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Zinc significantly changes the aggregation pathway and the conformation of aggregates of human prion protein

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ABSTRACT

Prion diseases are caused by the conformational change of cellular prion protein PrP^C into pathological prion protein PrP^{Sc}. Here we study the effect of zinc on the aggregation and conformational change of human prion protein (PrP). As revealed by thioflavin T binding assays, Sarkosyl-soluble SDS-PAGE, and transmission electron microscopy, aggregation of wild-type PrP in the absence of Zn^{2+} undergoes four steps: amorphous aggregates, profibrils, mature fibrils, and fragmented fibrils. When the molar ratio of Zn^{2+} to PrP was 9:1, however, aggregation of wild-type PrP undergoes another pathway in which wild-type PrP forms oligomers quickly and then forms short-rod aggregates. Unlike wild-type PrP, the octarepeats deletion mutant PrPΔocta forms typical mature fibrils either with or without zinc. As evidenced by isothermal titration calorimetry, Fourier transform infrared spectroscopy, and proteinase K digestion assays, Zn^{2+} strongly binds to wild-type PrP monomers with the first binding constant exceeding 10^7 M^{-1} under denaturing conditions, and changes the conformation of wild-type PrP aggregates remarkably, but weakly binds to PrPΔocta with binding affinity around 10^4 M^{-1} and has no obvious effects on the conformation of PrPΔocta aggregates. Our data demonstrate that zinc significantly changes the aggregation pathway and the conformation of wild-type PrP aggregates mainly *via* interaction with its octarepeat region. Our findings could explain how zinc modifies pathological PrP conformation associated with prion diseases.

1. Introduction

Prion diseases, such as bovine spongiform encephalopathy, scrapie of sheep, and Creutzfeldt–Jakob disease of humans, are caused by the misfolding and conformational change of the normal cellular form of prion protein (PrP^C) into an abnormal disease-causing isoform of prion protein (PrP^{Sc}) [1–4]. The protein-only hypothesis points out that the infectious agents of prion diseases mainly consist of pathological prion protein PrP^{Sc} without nucleic acids [1]. PrP^C is a cell surface glycoprotein anchored to cell surface *via* glycosylphosphatidylinositol (GPI) [4]. There is no difference in sequence between PrP^C and PrP^{Sc} [1–4]. PrP^C and PrP^{Sc} differ in their conformations and three-dimensional structures, and PrP^C is largely α-helical contained but PrP^{Sc} is largely β-sheet contained [1]. There are a number of NMR structures of PrP^C from different species published so far, but the high-resolution structure of PrP^{Sc} is lacking due to its insolubility and heterogeneity [2,5].

The mature human PrP^C consists of residues 23–231, with an unstructured N-terminal half 23–120 and a global C-terminal half 121–231. The residues 58–89 are an octapeptide repeats sequence, with GQPHGGGW repeated four times. This sequence can bind to several bivalent metal ions, such as copper, zinc, and manganese [6–9]. Many researches point out that prion protein (PrP) is a copper-binding protein [10,11]. There are several copper binding sites, four of which are in its octarepeat region, two of which just after the octarepeats, and two of which in the helical region [6,7]. However, how copper affects prion diseases is complicated and controversial. Some experiments show that interaction between copper and PrP can accelerate the onset of prion diseases [12–15], but other experiments show that copper can prevent the conversion of PrP into amyloid fibrils and delay the onset of prion diseases [16,17]. The affinity of copper binding to PrP can be above 10^{10} M^{-1}, much higher than that for zinc binding to PrP (about 10^4 M^{-1}) [10,11,18,19].

Zinc, as one of the most abundant transition metals in the brain, is present in and essential to all forms of life [20,21]. In the synaptic cleft, where PrP is enriched, the concentration of Zn^{2+} can be up to 100–300 μM [20,21], remarkably higher than that of copper [6]. Therefore, we want to know whether and how micromolar Zn^{2+} affects the misfolding and conformational change of PrP, associating with prion

Abbreviations: CD, circular dichroism; GPI, glycosylphosphatidylinositol; FTIR, Fourier transform infrared; ITC, isothermal titration calorimetry; PrP, prion protein; PrPΔocta, human prion protein with the deletion of octarepeats (58–89); PrP^C, the normal cellular PrP molecule; PrP^{Sc}, an abnormal disease-causing isoform of prion protein; IAPP, islet amyloid polypeptide; SDS, sodium dodecyl sulfate; TEM, transmission electron microscopy; ThT, thioflavin T; GdnHCl, guanidine hydrochloride; PK, proteinase K

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diseases. A valuable evidence of PrP^C–zinc interaction is that not only neuronal zinc potently stimulates the endocytosis of PrP^C [22,23], but also PrP^C enhances neuronal zinc uptake [24]. Clearly, PrP is involved in neuronal zinc homeostasis [21]. In addition, it has been reported that different conformations of PrP^{Sc} can lead to different pathological symptoms of prion diseases, and PrP^{Sc} conformation is the foundation of prion strain diversity [1,3,25]. The ability of Zn^{2+} to interact with PrP^{Sc} and influence PrP^{Sc} conformation directly has widespread implications for understanding the pathogenesis of prion diseases [25]. However, how zinc affects PrP fibrillization and conformational change is still poorly studied. One report shows that the fibrillization of human PrP106-126 is completely inhibited in an environment depleted of transition metals, and Zn^{2+} can restore PrP106-126 aggregation [26], but another report shows that Zn^{2+} can inhibit conversion of full-length mouse PrP into amyloid fibrils [16].

Zinc plays an important role in the misfolding and amyloid properties of many different amyloidogenic proteins and peptides like islet amyloid polypeptide (IAPP) [27–32], amyloid β [31–33], and insulin [30,31] in addition to PrP [16,25,26]. Previous studies on IAPP's interactions with zinc have indicated that zinc clearly suppresses the aggregation of IAPP [27–29]. Recent studies have shown that zinc can alter the aggregation pathway of IAPP and amyloid β [32]. Related NMR studies have shown the structural changes accompanied by zinc binding [27–29,32].

In this study, we investigated the effect of zinc on the aggregation and conformational change of wild-type human PrP and its octarepeats deletion mutant PrPΔocta. By using thioflavin T (ThT) binding assays, Sarkosyl-soluble SDS-PAGE, and transmission electron microscopy (TEM), we demonstrated that in the presence of 100 μM Zn^{2+}, wild-type PrP formed oligomers quickly and then formed short-rod aggregates. In the absence of Zn^{2+}, however, aggregation of wild-type PrP underwent four different stages of amorphous aggregates, profibrils, mature fibrils, and finally fragmented fibrils. By using isothermal titration calorimetry (ITC), circular dichroism (CD), Fourier transform infrared (FTIR) spectroscopy, and proteinase K (PK) digestion assays, we found that Zn^{2+} strongly bound to the apo form of wild-type PrP under denaturing conditions. We also reported that the binding was accompanied by a remarkable conformational change of wild-type PrP aggregates, whereas such phenomena were not observed for PrPΔocta. Our results indicated that zinc significantly changed the aggregation pathway and the conformation of wild-type PrP aggregates mainly *via* interaction with its octarepeat region. Our findings could explain how zinc modifies pathological PrP conformation associated with prion diseases and why so many prion strains existed.

2. Materials and methods

2.1. Materials

ThT was purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile was obtained from Fisher Scientific (Fairlawn, NJ). Guanidine hydrochloride (GdnHCl) was obtained from Promega (Madison, WI). Proteinase K, Triton X-100 and Sarkosyl were purchased from Amresco (Solon, OH). All other chemicals used were made in China and were of analytical grade.

2.2. Plasmid construction and protein purification

Wild-type human PrP (23–231) and its octarepeats deletion mutant PrPΔocta (23–57, 90–231) were constructed into the pET30a plasmids. PrPΔocta was generated by polymerase chain reaction (PCR) using wild-type PrP plasmid as a template with primers 5′GGTCAAGGAGGT GGCACCCACAGTCAGT/5′CCAGCCACCACCGC CCTGAGGTG. Then ligation reaction was employed to link the two blunt ends of PCR product. The plasmids for wild-type PrP and PrPΔocta were transformed into *Escherichia coli*. Recombinant wild-type human PrP and its mutant were expressed in *E. coli* BL21 (DE3) (Novagen), and purified by HPLC on a C4 reversed-phase column (Shimadzu, Kyoto, Japan) as described by Bocharova and co-workers [16,34] with minor changes. Briefly, the inclusion body was dissolved in 8 M urea, purified by Ni-sepharose, refolded in 6 M urea containing 2 mM ethylenediaminetetraacetic acid over night, and further purified by C4 reversed-phase column. After purification, human PrP was dialyzed against 5 mM MES buffer for two times, concentrated and filtered. Purified human PrP was confirmed by SDS-PAGE and mass spectrometry to be single species with an intact disulfide bond. The concentration of human PrP was determined by its absorbance at 280 nm using the molar extinction coefficient value deduced from the composition of the protein online (http://web.expasy.org/protparam/). Metal content was determined by atomic absorption using an AAnalyst-800 atomic absorption spectrometer (PerkinElmer Life Sciences, Shelton, CT) to confirm that human PrP samples were indeed in the apo state, in which the molar ratios of Cu^{2+}, Zn^{2+}, and Ni^{2+} to human PrP monomer were 0.002:1, 0:1, and 0:1, respectively.

2.3. Fibril formation

To form fibrils, freshly prepared (within two weeks) wild-type human PrP and its octarepeats deletion mutant PrPΔocta were diluted to a final concentration of 11 μM in 5 mM MES buffer (pH 7.0) containing 2 M GdnHCl and related concentration of zinc, incubated at 37 °C and shaking at 220 rpm.

2.4. ThT binding assays

A 2.5 mM ThT stock solution was freshly prepared in 5 mM MES buffer (pH 7.0) and passed through a 0.22-μm pore size filter before use to remove insoluble particles. A serial of 50 μl samples were taken in chronological order, and mixed with 2.5 ml of 5 mM MES buffer and 12.5 μl of ThT stock solution before measurements. The fluorescence of ThT was excited at 450 nm with a slit-width of 5.0 nm and the emission was measured at 480 nm with a slit-width of 5.0 nm on an LS-55 luminescence spectrometer (PerkinElmer Life Sciences, Shelton, CT). Kinetic parameters were determined by fitting ThT fluorescence intensity *versus* time to a sigmoidal equation [35–38]:

$$F = F_0 + (A + ct)/\{1 + \exp[k(t_m - t)]\} \quad (1)$$

where F is the fluorescence intensity, k the rate constant for the growth of fibrils, and t_m the time to 50% of maximal fluorescence. F_0 describes the initial baseline during the lag time. $A + ct$ describes the final baseline after the growth phase has ended. The lag time is determined to be $t_m - 2/k$.

2.5. Sarkosyl-soluble SDS-PAGE

A serial of 20 μl human PrP samples were taken in chronological order during fibril formation corresponding to ThT binding assays. 20 μl samples were added with 2.5 μl 20% Sarkosyl. The mixture was left at room temperature for 30 min, and then mixed with 5× loading buffer (without β-mercaptoethanol and SDS, and no heating) and separated by 15% SDS-PAGE. Gels were stained by Coomassie Blue R250.

2.6. Transmission electron microscopy (TEM)

The formation of fibrils by human PrP was confirmed by electron microscopy of negatively stained samples. The preparation for negatively stained samples was described in detail previously [36,38]. Sample aliquots of 10 μl were placed on copper grids and left at room temperature for 1–2 min, rinsed twice with H_2O, and then stained with 2% (w/v) uranyl acetate for another 1–2 min. The stained samples were examined using an H-8100 (or an H-7000 FA) transmission electron microscope (Hitachi, Tokyo, Japan) operating at 100 kV.