Electron Paramagnetic Resonance Evidence for Binding of Cu^{2+} to the C-terminal Domain of the Murine Prion Protein

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ABSTRACT Transmissible spongiform encephalopathies in mammals are believed to be caused by scrapie form of prion protein (PrP^{Sc}), an abnormal, oligomeric isoform of the monomeric cellular prion protein (PrP^{C}). One of the proposed functions of PrP^{C} in vivo is a Cu(II) binding activity. Previous studies revealed that Cu^{2+} binds to the unstructured N-terminal PrP^{C} segment (residues 23–120) through conserved histidine residues. Here we analyzed the Cu(II) binding properties of full-length murine PrP^{C} (mPrP), of its isolated C-terminal domain mPrP(121–231) and of the N-terminal fragment mPrP(58–91) in the range of pH 3–8 with electron paramagnetic resonance spectroscopy. We find that the C-terminal domain, both in its isolated form and in the context of the full-length protein, is capable of interacting with Cu^{2+}. Three Cu(II) coordination types are observed for the C-terminal domain. The N-terminal segment mPrP(58–91) binds Cu^{2+} only at pH values above 5.0, whereas both mPrP(121–231) and mPrP(23–231) already show identical Cu(II) coordination in the pH range 3–5. As the Cu^{2+}-binding N-terminal segment 58–91 is not required for prion propagation, our results open the possibility that Cu^{2+} ions bound to the C-terminal domain are involved in the replication of prions, and provide the basis for further analytical studies on the specificity of Cu(II) binding by PrP.

INTRODUCTION

The cellular prion protein (PrP^{C}) is a host-encoded cell surface glycoprotein expressed mainly in the brain and to a lesser extent in many other tissues (Prusiner, 1991; Prusiner, 1998). Prion-related diseases, known as transmissible spongiform encephalopathies, such as bovine spongiform encephalopathy in cattle and Creutzfeldt-Jakob disease in humans, are fatal diseases supposed to be caused by a conformational isomer of PrP^{C} (PrP^{Sc}) (Prusiner, 1998; Griffith 1967). PrP^{Sc} is an insoluble, proteinase K-resistant oligomer of PrP that accumulates in the brain during infection with prions (Weissmann et al., 1996). The covalent structure of the PrP^{Sc} subunits is supposed to be identical with that of PrP^{C} (Stahl and Prusiner, 1991). The mechanism of the conversion of PrP^{C} to its abnormal isoform PrP^{Sc} is presently unknown, and it is still a matter of debate whether the infectious scrapie agent is identical to PrP^{Sc} (Griffith, 1967; Alper et al., 1967; Prusiner, 1982). Also the function of PrP^{C} remains unknown; several potential roles of PrP have been postulated, including a possible interaction with a ligand, which results in a signal essential for proper neuronal function (Shmerling et al., 1998), a role in targeting the neuronal nitric oxide synthase to its normal subcellular localization in cholesterol-rich membrane microdomains (Keshet et al., 1999), and a role in copper metabolism, related to the finding, that the prion protein can bind copper ions (Brown et al., 1998; Pauly and Harris, 1998; Brown and Mohn, 1999; Wong et al., 1999; Herms et al., 1999; Brown et al., 1999; Brown, 1999).

A first hint of a Cu^{2+} binding ability of PrP^{C} came from the observation that hamster PrP^{C} can be enriched by Cu^{2+} chelate affinity chromatography (Pan et al., 1993). Subsequently, it was proposed that PrP^{C} is also a Cu^{2+} binding protein in vivo, because the content of Cu^{2+} in membrane preparations of brains from prion protein deficient ($PrP^{0/0}$, Büeler et al., 1992) mice was found to be only 20% of that in wild-type mice (Brown et al., 1997a), although Waggoner et al. (2000) recently claimed that these data could not be reproduced.

Nevertheless, binding of Cu^{2+} to the prion protein has been supposed to play a role in the pathogenesis of prion diseases because $PrP^{0/0}$ mice, albeit otherwise normal in their phenotype (Büeler et al., 1992), show a higher sensitivity to copper toxicity and oxidative stress conditions compared to the wild-type animals (Brown et al., 1996, 1997b). In addition, a decreased activity of the cuproenzyme superoxide dismutase (SOD) was found in brain extracts from $PrP^{0/0}$ mice, and it was suggested that PrP^{C} acts as a Cu^{2+} storage protein guaranteeing sufficient biological activity of SOD (Brown et al., 1997b; Brown and Besinger, 1998). Several other findings support the idea of a correlation between Cu^{2+} binding to PrP and prion diseases. A 32-amino acid peptide representing residues 59–91 of the human PrP, containing the four characteristic octapeptide repeats of the N-terminal segment, has been shown to promote survival of $PrP^{0/0}$ cerebellar cells in the presence of Cu^{2+} (Brown et al., 1998). In addition, Cu^{2+} ions apparently enhance recovery of proteinase K resistance after treatment of PrP^{Sc} with guanidine hydrochloride, followed by dilution to low guanidine hydrochloride concentrations (McKenzie et al., 1998). Cu^{2+} was also shown to stimulate

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endocytosis of the prion protein in cultured neuroblastoma cells, suggesting that PrP^C could serve as a receptor for the uptake of copper ions in neuronal cells from the extracellular milieu (Pauly and Harris, 1998). Furthermore, a possible localization of PrP^C in the presynaptic membrane is discussed, at which PrP^C may modulate synaptic transmission by recapturing copper ions released into the synaptic cleft (Herms et al., 1999). Very recently, Brown et al. (1999) suggested that the recombinant mouse prion protein itself has an activity like that of superoxide dismutase, but only after refolding of PrP in the presence of excess copper ions.

In contrast to the uncertainties about the function of PrP^C in vivo, a large body of structural information on PrP^C has become available from the NMR structure determination of recombinant mouse (Riek et al., 1996), hamster (Donne et al., 1997), and human (Zahn et al., 2000) PrP^Cs. The common structural feature of these mammalian PrP^Cs, which are composed of 210 amino acids, is a well structured C-terminal domain, extending from residues 125–228, and a flexibly disordered N-terminal domain (residues 23–124). The C-terminal domain consists of three α-helices and a two-stranded antiparallel β-sheet; the only two cysteine residues of the protein form a disulfide bridge connecting helix 2 and 3.

Biophysical investigations on copper binding to PrP^C were so far mainly focused on peptides from the unstructured, N-terminal segment of PrP, which is characterized by a highly conserved repeat of four octapeptide units with the consensus sequence PHGGGWGQ. The histidine residue contained in each repeat is the most obvious candidate for Cu^{2+} binding.

Regarding the stoichiometry of Cu^{2+} binding to the N-terminal segment of PrP, 2–6 copper ions have been proposed to bind per PrP^C molecule (Brown et al., 1997a; Hornshaw et al., 1995a; Hornshaw et al., 1995b; Stöckel et al., 1998; Viles et al., 1999; Miura et al., 1999; Aronoff-Spencer et al., 2000). These differences may be caused by different pH-dependent protonation states of the histidine residues and by buffer artifacts. The copper binding model from Viles et al. (1999) proposes cooperative binding of four copper(II) ions per PrP(58–91) peptide. In this model, the coordination sphere is supposed to be practically identical for all of the four copper(II) bound, consisting of a square-planar geometry with three nitrogen and one oxygen ligand. Later, Whittal et al. (2000), working on fragments from the unstructured, N-terminal domain of Syrian hamster PrP (SHaPrP), found that the binding stoichiometry of Cu^{2+} to PrP^C is pH dependent, with two copper(II) bound at pH 6 to the octapeptide repeat region, and four copper(II) bound at pH 7.4. The most recent copper binding model from Aronoff-Spencer et al. (2000) confirmed binding of four copper ions per PrP(57–91) peptide proposed by Viles et al. (1999), with a detailed electron paramagnetic resonance and circular dichroism analysis of copper(II) binding. Here, an alternative mode of binding is suggested, in which the coordinating water molecules proposed by Viles et al. (1999) in the coordination sphere are replaced by backbone carbonyl oxygens from glycine residues; furthermore, the nitrogen ligands are provided by only one histidine residue per Cu(II) ion and by two backbone nitrogens, in contrast to the cooperative binding of Cu(II) by the nitrogens from two histidine residues in neighboring repeat units and one backbone nitrogen as postulated by Viles et al (1999).

In this study, we examine the Cu^{2+} binding to the recombinant full length mouse prion protein mPrP(23–231), its structured C-terminal fragment mPrP(121–231), and the N-terminal segment mPrP(58–91), containing the four conserved octapeptide repeats, at different pH values with continuous wave electron paramagnetic resonance (CW EPR) spectroscopy. This technique permits to selectively detect protein-bound copper(II) ($[Cu^{2+}] > 20\ \mu M$) and to obtain information about the nature of its coordination sphere. Copper(II) binding to the C-terminal structured part of recombinant PrP^C was observed. These unexpected results are discussed in detail and compared to earlier observations. Furthermore, the general problem of estimating the biological relevance of Cu(II) binding studies performed at "high" protein concentrations in vitro is discussed.

MATERIALS AND METHODS

Protein purification

Recombinant mPrP(23–231) and mPrP(121–231) were purified as described (Liemann and Glockshuber, 1999). After purification, the proteins were dialyzed against distilled water and stored at −20°C after freezing in liquid nitrogen. Protein concentrations were determined by the specific absorbance of the proteins according to Gill and von Hippel (1989) with $\epsilon = 19'890\ M^{-1}\ cm^{-1}$ for mPrP(121–231) and $\epsilon = 62'280\ M^{-1}\ cm^{-1}$ for mPrP(23–231).

The peptide mPrP(58–91), corresponding to the octapeptide repeat containing region of mouse PrP, was purchased from Primm (Milan, Italy). The peptide was synthesized by FMOC solid phase synthesis, and acetylated at the N terminus and amidated at the C terminus (sequence: GQPHGGGWGQPHGGSWGQPHGGSWGQPHGGGWGQ). The peptide was purified by reversed phase HPLC and its molecular mass verified by matrix assisted UV laser desorption/ionization time-of-flight mass spectrometry (calculated mass: 3409.42 Da; found: 3408.09 Da). The peptide concentration was determined by acquiring UV spectra of samples in 6 M guanidine hydrochloride, using an extinction coefficient at 280 nm of $22760\ M^{-1}\ cm^{-1}$.

Sample preparation

pH-dependent EPR measurements

The following buffers in the range of pH 3–8 were used (10 mM each): formic acid/NaOH (pH 3.0 and 4.0), sodium acetate/HCl (pH 5.0), sodium cacodylate/HCl (pH 6.0) and 3-(*N*-morpholino)propane-sulfonic acid/NaOH (pH 7.0, 7.4 and 8.0). For the measurements of the Cu(II) binding of mPrP(58–91), 2 or 4 molar equivalents $CuCl_2$ were added to a stock solution of the peptide to reach a final 0.1 mM peptide concentration at pH 3, 4, 5, 6 and 7.4. For the measurements of the Cu(II) binding of mPrP(121–231) and mPrP(23–231) at pH 3.0 to 6.0, 3 molar equivalents of

$CuCl_2$ were added to 0.1 mM of the peptide in 10 mM buffer. Due to the low solubility of Cu(II) at pH 7.0 to 8.0, the samples for measurements at these pH values were prepared by dialysis of the metal-free protein against the same buffer without $CuCl_2$ to remove free Cu^{2+}. Because mPrP(121–231) shows a strong aggregation tendency upon addition of Cu^{2+} at pH 7–8, the concentration of soluble protein after dialysis was too low to give interpretable EPR spectra. However, by addition of 0.8 molar equivalents of $CuCl_2$ to 0.1 mM PrP(121–231) at pH 7.4, an EPR spectrum with good signal-to-noise ratio was obtained. Buffer solutions with free Cu^{2+} (0.3 mM $CuCl_2$) were prepared as control.

Titration experiments at pH 4.0 and 6.0

A stock solution of mPrP(23–231) or mPrP(121–231) (70 μl, 0.143 mM in H_2O) was mixed with 20 μl of 50 mM sodium cacodylate/HCl (pH 6.0), or 50 mM formic acid/NaOH (pH 4.0) and 10 μl of a $CuCl_2$ solution (0.25–20 mM in H_2O) (final protein concentration, 0.1 mM; final Cu^{2+} concentration, 0.025–2 mM).

Samples of refolded PrP

mPrP(23–231) or mPrP(121–231) (0.8 mM each) in 25 mM sodium acetate/HCl, pH 4.0 (same conditions as in Negro et al., 1997), 8 M urea, was allowed to refold for 1 h at 15°C after a 1:30 dilution with 25 mM sodium acetate/HCl (pH 4.0). The refolded protein was extensively dialyzed against distilled water at 4°C. The final protein solution had a pH of 5.6. For EPR measurements, the protein solutions were again concentrated to 0.1 mM. In another set of experiments, refolding was performed under the same conditions, but in the presence of 1 mM $CuCl_2$, followed by dialysis against distilled water as described above. A final glycerol content of 10% (v/v) was used for the EPR measurements.

Samples for circular dichroism measurements

A stock solution of PrP(23–231) was used to prepare samples of 10 μM PrP(23–231) with or without 0.1 mM $CuCl_2$ in distilled water without buffer or with 10 mM formic acid/NaOH, pH 4.0. The sample of PrP(23–231) refolded in the presence of 1 mM $CuCl_2$ and dialyzed against distilled water was prepared as described in the previous paragraph.

Continuous wave EPR spectroscopy

The EPR spectra were recorded on a Bruker ESP300 spectrometer (microwave frequency 9.43 GHz), equipped with a ER4131 VT digital temperature control system, making use of gaseous nitrogen as a coolant. A microwave power of 20 mW, a modulation amplitude of 0.5 mT, and a modulation frequency of 100 kHz were used. All spectra were recorded at a temperature of 120 K. The EPR spectra were simulated using the EasySpin program, a MATLAB toolbox developed for EPR simulations (http://www.esr.ethz.ch).

In order to evaluate the titration results, the double integrals of the EPR spectra were calculated after second order base-line correction. All values were normalized to the same amount of protein.

Circular dichroism spectra

Far-UV circular dichroism (CD) spectra were measured at 25° C on a Jasco 710 CD spectropolarimeter in 0.1-cm quartz cuvettes, accumulated 8 times and corrected for the corresponding buffer. Protein samples were centrifuged (5 min, 14,000 rpm, 25° C) before the concentration and CD measurements in order to remove possible aggregates.

RESULTS

The EPR spectra of the full-length murine prion protein, mPrP(23–231) in the presence of Cu^{2+} (Figs. 1, 3) are typical for type-2 protein-copper(II) complexes (axial g matrix, copper hyperfine coupling, $A_{\|} > 400$ MHz) (Messerschmidt, 1998; Kaim and Rall, 1996). Type-2 complexes are largely square-planar with a possible fifth weak coordination. Naturally occuring type-2 copper proteins do not contain sulfur ligands, although type-2 copper sites with cysteinate ligation have been generated using genetic engineering (Messerschmidt, 1998). For type-2 copper complexes, the ($g_{\|}$, $A_{\|}$) values correlate with the type of the equatorially coordinating atoms (Peisach and Blumberg, 1974). Unfortunately, the $g_{\|}$ and $A_{\|}$ values depend also on the charge of the surrounding ligands and a possible fifth ligand, so that a clear-cut determination of the coordination sphere on the basis of the EPR data alone is not possible. Nevertheless, the $g_{\|}$ and $A_{\|}$ values can be used to get a first hint about the coordinating atoms.

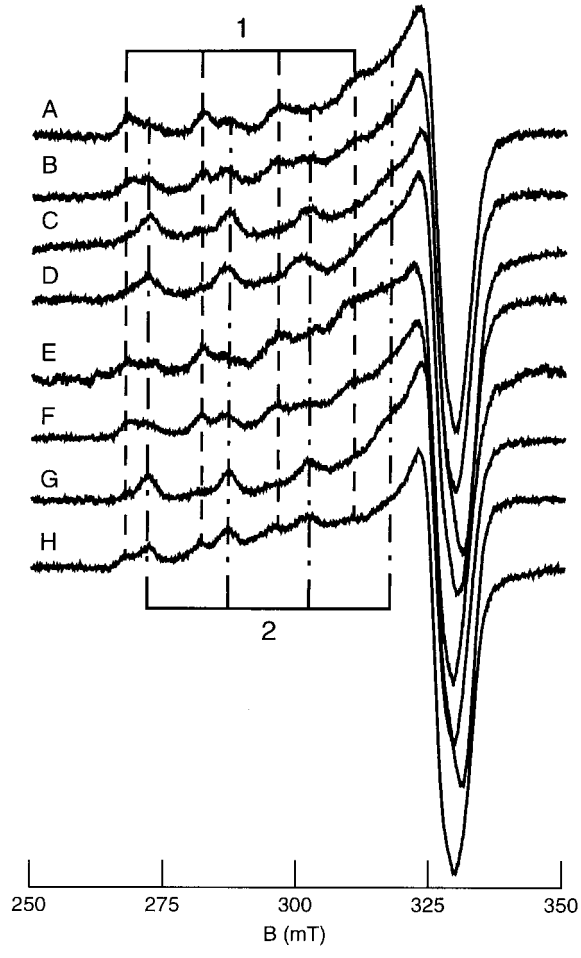

FIGURE 1 X-band EPR. spectra of 0.1 mM mPrP(23–231) with 3 molar equivalents, $CuCl_2$ at pH 3 (*A*), 4 (*B*), 5 (*C*), and 6 (*D*), and of 0.1 mM mPrP(121–231) with 3 molar equivalents, $CuCl_2$ at pH 3 (*E*), 4 (*F*), 5 (*G*), and 6 (*H*).

The pH dependence of the copper(II)-PrP interaction

Fig. 1 (*A–D*) shows the EPR spectra of 100 μM mPrP(23–231) in the presence of 3 molar equivalents of $CuCl_2$ at pH 3.0–6.0. Buffer solutions with 0.3 mM $CuCl_2$ were also examined to confirm that the observed EPR spectra were due to copper binding to the protein. The EPR spectra of copper(II) with two different coordination spheres (1 and 2 in Fig. 1) are observed; the ratio of both binding modes depends on pH. The $g_\parallel$ and $A_\parallel$ values of complex 1 (Table 1) are in agreement with three types of copper ligation, namely ligation to two nitrogens and two oxygens (2N2O), to one nitrogen and three oxygens (1N3O), or to four oxygens (4O). For complex 2, the EPR parameters (Table 1) are in agreement with the four combinations 3N1O, 2N2O, 1N3O, or 1N2O1S (Peisach and Blumberg, 1974; den Blauwen and Canter, 1993).

Fig. 1 (*E—H*) shows the corresponding EPR spectra of the isolated C-terminal domain of murine PrP, mPrP(121–231), with 3 molar equivalents of $CuCl_2$ in the same pH range. The same complexes 1 and 2 are found as in the case of mPrP(23–231) (Fig. 1 *A—D*).

Fig. 2 *B*, *D*, and *F* show the X-band EPR spectra of mPrP(58–91) with four equivalents of Cu^{2+} at pH 4, 5, and 6. Fig. 2, *A*, *C*, *E* depict the corresponding buffer controls with 0.3 mM Cu^{2+}. The spectra reveal that mPrP(58–91) does not bind Cu(II) below pH 6 in contrast to mPrP(121–231) and mPrP(23–231) (Fig. 1). At pH 6, the EPR spectrum of mPrP(58–91) with Cu^{2+} (Fig. 2 *F*) consists of two components, the Cu(II)-buffer signal (Fig. 2 *E*) and the Cu(II)-bound mPrP(58–91) signal (complex 4, Table 1). The EPR parameters of complex 4 are different from those observed for complex 1 and 2. This indicates that, up to pH 6, Cu^{2+} preferably binds to the C-terminal segment of the mouse prion protein and not, as expected (Hornshaw et al., 1995a,b; Stöckel et al., 1998; Viles et al., 1999; Miura et al., 1999; Whittal et al., 2000), to the histidine-rich N-terminal region.

At pH 7.0–8.0, Cu^{2+} salts have a low solubility. To analyze Cu^{2+} binding to PrP at pH 7–8, metal-free mPrP(23–231) and mPrP(121–231) were dialyzed at concentrations of 0.1 mM against a buffer solution containing 50 μM $CuCl_2$. Thereafter the protein solutions were dialyzed against the same buffers without copper to remove unbound Cu^{2+}. The corresponding EPR spectra are shown in Fig. 3 (*A–C*). The EPR spectra of mPrP(23–231) at pH 7.0–8.0 lack the features of complex 1, but exhibit an additional contribution corresponding to a new coordination sphere (complex 3, table 1). Complex 2 is still observed. The $g_\parallel$ and $A_\parallel$ values of complex 3 are in agreement with the ligations 4N, 3N1O, 2N2O, or 2N1O1S (Peisach and Blumberg, 1974; den Blauwen and Canter, 1993; van Pouderoyen et al., 1996).

Because mPrP(121–231) shows a strong aggregation tendency upon addition of $CuCl_2$ at pH 7.0–8.0, the effective concentration of the remaining soluble protein in solution after dialysis was too low to give interpretable EPR spectra. However, by addition of less than 1 molar equivalent Cu^{2+} to mPrP(121–231) at pH 7.4, a spectrum with sufficient signal-to-noise ratio was obtained (Fig. 3 *D*). Complex 2 and complex 3 contributing to this EPR spectrum are the same as those found for mPrP(23–231) at pH 7.4. The EPR spectrum of Cu(II)-bound mPrP(58–91) (Fig. 2 *G*) consists of a complex 3-type coordination and complex 4. The finding that a complex 3-type coordination sphere is also formed for mPrP(58–91) at pH 7.4 will be treated in the Discussion section.

Titration of mPrP(23–231) and mPrP(121–231) with increasing amounts of Cu^{2+}

To investigate further the finding that copper(II) binds to the C-terminal part of the prion protein at pH < 7, different molar equivalents of $CuCl_2$ were added to mPrP(23–231) and mPrP(121–231) at pH 4.0 and pH 6.0. The pH-value in the lysosomes, where the scrapie isoform of the prion protein accumulates, is 4.0–6.0 (Lee et al., 1996), hence the choice of the pH values for the titration.

Fig. 4 *A* shows the control EPR spectrum of the formic acid/NaOH buffer solution (pH 4.0) with 0.3 mM $CuCl_2$.

TABLE 1 EPR parameters of the type-2 Cu(II) complexes observed in copper containing full-length mPrP(23–231).

Complex	$g_\perp$ ($\pm$0.005),	$g_\parallel$ ($\pm$0.005)	$A_\perp$ ($\pm$10) (MHz)	$A_\parallel$ ($\pm$10) (MHz)	Observed in	pH
1	2.068	2.332	12	452	mPrP(121–231) mPrP(23–231)	3–6
2	2.068	2.295	20	457	mPrP(121–231) mPrP(23–231)	3–8
3	2.055	2.230	50	495	mPrP(121–231) mPrP(58–91) mPrP(23–231)	7–8
4	2.055	2.270	50	520	mPrP(58–91)	6, 7.4
5	2.114		Not resolved		mPrP(23–120) $>$ 4 equivalents Cu^{2+}	4 + 6

The hyperfine data are given for ^{63}Cu. The location of the Cu(II) complexes in the protein and the pH values at which they are observable are also given.

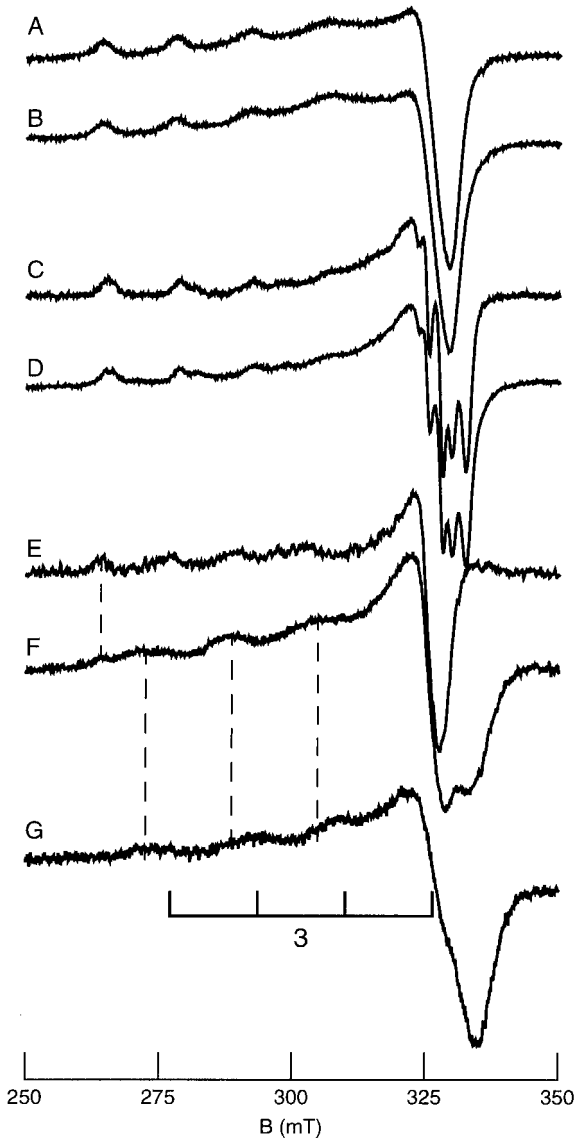

FIGURE 2 X-band EPR spectra of the peptide mPrP(58–91) between pH 3 and 7.4 in the presence of Cu^{2+}. Spectra of buffer at pH 4 with 0.3 mM $CuCl_2$ (*A*), mPrP(58–91) with 4 molar equivalents of Cu^{2+} at pH 4 (*B*), buffer at pH 5 with 0.3 mM $CuCl_2$ (*C*), mPrP(58–91) with 4 molar equivalents of Cu^{2+} at pH 5 (*D*), buffer at pH 6 with 0.3 mM $CuCl_2$ (*E*), mPrP(58–91) with 4 molar equivalents of Cu^{2+} at pH 6 (*F*), mPrP(58–91) with 2 molar equivalents of Cu^{2+} at pH 7.4 (*G*) are shown.

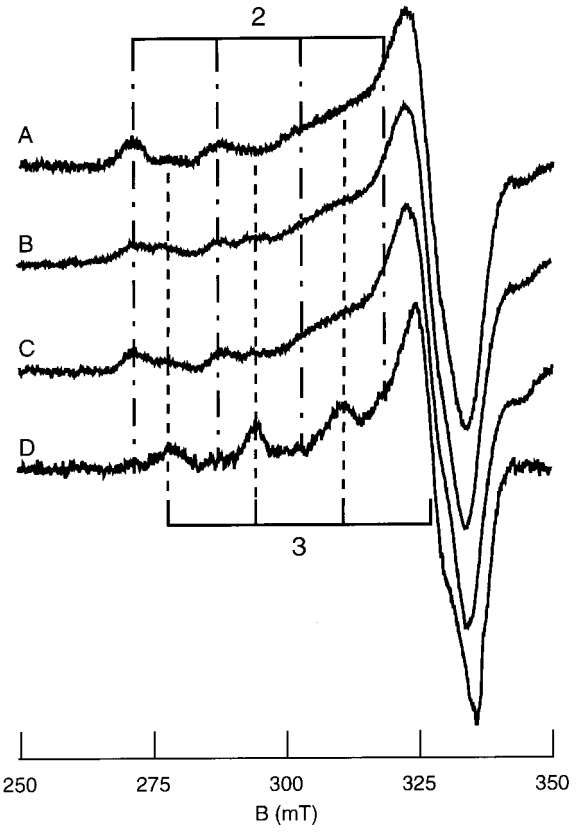

FIGURE 3 X-band EPR spectra of mPrP(23–231) dialyzed against a buffer solution at pH 7.0 (*A*), 7.4 (*B*), and 8.0 (*C*) with 50 μ. M. $CuCl_2$, with subsequent dialysis against the same buffers lacking Cu^{2+}. As a comparison, the EPR spectrum of mPrP(121–231) with less than one molar equivalent copper added at pH 7.4 is also shown (*D*).

Upon addition of 0.25 to 4 molar equivalents of $CuCl_2$ to a 0.1 mM mPrP(121–231) solution at pH 4, the EPR spectra show a mixture of contributions from complexes 1 and 2 (Fig. 4, *B* and *C*). There seems to be no special preference for one of these complexes, because the EPR spectra of both complexes are already visible when <1 molar equivalent of Cu^{2+} is added. The EPR signal intensities increase linearly with the number of molar equivalents $CuCl_2$ added to the solution. From 5 molar equivalents of Cu^{2+} onwards, three copper(II) complexes contribute to the EPR spectrum of mPrP(121–231) at pH 4 (Fig. 4 *D*). The additional EPR spectrum is due to the copper(II)-formate complex (Fig. 4 *A*). This contribution increases with increasing concentration of $CuCl_2$, indicating that mPrP(121–231) binds a maximum of four copper(II) ions at pH 4.

When up to 4 molar equivalents of $CuCl_2$ were added to a 0.1 mM solution of full-length mPrP(23–231) at pH 4, the same behavior as for mPrP(121–231) was observed (Fig. 4, *E* and *F*). Upon addition of 5 to 10 molar equivalents of Cu^{2+} to mPrP(23–231), however, the overall EPR spectrum changed in the $g_\perp$-region, but no feature was found that corresponds to the copper(II)-formate complex (Fig. 4, *G* and *H*). Only from 12 molar equivalents of $CuCl_2$ onwards, a contribution of non-protein-bound Cu^{2+} in the form of the copper(II)-formate complex became visible (Fig. 4 *I*). Moreover, from 4 to 10 molar equivalents of $CuCl_2$, the signal intensity did not change in the 260 to 280 mT region. Fig. 5 shows the difference between the EPR spectra of mPrP(23–231) with 10 and 4 molar equivalents of $CuCl_2$. The resulting EPR signal does not show resolved hyperfine splittings (Table 1, complex 5), indicating that the Cu(II) complexes are in close vicinity to each other. Large local

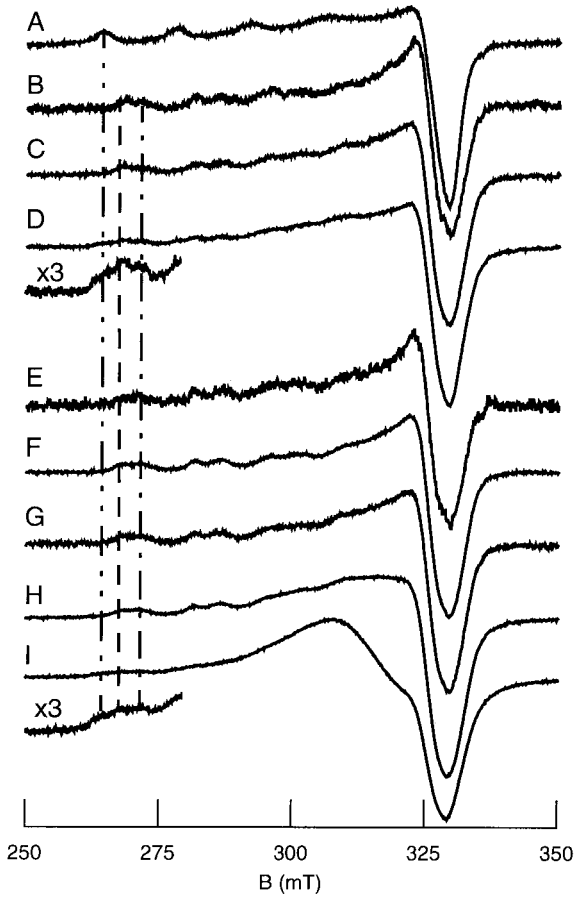

FIGURE 4 Titration experiments at pH 4.0. X-band EPR spectra of the following samples are shown: buffer control (formic acid/NaOH buffer (pH 4.0) and 0.3 mM $CuCl_2$) (*A*), mPrP(121–231) with 0.75 (*B*), 4 (*C*), and 5 (*D*) molar equivalents $CuCl_2$ (pH 4) and mPrP(23–231) with 0.5 (*E*), 4 (*F*), 5 (*G*), 10 (*H*), and 12 (*I*) molar equivalents $CuCl_2$ (pH 4). For clarity, the low-field part of the spectra, *D* and *I*, are shown in triple magnification.

concentrations of Cu(II) complexes are shown to cause inevitable broadening of the EPR lines (Brüggeller and Mayer, 1980).

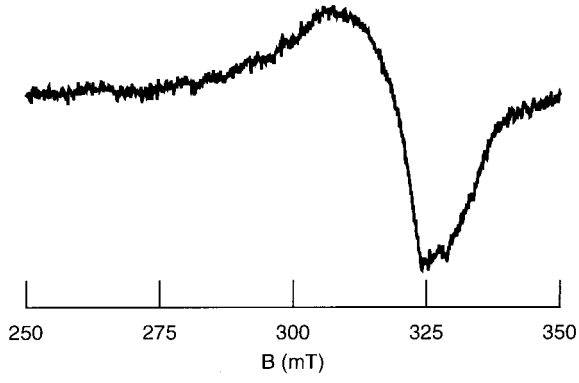

FIGURE 5 Difference EPR spectrum of mPrP(23–231) in the presence of 10 molar equivalents $CuCl_2$, and mPrP(23–231) in the presence of 4 molar equivalents $CuCl_2$ at pH 4 (cf. Fig. 3).

Analogous results were obtained at pH 6.0. In the presence of 0–3 molar equivalents of $CuCl_2$ relative to mPrP(121–231), the EPR intensity increased linearly with Cu^{2+} concentration, but the C-terminal domain aggregated upon addition of 4 molar equivalents of Cu^{2+}. On the contrary, the full-length prion protein did not aggregate, even in the presence of 20 equivalents of Cu^{2+}. In the range of 0–4 equivalents of $CuCl_2$, the typical EPR spectrum shown in Fig. 1 (pH 6) was observed. For 5 or more molar equivalents of Cu^{2+}, the spectrum altered, although the changes were not as clearly interpretable as in the pH 4 case. At concentrations corresponding to >12 equivalents of Cu^{2+}, the Cu^{2+}-buffer EPR spectrum became visible, indicating that also at pH 6, the prion protein can bind up to 12 equivalents of Cu^{2+}.

Pulse EPR and electron-nuclear double resonance results

In order to analyze the above findings further, pulse EPR and electron-nuclear double resonance (ENDOR) measurements were undertaken (Van Doorslaer et al., 2001). Here we mention those results which are of importance for the present investigation. All pulse EPR and ENDOR spectra of Cu(II)-bound mPrP(121–231) and of Cu(II)-bound mPrP(23–231) at pH 5.6 were identical, confirming again that the observed Cu(II) binding takes place in the C-terminal part of the protein. For complex 2, the hyperfine sublevel correlation spectroscopy and Davies-ENDOR spectra revealed the interactions with the remote and directly coupled nitrogen of one histidine ligand. For complex 1, no interactions with directly coordinating nitrogens were observed. Finally, the Davies-ENDOR analysis of mPrP(23–231) at pH 7.4 showed that more than one nitrogen atom is directly bound to copper in complex 3.

Influence of Cu^{2+} on the refolding of oxidized mPrP(23–231)

To analyze the influence of Cu^{2+} on the conformation of mPrP(23–231) after refolding from the urea-denatured state, unfolded mPrP(23–231) (pH 4, 8 M urea) was allowed to refold in the presence of a 10-fold molar excess of $CuCl_2$ for 1 h and was then extensively dialyzed against distilled water at 4°C to remove unbound copper ions. The final pH was 5.6. The resulting CW EPR spectrum of the concentrated sample (0.1 mM) is shown in Fig. 6 *A*. In a parallel experiment, $CuCl_2$ was added to a solution of native mPrP(23–231) at pH 4 in the absence of urea, and the sample was then dialyzed extensively against distilled water as described above. The resulting EPR spectrum at pH 5.6 is identical (Fig. 6 *B*) and proves that Cu^{2+} binds to mPrP(23–231) independent of whether Cu^{2+} is added before or after re-

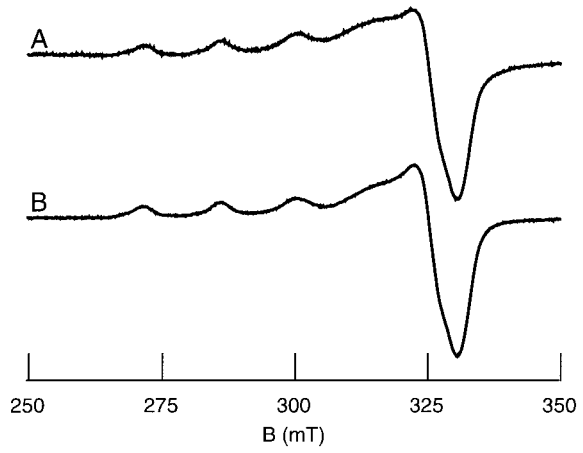

FIGURE 6 X-band EPR spectra of Cu(II)-bound mPrP(23–231). (*A*) mPrP(23–231) after refolding in the presence of $CuCl_2$ and subsequent dialysis to a final pH 5.6. (*B*) mPrP(23–231) after addition of $CuCl_2$ and subsequent dialysis to a final pH 5.6. Both samples contained 10% (v/v) glycerol.

folding. The same result was found for mPrP(121–231) (data not shown).

In addition, the CD spectra of mPrP(23–231) in water and 25 mM sodium acetate/HCl (pH 4.0) are identical to those of mPrP(23–231) in the presence of 10 molar equivalents of $CuCl_2$, as well as to that of mPrP(23–231) refolded in presence of Cu^{2+} and dialyzed against distilled water. This indicates that there is no substantial change in protein conformation due to refolding in the presence of Cu^{2+} (Fig. 7).

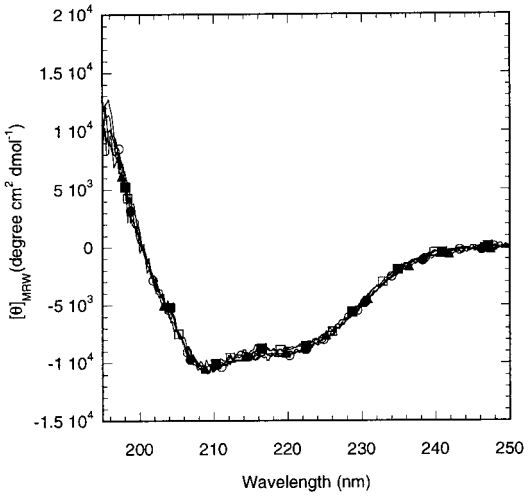

FIGURE 7 Circular dichroism (CD) spectra of 10 μM mPrP(23–231) in absence or presence of 10 molar equivalents $CuCl_2$. Ten-μM mPrP(23–231) in water without (○) or with Cu^{2+} (●), in 25 mM sodium acetate, pH 4.0, without (□) or with Cu^{2+} (■), and refolded in the presence of 10 molar equivalents Cu^{2+} at pH 4.0 and thereafter extensively dialyzed against water (▲). The symbols depict 7–11 data points from each measurement.

DISCUSSION

One has to be cautious in evaluating and comparing different studies of in vitro Cu(II) binding to prion proteins. First of all, the used analytical methods are usually sensitive in different concentration ranges. On the one hand, techniques suitable for structure analysis, such as NMR, Raman or EPR spectroscopy, are only sensitive at "high" protein and/or Cu^{2+} concentrations (25 μM–20 mM) (Viles et al., 1999; Miura et al., 1999; Aronoff-Spencer et al., 2000). On the other hand, analytical techniques such as CD, fluorescence and electron spray ionization mass spectrometry (ESI-MS) spectroscopy are sensitive to peptide and Cu^{2+} concentrations in the 1–10 μM region, but cannot give detailed structural information on the binding sites (Hornshaw et al., 1995a,b; Stöckel et al., 1998; Viles et al., 1999; Whittal et al., 2000). One of the missing links is a test to which extent the observed Cu(II) binding avidity of PrP is dependent on protein concentration. In this work, we tested the stability of the observed Cu(II) complexes by dialyzing the solutions of mPrP(23–231) and mPrP(121–231) with Cu^{2+} at pH 4 against water. During dialysis the protein concentration was lowered to 3.3 μM. Under these conditions, weakly and nonspecifically bound Cu(II) ions are expected to be removed. After dialysis, the protein was again concentrated to a final concentration of 100 μM. The fact that the same Cu(II) complexes were observed for these samples as for those in which Cu^{2+} was directly added to the protein solution provides strong evidence for specific binding.

Second, several in vitro studies on Cu(II)-binding to the prion protein were done with small synthetic peptide segments of the protein (Hornshaw et al., 1995a,b; Miura et al., 1999; Viles et al., 1999; Whittal et al., 2000; Aronoff-Spencer et al., 2000) and the question remains whether the behavior of these peptides is representative for the full-length protein. In this work, we therefore compared the results of the protein segments mPrP(121–231) and mPrP(58–91) with those of the full-length protein.

Finally, the biological interpretation of the different analytical results is rendered difficult by the fact that PrP is a membrane protein. As PrP^C is not an integral membrane protein, but attached to the cell surface via a GPI anchor, it is supposed to have a high lateral mobility within the membrane. Therefore, the effective concentration of the protein on the surface of the living cell is certainly high, not only in general terms due to its exclusive localization in a two-dimensional membrane, but also in terms of relative PrP^C concentrations. Moreover, the prion protein might interact with another protein at the membrane that stabilizes PrP^C-Cu(II) complexes. Futhermore, although the copper concentration is relatively high in the brain compared to other tissues (23 μg/g dry weight), the total copper content of different brain regions varies widely. The highest values are found in the locus ceruleus (1.4 mM), whereas the concentration of free copper ions in the cerebrospinal fluid

is only 0.24–0.28 μM (Geigy Scientific Tables, 1981). In view of all these factors it is principally difficult to correlate different in vitro results with Cu(II) binding of the prion protein in vivo.

In this work, different pH-dependent modes of copper complexation were observed in vitro for mPrP(23–231). Because the EPR spectra of complex 1 and 2 are observed in Cu(II)-bound mPrP(23–231) and mPrP(121–231), but not in Cu(II)-bound mPrP(58–91), they can be ascribed to Cu(II) binding sites in the structured C-terminal part of the protein.

Complex 1 is mainly observed at pH values < 7.0. Its EPR parameters suggest strong oxygen ligation (4O, 3O1N, or 2O2N). The fact that complex 1 forms the major fraction at pH 3.0 suggests that, for this complex, glutamic or aspartic acid are involved in the ligation of Cu(II). This agrees with the lack of nitrogen interactions in Davies-ENDOR or hyperfine sublevel correlation spectroscopy experiments (Van Doorslaer et al., 2001).

Complex 2 is visible throughout the investigated pH range of 3–8, although the contribution of this complex to the CW EPR spectrum decreases strongly below pH 5.0. The EPR parameters indicate that at least one nitrogen atom binds to the Cu^{2+} ion. The pH dependence of this complex suggests ligation of a histidine residue, which was confirmed by the pulse EPR and ENDOR studies (Van Doorslaer et al., 2001). mPrP(121–231) contains three histidines, H140, H177, and H187, that are too far apart in the structure to allow concerted Cu^{2+} binding. The possible involvement of the N-terminal amino group of mPrP(23–231) in the metal ligation, as observed for octapeptide repeat peptides (Whittal et al., 2000), can be ruled out for complexes 1 and 2 based on the pulse EPR/ENDOR results (Van Doorslaer et al., 2001) and on the fact that both complexes are observed at low pH.

The observation that mPrP(58–91) does not bind Cu^{2+} at pH values below 6 is in agreement with the studies of Cu(II) binding to the octarepeat peptides of the N-terminal part of the mature Syrian hamster prion protein SHaPrP (Viles et al., 1999) and of the human PrP (Aronoff-Spencer et al., 2000). At pH 6, mPrP(58–91) binds Cu(II) (complex 4, table 1). The EPR parameters of complex 4 are clearly different from those of complexes 1 and 2, but bear strong resemblance with those observed for Cu(II)-bound PrP(23–28, 57–91) at pH 6.75 ($g_{\|} = 2.27 \pm 0.01$, $g_{\perp} = 2.06 \pm 0.01$, $A_{\|} = 536 \pm 6$ MHz) (Aronoff-Spencer et al., 2000). Complex 4 is still visible at pH 7.4 (Fig. 2 *G*), in accordance with the observations of Aronoff-Spencer et al (2000).

The EPR parameters of complex 3 indicate a large involvement of nitrogen atoms in the Cu^{2+} ligation (2O2N, 1O3N, 4N, or 2N1S1O). Analogous data were found for Cu(II)-bound PrP(23–28, 57–91) at pH 7.45 ($g_{\|} = 2.23 \pm 0.01$, $g_{\perp} = 2.06 \pm 0.01$, $A_{\|} = 493 \pm 6$ MHz) (Aronoff-Spencer et al., 2000). Davies-ENDOR measurements of Cu(II)-bound mPrP(23–231) at pH 7.4 confirmed that the Cu(II) ion is directly coupled to more than one nitrogen atom (Van Doorslaer et al., 2001). Complex 3 is only visible at pH $\geq$ 7, suggesting that backbone nitrogens can be involved, because 1:2 Cu-peptide complexes in alkaline solutions, in which Cu(II) directly binds to backbone nitrogens, have similar *g* and copper hyperfine values (Szabó-Plánka et al., 1989). The fact that complex 3 is found in the EPR spectra of all three Cu(II)-bound peptides indicates that at pH $\geq$ 7 both mPrP(121–231) and mPrP(51–85) have very similar Cu(II) binding sites giving rise to quasi the same EPR spectrum. Further investigations will be necessary to elucidate the exact nature of the coordination spheres.

Whittal et al. (2000) reported that only weak and apparently nonspecific binding of Cu^{2+} could be found for SHaPrP(90–144) and mPrP(177–230) using ESI-MS. Protein concentrations of 10 μM and pH values 6 and 7.4 were used in this study, which makes a direct comparison with our data difficult. However, the ESI-MS findings do not necessarily imply that the Cu(II) complexes observed with EPR are nonspecific, as the isolated structured domain mPrP(121–231) was not investigated by Whittal et al. Furthermore, our dialysis tests support the model of specific binding of Cu^{2+} to the structured part of the protein.

Complex 5 is exclusively observed with full-length PrP^C upon addition of >4 molar equivalents of Cu^{2+}. The observation that full-length PrP^C may bind 10–11 Cu^{2+} ions at pH 4, whereas the N-terminal mPrP(58–91) does not bind Cu^{2+} and mPrP(121–231) binds only 4 equivalents of Cu^{2+} seems to indicate that most of the binding sites contributing to signal 5 are non-specific. This is corroborated by the fact that complex 5 is not found after dialysis of the sample. At pH 6, mPrP(58–91) binds specifically Cu^{2+} (4). However, the large uptake of Cu^{2+} found for mPrP(23–231) at this pH and the observed broadening of the EPR signals when more than 4 equivalents of Cu^{2+} are added suggest that besides the specific binding also non-specific binding is taking place. This agrees with the earlier observation that the octarepeats nonspecifically bind Cu(II) at high Cu/peptide ratios (Whittal et al., 2000).

Due to the observed cooperative binding of Cu^{2+} ions to the four histidines contained in the octapeptide repeats of the unstructured, N-terminal region 60–91 of PrP^C it was so far assumed that the copper binding properties of the prion protein are mainly attributable to this region of the protein (Hornshaw et al., 1995a,b; Stöckel et al., 1998; Viles et al., 1999; Miura et al., 1999; Whittal et al., 2000; Aronoff-Spencer et al., 2000). Our result that the C-terminal part of the prion protein binds Cu(II) raises the possibility of Cu^{2+} binding to the recently described prion analog (prion doppel), which has been proposed to share structural similarities with the C-terminal domain of PrP^C, but lacks the N-terminal octapeptide repeats (Moore et al., 1999; Weissmann and Aguzzi, 1999). In addition, because the structured C-terminal domain of

mouse PrP^C can bind Cu^{2+}, this Cu^{2+} binding ability must consequently be attributed also to the fragment of the prion protein ranging from residue 90 to residue 231. Because this fragment is still capable of propagating prion disease (Shmerling et al., 1998), our results open the possibility that Cu(II)-bound PrP^C may be involved in the transition to PrP^{Sc}. Furthermore, our present finding that the full-length prion protein can bind Cu(II) at pH values <6 is interesting in view of the fact that the scrapie isoform of the prion protein accumulates in the lysosomes (pH 4–6) (Lee et al., 1996). Finally, the observed affinity of mPrP(23–231) for Cu^{2+} principally supports the hypothesis of a copper receptor or storage function of the prion protein in neuronal cells (Pauly and Harris, 1998; Herms et al., 1999), although we have to consider contributions of nonspecific binding.

In this study, we also analyzed the influence of Cu^{2+} on the refolding of urea-denatured, disulfide-intact mPrP(23–231) and mPrP(121–231). Brown et al. (1999) reported that only acquisition of Cu^{2+} by PrP^C during protein folding endowed SOD activity on the protein, but the addition of copper after refolding did not. Our present study shows, however, that binding of Cu^{2+} to the full-length mouse prion protein is independent of whether the protein was allowed to refold in the presence of Cu^{2+}, or Cu^{2+} was added after refolding. The discrepancy between our results and those of Brown et al. (1999) may result from different protein preparations. Specifically, the disulfide bond in all proteins used in the present study was quantitatively formed (Liemann and Glockshuber, 1999). Brown and co-workers did not first oxidize the two cysteine thiols of PrP before the copper-addition experiments. Thus, if the buried disulfide bond is not formed, the free cysteines may interact with the Cu^{2+} ions, leading to a non-native protein conformation and a non-native copper coordination sphere.

In conclusion, analysis of Cu^{2+} binding to the recombinant mouse prion protein with EPR shows that Cu^{2+} ions can bind to the structured C-terminal part of the protein. Cu^{2+} ions bound to this domain show three different coordination geometries, one of which involves the nitrogen atom of one of the three histidines contained in mPrP(121–231). This finding opens again the discussion on the possibility that Cu^{2+} is involved in the process of PrP^{Sc} formation and a putative function of PrP^C in the Cu^{2+} metabolism. Furthermore, our observations provide the basis for further investigations of the Cu(II) binding avidity and specificity of the C-terminal domain of the prion protein with different analytical techniques.

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