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Copper(II) Inhibits *In vitro* Conformational Conversion of Ovine Prion Protein Triggered by Low pH

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To gain insight into the conformational conversion of ovine prion protein ($OvPrP^C$) at different pH values and/or in the presence of $CuCl_2$, the secondary structure of $OvPrP^C$ was analysed by circular dichroism (CD) spectroscopy. Copper treatment of $OvPrP^C$ under moderately acidic conditions (pH ~5.0–6.0) as well as physiological conditions (pH 7.4) also makes $OvPrP^C$ adopt protease-resistant and β-sheet-rich conformation. However, under lower pH conditions (2.0–4.5) with copper treatment, $OvPrP^C$ gained higher α-helix structure. This study demonstrated that Cu^{2+} can significantly modulate conformational conversion triggered by acidic pH, and this will provide therapeutic intervention approaches for prion diseases.

Key words: circular dichroism spectra, conformational conversion, copper, ovine prion protein, pH, protease K.

Abbreviations: BSE, bovine spongiform encephalopathy; CD, circular dichroism; $OvPrP^C$, ovine prion protein; PK, protease K ovine prion; PrP, prion protein; TSE, transmissible spongiform encephalopathies.

Prion diseases are a variety of neurodegenerative diseases that are genetic, sporadic, infectious and affect numerous mammal species. The prion protein (PrP) is a highly conserved cellular protein attached to the neuronal cell surface through a glycosylphosphatidylinositol anchor (*1*). PrP is misfolded to cause the pathogenesis of transmissible spongiform encephalopathies (TSE) such as scrapie in sheep, bovine spongiform encephalopathy (BSE) in cow and various Creutzfeldt–Jakob diseases in humans (*2–6*). The crucial pathogenic event in prion disease propagation is the structural conversion of the benign, α-helix rich form of PrP (PrP^C) into the highly stable, β-sheet-rich PrP^{Sc} isoform associated with infectivity (*4*, *7*, *8*). The widely accepted 'protein-only' hypothesis holds that PrP^{Sc} is itself infectious, interacting with normal PrP^C molecules and inducing them to switch their conformations in a fatal chain reaction.

The $PrP^C \rightarrow PrP^{Sc}$ conversion results in the alteration of some of the physical and biochemical traits of the protein including a reduction in solubility, an increase in resistance to protease K (PK) hydrolysis and change from monomeric form of PrP^C to multimeric form of PrP^{Sc}. In cells infected with the TSE agent, PrP^C is converted to PrP^{Sc} on the cell surface or in endosomes as a result of endocytosis. This working model is supported by the results of *in vitro* studies in which recombinant forms of human and murine PrP^C undergo a pH-dependent conformational change at low pH ranging from 4.4 to 6.0 in a cell-free conversion assay (*9*), with a loss of α-helix and gain of β-sheet structure. Thus, low pH may play a role in facilitating the conformational change that ultimately results in PrP^{Sc} formation.

Recent advances in PrP structural studies have revealed that the N-terminal half of the PrP protein is largely unstructured, and the C-terminal half is well folded and consists of three α-helices and two short β-sheets (*8*, *10–12*). The N-terminus of PrP^C contains multiple highly conserved repeating sequences of eight amino acid residues, named 'octapeptide repeats' (OP) that can bind up to four Cu^{2+} with high specificity (*13–17*). In addition to OP units, more Cu^{2+}-binding sites identified in the central region of PrP can also bind Cu^{2+}. The binding of PrP to Cu^{2+} is pH-dependent (*14*, *18–20*). The biological significance of the PrP-specific binding affinity for Cu^{2+} has been suggested by both *in vivo* and *in vitro* studies. The presence of copper could reduce the accumulation of PrP^{Sc} in scrapie-infected neuroblastoma cells, and scrapie-infected animals with copper treatment resulting in a substantial delay in prion disease onset (*4*, *15*, *21*). In addition, studies *in vitro* showed that Cu^{2+} can induce PrP conformational changes and convert PrP into a protease-resistant species distinct from the scrapie isoform and inhibit the formation of amyloid fibrils of PrP (*22–24*).

In this study, the recombinant ovine prion protein ($OvPrP^C$), cloned from Chinese little-fat-tail sheep with the scrapie-susceptible genotype ARQ (Ala136/Arg154/Gln171), was expressed in *Escherichia coli*, and was used to characterize the conformational properties of $OvPrP^C$ under different pH conditions in the presence of Cu^{2+}, to investigate the physiological relevance of $OvPrP^C$ binding to copper.

MATERIALS AND METHODS

Unless stated otherwise, all chemicals were purchased from Sigma (USA).

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Expression and Refolding of Recombinant OvPrP^C—The cDNA encoding the ARQ (Ala136/Arg154/Gln171) genotype of Chinese little-fat-tail OvPrP^C domain (residues 23–256), which was marked by OvPrP^C, was expressed and purified following the method of Eghiaian *et al*. (*10*). The protein was as inclusion body and need to be refolded. Refolding was done with chaotropic agents' concentration gradient dialysis. The solution of denatured protein (0.2 mg/ml) was dialysed against 2 l of freshly made 4, 2, 1, 0.5 and 0 M guanidine–HCl; or 6, 4, 2, 1, 0.5 and 0 M urea, respectively, with 5 mM Tris (pH 7.4). With each concentration, the protein was dialysed 12 h at 4°C in dialysed sack.

CD Spectrum of OvPrP^C in PBS Buffer at Various pH—In order to probe if there is conformational conversion of the OvPrP^C at various pH, the buffer containing the protein was adjusted by HCl or NaOH to reach certain point (pH 10.0, 8.0, 7.4, 6.0, 5.5, 5.0, 4.5, 4.0, 3.6, 3.0, 2.6 and 2.0). The proteins at concentrations of 0.3–0.4 mg/ml were placed in a 0.1 cm quartz cuvette, and CD spectra were recorded at wavelengths ranging from 190 to 250 nm at 25°C, under constant nitrogen gas purge, using a Jascow J715 spectrophotometer (Japan). At least eight scans of every spectrum were accumulated, and quantitative conformation content was determined using the CONTINLL Program of Jascow32 for Windows Secondary Structure Estimation.

Effect of pH and Copper(II) Chloride on the Conformational Conversion of OvPrP^C—To investigate whether $CuCl_2$ can modulate the conformational conversion of OvPrP^C at various pH values, the OvPrP^C protein was treated in PBS (phosphate-buffered saline) buffer under different pH conditions (2.6, 3.0, 4.0, 4.5, 5.0, 5.5, 6.0, 7.4), followed by the addition of $CuCl_2$ to achieve a copper ion concentration of 20 μM, incubated at 37°C for 1 h, and then was subjected to CD spectral analysis. To indicate the time dependent, the conformational changes of OvPrP^C proteins treated by copper and pH at different incubation time (5 min, 10 min, 15 min, 30 min, 1 h, 6 h, 12 h) were also analysed by CD spectra.

Data Analysis of Structural Content—Structural content data were analysed using statistical analysis software SPSS 13.0 (SPSS Inc, Chicago, IL, USA). Data is expressed as mean ± SEM (standard error mean). To analyse the results, one-way analysis of variance (ANOVA) was used. Differences with $P < 0.05$ were considered statistically significant.

Proteinase K Digestion Assay—It has been shown by others that acidic conditions and copper treatment can induce PrP conformational changes and lead to the PK digestion-resistant phenotype of mouse and human PrP. To determine whether Cu^{2+} treatment of OvPrP^C and acidic conditions could cause similar protease digestion resistance, OvPrP^C treated at different pH conditions, or in the presence of Cu^{2+}, was subjected to digestion by proteinase K. OvPrP^C at 0.4 mg/ml was mixed with proteinase K at final concentration of 20 μg/ml, and incubated in digestion buffer (0.1% $CaCl_2$, 20 mM Tris–Cl buffer, pH 8.0) for 40 min at 48°C. The proteolytic reaction was stopped by the addition of digestion-stopping buffer (0.174 g PMSF solved in 50 ml isopropyl alcohol), and the digested samples were analysed by western blot assay to test for recognition by the PrP conformation-specific monoclonal antibody, BE12 (Compton, UK; 1:1000 dilution) and the membrane was stained with DAB (3,3′-diaminobenzidine) (Roche, USA) and scanned to collect image data.

RESULTS AND DISCUSSION

In the current study, in order to reveal the pathogenetic mechanism of prion diseases (*2–4*, *15*), we have shown that recombinant ovine PrP^C undergoes a pH-dependent conformational change with a drastic loss of α-helix and gain of β-structure at pH 2.0–5.5. Compared to the CD spectra of OvPrP^C at pH 7.4 with double intensive negative band at 208 and 222 nm respectively, the CD

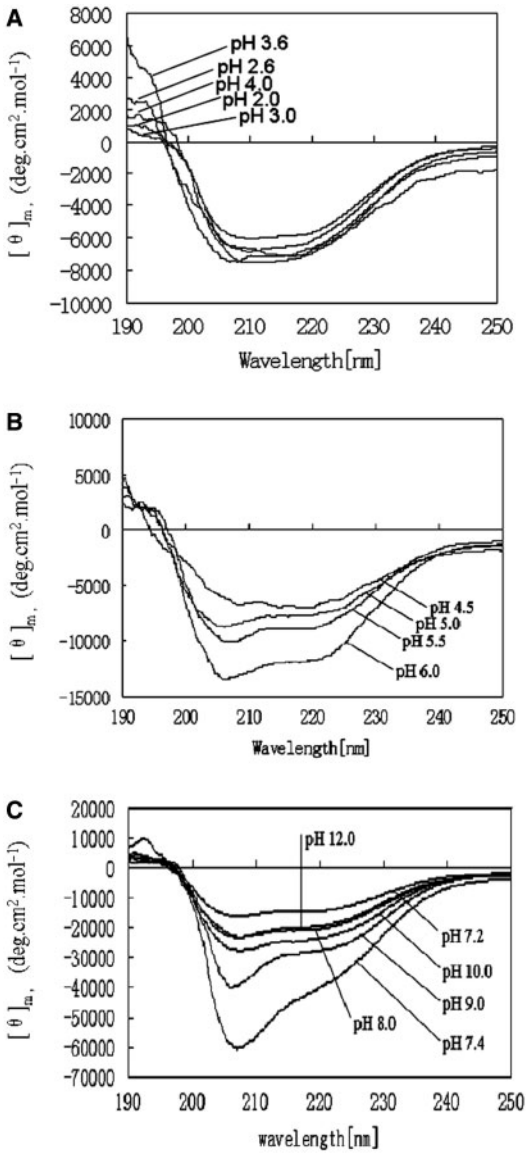

Fig. 1. **Circular dichroism spectra of OvPrP^C at various pH.** (Panel A): Spectra at acidic pH (2.6, 3.0, 3.6, 4.0); (Panel B): Spectra at moderate acidic pH (4.5, 5.0, 5.5, 6.0); (Panel C): spectra at neutral and basic pH (7.2, 7.4, 8.0, 9.0, 10.0, 12.0).

Table 1. **Effect of $CuCl_2$ treatment at pH 7.4 on $OvPrP^C$ secondary structure content.***

Treatment	Secondary structure component (%)			
	α Helix	β Sheet	Turn	Freedom curl
No Cu^{2+}	35.77 ± 4.737^a	1.80 ± 1.800^b	10.17 ± 10.167^a	52.23 ± 7.670^a
Cu^{2+}	16.83 ± 0.203^b	35.77 ± 1.484^a	17.53 ± 0.689^a	29.87 ± 1.011^b

*Secondary structure content was determined by the CD spectra analysis as described in the METHODS AND MATERIALS section. Each measurement is repeated at least three times. The secondary structure content is presented as the mean ± SEM (standard error mean). The different letters indicated that differences were statistically significant with $P < 0.05$ within each column.

spectra of $OvPrP^C$ at acidic pH (2.0, 2.6, 3.0, 3.6, 4.0) displayed a single even negative band at 215 nm (Fig. 1A), which reflect the typical profiles of β-sheet-rich conformation. At modest acidic pH (4.5, 5.0, 5.5, 6.0), the spectra showed a decreased yet detectable maxima UV absorbance peaks at 208 and 222 nm, compared to that at pH 7.4 (Fig. 1B). In contrast, at basic pH (7.2, 7.4, 8.0, 9.0, 10.0 and 12.0), the spectra were similar, with double maxima UV absorbance peaks at 208 and 222 nm (Fig. 1C), suggesting α-helix pre-dominant secondary structures. This is consistent with the previous studies, which reported that acidic pH can accelerate the conformational conversion of recombinant forms of human and murine PrP^C at pH 4.4–6 (*11*, *22*). Moreover, in our study, the recombinant $OvPrP^C$ can also be used to disclose the conformational changes of PrP^C binding to RNA *in vitro* study (*25*).

Conformational conversion of the $OvPrP^C$ in the presence of $CuCl_2$ in PBS buffer (pH 7.4) was analysed by CD spectroscopy, which suggested considerable secondary structure rearrangement. The secondary structure content analysis data indicated that the β-sheet conformation increased overtly from 1.8% to 35.77% in $OvPrP^C$ after copper treatment (Table 1). This observed conformational conversion of $OvPrP^C$ in the presence of $CuCl_2$ is consistent with earlier studies (*26*).

The CD spectra of $OvPrP^C$ binding to copper at pH 5.5 and 7.4 showed a single even negative band at 210–215 nm, with maxima UV absorbance peaks decreased at 208 and 222 nm, which suggested a β-sheet-rich conformation (Fig. 2A). However, at lower pH (4.0, 4.5), the CD spectra showed double negative band at 208 and 222 nm, α-helix content tends to increase and β-sheet content significantly decrease (Fig. 2B). At even lower pH (2.6, 3.0), the CD spectra showed typical double negative band at 208 nm and 222 nm (Fig. 2B), the α-helix content holds the same, with the abolishment of β-sheet content and relatively high content of random curl (Table 2). These data suggested that Cu^{2+} could inhibit the β-sheet-rich conformation of $OvPrP^C$ at acidic pH condition. However, the β-sheet-lacking conformation of $OvPrP^C$ in the presence of Cu^{2+} and acidic pH seems to be distinct from the native conformation of $OvPrP^C$ at pH 7.4, with the former having lower α-helix and higher turn contents (Table 2). CD spectra of $OvPrP^C$ at different incubation time after copper and pH treatment showed that conformational changes with high β-sheet at 1 h, and the difference was not statistically significant at 1, 6 and 12 h. CD spectra is shown in Fig. 3. The β-sheet-rich conformation increased as time dependent treated by Cu^{2+} and pH, which is consistent with previous research (*19*).

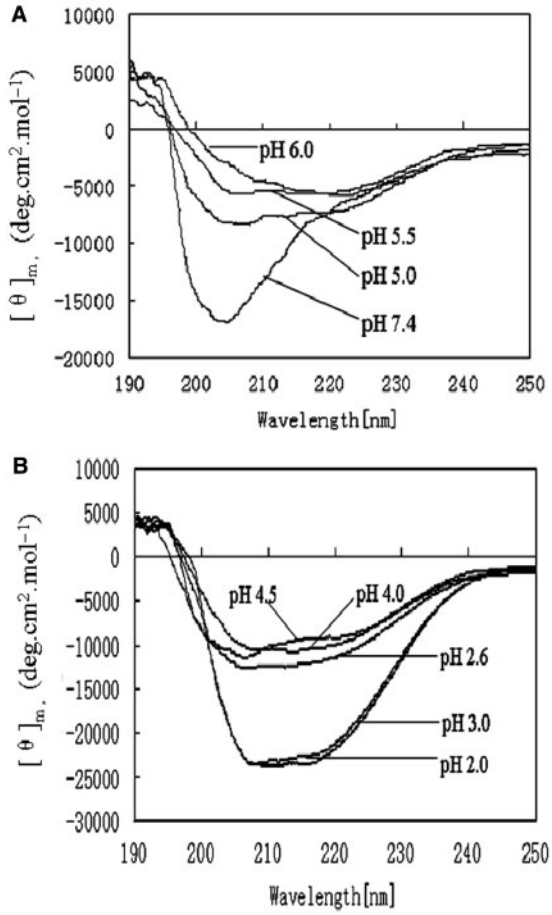

Fig. 2. **Circular dichroism spectra of $OvPrP^C$ with both pH and $CuCl_2$ treatments.** (A) Cu^{2+} treatment at pH 5.0, 5.5, 6.0 and 7.4; (B) Cu^{2+} treatment at pH 2.0, 2.6, 3.0, 4.0 and 4.5.

Taken together, our study suggests that Cu^{2+} could prevent the β-sheet-rich conformation of $OvPrP^C$ at acidic pH condition.

The $OvPrP^C$ treated with Cu^{2+} and/or moderately acidic conditions, was tested for the PK digestion-resistant phenotype as showed in Fig. 4. The native $OvPrP^C$ at pH 7.4 was readily degraded by PK, with the disappearance of the 25 kDa band (Fig. 4, Lane 2) probed by monoclonal antibody BE12. However, $OvPrP^C$ acquired PK resistance after Cu^{2+} treatment, as revealed by the appearance of PK-resistant bands at the size of 25 kDa (Fig. 4, Lane 3). This PK-resistant phenotype of $OvPrP^C$ can also be observed under moderately acidic conditions (pH 5.5) alone (Fig. 4, Lane 4) and coupled

Table 2. **Effect of pH and $CuCl_2$ on $OvPrP^C$ secondary structure content.**[a]

pH value	Secondary structure component (%)			
	α Helix	β Sheet	Turn	Freedom curl
2.6	26.20 ± 0.231*	0.00*	21.10 ± 1.563	52.70 ± 1.778
3.0	29.50 ± 0.577*	0.00*	13.17 ± 0.441	57.37 ± 0.448
4.0	26.40 ± 0.115*	5.60 ± 0.115*	23.03 ± 0.606	44.97 ± 0.745
4.5	20.90 ± 0.115	11.20 ± 0.519*	18.00 ± 0.625	49.90 ± 0.153
5.0	15.60 ± 0.289	31.60 ± 0.346	11.50 ± 0.361	41.30 ± 0.987
5.5	14.10 ± 0.577	39.20 ± 0.625	12.53 ± 0.291	34.17 ± 0.869
6.0	15.90 ± 0.971	38.13 ± 0.984	13.00 ± 0.577	32.97 ± 2.313
7.4	12.80 ± 2.364	37.50 ± 2.050	9.67 ± 5.356	40.03 ± 6.190

[a]Secondary structure content was determined by the CD spectra analysis as described in the METHODS AND MATERIALS section. Each measurement is repeated at least three times. The secondary structure content is presented as the mean value of secondary content plus standard deviation. *$P < 0.05$ when compared with the content without Cu^{2+} treatment, at pH 7.4.

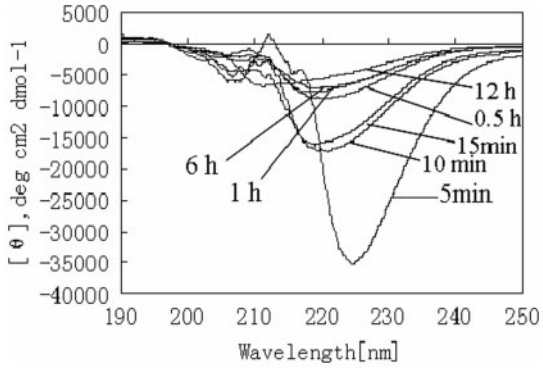

Fig. 3. **Circular dichroism spectra of $OvPrP^C$ binding $CuCl_2$ at different pH for different incubation time (5 min, 10 min, 15 min, 30 min, 1 h, 6 h, 12 h).**

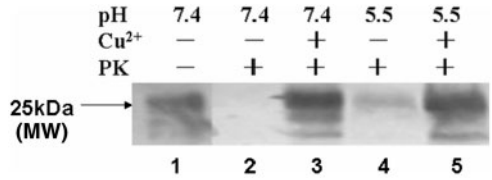

Fig. 4. **Western blot analysis of $OvPrP^C$ proteinase K (PK) degradation products after acidic pH and copper treatment.** MW, molecular weight.

with Cu^{2+} treatment (Fig. 4, Lane 5), which makes the PK-resistant phenotype more pronounced. These results are in agreement with the related study (*22–24*, *26*). Structural analysis by Raman spectroscopy has revealed that Cu^{2+} binding was over a pH range from 5.0 to 8.2 (*20*).

Our study demonstrated that, like the PrP in other species, the presence of copper ion at pH 5.0–7.4 could also promote ovine PrP to undergo conformational conversion with a great gain of β-sheet-rich conformation and PK-resistant phenotype. However, whether this β-sheet-rich conformation and PK-resistant phenotype form of ovine PrP is similar to or distinct from the scrapie isoform of ovine PrP still remains unclear. Further investigations of the biological function of these conformational isoforms would help to delineate the effects of copper ion on ovine PrP conformation. This modulation may be due to Cu^{2+} binding directly to ovine PrP under acidic conditions such as pH 4.5 and subsequently stabilizing the N-terminus of PrP. It has been demonstrated that PrP from different species could coordinate Cu^{2+} with different capabilities under lower pH conditions (*27*, *28*).

CONCLUSION

In summary, our study demonstrated that Cu^{2+} can significantly modulate conformational conversion triggered by acidic pH conditions in ovine PrP, which has scrapie-like phenotypes such as PK resistance and β-sheet-rich structure. These results could provide new insight into the PrP conformation conversion process and help to develop therapeutic intervention approaches for prion diseases.

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