Distinct effects of Cu²⁺-binding on oligomerization of human and rabbit prion proteins

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Received 22 April 2015; Accepted 12 June 2015

Abstract

The cellular prion protein (PrPC) is a kind of cell-surface Cu²⁺-binding glycoprotein. The oligomerization of PrPC is highly related to transmissible spongiform encephalopathies (TSEs). Cu²⁺ plays a vital role in the oligomerization of PrPC, and participates in the pathogenic process of TSE diseases. It is expected that Cu²⁺-binding has different effects on the oligomerization of TSE-sensitive human PrPC (HuPrPC) and TSE-resistant rabbit PrPC (RaPrPC). However, the details of the distinct effects remain unclear. In the present study, we measured the interactions of Cu²⁺ with HuPrPC (91–230) and RaPrPC (91–228) by isothermal titration calorimetry, and compared the effects of Cu²⁺-binding on the oligomerization of both PrPs. The measured dissociation constants (K_d) of Cu²⁺ were 11.1 ± 2.1 μM for HuPrPC and 21.1 ± 3.1 μM for RaPrPC. Cu²⁺-binding promoted the oligomerization of HuPrPC more significantly than that of RaPrPC. The far-ultraviolet circular dichroism spectroscopy experiments showed that Cu²⁺-binding induced more significant secondary structure change and increased more β-sheet content for HuPrPC compared with RaPrPC. Moreover, the urea-induced unfolding transition experiments indicated that Cu²⁺-binding decreased the conformational stability of HuPrPC more distinctly than that of RaPrPC. These results suggest that RaPrPC possesses a low susceptibility to Cu²⁺, potentially weakening the risk of Cu²⁺-induced TSE diseases. Our work sheds light on the Cu²⁺-promoted oligomerization of PrPC, and may be helpful for further understanding the TSE-resistance of rabbits.

Key words: Cu²⁺, prion oligomerization, affinity, conformational stability, TSE-resistance

Introduction

Prion proteins (PrPs) are cell-surface Cu²⁺-binding glycoproteins that are responsible for prion diseases, the transmissible spongiform encephalopathies (TSEs) [1,2]. TSEs are fatal neurodegenerative diseases that are associated with the conversion of α-helix-rich cellular PrP (PrPC) into β-sheet-rich scrapie form (PrPSc) [1,3–5]. The insoluble PrPSc has been treated as the pathogenic agent for many years. However, growing investigations suggest that the infectious prion is probably the soluble oligomer, the intermediate of the conformational transformation from PrPC to PrPSc [6–8]. Thus, the oligomerization of PrPC is believed to be a vital step in the pathogenic processes of prion diseases.

As an essential trace metal, copper plays fundamental roles in biological functions of the nervous system [9,10]. The central nervous system has high level of copper, and the brain comprises 2% of the total body mass but containing 7.3% of total body copper [11,12]. The imbalance of copper has been linked to neurodegenerative diseases, including prion diseases [13,14]. Increasing evidence supports the
participation of Cu²⁺ in the pathogenic processes of prion diseases [15–17]. Previous studies showed that Cu²⁺-binding-induced protease resistance of PrP [18–21], and promoted conformational conversion of monomeric PrP⁰ into neurotoxic PrP oligomer [22]. At acidic condition, Cu²⁺ could promote the conversion of α-helical PrP⁰ into β-sheet oligomer with neurotoxicity. Furthermore, the Cu²⁺-induced reactive oxygen species could also lead to conformational transition and aggregation of PrP [23]. Therefore, Cu²⁺-binding plays a vital role in the oligomerization process of PrP⁰.

Many mammals have been reported to be TSE-sensitive species with the potential of converting conformation of PrP from PrP⁰ to PrP²⁰, such as human, cattle, sheep, and mice [24–26]. On the contrary, rabbits are one of the few species that are resistant to prion diseases and have a low susceptibility to be infected by PrPSc [27]. The resistance of rabbits to TSEs is highly associated with the difference of the conformational conversion of rabbit PrP²⁰ (RaPrP²⁰) mostly due to the unique structural characteristics of RaPrP⁰ [33,34]. The local structures of the copper-binding globular regions of HuPrP⁰ and RaPrP⁰ have been accessed by X-ray absorption fine structure technique and X-ray absorption near-edge structure technique [35,36]. The three-dimensional structures of copper-binding sites in HuPrP⁰ (91–231) and RaPrP⁰ (91–228) show substantial differences in both the coordination of amino acid residues and the copper bond. The copper bond in RaPrP⁰ is longer than that in HuPrP⁰, implying less Cu²⁺-binding-induced conformational change of RaPrP⁰ compared with that of HuPrP⁰ [36]. It is thereby expected that Cu²⁺-binding has distinctly different effects on the oligomerization of TSE-sensitive HuPrP⁰ and TSE-resistant RaPrP⁰. However, so far the distinct effects have not been addressed in detail. Obviously, the detailed roles played by Cu²⁺ in promoting oligomerization processes of both PrPs would be of great benefit to the understanding of the mechanism of TSE-resistance of rabbits.

In the present study, we measured the interaction of Cu²⁺ with HuPrP⁰ and RaPrP⁰ by isothermal titration calorimetry (ITC), and analyzed the Cu²⁺-binding-promoted oligomerization of HuPrP⁰ and RaPrP⁰ by fast protein liquid chromatography (FPLC). We then compared the distinct effects of Cu²⁺-binding on the secondary structures and conformational stabilities of HuPrP⁰ and RaPrP⁰ by circular dichroism (CD) spectroscopy.

Materials and Methods
Plasmid construction, protein expression, and purification
The gene fragment encoding either HuPrP⁰ (91–230) or RaPrP⁰ (91–228) was inserted into the vector pET-30a via NdeI and XhoI restriction sites and then transformed into Escherichia coli BL21 (DE3) strain. The cells were cultured in 2x YT medium containing 0.05 mg/ml kanamycin at 37°C. When the A₆₀₀nm reached between 0.8 and 1.0, 1.0 mM isopropyl-β-D-thiogalactoside was added and cells were grown for another 4 h. Then, cells were harvested by centrifugation at 9000 rpm for 4 min at 4°C, and re-suspended in the buffer (20 mM Tris, 2 mM EDTA, 150 mM NaCl, 100 μg/ml lysozyme, 0.1% Triton X-100, 1 mM PMSF, pH 7.4), followed by sonication for 30 min. After centrifugation at 11,000 rpm for 25 min at 4°C, the inclusion bodies were collected and washed with the buffer (20 mM Tris, 150 mM NaCl, 0.5% Triton X-100, 2 M NaCl, 4 M urea, pH 7.4). The resulting pellets were dissolved in the buffer (10 mM Tris, 100 mM Na₂HPO₄, 0.5% Triton X-100, 10 mM β-Mercaptooethanol, 6 M GdnHCl, pH 8.0) and refolded by dialysis using the buffer (10 mM Tris, 100 mM Na₂HPO₄, pH 8.0) with a linear gradient 6–0 M GdnHCl. The resulting protein solution was purified by FPLC on a 10/300 Superdex 75 column (GE Healthcare, Wisconsin, USA) with the elution buffer (20 mM NaOAc, 0.02% NaN₃, pH 5.5). The protein purity was assessed by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The purified protein solution was concentrated by ultra centrifugation and the protein concentration was determined using NanoVue plus (GE Healthcare) at 280 nm with the extinction coefficient of 22 × 10³ M⁻¹ cm⁻¹ calculated from the amino acid sequences of HuPrP⁰ (91–230) and RaPrP⁰ (91–228) by the web-based tool provided by ExPasy.

Size exclusion chromatography
Size exclusion chromatography was performed using AKTA FPLC equipment (GE Healthcare). Five column volumes of elution buffer were used to equilibrate the column prior to the experiments. The flow rate was 0.4 ml/min and the protein elution was monitored by ultraviolet absorption at 280 nm.

Oligomeric prion preparation
The purified HuPrP⁰ (91–230) and RaPrP⁰ (91–228) proteins were diluted to 40 μM in the buffer (20 mM NaOAc, 150 mM NaCl, 0.02% NaN₃, pH 4.0). The samples were kept in the thermostatic water bath at 37 and 42°C for 80 min without or with 40–480 μM of Cu(OAc)₂, respectively. Products were further separated by 10/300 Superdex 75 column (GE Healthcare) for the subsequent experiments. Data were processed with the software Unicorn 5.2.

Isothermal titration calorimetry
The dissociation constant (Kd) and stoichiometry (N) of the interaction between Cu²⁺ and PrP⁰ was measured by ITC using an ITCA200 calorimeter (GE Healthcare). Calorimetric titration of Cu²⁺ to PrP⁰ was performed at 25°C in acetate buffer (20 mM NaOAc, pH 5.5). Time between injections was 150 s. ITC data were analyzed by integrating the heat effects after the data were normalized to the amount of injected protein. Data fitting was conducted to determine the dissociation constant and stoichiometry based on a single-site binding model using the Origin software package (GE Healthcare).

CD spectroscopy
All CD spectra were recorded on a Jasco J-810 spectropolarimeter (Jasco, Tokyo, Japan) interfaced with a Peltier-type temperature control at 25°C. The reported spectra were an average of three consecutive scans and blanked with respective buffers. Far-UV CD spectra were collected in the wavelength range of 200–250 nm using 1-mm path length. The spectra were recorded in continuous scanning mode at a scanning rate of 50 nm/min with a step size of 0.2 nm and a bandwidth of 1 nm. To conduct urea-induced unfolding transition experiments, the range of urea concentrations up to 10 M was used with 0.5 M gradient. The Cu(OAc)₂ stock solution was added into the protein solution prior to titrating the stock solution of urea.
Results

Cu2+ bound to PrPC with moderate affinity

For the experiments of metal binding to PrP, it is very important that the protein should be in its apo state. We thus measured the metal content by using inductively coupled plasma mass spectrometry. Our results showed that the protein samples did not contain any metal atoms and were indeed in the apo state (data not shown).

The thermodynamic parameters of equilibrium interaction between protein and ligand can be accurately determined by ITC [37]. ITC experiments were carried out to measure the interactions of Cu2+ with HuPrPC (91–230) and RaPrPC (91–228) at pH 5.5 (Fig. 1 and Table 1). The Cu2+ titration yielded positive heat deflection in the ITC curve, indicating that the Cu2+ titration progress was associated with the endothermic reaction (Fig. 1). Both HuPrPC (91–230) and RaPrPC (91–228) bound Cu2+ with a binding stoichiometry (N) close to 1, suggesting that one PrPc molecule bound one Cu2+ molecule (Table 1). The results derived from the ITC curves showed that Cu2+ had moderate affinities for both proteins at mild acidic condition (pH 5.5). The dissociation constant (Kd) of HuPrPC–Cu2+ interaction was 11.1 ± 2.1 μM, while that of RaPrPC–Cu2+ was 21.1 ± 3.1 μM, indicating that HuPrPC bound Cu2+ with an affinity higher than RaPrPC.

To confirm that the proteins remained monomeric during ITC experiments with very rapid stirring, size exclusion chromatography was used to analyze the protein samples. The results showed that the proteins did remain monomeric (Supplementary Fig. S1), indicating that both HuPrPC (91–230) and RaPrPC (91–228) did not aggregate during the ITC experiments.

Cu2+-binding promoted the oligomerization of PrPc

The size exclusion chromatography was applied to analyze the percentages of PrPc monomers and oligomeric PrP (PrPO) oligomers. The peaks around 7 and 13 ml represented the oligomeric PrPc and monomeric PrPc, respectively (Fig. 2A). The absence of Cu2+, the oligomerization was observed in acidic buffer (20 mM NaOAc, 150 mM NaCl, 0.02% NaN3, pH 4.0). The percentages of HuPrPO and RaPrPO were 62.2% and 23.8%, respectively. This result illustrated that more HuPrPc proteins were formed compared with RaPrPc proteins. Expectedly, in the presence of Cu2+, the percentage of monomer was decreased significantly whereas the percentage of oligomer was increased markedly (Fig. 2A), indicating that Cu2+-binding significantly promoted the oligomerization of PrPc. Moreover, the oligomerization of PrPc was dependent on the Cu2+ concentration (Fig. 2B). After incubation with 1 molar equivalent (eq.) of Cu2+ (40 μM) at 42°C, the HuPrPO percentage was 81.9% with an increase of 19.7%, while the RaPrPO percentage was 32.6% with an increase of 8.8% only (Fig. 2C). Furthermore, when incubated with 12 eq. of Cu2+, the HuPrPO percentage was increased by 29.2%, whereas the RaPrPO percentage was increased by only 24.8%. These results showed that Cu2+-binding promoted the oligomerization of HuPrPc more significantly than that of RaPrPc at 42°C.

Table 1. Binding parameters derived from the ITC curves of monomeric HuPrPC (91–230) and RaPrPC (91–228) titrated with Cu2+

<table>
<thead>
<tr>
<th>PrPC</th>
<th>n</th>
<th>Kd (μM)</th>
<th>ΔH (cal/mol)</th>
<th>ΔS (cal/mol/deg)</th>
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<tr>
<td>HuPrPC (91–230)</td>
<td>1.07 ± 0.02</td>
<td>11.1 ± 2.1</td>
<td>3833 ± 95.0</td>
<td>35.6</td>
</tr>
<tr>
<td>RaPrPC (91–228)</td>
<td>0.99 ± 0.02</td>
<td>21.1 ± 3.1</td>
<td>4180 ± 95.4</td>
<td>35.4</td>
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</table>

The ITC experiments were performed at 25°C with acetate buffer (20 mM NaOAc, pH 5.5). The concentrations of HuPrPc (91–230), RaPrPc (91–228), and Cu2+ are 0.4, 0.4, and 4.5 mM, respectively.

The effects of Cu2+-binding on the oligomerization of PrPs
Monomeric PrPC and oligomeric PrPO adopted different conformations

CD spectroscopy was applied to compare the secondary structures of monomeric and oligomeric PrPs (Supplementary Fig. S2). The CD spectra indicated that both HuPrPC and RaPrPC adopted typical α-helical structures with well-defined negative peaks at 208 and 222 nm. However, the CD spectra showed one negative peak at 217 nm for HuPrPO and RaPrPO prepared either in the presence or in the absence of Cu2+, indicating that both HuPrPO and RaPrPO adopted β-sheet-rich structures. These results show the distinct difference of conformation between the monomeric PrPC and oligomeric PrPO.

Cu2+-binding changed the secondary structure of PrPC

CD experiments were conducted at 25°C to measure the Cu2+-binding-induced changes in secondary structures of both HuPrPC (91–230) and RaPrPC (91–228) in acetate buffer (20 mM NaOAc, pH 5.5). The oligomerization of PrPC was negligible at such a low temperature (data not shown). In the absence of Cu2+, the far-UV CD spectra showed two negative peaks at 208 and 222 nm, indicating that both HuPrPC and RaPrPC adopted α-helical structures (Fig. 3). When 1 eq. of Cu2+ was added, the CD spectrum of HuPrPC began to shift up (Fig. 3A), whereas that of RaPrPC had a small shifting-down (Fig. 3B). With the addition of Cu2+ up to 4 eq., the CD spectra of HuPrPC shifted more significantly than those of RaPrPC. In the presence of 8–16 eq. of Cu2+, the CD spectra gradually became one negative peak at 217 nm, implying the generation of β-sheet-rich structure (Fig. 3A, right). Compared with those of HuPrPC, the CD spectra of RaPrPC shifted up more slowly, although the characteristic of β-sheet-rich structure was observed with the addition of Cu2+ up to 16 eq. (Fig. 3B, right). Furthermore, the CD spectra of RaPrPC did not reach a roughly stable level like HuPrPC even with the addition of 16 eq. of Cu2+ (Fig. 3B, right). These results indicated that Cu2+-binding can significantly change the secondary structures of both HuPrPC and RaPrPC, and induce the conformational conversion of PrPC from α-helix-rich structure to β-sheet-rich structure. The Cu2+-binding-induced secondary structural change of HuPrPC is more remarkable than that of RaPrPC.

Cu2+-binding decreased the conformational stability of PrPC

To address the effects of Cu2+ on the conformational stabilities of HuPrPC and RaPrPC, urea-induced unfolding transition experiments were performed by far-UV CD spectroscopy (Fig. 4). Both HuPrPC and RaPrPC completely lost their secondary structures in the presence of urea.
of 10 M urea. The apparent thermodynamic parameters of urea-induced unfolding transition for rabbit PrPC and its variants were described previously [33,34].

\[ C_m \] is the concentration of urea required to denature 50% of a protein, and \( \Delta G_{H2O}^{N\rightarrow U} \) represents the apparent estimated free energy change of unfolding extrapolated to zero concentration of denaturant.

In the absence of Cu\(^{2+}\), the measured \( C_m \) values of HuPrPC and RaPrPC were 5.37 ± 0.07 and 5.83 ± 0.09 M, respectively (Table 2), indicating that the conformational stability of HuPrPC was lower than that of RaPrPC. In the presence of Cu\(^{2+}\), the \( C_m \) values of HuPrPC and RaPrPC were 4.27 ± 0.04 and 4.98 ± 0.07 M, respectively. Cu\(^{2+}\)-binding decreased the \( C_m \) values of HuPrPC and RaPrPC by Figure 3. Far-UV CD spectra of HuPrPC (91–230) (A) and RaPrPC (91–228) (B) titrated by different concentrations of Cu\(^{2+}\). The CD experiments were performed at 25°C with acetate buffer (20 mM NaOAc, pH 5.5). The concentrations of Cu\(^{2+}\) are from 0 to 160 μM.

Figure 4. Effects of Cu\(^{2+}\) on the urea-induced unfolding transitions of monomeric HuPrPC (91–230) (A) and RaPrPC (91–228) (B). The CD experiments were performed at 25°C with acetate buffer (20 mM NaOAc, pH 5.5). The concentrations of HuPrPC (91–230), RaPrPC (91–228), and Cu\(^{2+}\) are 10 μM, respectively.
The effects of Cu$^{2+}$-binding on the oligomerization of PrPs

Table 2. Apparent thermodynamic parameters of urea-induced unfolding of monomeric HuPrPC (91–230) and RaPrPC (91–228) in the presence and absence of Cu$^{2+}$

<table>
<thead>
<tr>
<th></th>
<th>$\Delta G_{\text{m}}^{(0)}$ (kJ/mol)</th>
<th>$m_{\text{N,at}}$ (kJ/mol/M)</th>
<th>$C_{\text{m}}$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu$^{2+}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HuPrPC</td>
<td>$13.35 \pm 0.85$</td>
<td>$12.77 \pm 1.45$</td>
<td></td>
</tr>
<tr>
<td>RaPrPC</td>
<td>$14.91 \pm 0.88$</td>
<td>$13.20 \pm 0.52$</td>
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The CD experiments were conducted with acetic buffer (20 mM NaAc, pH 5.5) at 25°C. $\Delta G_{\text{m}}^{(0)}$ is designated as the apparent free energy change of unfolding extrapolated to zero concentration of denaturant, $m_{\text{N,at}}$ is the cooperativity of the unfolding transition, and $C_{\text{m}}$ is the concentration of urea required to denature 50% of the protein. The concentrations of HuPrPC (91–230), RaPrPC (91–228), and Cu$^{2+}$ are 10 μM, respectively.

Discussion

Previous studies have demonstrated that the central nervous system has a high level of copper [11,12] and copper plays crucial roles in TSE diseases [38–41]. However, the underlying mechanisms of Cu$^{2+}$-participating in the pathogenic processes of prion diseases have not been well addressed. Recently, Wu et al. [22] demonstrated that Cu$^{2+}$ could promote the formation of neurotoxic PrP oligomer and play a vital role in the oligomerization of PrPC. Their work showed that Cu$^{2+}$ could promote α-helical HuPrPC (91–230) converting to soluble β-sheet oligomer at acidic environment (pH 5.0), and the Cu$^{2+}$-induced oligomer generated significant damage in human SK-N-SH neuroblastoma cells. However, the details of Cu$^{2+}$-induced oligomerization process of PrPC remain unclear. It is expected that Cu$^{2+}$ regulates the pathogenic processes of prion diseases via participating in the oligomerization process of PrPC. Thus, exploring the relationship between Cu$^{2+}$ and the oligomerization of PrPC could be helpful for the mechanistic understanding of prion diseases. Previous work showed that human beings are susceptible to TSE diseases, but rabbits are resistant to TSE diseases. It could be speculated that Cu$^{2+}$-binding promoted oligomerization of PrP differently between TSE-sensitive HuPrPC and TSE-resistant RaPrPC.

In the present study, we confirmed that Cu$^{2+}$-binding induces conformational transformation of PrPC [23,42–44], and promotes the oligomerization of PrPC [22]. More importantly, our results also demonstrated that Cu$^{2+}$-binding promotes the oligomerization of HuPrPC more significantly than that of RaPrPC (Fig. 2).

ITC experiments under acidic condition were conducted to mimic the environment in the endosomal–lysosomal system which is related to the pathogenesis of prion diseases [45]. The results showed that Cu$^{2+}$ bound to HuPrPC (91–230) and RaPrPC (91–228) with moderate affinities (10$^3$ M$^{-1}$) at pH 5.5. However, a previous study showed that Cu$^{2+}$ bound to mouse PrPC (91–231) with 10-fold higher affinity (10$^4$ M$^{-1}$) at pH 6.6 [46]. Nevertheless, this study also showed that the affinity of Cu$^{2+}$ with PrPC was pH-dependent, and low pH could decrease the affinity of Cu$^{2+}$ with PrPC [46]. The affinity difference between our study and the previous study might be mostly due to the different pH conditions used.

Furthermore, our ITC data showed that the affinity of HuPrPC (91–230) for Cu$^{2+}$ is higher than that of RaPrPC (91–228) (Table 1). It could thus be speculated that HuPrPC (91–230) potentially underwent more significant conformational change than RaPrPC (91–228) due to Cu$^{2+}$-binding (Fig. 3). Previous works indicated that Cu$^{2+}$ mainly binds to the amyloid fragment of PrPC containing the residues His 95/96, His 110/111, and Met 108/109 [35,36]. It has been suggested that the flexible region might make significant contribution to the misfolding of PrP and forming toxic agent [47–49]. Cu$^{2+}$ binding to PrP would induce significant change in the spatial structure of this region and thereby promote the oligomerization of PrPC.

Jones et al. [50] found that Cu$^{2+}$-binding to His-96 and His-111 resulted in an increase in β-sheet, which coupled with a loss of irregular structure in this fragment (residues 90–126) in PrP$.Cu^{2+}$. Our CD results indicated that Cu$^{2+}$-binding promoted the conformational conversion of PrP$^\beta$ from α-helix-rich structure to β-sheet-rich structure at mild acidic environment (pH 5.5; Fig. 3). Younan et al. [43] found that Cu$^{2+}$-binding could lead to the loss of α-helical structures but could not induce the increase in β-sheet content of PrP (23–231) at neutral environment (pH 7.4). These results may suggest that acidic environment facilitates Cu$^{2+}$-binding-induced conformational change in PrP$^\beta$ [22].

The ITC data indicated that Cu$^{2+}$ bound to HuPrPC (91–230) and RaPrPC (91–228) with moderate affinities (Table 1). The thermodynamic parameter derived from an ITC experiment includes contributions from all the events that might occur in the molecules going from free to bound state [51], such as the protein structural change. Therefore, it is possible that our ITC results also include certain contributions made by some protein structural changes. Furthermore, no significant secondary structure change was detected in far-UV CD experiments when 1 eq. of Cu$^{2+}$ was added to either HuPrPC or RaPrPC (Fig. 3). However, Cu$^{2+}$-binding-induced conformational conversion of PrP$^\beta$ from α-helix-rich structure to β-sheet-rich structure was obviously observed when 8 eq. of Cu$^{2+}$ was added (Fig. 3). The ITC data showed that the affinity of Cu$^{2+}$ for HuPrPC$^\beta$ was higher than RaPrPC$^\beta$, probably explaining the observation that CD spectra of HuPrPC$^\beta$ changed more significantly than those of RaPrPC$^\beta$ when Cu$^{2+}$ was titrated to the PrP$^\beta$ solutions (Fig. 3). These results showed that HuPrPC$^\beta$ is more readily to change its conformation upon Cu$^{2+}$-binding compared with RaPrPC$^\beta$. In other words, HuPrPC$^\beta$ is more susceptible to Cu$^{2+}$-binding than RaPrPC$^\beta$.

The urea-induced unfolding transition experiments demonstrated that Cu$^{2+}$-binding decreased the conformational stabilities of HuPrPC$^\beta$ and RaPrPC$^\beta$ (Fig. 4), which might be due to the secondary structure changes induced by Cu$^{2+}$-binding. Previous work demonstrated that Cu$^{2+}$-binding-induced protein destabilization could facilitate the conformational conversion and oligomerization of PrP$^\beta$ [20,52]. In the presence of Cu$^{2+}$, the conformational stability of HuPrPC$^\beta$ decreased more significantly than that of RaPrPC$^\beta$ (Table 2). These results might explain the observation that the percentage of HuPrPO was increased more significantly in the presence of Cu$^{2+}$ than that of RaPrPO (Fig. 2).

In addition, we explored the conformational stabilities of oligomeric PrP$^\beta$ in the presence or in the absence of Cu$^{2+}$ by urea-induced unfolding transition experiments (Supplementary Fig. S3 and Table S1). The measured $C_{\text{m}}$ values of HuPrPO with and without Cu$^{2+}$ were 6.28
Studies on the interaction of Cu²⁺ with other misfolded proteins that are becomes due to its misfolding [59]. Previous works demonstrated that Cu²⁺ exists a minimum of 0.25, and 4.37 ± 0.12 M, respectively. Those values of RaPrP⁰ with and without Cu²⁺ were 5.03 ± 0.14 and 4.33 ± 0.08 M, respectively. In the present study, the thermal-induced PrP⁰ without Cu²⁺ was found to be less stable than PrP⁰. However, previous studies showed that the denaturant-induced β-rich isomorph of PrP was thermodynamically more stable than the native PrP [53,54], which contradicted our results of this work. We think that the contradiction was resulted from different experimental conditions. The oligomers formed under different experimental conditions (pH, salt concentration, and denaturant) were pleomorphic [7,55–57], and could have somewhat different properties and possess different stabilities. In the previous studies, the prion oligomers were prepared in several days with denaturants (either urea or GdnHCl) under acidic conditions at room temperature (23°C). In the present study, oligomers were prepared by incubating proteins in the buffer without denaturants at 42°C, and obtained in much shorter time (80 min). It is expected that the stability of denaturant-induced oligomers is somewhat different from that of thermal-induced oligomers, and the status of denaturant-induced oligomers might be more similar to that of PrP⁰ which is more stable than PrP⁰. Moreover, different buffers were used for the equilibrium denaturation of PrP in the previous studies and our study. In this study, we used the buffer without NaCl (20 mM NaOAc, pH 5.5) in urea-induced unfolding transition experiments, which could significantly reduce the thermodynamic stability of PrP⁰ [58], while in the previous studies, authors used the buffer with NaCl (20 mM NaOAc, 0.2 M NaCl, pH 3.6) [54]. In the absence of Cu²⁺, HuPrP⁰ possesses similar conformational stability to RaPrP⁰. However, in the presence of Cu²⁺, HuPrP⁰ possesses significantly higher conformational stability than RaPrP⁰. These results showed that Cu²⁺ could enhance the conformational stability of PrP⁰, and Cu²⁺ enhanced the conformational stability of HuPrP⁰ more significantly than that of RaPrP⁰. However, the underlying mechanisms have not been addressed. Studies on the interaction of Cu²⁺ with other misfolded proteins that are highly associated with neurodegenerative disorders will provide a better understanding of the conformational stability of PrP⁰ enhanced by Cu²⁺. As well known, amyloid-β (Aβ) is responsible for Alzheimer’s diseases due to its misfolding [59]. Previous works demonstrated that Cu²⁺ could destabilize Aβ, promote protein oligomerization, and stabilize the Aβ-oligomer [60,61]. Cu²⁺ enhances the stability of the Aβ-oligomer by increasing the peptide–peptide binding forces [61]. It was further suggested that Cu²⁺ could bridge the peptides in the oligomers and form a Aβ–Cu–Aβ–Cu···· structure by binding to the His residues from the two peptides, and thereby stabilize the Aβ oligomer. Based on the previous results, we herein make a hypothesis that Cu²⁺ could bridge monomeric PrP units in the PrP oligomer and form a PrP–Cu–PrP–Cu···· structure. Nevertheless, further theoretically modeling and experimental studies are required to confirm this hypothesis.

Based on our results, the mechanism by which Cu²⁺-binding promotes the oligomerization of PrP⁰ in prion diseases could be described as follows: Cu²⁺ binds to PrP⁰ and then induces significant conformation changes of PrP⁰, which decreases the conformational stability of PrP⁰, and thus facilitates the oligomerization of PrP⁰. The formed PrP⁰ oligomers possess significant neurotoxicity. In addition, the presence of Cu²⁺ increases the stability of PrP⁰. As reported previously, the central nervous system has high level of copper, and Cu²⁺ could increase the risk of neurodegenerative disorders, including prion diseases [11,12,62]. The present study demonstrated that Cu²⁺-binding promotes the oligomerization of HuPrP⁰ more significantly than that of RaPrP⁰. Our results indicated that HuPrP⁰ is much more susceptible to Cu²⁺ than RaPrP⁰, which might make significant contribution to the conformational conversion of HuPrP⁰ and the pathogenic process of human prion diseases. On the other hand, as one of the few species resistant to TSE diseases, rabbits are not infected by PrP⁰ under regular conditions [24], suggesting that the TSE-resistance of rabbits is highly associated with the crucial factors that significantly regulate the unique oligomerization process of RaPrP⁰. Our results showed that RaPrP⁰ possesses a low susceptibility to Cu²⁺ when compared with HuPrP⁰. The low susceptibility of RaPrP⁰ to Cu²⁺ may contribute to the difficulty of conformational conversion from normal cellular form to pathogenic scrapie isoform, which potentially weakens the risk of Cu²⁺-induced TSE diseases.

In summary, Cu²⁺ plays vital roles in pathogenic prion diseases via participating in the oligomerization process of the PrP. Our results demonstrated that Cu²⁺ binds to both HuPrP⁰ and RaPrP⁰ with moderate affinity. Cu²⁺-binding induces significant conformational change of PrP⁰, and decreases the conformational stability of PrP⁰, thereby promoting the conversion of α-helix-rich PrP⁰ into β-sheet-rich form and thus facilitating the oligomerization of PrP⁰. Moreover, our results also indicated that RaPrP⁰ has a lower susceptibility to Cu²⁺ than HuPrP⁰. The unique properties of RaPrP⁰ mostly contribute to the TSE-resistance of rabbits. This work is of great benefit to the mechanistic understanding of Cu²⁺-binding-induced conformational conversion of PrP, and sheds light on the resistance of rabbits to prion diseases.

Supplementary Data

Supplementary data is available at ABRS online.

Funding

This work was supported by the grants from the National Natural Science Foundation of China (Nos. 31170717, 31470034, and 31270777).

References

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