catalogue no. B-7264) at 1/1000 was added for 60 min at 21 °C, followed by three washes with PBS-T then by a 1/1000 dilution of avidin–alkaline phosphatase (Sigma, catalogue no. A-7294) for 60 min at 21 °C. Plates were washed three times in PBS-T and once with ELISA buffer (0.05 M glycine/0.03 M NaOH/0.25 mM each of $ZnCl_2$ and $MgCl_2$) before addition of the substrate *p*-nitrophenyl phosphate (Sigma, catalogue no. N-2765) at 0.5 mg/ml for 30–60 min at 21 °C. ELISA plates were read at an A_{405} nm on a Dynatech MR5000 micro ELISA plate reader.

Western blot analysis of recombinant PrP

For Western blot analysis of recombinant PrP, proteins were subjected to SDS/PAGE run under reducing conditions, and were subsequently transferred to nitrocellulose membranes by semi-dry blotting. Membranes were blocked with TBS-T [10 mM Tris/HCl (pH 7.8)/100 mM NaCl/0.05 % Tween 20] plus 5 % non-fat milk, and were subsequently incubated with purified antibody for 60 min at 21 °C. This was followed by goat anti-mouse IgG–biotin (Sigma, catalogue no. B-7264) (1/1000), and finally extravidin–horseradish peroxidase (Sigma, catalogue no. E-2886) (1/1000). All antibody dilutions were in 1 % non-fat milk in TBS-T. PrP bands were detected by enhanced chemiluminescence.

Metal-ion treatment of PrP

Recombinant PrP (10 μM) was dialysed into water at 4 °C and subsequently incubated with 100 μM copper sulphate, copper

chloride, manganese chloride or nickel chloride at 37 °C for 24 h to induce β-sheet formation. Metal-ion-treated samples were transferred to 4 °C for a further 24 h incubation prior to use. Control samples of recombinant PrP were incubated with metal ions at 4 °C for 48 h in order to maintain an α-helical rich conformation. All experiments were carried out using at least two different preparations of recombinant PrP.

CD spectra

Samples of recombinant PrP at concentrations of 10 μM, with or without metal-ion treatment, were used for CD spectroscopic analyses. All samples were centrifuged at 13 000 ***g*** at 4 °C for 30 min prior to analysis. CD spectra were recorded in a 0.1 cm-length quartz cuvette at either 20 °C, or the specified temperature, under constant nitrogen flushing using a Jasco J720 spectrophotometer. At least 10 spectra were accumulated and the appropriate blanks were subtracted. Values are expressed as molar ellipticity, $[\theta]_M$. Quantitative β-sheet content was determined from deconvoluted CD spectroscopic data using the CONTINLL program [45].

PK digestion

Recombinant PrP at 200 μg/ml was treated with 10 μg/ml of PK at 37 °C for 20 h. The reaction was stopped by the addition of SDS/PAGE sample buffer and boiling. The reaction mix was assessed by SDS/PAGE and Western blotting using monoclonal anti-PrP antibodies.

SEC (size exclusion chromatography) fractionation

SEC was carried out with a Superdex 75-prep grade column (Amersham Biosciences). The column was equilibrated with 50 mM sodium acetate buffer (pH 5.5) containing 150 mM NaCl before the application of 500 μl of recombinant PrP (1 mg/ml). Separation of oligomeric states of recombinant PrP was conducted at 21 °C in 50 mM sodium acetate buffer (pH 5.5) containing 150 mM NaCl.

RESULTS

Expression and purification of ovine recombinant PrP

Prion disease-susceptible (PrP^{VRQ}) and -resistant (PrP^{ARR}) forms of ovine PrP were generated for structural analysis and metal-ion-interaction studies. Ovine full-length recombinant PrP (residues 25–232) and truncated ovine PrP (residues 89–233) were cloned by PCR, expressed in the bacterial expression vector pET-23b, and purified by metal chelate and ion-exchange chromatography using a method adapted from Hornemann et al. [44]. Murine full-length recombinant PrP (residues 23–231) was generated as control protein. Figure 1(A) shows that oxidatively refolded ovine and murine recombinant PrP, and truncated forms of ovine recombinant PrP were highly purified and of the correct apparent molecular masses, 23 kDa and 17 kDa respectively [46]. Furthermore, the samples were predominantly in the monomeric state, as determined by SEC, which verified the solubility of these forms of PrP at pH conditions used in the present study (results not shown). Far-UV CD spectral analysis was used to determine secondary protein structure of the refolded recombinant PrP molecules. Figures 1(B) and 1(C) show that the CD spectra of ovine full-length recombinant PrP and truncated ovine PrP displayed maxima UV absorbance peaks at 208 and 222 nm respectively, typical of the predominantly α-helical structure expected for ovine PrP [44,46].

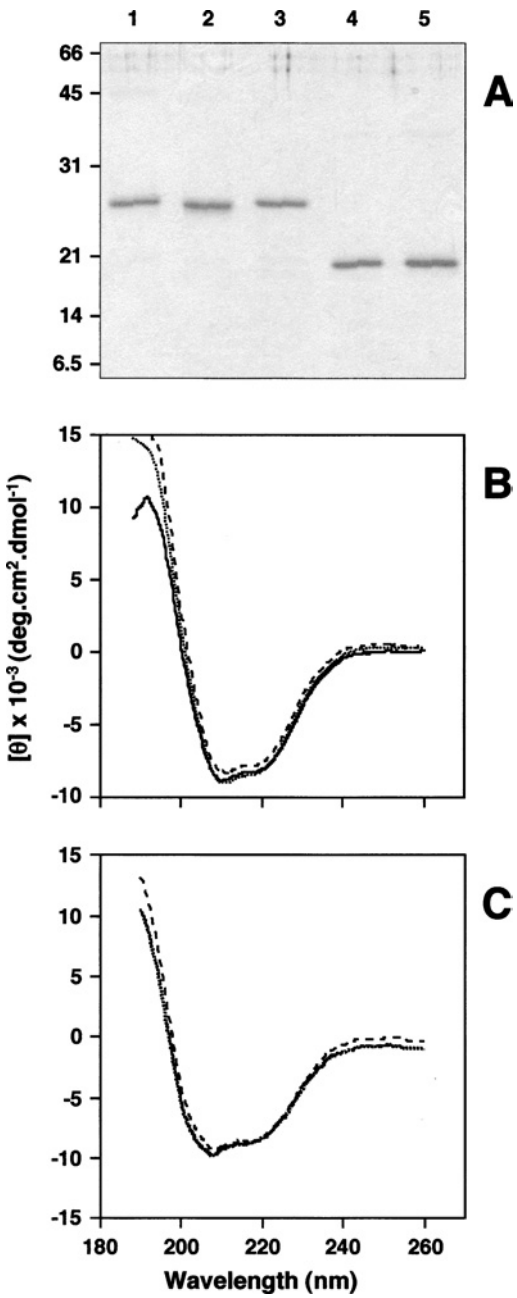

Figure 1 Purity and CD spectral analysis of refolded α-helical recombinant PrP

(**A**) PrP (200 ng) was analysed by Coomassie Blue stained SDS/PAGE. Lane 1, murine (residues 23–231); lane 2, PrP^{ARR} (residues 25–232); lane 3, PrP^{VRQ} (residues 25–232); lane 4, truncated PrP^{ARR} (residues 89–233); lane 5, truncated PrP^{VRQ} (residues 89–233). Molecular-mass-marker values (kDa) are shown on the left-hand side. CD spectral analysis was carried out on (**B**) 10 μM ovine PrP^{VRQ} (residues 25–232; dashed line); PrP^{ARR} (residues 25–232; dotted line); murine PrP (residues 23–231; continuous line), and (**C**) truncated PrP^{VRQ} (residues 89–233; dashed line); truncated PrP^{ARR} (residues 89–233; dotted line).

Although the CD spectra of both the disease-susceptible and -resistant forms of ovine PrP showed similar α-helical structure, a difference was seen in the thermal stability of these two proteins. CD spectra of full-length PrP proteins heated from 20 °C to 95 °C showed that conversion of the α-helical structure to random coil in PrP^{VRQ} occurred at a transition temperature (T_m) of 73 °C and for PrP^{ARR} at a T_m of 69 °C (results not shown). Murine recombinant PrP, which contains the amino acid residues alanine,

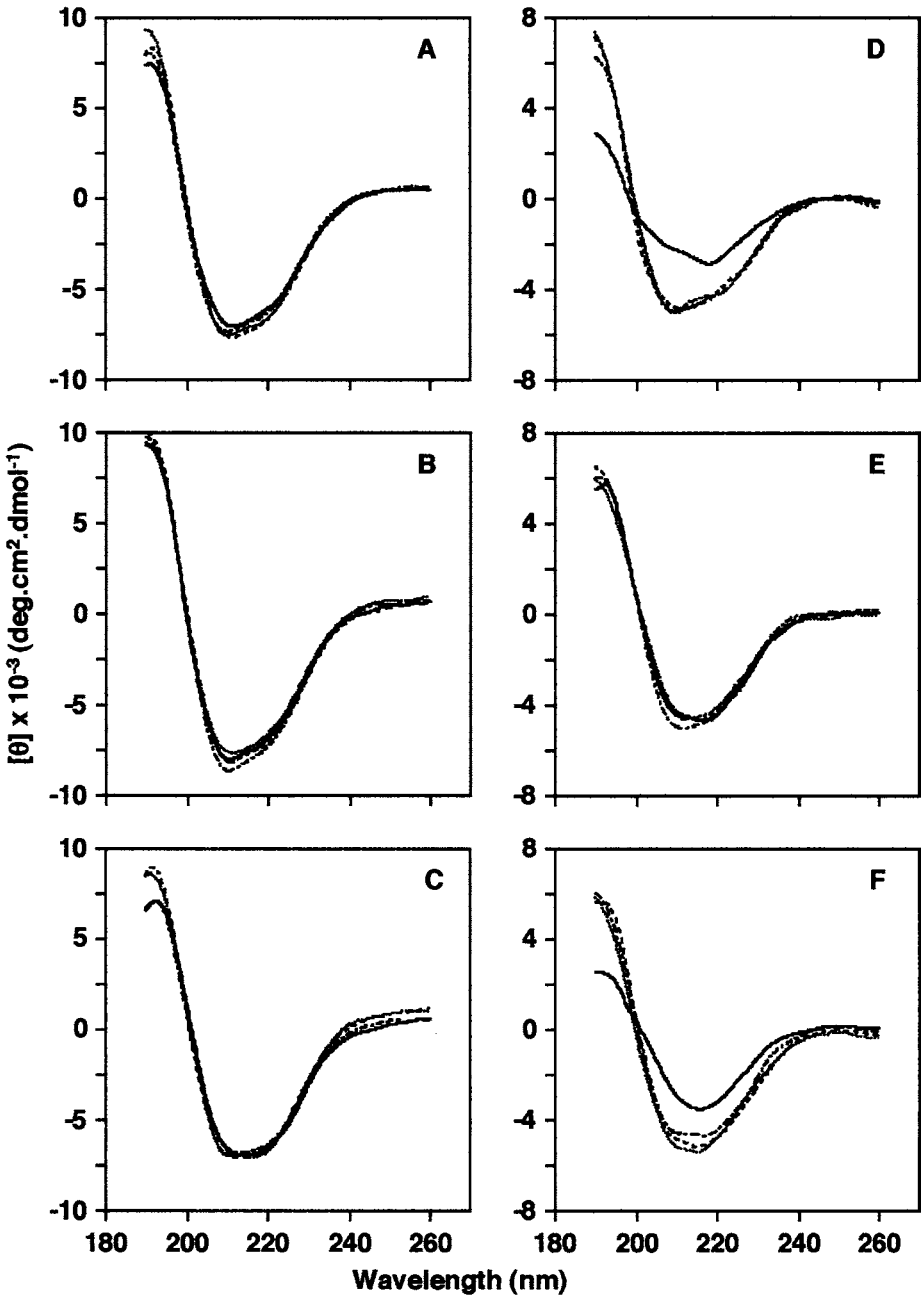

Figure 2 Effect of metal ions on the structure of ovine recombinant PrP

Full-length recombinant PrP was treated with water (dotted line), nickel (dashed line), manganese (elongated dashed line) and copper (continuous line). This was carried out at 4 °C (**A**–**C**) or 37 °C (**D**–**F**) as described in the Experimental section. Proteins were subsequently analysed by CD spectroscopy. (**A** and **D**) PrP^{VRQ}, (**B** and **E**) PrP^{ARR}, and (**C** and **F**) murine PrP.

arginine and glutamine at positions 136, 154 and 171 respectively, had a T_m of 69 °C. These results show that while the overall structure of the two allelic variants of ovine PrP was similar, PrP^{VRQ} was thermodynamically more stable and probably a more compact molecule compared with that of PrP^{ARR}. The stability of PrP^{VRQ} was a feature of the core structure of the molecule, as the truncated form of this protein displayed a similar T_m value (results not shown).

Copper induces extensive β-sheet conformation in PrP^{VRQ}

It is likely that the N-terminus region of the PrP molecule plays an important role in the overall structure of the protein. As the N-terminal of PrP contains the copper-binding octapeptide repeat region, we investigated the effect of metal ions on ovine PrP conformation. Figures 2(A)–2(C) show that full-length PrP^{VRQ}, PrP^{ARR} (residues 25–232) and murine PrP (residues 23–231) retained a predominantly α-helical conformation following incubation with various metals at 4 °C. The effect of metals on PrP was further investigated at 37 °C, as this was predicted to mimic conditions *in vivo*. When incubated with copper at 37 °C PrP^{VRQ} underwent considerable secondary structure rearrangement (Figure 2D), more so than PrP^{ARR} (Figure 2E) treated under similar conditions. The data in Table 1 show that the conformational change induced by incubation with copper at 37 °C in PrP^{VRQ} protein was reflected by an increase in β-sheet content from

Table 1 Effect of copper on the β-sheet content of PrP

Full-length recombinant PrP was treated with or without copper at 37 °C, as described in the Experimental section. Proteins were subsequently analysed by CD spectroscopy, and the β-sheet content determined from the deconvoluted data [45]. The values shown are the means ± S.D. for the percentage of β-sheet content ($n = 3$).

PrP	β-sheet content (%)		Increase in β-sheet (%)
	Without copper	With copper	
PrP^{VRQ}	21.3 ± 6.9	38.8 ± 0.3*	82.2
PrP^{ARR}	18.8 ± 7.6	21.8 ± 2.3	16.0
Murine	19.6 ± 4.0	27.7 ± 9.7	41.3

*$P < 0.05$ (PrP^{VRQ} without copper compared with PrP^{ARR} with copper).

21.3 % to 38.8 %. In contrast, PrP^{ARR} showed significantly less conformational change as a result of exposure to copper at 37 °C, as the β-sheet content was raised from 18.8 % to only 21.8 %. The level of β-sheet content in PrP^{VRQ} following treatment with copper was significantly greater ($P < 0.05$) than that in PrP^{ARR} treated in a similar manner. In response to incubation with copper at 37 °C, the β-sheet content of murine PrP increased from 19.6 % to 27.7 %. Murine PrP resembled PrP^{VRQ} protein in its response to incubation with copper at 37 °C. In this respect, it is significant that these prion disease-susceptible genotypes of PrP possess a glutamine residue at the equivalent amino acid positions, namely residue 171 of ovine PrP and residue 169 of murine PrP. The ability of copper to induce these structural changes was dependent upon incubation at 37 °C as copper treatment at 4 °C was unable to induce the changes in β-sheet content of either ovine PrP^{VRQ} or murine PrP. Significantly, other metal ions, such as nickel and manganese, which have both been reported to bind to PrP [38], failed to promote a change in the β-sheet content of either ovine PrP or murine PrP when incubated with these proteins at 37 °C or 4 °C.

It has been shown that copper can induce a conformational change in PrP that renders the protein resistant to protease digestion [29,47–50]. In the present study we investigated whether ovine PrP could adopt a similar phenotype after incubation with copper at 37 °C. Figure 3 shows that following incubation without metal ions, both PrP^{VRQ} (Figure 3A) and PrP^{ARR} (Figure 3B) recombinant proteins were readily degraded by PK. However, both forms of ovine PrP acquired PK resistance after incubation with copper at 37 °C, as revealed by the appearance of PK-resistant bands at 14 kDa and 12 kDa. These PK-resistant bands of PrP lacked the N-terminus portion of the molecule, as they were not detected in Western blot analysis using an anti-PrP monoclonal antibody reactive with an epitope within the amino acid region 25–88 of ovine PrP (results not shown). PK-resistant bands were seen with murine PrP following copper treatment at 37 °C (Figure 3C) in accordance with similar results reported by others [47]. Nickel or manganese did not induce any PK resistance in either ovine or murine PrP (results not shown).

Immunological detection of copper-induced conformational change in ovine PrP

The increase in β-sheet structure of ovine recombinant PrP^{VRQ} as a consequence of incubation with copper at 37 °C is likely to be accommodated by structural changes in the region of helix-1 and helix-2 within the central portion of PrP. This would be consistent with the view that this region of the molecule undergoes the major conformational event associated with conversion of PrP^{C} into PrP^{Sc}, believed to be reflected by conversion from the α-helical

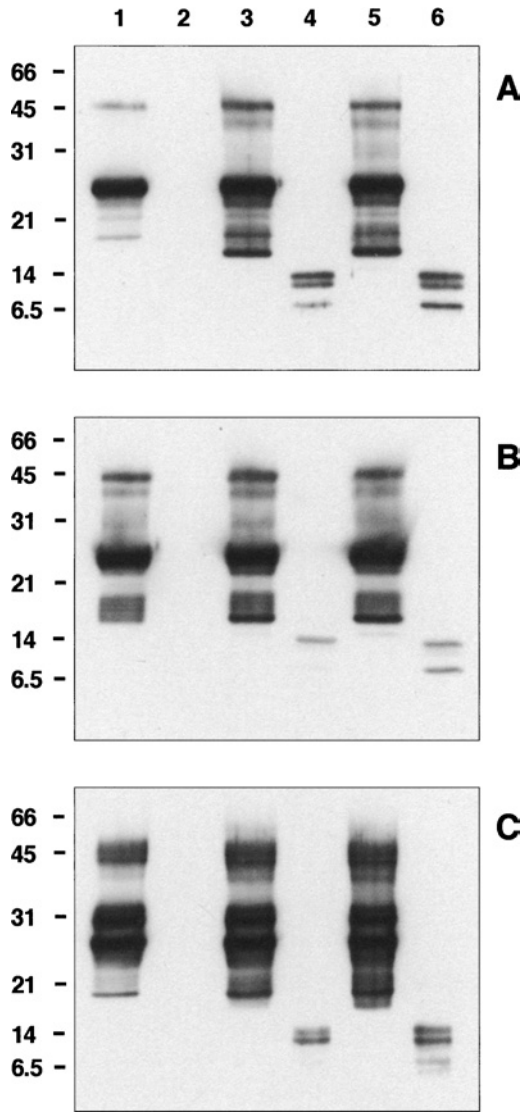

Figure 3 Copper induces the formation of PK-resistant ovine recombinant PrP

Full-length recombinant PrP (**A**) PrP^{VRQ}, (**B**) PrP^{ARR} or (**C**) murine PrP was incubated in the absence (lanes 1 and 2) or presence of copper sulphate (lanes 3 and 4) or copper chloride (lanes 5 and 6) at 37 °C as described in the the Experimental section, and analysed by Western blotting with monoclonal antibody 683 (**A** and **C**), or monoclonal antibody 249 (**B**). Lanes 1, 3 and 5, without PK digestion; lanes 2, 4 and 6, with PK digestion. Molecular-mass-marker values (kDa) are shown on the left-hand side.

into more β-sheet form of recombinant PrP. The conformational changes in PrP induced by copper were investigated by ELISA with C-terminal reactive monoclonal antibodies raised to refolded PrP [42]. Figures 4(A) and 4(B) show that monoclonal antibody 241 reacted similarly with ovine PrP^{VRQ} and PrP^{ARR} previously incubated with copper at 4 °C. In contrast, following incubation of PrP with copper at 37 °C, reactivity with monoclonal antibody 241 was diminished. The decrease in reactivity with monoclonal antibody 241 was greater with copper-treated PrP^{VRQ} (Figure 4D) compared with PrP^{ARR} (Figure 4E). This was judged by the increased amount of recombinant PrP required to achieve half-maximal binding (3.0-fold increase for ovine PrP^{VRQ} and 2.4-fold increase for ovine PrP^{ARR}). This trend was seen in two repeat experiments and is further supportive evidence that ovine PrP^{VRQ} undergoes a greater degree of conformational change than ovine

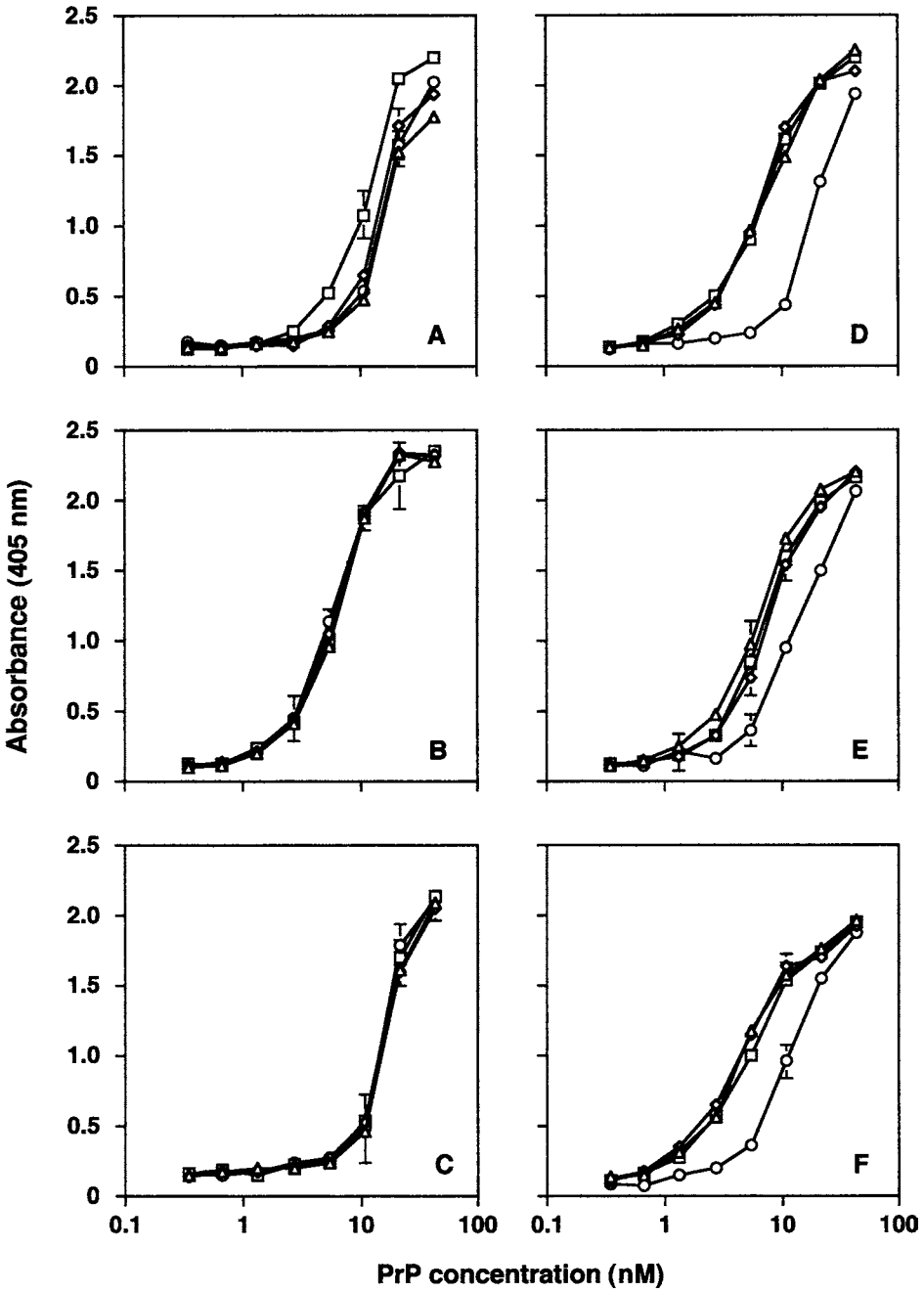

Figure 4 Immunological detection of the conversion to the β-sheet form of ovine recombinant PrP

Full-length recombinant PrP was treated with water (△), nickel (◇), manganese (□) or copper (○) at 4 °C (**A**–**C**) or 37 °C (**D**–**F**) as described in the Experimental section. Subsequently, 2-fold dilutions of PrP were analysed by ELISA using monoclonal antibody 241. (**A** and **D**), PrP^{VRQ}; (**B** and **E**), PrP^{ARR}; (**C** and **F**), murine PrP.

PrP^{ARR} does as a consequence of the interaction with copper at 37 °C. The reactivity of monoclonal antibody 241 with copper-treated murine PrP (Figures 4C and 4F) showed the same trend as seen with ovine PrP^{VRQ}. Since monoclonal antibody 241 binds in the region of helix-2 of the PrP protein [42], we predict the loss of antibody binding is a consequence of unfolding of this helix to accommodate the increased β-sheet structure within the protein.

Several other C-terminal-specific anti-PrP monoclonal antibodies reacted with copper-treated PrP in a similar manner to that seen with antibody 241 (results not shown). In contrast, monoclonal antibody 245 reacted equally well with the α-helical and the unfolded copper-treated forms of ovine PrP (results not shown). This indicates that the conversion from the α-helical to the more extensive β-sheet form of ovine PrP involves selective epitope modulation within the protein. It has been suggested that copper is capable of precipitating PrP from solution [49]. To exclude the possibility that the decrease in ELISA reactivity with copper-treated ovine PrP was due to loss of protein, similarly treated samples were analysed by Western blotting using monoclonal antibody 249 to verify the amount of PrP present in each tube. Figure 5 shows equal amounts of PrP^{VRQ} were present in all the metal-ion-treated samples and were comparable with that seen in water-treated PrP samples. Similar results were seen for ovine PrP^{ARR} and murine PrP (results not shown). This strongly suggests

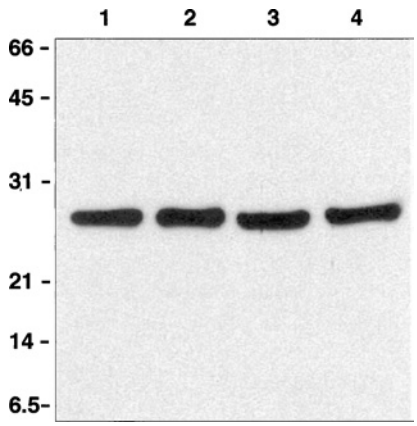

Figure 5 Copper does not induce non-specific degradation of ovine recombinant PrP

Full-length recombinant PrP^{VRQ} was incubated in the presence of water (lane 1), nickel (lane 2), manganese (lane 3) or copper (lane 4) at 37 °C, as described in the Experimental section, and analysed by Western blotting with monoclonal antibody 249. Molecular-mass-marker values (kDa) are shown on the left-hand side.

that the alteration in ELISA reactivity of PrP^{VRQ} following copper treatment at 37 °C was due to a specific conformational change within the protein, rather than a non-specific loss of protein.

Lack of structural changes in truncated PrP following copper treatment

The structural changes in PrP induced by incubation with copper at 37 °C most likely resulted in the binding of this metal ion to the N-terminal octapeptide repeat region. This was strongly supported by the fact that truncated ovine PrP, which lacked residues 25–88, did not undergo significant structural changes as a consequence of an interaction with copper. Figure 6 shows that although truncated ovine PrP^{VRQ} and PrP^{ARR} differ to some extent in overall structure, there was no change in secondary structure composition following copper treatment at 37 °C (Figures 6C and 6D) or 4 °C (Figures 6A and 6B). In addition, truncated ovine PrP^{VRQ} and PrP^{ARR} showed no alteration in reactivity with the anti-PrP monoclonal antibody 241 as a consequence of incubation with copper at 37 °C (results not shown). Collectively, these data indicate that the copper-induced changes in conformation of full-length ovine PrP reflect the binding of this metal ion to structurally relevant sites located in the N-terminus region of the molecule.

DISCUSSION

Ovine recombinant PrP^{ARR} and PrP^{VRQ} were generated via a prokaryotic expression system to investigate the stability and structural differences between these scrapie-susceptible and -resistant forms of the prion protein. Purified and oxidized forms of these

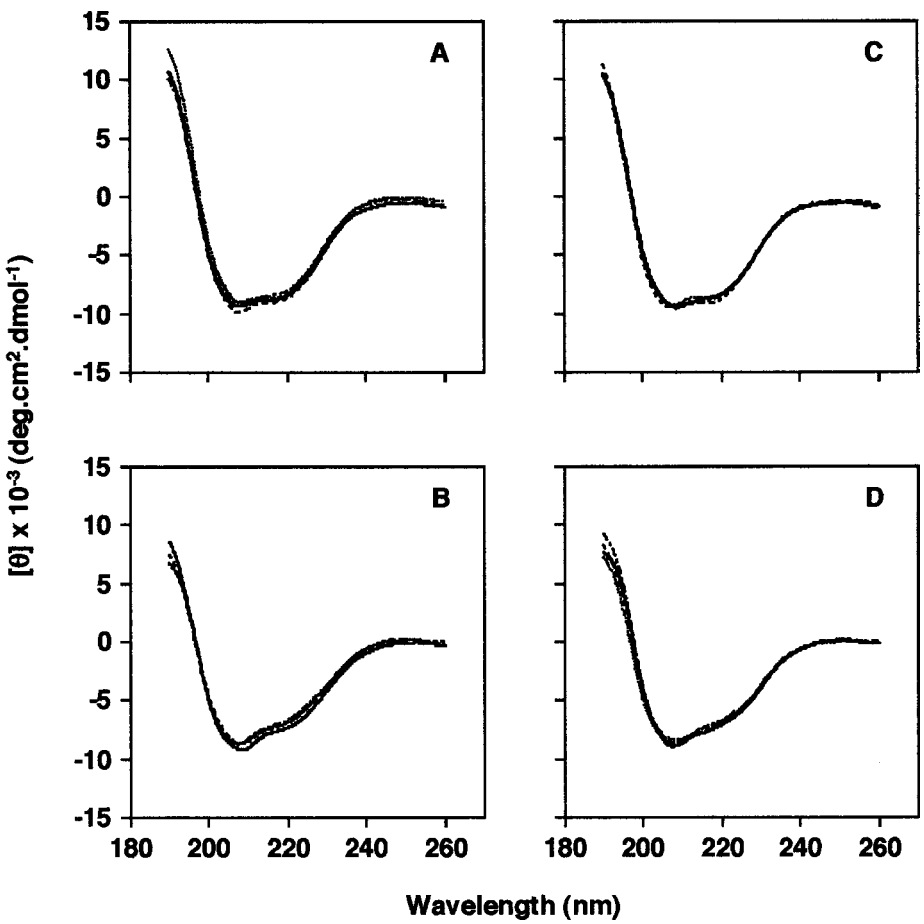

Figure 6 Lack of effect of metal ions on the structure of truncated ovine recombinant PrP

Truncated ovine PrP (residues 89–233) was incubated with water (dotted line), nickel (dashed line), manganese (elongated dashed line) or copper (continuous line) at 4 °C (**A** and **B**) or 37 °C (**C** and **D**), as described in the Experimental section. Proteins were subsequently analysed by CD spectroscopy. (**A** and **C**) truncated PrP^{VRQ}; (**B** and **D**) truncated PrP^{ARR}.

recombinant proteins were correctly refolded into predominantly α-helical conformation, as shown by CD spectral analysis, in accordance with other species forms of PrP [6,46]. SEC demonstrated that the recombinant proteins in solution were principally monomeric. The initial structural analysis of these proteins investigated their thermal stability, as judged by unfolding of the α-helical structure. Melting curves showed that PrP^{VRQ} had a higher T_m than PrP^{ARR}, indicating that the scrapie-susceptible PrP protein was thermodynamically more stable than the scrapie-resistant form, which is in agreement with previously published data for similar proteins [46]. Differences in the unfolding of the scrapie-susceptible and -resistant forms of PrP are known to exist. PrP^{VRQ} intermediates in this process exhibit β-sheet structure, whereas PrP^{ARR} display random-coil structure [51]. The stability difference between the various allelic forms of ovine PrP would appear to be a feature of the core structure of the molecule, as truncated PrP^{VRQ}, lacking the N-terminus region of the molecule, displayed a similar T_m value to that of full-length protein. Collectively, our observations, together with other reported T_m values for PrP, indicate that amino-acid substitutions in PrP that predispose a particular allelic variant to prion disease do not necessarily lead to expression of a thermodynamically unstable α-helical form of prion protein [52].

We subsequently investigated the effect of copper on the overall conformation of ovine PrP as this metal ion is believed to be a natural ligand for this protein. We compared the effect of copper on ovine PrP at 37 °C and 4 °C as others have shown that an increase in the β-sheet content of PrP may occur as a consequence of thermal incubation [29]. The CD spectral analysis of α-helical PrP incubated at 37 °C in the absence of copper showed that whereas both allelic forms of ovine PrP acquired some increase in β-sheet content, PrP^{VRQ} retained more α-helical conformation compared with ovine PrP^{ARR}. However, incubation with copper at 37 °C resulted in a significant increase in β-sheet conformation within PrP^{VRQ}, whereas PrP^{ARR} remained relatively unchanged. This effect of copper was not peculiar to PrP^{VRQ} as murine recombinant PrP also displayed a similar increase in β-sheet content compared with PrP^{ARR}. The primary structure of murine PrP resembles that of ovine PrP^{ARQ} and has the same amino acid residues at the equivalent amino acid positions 136, 154 and 171. In addition, murine PrP, similar to ovine PrP^{ARQ} and PrP^{VRQ}, all contain a glutamine residue at the equivalent amino acid position 171 in ovine PrP. As ovine PrP^{ARR} possesses arginine at amino acid 171, our data imply that this residue plays a critical role in determining the extent of β-sheet formation in ovine and other genotypes of PrP that are associated with susceptibility to prion disease. Conversion of PrP into the β-sheet form seen in the present study only occurred in the presence of copper and was not induced by other metal ions, such as nickel or manganese. In addition, no conversion to the β-sheet form was seen when PrP was incubated with any of these metal ions at 4 °C. As the conversion of α-helical recombinant PrP to the more β-sheet form is believed to represent conversion of PrP^{C} into PrP^{Sc}, these data suggest that factors other than metal-ion binding influence the rate and extent of PrP^{Sc} formation during prion disease. Furthermore, our data seem to support the view that the stability of certain intermediate structures plays a critical role in the conversion pathway of PrP^{C} to PrP^{Sc} [14,29,53–55]. In the case of ovine PrP^{VRQ}, the native cellular form of this copper-depleted protein may possess a β-sheet content similar to that of PrP^{ARR}, but may be more responsive, in terms of conformational change, when copper-loaded.

It is uncertain as to what effects copper may have on the modification of specific amino acids within PrP or to the underlying mechanism of action by copper in converting PrP from the predominantly α-helical form to that which contains more β-sheet. Qin et al. [47] proposed that copper coupled with ageing of the protein invokes the deamidation of asparagine residues (specifically Asn^{107} in mice) into aspartic acid or iso-aspartic residues, and that this is thought to be the contributing factor in the mechanism of copper-induced β-sheet-rich PrP. The differences in response to copper treatment between ovine PrP^{ARR} and ovine PrP^{VRQ} strongly imply involvement of amino acids at positions 136 and 171 in ovine PrP in structural conversions. Somewhat surprisingly, both the scrapie-susceptible and -resistant allelic variants of ovine PrP acquired PK resistance following incubation with copper at 37 °C. However, while the non-PK digested bands in the PrP^{VRQ} and PrP^{ARR} gels were of similar intensity, the PK-resistant bands for these two proteins were different. Significantly, the lower band of the 14 kDa doublet in the PK-digested PrP^{ARR} track was considerably lower in intensity than the equivalent band in the corresponding PrP^{VRQ} track. This indicates that the two genotypes of ovine PrP show different levels of PK-resistant material, which would complement the quantitative differences in β-sheet content seen with these copper-treated proteins. With respect to both allelic forms of ovine recombinant PrP, the molecular mass of the PK-resistant fragments differed from the predicted molecular mass for unglycosylated PK-digested ovine PrP^{Sc}. This suggests that the PK-resistant peptides of ovine recombinant PrP differ in conformation to ovine PrP^{Sc}. Instead, the conformation adopted by recombinant PrP following incubation with copper at 37 °C may represent an intermediate state prior to complete PrP^{Sc} formation, as exemplified with murine PrP by Quaglio et al. [49]. The acquisition of PK resistance by ovine recombinant PrP^{ARR} indicates that this allelic form of PrP, which is associated with resistance to natural scrapie, is a potential substrate for PrP^{Sc} accumulation. This has been shown to be the case with the recent report that PrP^{ARR} sheep develop prion disease following experimental inoculation with tissue that has bovine spongiform encephalopathy [56].

As the N-terminus of PrP is regarded as the principal binding site for copper ions, it would seem quite likely that the conformational changes seen in the experiments reported here arise as a consequence of the metal-ion binding to this region of the molecule. Structural events in the N-terminal region of PrP do influence conformational changes or binding events elsewhere in the molecule [43]. In the present study we have shown that the truncated form of ovine PrP^{VRQ}, which lacks amino acid residues 25–88, did not undergo the same copper-induced structural changes seen with full-length PrP^{VRQ} (25–232). Furthermore, truncated ovine PrP^{VRQ} retained a predominantly α-helical conformation following incubation with copper at 37 °C. This implies that the octapeptide repeat region within the N-terminus of PrP plays an important role, with respect to copper binding, in the conversion from α-helical to β-sheet conformation. This may account for the reduced efficiency in conversion of truncated PrP^{C} to PrP^{Sc}, and subsequently prolonged incubation time for prion disease in mice transgenic for N-terminal depleted PrP [57]. In addition, the lack of metal effect on the truncated ovine PrP proteins suggest that additional metal-binding sites in the C-terminus have limited influence on conformational conversion. A normal function of PrP has been proposed that involves the trafficking of copper-loaded protein from the cell membrane to an endosomal compartment and the recycling of copper-depleted PrP back to the cell surface. In accordance with this proposed function, our results suggest that during this trafficking event, the native structure of PrP^{C} may oscillate between a predominantly α-helical conformation and one with more β-sheet structure. The presence of copper would appear to exacerbate the degree of β-sheet formation within PrP^{C} and enable the protein to acquire PK resistance. Presumably, this event would be tightly regulated

in vivo by other cellular factors that normally prevent the accumulation of non-metabolizable PrP. The increase in PrP^{VRQ} β-sheet conformation in the presence of copper compared with that of PrP^{ARR} implies that the disease-susceptible form of ovine PrP can adopt potential intermediates in the $PrP^{C} \rightarrow PrP^{Sc}$ conversion pathway that, under some conditions, are unobtainable by the disease-resistant form.

This study shows that scrapie-susceptible PrP^{VRQ} is more compact and therefore more thermodynamically stable than the scrapie-resistant allelic form PrP^{ARR}. Studies by others have shown that PrP^{VRQ} is more resistant to enzymic degradation than PrP^{ARR}. Similarly, brain homogenate from PrP^{ARR} sheep is more susceptible to PK digestion than PrP^{VRQ} brain homogenate [58]. Furthermore, we have seen by immunostaining that more PrP^{Sc} accumulates in sheep of the PrP^{VRQ} allelic form than any other scrapie allele. This may reflect the fact that the PrP^{VRQ} allelic form of ovine PrP^{Sc} is more resistant to degradation and accumulates more quickly leading to shorter incubation time in sheep of this genotype. The implication of this, with respect to natural scrapie, is that sheep of scrapie-resistant allelic forms do not readily accumulate PrP^{Sc} because it is either unstable or metabolized by the host.

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