Biochemical and Biophysical Research Communications **267,** 398–402 (2000)
doi:10.1006/bbrc.1999.1944, available online at http://www.idealibrary.com on IDEAL®

The Octapeptide Repeat Region of Prion Protein Binds Cu(II) in the Redox-Inactive State

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Received November 24, 1999

The octapeptide repeat region of human prion protein is known to bind four Cu(II) ions per molecule. A peptide, $Octa_4$, representing this region was tested for inhibitory effects on copper-catalyzed oxidation of L-ascorbate or glutathione and on generation of $OH^{\cdot}$ during the former reaction. The result indicated that the catalytic activity of the first Cu(II) ion bound to an $Octa_4$ molecule was completely suppressed. The valence state of the copper under reducing conditions was Cu(II), as determined by a newly developed method using bathocuproinedisulfonate under acidic conditions. Furthermore, it was shown that *Escherichia coli* cells expressing the octapeptide repeat region were significantly resistant to copper treatment compared with control cells. The results taken together indicate that prion protein can function to sequester copper ions in the redox-inactive state, rendering copper-induced generation of reactive oxygen species impossible.

Key Words: **prion protein; octapeptide repeat region; copper; L-ascorbate; glutathione.**

Prion diseases are a family of neurodegenerative diseases, including scrapie in sheep, bovine spongiform encephalopathy in cattle, and Creutzfeldt-Jacob disease in humans (1). They are characterized by the accumulation in the affected brains of an abnormal isoform derived from cellular prion protein (PrP) of the normal brain (1). Although little is known about the function of PrP, a current hypothesis is that PrP plays some roles in copper metabolism and detoxification. First, studies with synthetic peptides and recombinant PrP have demonstrated that these can bind Cu(II) *in vitro* (2–6). Second, membrane-rich brain fractions from $Prnp^{0/0}$ mice was shown to possess reduced levels of copper compared to those from wild-type mice (6), indicating that PrP is a major copper-binding protein in the brain. Also, Cu–Zn superoxide dismutase in the brain of $Prnp^{0/0}$ mice was found to be enzymatically less active and incorporate less radioactive copper than that in normal mouse brain (7, 8). Third, it was reported that PC12 cells that had been selected for resistance to copper toxicity expressed elevated levels of PrP (9), and that cerebellar cells from $Prnp^{0/0}$ mice were more sensitive to copper toxicity than those from wild-type mice (10). Finally, it is also observed that copper at its increased extracellular concentrations stimulates endocytosis of PrP from the cell surface (11).

Copper is an essential element that serves as a cofactor for a number of enzymes. However, it is a transition metal with multiple valences, Cu(I) and Cu(II), and thus is a powerful catalyst of autooxidation reactions (e.g., oxidation of ascorbate, thiols and catecholamines) that generate various reactive oxygen species (12). In view of the property of PrP as a copper-binding protein, one can reason that PrP may sequester Cu(II) and prevent oxidative damage that would be induced by the coexistence of Cu(II) and autooxidizable compounds. For PrP to do so, the Cu(II) bound to it should preferably be maintained in the redox-inactive state; otherwise, the protein itself would be subjected to oxidative degradation. With this view in mind, we have here addressed a question of whether the redox activity of the copper bound to PrP is suppressed in the presence of physiologically-relevant reducing substance L-ascorbate or glutathione. A peptide, $Octa_4$, representing the copper-binding region (amino acid residues 60–92) of human prion protein was tested for inhibitory effects on copper-catalyzed oxidation of L-ascorbate or glutathione and on generation of $OH^{\cdot}$ during the former reaction. The valence state of the bound copper in the presence of the reducing substance L-ascorbate or glutathione was also determined by a newly-developed method using bathocuproinedisulfonate as a Cu(I) chelator. Furthermore, by expressing a portion of human PrP representing amino acid residues 23–98 ($PrP_{23–98}$) in *Escherichia coli,* we investi-

Abbreviations used: PrP, prion protein; $Octa_4$, peptide comprising four octapeptide repeats; Mes, 2-morpholinoethanesulfonic acid; GST, glutathione *S*-transferase.

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0006-291X/00 $35.00

gated whether PrP_{23-98} could confer the resistance to cytotoxicity induced by copper.

EXPERIMENTAL PROCEDURES

Materials. Commercial sources of materials are as follows: pGEX-5X-3 vector from Pharmacia Biotech (Uppsala, Sweden); primate PCRable DNAs from BIOS Laboratories (New Heaven, CT); *Pfu* DNA polymerase from Stratagene (La Jolla, CA); synthetic oligonucleotides from Biotechnologies, Inc. (Gaithersburg, MD); restriction enzymes from New England Biolabs (Beverly, MA); *E. coli* strain BL21 (DE3) pLysS from Novagen, Inc. (Madison, WI). $Octa_4$ (four repeats of sequence PHGGGWGQ) was purchased from TANA Laboratories (Houston, TX). The concentration of $Octa_4$ were determined spectrophotometrically by using a molar extinction coefficient at 278 nm of 22,200 M^{-1} cm^{-1}, which was calculated by multiplying 5550 M^{-1} cm^{-1} (a molar extinction coefficient of tryptophan) multiplied by 4 (the number of tryptophan residues). All other chemicals were of analytical grade. Distilled water was purified by passage through a Milli-Q academic A10 system (Millipore Corp., Bedford, MA). The resistance of the water was 1.8×10^7 $\Omega \cdot$ cm at 20°C.

Copper binding study by ultrafiltration technique. Mixtures (400 μl) containing 20 mM 2-morpholinoethanesulfonic acid (Mes) buffer (pH 7.5), 5 μM $Octa_4$, and varying concentrations (10–30 μM) of $CuCl_2$ were dispensed into the cups of NANOSEP centrifugal concentrators (3K, Pall Filtron Corp., MA). After the filter units were allowed to stand at room temperature for 5 min, they were centrifuged at 6700*g* at 5°C for 2 min, and the copper concentration of the resulting filtrates was measured by the flameless mode with a Shimadzu atomic absorption spectrophotometer AA-6800 (Shimadzu, Kyoto, Japan).

Copper-catalyzed oxidation of L-ascorbate. L-Ascorbate (500 μM) was allowed to react with varying concentrations (5–80 μM) of $CuCl_2$ in 100 μl of 20 mM Mes buffer (pH 7.5) in the presence and absence of 20 μM $Octa_4$ at 25°C for 2 min, and then 900 μl of 20 mM Mes buffer (pH 7.5) containing 1.1 mM diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid was added into the reaction mixtures to stop the reaction. The concentration of the remaining L-ascorbate was quickly determined by measuring absorbance at 265 nm with a Shimadzu UV-visible recording spectrophotometer UV-2500.

Copper-catalyzed oxidation of glutathione. Reaction mixtures (1 ml) containing 20 mM Mes buffer (pH 7.5), varying concentrations (1–5 μM) of $CuCl_2$, 10 μM glutathione, and $Octa_4$ (0 or 2 μM) were incubated at 25°C for 10 min, then 20 μl of 5 mM diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid was added to the reaction mixtures to stop the reaction, and the remaining glutathione concentration was determined by the Ellman method (13).

Determination of valence state of copper. The valence state of copper in the presence of L-ascorbate or glutathione was determined by use of bathocuproinedisulfonate on the following principle. Peptide–copper complexes were quickly disrupted by acidification with trichloroacetic acid, and at the same time, the released Cu(I) was detected with the Cu(I)-specific chelator bathocuproinedisulfonate. Because the reduction of Cu(II) by L-ascorbate or glutathione was quenched under acidic conditions, the amount of Cu(I) existing at the time of quenching can be quantitatively determined. Mixtures (100 μl) of the same composition as used in the above experiment of copper-catalyzed oxidation of L-ascorbate or mixtures (100 μl) containing 20 mM Mes buffer (pH 7.5), varying concentrations (10–40 μM) of $CuCl_2$, 100 μM glutathione, and $Octa_4$ (0 or 20 μM) were dispensed into a microcuvette, and incubated at room temperature for 1 min. Then 10 μl of a solution containing 55 mM bathocuproinedisulfonate and 44% trichloroacetic acid was added to the above mixtures, and absorbance at 485 nm was read.

Measurement of hydroxyl radical. $OH^{\cdot}$ formed during copper-catalyzed oxidation of L-ascorbate was estimated by including 0.5 mM coumarin-3-carboxylic acid in the reaction system (14). The reaction conditions were the same as in the experiment of copper-catalyzed oxidation of L-ascorbate. The fluorescent product 7-hydroxycoumarin-3-carboxylic acid was measured with a Shimadzu spectrofluorometer RF-5300 with excitation at 400 nm and emission at 450 nm.

Copper resistance by expression of PrP_{23-98} in E. coli cells. The DNA encoding PrP_{23-98} was amplified from human genomic DNA using primers 5′-CCGAATTCCTGCAAGAAGCGCCCGAA-3′ (sense primer which includes an artificial *Eco*RI site) and 5′-CCGTCGACTCACTGACTGTGGGTGCCA-3′ (antisense primer which includes an artificial *Sal*I site) under the same PCR conditions as described previously (15). After digestion with *Eco*RI and *Sal*I, the PCR product was ligated to the respective restriction sites of pGEX-5X-3, so that the resulting construct (designated pGEX-5X-3/PrP_{23-98}) could produce a fusion protein of glutathione *S*-transferase (GST) and human PrP_{23-98}. *E. coli* cells of the strain BL21 (DE3) pLysS were transformed with this plasmid or pGEX-5X-3, and the respective recombinant clones obtained were cultured at 37°C in LB containing 50 μg/ml ampicillin. When the OD_{600} of the culture became about 0.8, isopropylthio-β-D-galactopyranoside was added to a concentration of 1.0 mM, and the cells were cultured at 30°C for 30 min, when $CuCl_2$ was added to a concentration of 0.75, 1.0, or 2.0 mM and further cultured at 30°C for 30 min. The number of viable cells was counted after spreading an appropriate portion of the cell suspensions on LB plates containing 50 μg/ml ampicillin and culturing overnight. The results were subjected to statistical evaluation using Student's *t* test. For estimation of the amounts of the expressed proteins, SDS–PAGE was carried out as described previously (15).

RESULTS

Copper Binding with $Octa_4$

We studied copper binding with $Octa_4$ by a centrifugal ultrafiltration technique. A Scatchard plot of the data obtained indicated that 4.3 atoms of copper bound to a molecule of $Octa_4$ with a dissociation constant of 0.58 μM (data not shown). The number of copper ions bound per molecule of $Octa_4$ agrees well with the result of a circular dichroism study, which showed that a peptide comprising four octapeptide repeats binds four copper per molecule (5). However, the K_d value measured by us is considerably smaller than that obtained by the circular dichroism study (6 μM) (5). This discrepancy may be due to the difference of the experimental conditions: our measurement was carried out in Mes buffer, whereas the latter study was done without buffer.

Suppression of Copper-Catalyzed Oxidation of L-Ascorbate by $Octa_4$

Next, we studied whether the copper bound to $Octa_4$ is catalytically active for the oxidation of physiologically-relevant reducing substances such as L-ascorbate and glutathione. At Cu/$Octa_4$ molar ratios below 1, $Octa_4$ completely inhibited the copper-catalyzed oxidation of L-ascorbate, and at molar ratios between 1 and 2, the substantial, though not total, inhibition was observed (Fig. 1A). At molar ratios above 2, the inhibitory effect of $Octa_4$ was not

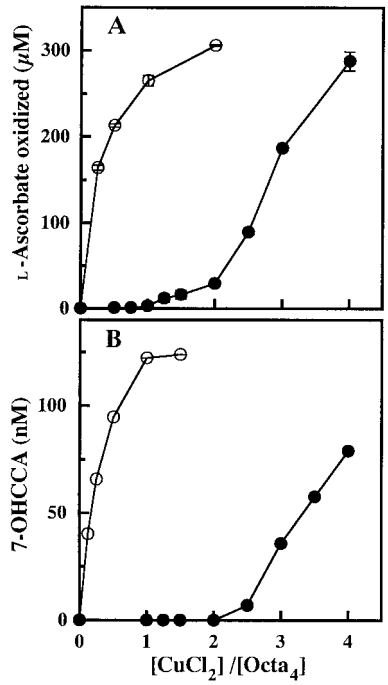

FIG. 1. Suppression of copper-catalyzed oxidation of L-ascorbate and production of $OH^{\cdot}$ by $Octa_4$. L-Ascorbate (500 μM) was allowed to react with varying concentrations (5–80 μM) of $CuCl_2$ in 20 mM Mes buffer (pH 7.5) in the presence (solid circles) and absence (open circles) of 20 μM $Octa_4$ at 25°C for 2 min, and the concentrations of the remaining L-ascorbate (A) were measured spectrophotometrically. $OH^{\cdot}$ formed during the copper-catalyzed oxidation of L-ascorbate (B) were measured with coumarin-3-carboxylic acid. Data in A indicate means ± SD of three determinations; data in B, mean of two determinations.

marked. The dependency of $OH^{\cdot}$ formation on the $Cu/Octa_4$ molar ratio was somewhat different: complete inhibition was observed at $Cu/Octa_4$ molar ratios below 2 (Fig. 1B). These results taken together clearly indicate that the first copper ion binds to an $Octa_4$ molecule with its catalytic activity completely suppressed and that the catalytic activity of the second copper bound to $Octa_4$ is also suppressed to a near complete degree.

To examine how the catalytic activity of the copper bound to $Octa_4$ was suppressed in the above experiment, we determined the valence state of the copper by a newly-developed method in which Cu(I) was determined by chelation to bathocuproinedisulfonate under such acidic conditions as the redox cycling of copper could be quenched. In the early phase of the copper-catalyzed oxidation of L-ascorbate, approximately 60% of the copper was in the Cu(II) form through the range of $Cu/Octa_4$ molar ratios examined (Fig. 2, open circles), indicating that the reduction and oxidation of copper ions occur at a nearly same rate. On the other hand, when $Octa_4$ was included in the reaction system, the valence state of the copper changed as a function of the $Cu/Octa_4$ molar ratio (Fig. 2, solid circles). At $Cu/Octa_4$ molar ratios below 1, the copper bound to $Octa_4$ was totally in the Cu(II) form. This result taken together with the complete inhibition of L-ascorbate oxidation in the same range of $Cu/Octa_4$ molar ratios indicate that the first copper bound to an $Octa_4$ molecule is catalytically inactive and remain in the Cu(II) state even in the presence of the reducing compound L-ascorbate. At $Cu/Octa_4$ molar ratios above 1, the Cu(I) form increased with an increase in the $Cu/Octa_4$ molar ratio. This finding is consistent with the observation that the L-ascorbate oxidation becomes appreciable above a $Cu/Octa_4$ molar ratio of 1.

Suppression of Copper-Catalyzed Oxidation of Glutathione by $Octa_4$

We extended the above study to see whether the copper-catalyzed oxidation of glutathione is likewise inhibited by $Octa_4$. At $Cu/Octa_4$ molar ratios below 1, the oxidation of glutathione did not occur at all, whereas no inhibitory effect of $Octa_4$ was observed with the copper added above a molar ratio of 1 (Fig. 3A). Measurement of the valence state of the copper in the early phase of the glutathione oxidation showed that all copper was in the Cu(I) form at all $Cu/Octa_4$ molar ratios examined (Fig. 3B, open circles), whereas upon addition of $Octa_4$, nearly all copper was in the Cu(II) form at $Cu/Octa_4$ molar ratios below 1 (Fig. 3B, solid circles). This finding indicate that $Octa_4$ forms a 1:1 chelate with Cu(II) in the presence of glutathione. The Cu(I) form increased above a $Cu/Octa_4$ molar ratio of 1, where glutathione oxidation was enhanced. Though glutathione forms stable complexes with Cu(I) with high affinity (16), it is clear that an $Octa_4$ molecule can bind one Cu(II) ion in competition with glutathione.

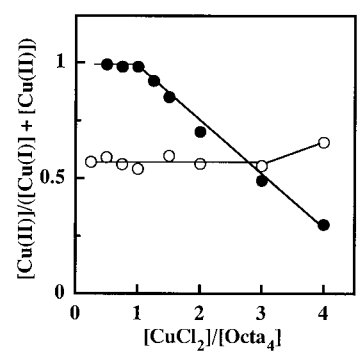

FIG. 2. Valence state of copper catalyzing oxidation of L-ascorbate with or without $Octa_4$. Copper-catalyzed oxidation of L-ascorbate (500 μM) was carried out in the presence (solid circles) and absence (open circles) of 20 μM $Octa_4$ as specified in the legend to Fig. 1, and the valence state of the copper was determined 1 min after the start of the reaction as specified under Experimental Procedures. Data indicate mean of two determinations.

Copper Resistance of E. coli Cells by Expression of $PrP_{23–98}$

To investigate whether copper-binding region of PrP, when expressed in *E. coli* cells, could confer the resistance to cytotoxicity induced by copper, we constructed an expression plasmid, pGEX-5X-3/$PrP_{23–98}$, which enabled the expression of $PrP_{23–98}$ as a fusion with GST in *E. coli* cells. When *E. coli* cells harboring this plasmid were induced to produce the fusion protein, they produced as much amount of the fusion protein as the GST produced by cells harboring pGEX-5X-3 (data not shown). Without the copper treatment, the cells expressing the GST-$PrP_{23–98}$ fusion protein showed a slightly lower cell viability than control cells; however, they were significantly resistant to the copper treatment at all the concentrations tested (0.75–2 mM) compared with control cells (Fig. 4). This result provides evidence that $PrP_{23–98}$ can suppress copper toxicity under *in vivo* conditions.

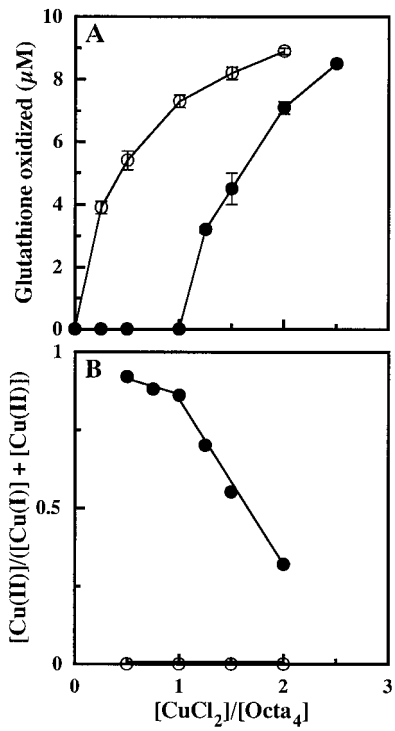

FIG. 3. Suppression of copper-catalyzed oxidation of glutathione by $Octa_4$ and valence state of the copper catalyzing the oxidation. (A) Reaction mixtures (1 ml) containing 20 mM Mes buffer (pH 7.5), varying concentrations (1–5 μM) of $CuCl_2$, and 10 μM glutathione were incubated at 25°C for 10 min with (solid circles) or without (open circles) 2 μM $Octa_4$, and the remaining glutathione concentration was determined by the Ellman method. Data indicate mean ± SD of three determinations. (B) Copper-catalyzed oxidation of glutathione (100 μM) was carried out in the presence (solid circles) and absence (open circles) of 20 μM $Octa_4$, and the valence state of the copper was determined 1 min after the start of the reaction as specified under Experimental Procedures. Data indicate means of two determinations.

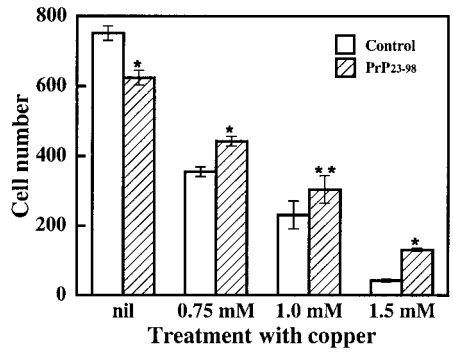

FIG. 4. Copper resistance of *E. coli* cells expressing human $PrP_{23–98}$ fusion protein. *E. coli* cells harboring pGEX-5X-3/$PrP_{23–98}$ (hatched bar) or pGEX-5X-3 (open bars) were induced to produce $PrP_{23–98}$ as a fusion with GST or GST alone, respectively, and then treated with the indicated concentrations of $CuCl_2$ at 30°C for 30 min. The number of viable cells was counted after inoculation of 100 μl of each of 100,000-fold diluted cell suspensions on LB plates containing 50 μg/ml ampicillin and culturing overnight. Data indicate means ± SD of three determinations. *Significant difference ($P < 0.01$) from control cells. **Significant difference ($P < 0.05$) from control cells.

DISCUSSION

Metallothioneins (17), copper-transporting ATPases (18, 19), and copper chaperons (20–24) are a group of copper-binding proteins, whose copper needs no valence shuttle for their functions. Interestingly, the copper-binding sites of these protein contain two or more cysteine residues and form complexes with Cu(I). It appears that the copper in such complexes are not ready to be oxidized in the presence of atmospheric oxygen, as it was reported that Atx1, a yeast copper chaperon, was stable in air at 4°C for 30 min (22). On the other hand, the copper-binding site of PrP has no cysteine residue and binds copper in the Cu(II) state. PrP probably exists *in vivo* in the copper-bound form (6), then if the Cu(II) in PrP were readily reduced with L-ascorbate and glutathione, its subsequent exposure to H_2O_2 would result in reoxidation of Cu(I) concomitant with production of highly reactive $OH^{\cdot}$. As demonstrated in the present study, such a redox cycling reaction does not take place unless the Cu/$Octa_4$ molar ratio exceeds 1. Thus, whatever the function of PrP, its copper-binding site coordinates copper ideally in that the bound Cu(II) remains catalytically inactive under physiologically-relevant reducing conditions, i.e., in the presence of L-ascorbate and glutathione.

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