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Copper binding to octarepeat peptides of the prion protein monitored by mass spectrometry

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Abstract

Electrospray ionization mass spectrometry (ESI-MS) was used to measure the binding of Cu^{2+} ions to synthetic peptides corresponding to sections of the sequence of the mature prion protein (PrP). ESI-MS demonstrates that Cu^{2+} is unique among divalent metal ions in binding to PrP and defines the location of the major Cu^{2+} binding site as the octarepeat region in the N-terminal domain, containing multiple copies of the repeat ProHisGlyGlyGlyTrpGlyGln. The stoichiometries of the complexes measured directly by ESI-MS are pH dependent: a peptide containing four octarepeats chelates two Cu^{2+} ions at pH 6 but four at pH 7.4. At the higher pH, the binding of multiple Cu^{2+} ions occurs with a high degree of cooperativity for peptides C-terminally extended to incorporate a fifth histidine. Dissociation constants for each Cu^{2+} ion binding to the octarepeat peptides, reported here for the first time, are mostly in the low micromolar range; for the addition of the third and fourth Cu^{2+} ions to the extended peptides at pH 7.4, K_D's are $<$100 nM. N-terminal acetylation of the peptides caused some reduction in the stoichiometry of binding at both pH's. Cu^{2+} also binds to a peptide corresponding to the extreme N-terminus of PrP that precedes the octarepeats, arguing that this region of the sequence may also make a contribution to the Cu^{2+} complexation. Although the structure of the four-octarepeat peptide is not affected by pH changes in the absence of Cu^{2+}, as judged by circular dichroism, Cu^{2+} binding induces a modest change at pH 6 and a major structural perturbation at pH 7.4. It is possible that PrP functions as a Cu^{2+} transporter by binding Cu^{2+} ions from the extracellular medium under physiologic conditions and then releasing some or all of this metal upon exposure to acidic pH in endosomes or secondary lysosomes.

Keywords: circular dichroism; copper binding; dissociation constants; electrospray ionization mass spectrometry; prion protein; PrP; PrP peptides

The prion protein (PrP) exists in two physically and biologically distinct isoforms, the normal cellular protein (PrP^C), and the pathogenic isoform (PrP^{Sc}). PrP has been identified in all mammalian and avian species studied to date. Transgenic mice lacking the PrP gene are resistant to prion infection (Büeler et al., 1993; Prusiner et al., 1993), but in most other respects they develop normally. A report that PrP was necessary for normal synaptic function (Collinge et al., 1994) was not supported by later observations (Herms et al., 1995; Lledo et al., 1996). Abnormal sleep patterns have been described in PrP knockout mice (Tobler et al., 1996), and there have been reports of impaired learning and long-term memory (Nishida et al., 1997), but swimming navigation ability was not impaired for $PrP^{0/0}$ mice up to 2 years of age (Lipp et al., 1998). Thus, the normal physiological function of PrP has not been determined, although recent evidence suggests that PrP is a copper-binding protein. NMR analysis of recombinant PrP, refolded to resemble PrP^C but devoid of the oligosaccharides and GPI anchor, revealed an N-terminal unstructured region of ~100 amino acids (Riek et al., 1996, 1997; Donne et al., 1997; James et al., 1997). This somewhat unusual feature suggests that secondary structure in this region of PrP^C may be induced by some external process. Metal ion binding could provide such a mechanism (Viles et al., 1999).

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Residues 57–91 of the N-terminus of Syrian hamster (SHa) PrP comprise a highly conserved region WGQ(PHGGGWGQ)$_4$ (Oesch et al., 1985). The repetition of the eight amino acid sequence PHGGGWGQ, each copy of which is referred to as one octarepeat, was identified as a potential Cu^{2+} binding motif, which might induce secondary structure and protect the N-terminus against adventitious proteolysis. The high histidine content suggested PrP might be a Cu^{2+} binding protein that could be purified by immobilized metal ion affinity chromatography (Sulkowski, 1985, 1989), which subsequently proved to be correct (Pan et al., 1992). Hornshaw et al. showed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) (Hornshaw et al., 1995a) and tryptophan fluorescence spectroscopy (Hornshaw et al., 1995b) that octarepeat synthetic peptides and chicken PrP hexarepeat peptides preferentially bind Cu^{2+} compared with nickel and zinc. Raman spectroscopy showed that the sequence HisGlyGlyGly binds Cu^{2+} and that a single octarepeat forms an α-helix in the presence of Cu^{2+}, with helix formation extending to further residues in the longer peptide HuPrP(84–103) (Miura et al., 1996). Equilibrium dialysis at pH 7.4 indicated that recombinant HuPrP(23–98) binds 5.6 moles of Cu^{2+} per mole of protein with 3.4 moles binding cooperatively (Brown et al., 1997a), whereas independent studies using circular dichroism (CD), tryptophan fluorescence-spectroscopy, and equilibrium dialysis determined that recombinant SHaPrP(29–231) and a synthetic peptide SHaPrP(57–91) selectively bind only 2 moles of Cu^{2+} at pH 6.0, two octarepeats being necessary for chelation of each Cu^{2+} ion (Stöckel et al., 1998). On the basis of more recent studies on octarepeat peptides using CD, ESR, and NMR, a novel planar structure has been proposed for the binding of four Cu^{2+} ions by the four octarepeats, which could confer a stable structure on the N-terminus of PrP^{C} in vivo (Viles et al., 1999). Other recent studies by Raman spectroscopy confirm the difference in copper binding at pH 6.0 and 7.4 and have lead to an alternative predicted structure at the higher pH involving one of the imidazole nitrogens and two deprotonated glycyl amide nitrogens per octarepeat (Miura et al., 1999). However, it has been reported that the N-terminus of chicken PrP is structured in its native state and does not bind Cu^{2+}, despite the behavior of isolated peptides, implying that copper binding may not be related to the normal physiological role of PrP (Marcotte & Eisenberg, 1999). It has also been reported that Cu^{2+} assists the in vitro regeneration of scrapie infectivity after partial denaturation with guanidine hydrochloride (McKenzie et al., 1998), although it seems unlikely this is related to any biological role of copper. The reported data for Cu^{2+} binding to mammalian PrP peptides and recombinant protein containing at least four copies of the octarepeat are summarized in Table 1.

Recent reports using electrospray ionization mass spectrometry (ESI-MS) have shown that noncovalent protein-metal interactions in solution can be maintained during desolvation and transfer to the gas phase; thus, solution phase complex formation can be monitored directly by mass spectrometry (for reviews see Loo, 1997; Last & Robinson, 1999). Several recent studies illustrate specific effects induced by metal binding or other noncovalent interactions monitored by ESI-MS. These include the observation that complexation of zinc into the two zinc fingers of the DNA-binding domain of the vitamin D receptor induces a conformational change only when both sites are occupied (Veenstra et al., 1998). Peptides were used to elucidate the differences in the metal binding characteristics of some ATP-ases. HisXxxHis or HisXxxXxxHis-containing peptides (where Xxx is any other amino acid) bound either Cu^{2+} or Ni^{2+} but not Zn^{2+}, whereas analogous peptides with only a single histidine bound only Cu^{2+}. By contrast, a peptide corresponding to a region of the Alzheimer's precursor protein having the motif HisXxxHisXxxHis bound all three divalent cations (Volz et al., 1998). Complexes between the trp repressor (TrpR) and its specific operator DNA were monitored in a competition experiment by ESI-MS. When TrpR was mixed with an equimolar mixture of DNA containing two consensus sequences separated by two, four, or six base pairs, 1:1 protein:DNA complexes were observed only with DNA having the 4-bp spacer (Potier et al., 1998). Thus, it has been established that noncovalent complexes observed in ESI-MS may be highly specific and frequently require well-defined structural characteristics for their formation.

In the work reported here, the interactions between Cu^{2+} and synthetic peptides spanning the entire PrP sequence were probed by ESI-MS under a variety of conditions. This method has identified the octarepeats as the primary site in PrP for Cu^{2+} binding and, unlike earlier approaches that have relied on inferences from spectral changes such as fluorescence and CD, ESI-MS has provided a direct measurement of the stoichiometry of the interactions at physiologically relevant concentrations, allowing measurement of dissociation constants, $K_D n$ for the addition of each Cu^{2+} ion. Cu^{2+} binding to PrP peptides showed a strong dependence on pH, which could be measured directly and unambiguously by ESI-MS. In addition to the octarepeats, the N-terminus of PrP and an addi-

Table 1. *Summary of copper binding data reported for mammalian PrP species containing at least four octarepeats*

PrP sequence[a]	Copper[b]	K_D (μM)[c]	pH	Method	Reference
60–91	4	6.7	7.4	MALDI-MS, fluorescence	Hornshaw et al. (1995)
23–98	5.6	5.9	7.4	Equilibrium dialysis	Brown et al. (1997)
29–231	1.8	14	6.0	Equilibrium dialysis	Stöckel et al. (1998)
58–91	4	6	7.4	CD	Viles et al. (1999)
57–91	2	See Table 4	6.0	ESI-MS	This work
57–91	4	See Table 4	7.4	ESI-MS	This work
23–98	3	See Table 4	6.0	ESI-MS	This work
23–98	5	See Table 4	7.4	ESI-MS	This work

[a]The species studied were based on either Syrian hamster or human PrP for which the octarepeat sequences are the same.
[b]Maximum stoichiometry of Cu^{2+} ions binding at reasonable maximum physiological concentrations of copper.
[c]Dissociation constants, mostly derived from observed half-maximal binding.

Table 2. *PrP peptides used in this study*[a]

Residues	Amino acid sequence
23–60	KKRPKPGGWNTGGSRYPGQGSPGGNRYPPQGGGTWGQP
23–98	KKRPKPGGWNTGGSRYPGQGSPGGNRYPPQGGGTWGQ(PHGGGWGQ)$_4$GGGTHNQ
57–91	WGQ(PHGGGWGQ)$_4$
73–91	WGQ(PHGGGWGQ)$_2$
57–98	WGQ(PHGGGWGQ)$_4$GGGTHNQ
90–144	QGGGTHNQWNKPSKPKTNMKHMAGAAAAGAVVGGLGGYMLGSAMSRPMMHFGND
142–177	GNDWEDRYYRENMNRYPNQVYYRPVDQYNNQNNFVH
178–231	DCVNITIKQHTVTTTTKGENFTETDVKMMERVVEQMCVTQYQKESQAYYDGRRS

[a]All peptides are based on the SHa numbering system and all except for residues 178–231 correspond to the SHa sequence. PrP(178–231) is based on the Mo sequence and has the following amino acid differences compared with SHa: I203V, I205M, and T215V.

tional histidine C-terminal to the octarepeats (His96) may play a role in the binding of Cu^{2+}.

Results

The majority of methods employed to identify metal binding to peptides or proteins are indirect, relying on a change in a physical property such as fluorescence or molar ellipticity. By contrast, mass spectrometry reveals the complexes directly, but there is a danger of interference from nonspecific binding. Na^+ and K^+ are frequently observed to attach to peptides examined by ESI-MS, although this binding seems to be nonspecific. Since other metal ions, including Cu^{2+}, might bind to peptides in a nonspecific manner, we sought to distinguish such artefactual complexes from peptide-metal ion complexes that might be meaningful biologically.

The peptides used in this study are listed in Table 2. All correspond to the Syrian Hamster (SHa) sequence except PrP(178–231), which is based on the mouse (Mo) sequence.

Specificity of binding Cu^{2+} ions to octarepeat peptides

ESI mass spectra were recorded for the four-octarepeat peptide SHaPrP(57–91) at pH 6 (Table 3; Fig. 1) and 7.4 (Table 3) without added metal ions and in the presence of the divalent metal salts, copper(II)sulfate, nickel(II)sulfate, or zinc(II)sulfate at 25 μM concentration. For all ESI-MS experiments described here, the peptide concentration was 10 μM. The Cu^{2+} concentrations stated for each experiment allow the Cu^{2+}:peptide ratios to be determined.

Figure 1 shows the original mass spectra as recorded without further processing. They are typical of ESI-MS in that they reveal an array of multiply charged ions. Thus, the peaks at m/z 699.7, 874.4, and 1,165.5 in the spectrum at pH 6 without metal ions (Fig. 1A) are attributable to the PrP peptide with five, four, and three protons attached, respectively. A shift to higher m/z of each peak due to strong metal binding was observed only with the Cu^{2+} ions (Fig. 1B), for which the most abundant species corresponded to the attachment of two metal ions and the peaks representing free peptide amounted to less than 0.5% of the total. By contrast, in the presence of nickel (Fig. 1C) or zinc (Fig. 1D), 80% or more peptide remained free of metal, the remainder of the peptide being associated with a single metal ion. The ESI spectra obtained at pH 7.4 showed even greater binding of Cu^{2+} and some increase in binding of Ni^{2+} and Zn^{2+}, but for Ni^{2+} and Zn^{2+} the concentration of free metal ion in solution greatly exceeded that of the bound species; therefore, the binding constants for these other divalent metal ions greatly exceeded 25 μM. Data derived from the ESI spectra obtained at both pH's (Table 3) confirmed earlier findings that octarepeat peptides form specific complexes with Cu^{2+} but not with Ni^{2+} or Zn^{2+} (Stöckel et al., 1998). These data argue that ESI-MS can distinguish between the specific binding of Cu^{2+} to the four-octarepeat peptide compared with the weaker, seemingly nonspecific association of other divalent metal cations with the peptide.

Calculation of K_D

To determine the equilibrium constants for Cu^{2+} binding to the octarepeat peptide SHaPrP(57–91), it was incubated with increasing concentrations of Cu^{2+} at pH 6, and the signal intensities for the free and Cu^{2+} complexed peptide were summed for all charge states. From the total peptide and Cu^{2+} added as well as the relative signal intensities, the concentrations of free peptide and Cu^{2+} complexed peptide were plotted as a function of the Cu^{2+} added to the solution (Fig. 2). It was assumed that the total signal response for each individual species was proportional to the con-

Table 3. *Relative proportions (%) for free peptide, peptide with one metal ion, and peptide with two metal ions obtained from measured ESI-MS peak heights for 10 μM PrP(57–91) mixed with 25 μM divalent metal cation at pH 6.0 and 7.4; derived concentrations (μM) of free and bound metal ion from the same data*

M^{2+}	pH	Free PrP	PrP·M	PrP·2M	[Free M^{2+}]	[Bound M^{2+}]
Cu^{2+}	6.0	0.4	38.0	61.6[a]	7.9	17.1
	7.4	0	23.6	76.4[a]	2.6	22.4
Ni^{2+}	6.0	84.2	15.8	0	23.4	1.6
	7.4	63.8	32.5	3.6	21.0	4.0
Zn^{2+}	6.0	79.9	20.1	0	23.0	2.0
	7.4	69.0	28.2	2.8	21.6	3.4

[a]Includes peptide with three and four metal ions.

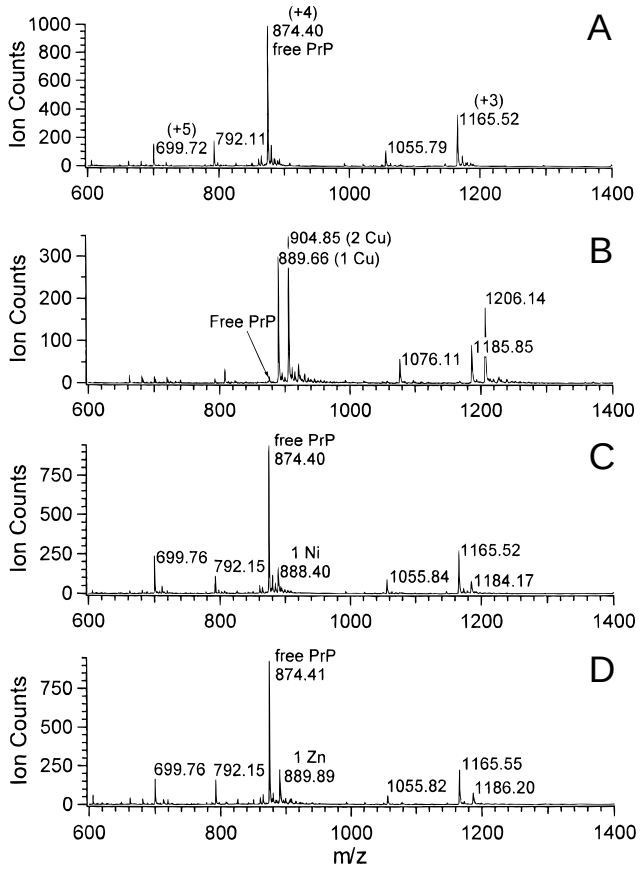

Fig. 1. ESI mass spectra of 10 μM SHaPrP(57–91) in 1 mM ammonium acetate, pH 6.0/10% methanol, (**A**) without added metal ions, (**B**) with 25 μM copper(II)sulfate, (**C**) with 25 μM nickel(II)sulfate, and (**D**) with 25 μM zinc(II)sulfate.

centration of that species in the gas phase, and by extension, in solution (see below). This also assumes that the free peptide and the peptide with metal bound give the same signal response.

As a control we established that the signal response remained the same for metal-free and metal-complexed octarepeat peptides, relative to the ion intensities for internal standard peptides that did not bind metal ions. From Figure 2, the total Cu^{2+} concentration at which the amount of free peptide was equal to the amount of singly complexed peptide was determined. At this point, the concentration of free Cu^{2+} was equal to the concentration of singly complexed peptide minus the amount of Cu^{2+} bound as $PrP \cdot Cu^{2+}$. Assuming an equivalence of concentrations and activities, from Equation 1, K_D1 is equal to the concentration of free Cu^{2+}.

$$K_D1 = \frac{[\text{Peptide}][Cu^{2+}]}{[\text{Peptide}\cdot Cu^{2+}]} = [Cu^{2+}]. \quad (1)$$

Similarly, further dissociation constants K_Dn can be obtained from Equation 2 as the concentration of free Cu^{2+} in solution when the amount of peptide with $n-1$ Cu^{2+} ions attached is equal to the amount of peptide with nCu^{2+} ions attached. Note that this is not the same as the overall dissociation constant for the equilibrium between a free peptide and the same peptide complexed with nCu^{2+} ions.

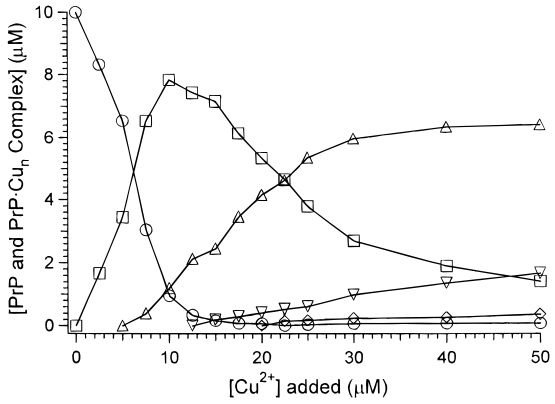

Fig. 2. Concentrations of free SHaPrP(57–91) (circles) and complexed SHaPrP(57–91)$\cdot n$Cu(II) for $n = 1$ (squares), 2 (triangles), 3 (inverted triangles), and 4 (diamonds), vs. the total Cu^{2+} added. Total peptide concentration was 10 μM. Data were derived from relative ion currents in ESI mass spectra, summing the ion currents for all charge states of each species.

$$K_Dn = \frac{[\text{Peptide}\cdot(n-1)Cu^{2+}][Cu^{2+}]}{[\text{Peptide}\cdot nCu^{2+}]} = [Cu^{2+}]. \quad (2)$$

It can be shown that the overall dissociation constant for the complex Peptide$\cdot nCu^{2+}$ is equal to the product of the individual stepwise K_D's, i.e., $K_D1 \times K_D2 \times \ldots \times K_Dn$. However, this has dimensions of $[\text{concentration}]^n$ and is not equivalent to the half-maximal binding values derived from saturation curves that have been reported previously for PrP peptides and Cu^{2+}. Stepwise K_Dn's for Cu^{2+} binding to PrP octarepeat peptides have never been reported before; the values calculated in this study are presented in Table 4.

An advantage of the ESI-MS method is the ability to obtain speciation information directly (Yu et al., 1993). For example, although at a fivefold molar ratio of Cu^{2+} there were on average 2.09 Cu^{2+} ions bound to the peptide, we could measure directly that 0.8% of the peptide was free of complexation, 14.3% had one Cu^{2+} bound, 64.3% had two Cu^{2+} ions bound, 16.8% had three Cu^{2+} ions bound, and 3.8% had four Cu^{2+} ions bound. This capability combined with the measured dissociation constants K_D1 and K_D2 contends that the attachment of two Cu^{2+} ions to SHaPrP(57–91) was avid and likely to be specific. By contrast, K_D3 was weak, suggesting the species with more than two Cu^{2+} ions attached arose from weak nonspecific interactions.

The extended peptide SHaPrP(57–98) contains four octarepeats with an additional seven C-terminal amino acid residues, including a fifth histidine at residue 96. We were interested to know whether His96 would complement the role of the octarepeat histidines in terms of the number of Cu^{2+} ions bound or the magnitude of the binding constants. With 25 μM Cu^{2+} at pH 6.0, the abundance of the species corresponding to the attachment of a single Cu^{2+} ion diminished from 38% of the total for SHaPrP(57–91) to 23% for SHaPrP(57–98), whereas the species with three Cu^{2+} ions increased from 7 to 12%. As listed in Table 4, K_D1 and K_D2 for SHaPrP(57–91) and SHaPrP(57–98) were similar. Although the binding of a third Cu^{2+} ion was somewhat stronger for the longer peptide, the magnitude of K_D3 for SHaPrP(57–91) (200 μM) and SHaPrP(57–98) (70 μM) confirmed only weak binding in both cases that would be irrelevant at physiological concentrations of Cu^{2+}, at least at pH 6.

Table 4. *Dissociation constants derived from ESI-MS data for the binding of Cu^{2+} to octarepeat-containing SHaPrP peptides* [a]

	K_D1	K_D2	K_D3	K_D4	K_D5
			At pH 6.0		
SHaPrP(73–91)	2.2 μM	69 μM			
SHaPrP(57–91)	0.7 μM	6.3 μM	~200 μM		
SHaPrP(57–98)	1.0 μM	6.0 μM	70 μM		
SHaPrP(23–98)	1.5 μM	2.8 μM	55 μM		
			At pH 7.4		
SHaPrP(57–91)	0.2 μM	0.7 μM	2.5 μM	12 μM	
SHaPrP(57–98)	1.7 μM	0.5 μM	<0.1 μM	<0.1 μM	4.3 μM
SHaPrP(23–98)	2.2 μM	0.5 μM	<0.1 μM	<0.1 μM	~5 μM

[a] Cu^{2+} was bound to the peptide in 1 mM ammonium acetate/10% methanol at room temperature at either pH 6.0 or 7.4.

Effect of pH and the role of histidine

SHaPrP(57–91) contains four octarepeats with the sequence PHG GGWGQ and a free N-terminal amino group. Each Cu^{2+} ion requires four ligands; thus, it is likely that binding two Cu^{2+} ions involves all four histidine imidazoles as well as other groups such as the amino terminus and perhaps the carbonyl oxygens of specific glycines (Stöckel et al., 1998). Since protonation should inhibit binding to histidine, we determined the pH dependence of Cu^{2+} binding. Figure 3A shows data extracted from spectra of SHaPrP(57–91) peptide with 10 μM Cu^{2+} in the pH range 3.2–8.0. At pH 3.2, only 5% of the peptide bound a single Cu^{2+} ion. At pH 3.9, the concentration of this species increased to slightly more than 50%. At higher pH the trend to increased Cu^{2+} binding continued, reaching a maximum of >80% from pH 6–7.4. Thus, although the binding of a single Cu^{2+} ion was suppressed below pH 5, above this value it was relatively independent of pH.

The data shown in Figure 1 established that at pH 6, SHaPrP(57–91) bound predominantly two Cu^{2+} ions in the presence of 25 μM Cu^{2+}. To determine whether the binding of multiple Cu^{2+} ions showed a different pH dependence than that of a single Cu^{2+}, we added 50 μM Cu^{2+} at a range of pH's. The resulting data showed a strong effect, with a dramatic difference between pH 6.0 and 7.4 (Fig. 3B). As anticipated, at pH 6.0 the major species formed was with two Cu^{2+} ions. By contrast, at pH 7.4 the most abundant species bound four Cu^{2+} ions with contributions from the addition of three and five Cu^{2+} ions. Under these conditions, 84% of the added Cu^{2+} was complexed, leaving only 8 μM free in solution.

Cu^{2+} binding was also monitored for SHaPrP(73–91), a peptide that has only two octarepeats and a free N-terminal amino group. At pH 6, this peptide bound a single Cu^{2+} ion strongly but a second Cu^{2+} ion was bound only weakly, even at high Cu^{2+} concentration. An analog of this peptide with the histidine imidazole rings replaced by cyclohexyl rings showed minimal Cu^{2+} binding at pH 6 with 10 μM Cu; only with 30 μM Cu^{2+} did the complex with a single Cu^{2+} ion become the most abundant species. Thus, as hypothesized previously, it appeared not only that two adjacent octarepeats were sufficient to bind one Cu^{2+} ion, but also that the histidines were essential ligands for this. However, at pH 7.4 the Cu^{2+} binding affinity of this peptide with mutated histidines was enhanced dramatically. In the presence of 10 μM Cu^{2+}, 64% of peptide molecules bound a single Cu^{2+} ion, and with 30 μM Cu^{2+} the fraction was virtually 100%. Thus, under conditions that would normally result in SHaPrP(73–91) binding two Cu^{2+} ions, this modified peptide was able to bind a single Cu^{2+} ion quite strongly via a histidine-independent mechanism, probably involving the free amino terminus together with other functional groups within the peptide.

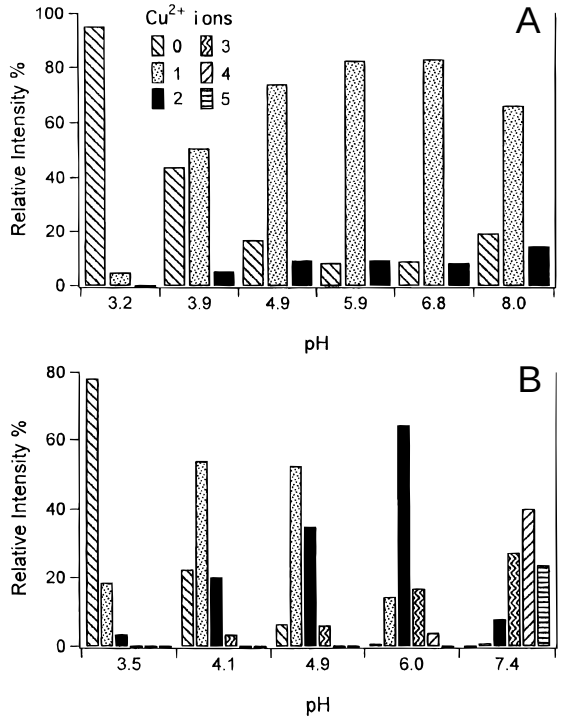

Fig. 3. The pH dependence of the binding of 0, 1, 2, 3, 4, and 5 Cu^{2+} ions to SHaPrP(57–91) (10 μM) in the presence of (**A**) 10 μM copper(II)sulfate and (**B**) 50 μM copper(II)sulfate.

Charge state of the bound copper

The use of ESI-MS has been reported to determine the redox state of iron in nonheme containing proteins (Lei et al., 1998). Although we added the copper in its +2 cupric state, we sought to establish whether or not it was reduced to +1 cuprous in the complex. The isotopic clusters of multiply charged ions were separated by the mass spectrometer, allowing accurate measurement of individual ion masses. At pH 6.0 the most abundant ion observed for SHaPrP(57–91) complexed with two copper ions had four positive charges. This was resolved into a cluster of closely spaced isotopic peaks, the mass/charge (m/z) value for the lowest mass component of which was measured to be 904.85. For comparison, theoretical masses were calculated based on the assumption that the four positive charges were derived from the addition of either: (1) two Cu^{2+} ions with no additional protons involved, $(Peptide \cdot Cu_2)^{4+}$; (2) one Cu^{+} plus one Cu^{2+} ion plus a single proton, $(Peptide \cdot Cu_2H)^{4+}$; or (3) two Cu^{+} ions plus two protons, $(Peptide \cdot Cu_2H_2)^{4+}$. For (1), the theoretical m/z value for the complex based on the so-called "monoisotopic mass" of ions of composition $^{12}C_{158}{}^{1}H_{200}{}^{14}N_{54}{}^{16}O_{40}{}^{63}Cu_2$ is 904.85, and for (2) and (3) the corresponding values are 905.10 and 905.35, respectively. Thus, the experimentally measured value was identical to the theoretical value for two Cu^{2+} ions and was clearly distinguishable from the alternatives. Furthermore, the observed isotope pattern closely matched the theoretical distribution for (1) with two Cu^{2+} ions rather than either (2) or (3), which confirmed that there was no significant contribution from species containing copper in the lower charge state together with additional protons. In Figure 4 the experimentally determined isotope ratios and isotopic masses are compared with theoretical predictions for the addition of zero, one and two Cu^{2+} ions to SHaPrP(57–91). It should be noted that data from the 4+ ions were presented here because they gave the most intense signals but analysis of the other charge states gave the same result.

Similar measurements were carried out at pH 7.4 for $(Peptide \cdot Cu_4)^{4+}$, the major species observed for the peptide at this pH in the presence of excess Cu^{2+}. These measurements established that the formation of this ion involved addition of four Cu^{2+} ions and the elimination of four protons from each peptide molecule, accounting for the overall charge of +4. This is consistent with the proposed formation of the imidazolate anion by ionization of the Nϵ2 NH of histidine, each of the four histidines acting as a bidentate ligand binding through Nδ1 and Nϵ2 to two copper ions (Viles et al., 1999). However, it is not compatible with the proposal that the backbone amides of two glycyl residues per octarepeat become deprotonated to act as copper binding sites (Miura et al., 1999).

Cu^{2+} binding to an N-terminally extended peptide

Cu^{2+} binding to the PrP N-terminal recombinant fragment HuPrP(23–98) at pH 7.4 has been reported to occur with a maximum molar ratio of 5.6 (Table 1) (Brown et al., 1997a). An analogous synthetic peptide was prepared using the Syrian hamster sequence. We observed that SHaPrP(23–98) strongly bound two Cu^{2+} ions at pH 6 with a much weaker affinity for a third Cu^{2+} ion. As with the smaller octarepeat peptides, Cu^{2+} binding by this extended N-terminal peptide showed a strong dependence on pH.

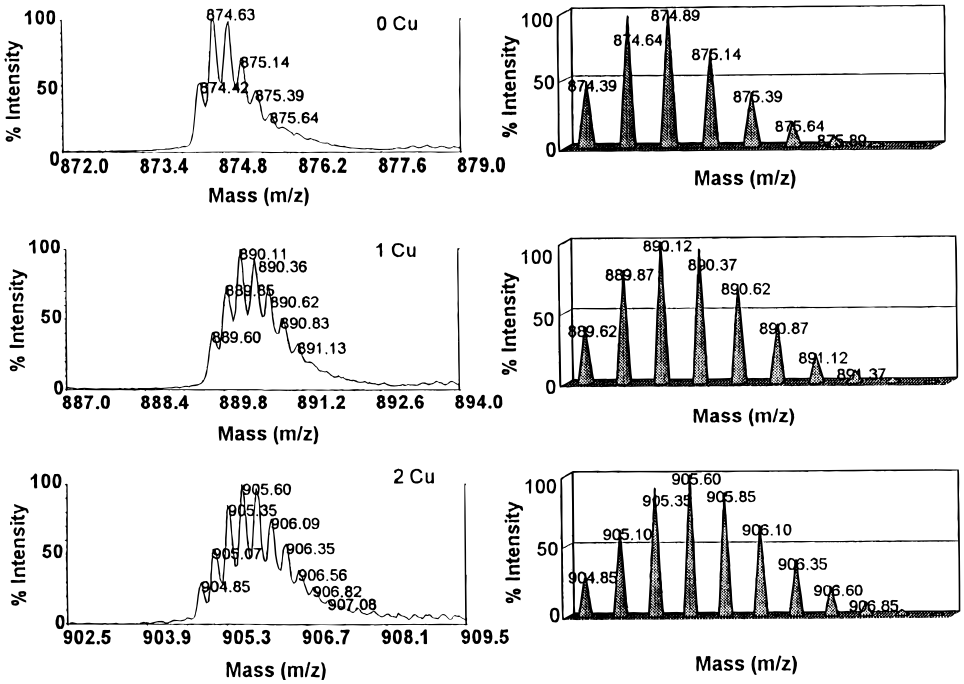

Fig. 4. ESI-MS isotope ratios and isotopic masses for the 4+ charge state of SHaPrP(57–91) with the addition of zero, one, and two copper ions at pH 6.0 (left-hand panel). The theoretical values (right-hand panel) were calculated assuming that each copper ion was Cu^{2+} and that the charges correspond to either $4H^{+}$ (top panel), $Cu^{2+}2H^{+}$ (center panel), or $2Cu^{2+}$ (lower panel). The lowest mass species for each isotopic cluster contains only the lowest mass isotopes, i.e., ^{1}H, ^{12}C, ^{14}N, ^{16}O, etc., and is defined as the monisotopic peak. All other species contain one or more heavier stable isotopes.

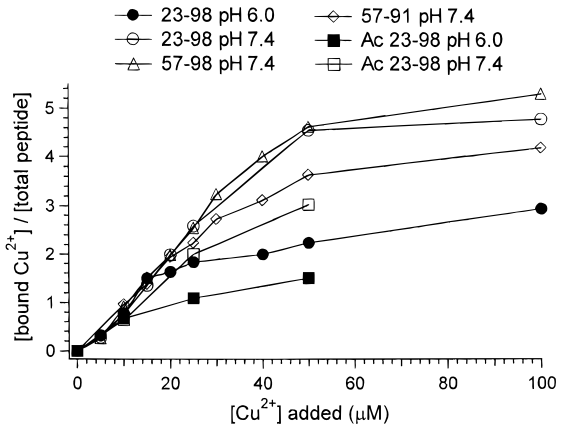

Fig. 5. The ratio of Cu^{2+} ions bound to total peptide as a function of Cu^{2+} added at pH 6.0 (solid symbols) and pH 7.4 (open symbols) for the following peptides: SHaPrP(57–91) (diamonds), SHaPrP(57–98) (triangles), SHaPrP(23–98) (circles), and SHaPrP(Ac23–98) (squares).

Figure 5 compares saturation curves measured at pH 6.0 and 7.4 over a wide concentration range. The ordinate represents the ratio of total Cu^{2+} bound in all complexes independent of stoichiometry relative to the total amount of peptide present. This is plotted as a function of total Cu^{2+} added. At the higher pH, there was a dramatic increase in multiple Cu^{2+} binding with strong cooperativity, manifest by the following observations. When 5 μM Cu^{2+} was added, 30% remained free in solution, but upon addition of 25 μM Cu^{2+}, 100% of this was complexed and there was no detectable free Cu^{2+} remaining in solution. In this situation with a 2.5 molar ratio of Cu^{2+}, only 2% of the peptide remained uncomplexed and the major species observed were Peptide$\cdot 2Cu^{2+}$ and Peptide$\cdot 3Cu^{2+}$. When the Cu^{2+} concentration was increased to 50 μM, 91% of the Cu^{2+} or 45.5 μM was bound and the balance of only 4.5 μM remained free in solution; thus, the average stoichiometry for the complex was 4.55 moles of Cu^{2+} bound per mole of peptide. Under these conditions the following species were observed: Peptide$\cdot 2Cu^{2+}$ (4%), Peptide$\cdot 3Cu^{2+}$ (13%), Peptide$\cdot 4Cu^{2+}$ (26%), Peptide$\cdot 5Cu^{2+}$ (33%), and Peptide$\cdot 6Cu^{2+}$ (23%). The extremely high level of cooperativity and the very low concentrations of free Cu^{2+} made it possible only to place an upper limit of 100 nM on some of the K_D's by the ESI-MS technique. Thus, although K_D1 was determined to be 2.2 μM and K_D2 was 0.5 μM, K_D3 and K_D4 for the addition of further Cu^{2+} ions were both $\leq$100 nM.

In addition to SHaPrP(23–98), Figure 5 also shows Cu^{2+} binding data for SHaPrP(57–91) and SHaPrP(57–98) at pH 7.4. At Cu^{2+}:peptide ratios from 0–2, all these peptides showed almost identical behavior. At higher Cu^{2+} concentration, the binding of further Cu^{2+} ions remained almost identical for the peptides having the C-terminal extension to residue 98, whereas SHaPrP(57–91) bound additional Cu^{2+} ions less strongly (see Table 4), and showed overall binding of one less Cu^{2+} ion. As was noted above at pH 6.0, this was consistent with additional binding to the C-terminal residues 92–98 containing His96.

The role of the N-terminal amino group

In positive ion ESI-MS, basic sites within a peptide accept protons from the solvent. In the absence of metal ions, the 5+ charge state of SHaPrP(57–91) at *m/z* 699.7 has an excess of five protons, presumably attached to basic sites corresponding to the four histidines and the amino terminus (Fig. 1A). At pH 6.0, attachment of one Cu^{2+} ion completely suppressed the 5+ charge state of the peptide although no charge state preference was observed with the other metal ions (Fig. 1B–D). This indicates that the first Cu^{2+} ion with its two charges was attached to the peptide in a manner that eliminated the protons from three of the five basic sites, perhaps from two histidine imidazoles as well as the N-terminal amine; the other two histidines could be protonated to give a maximum of four charges. When a second Cu^{2+} was bound to the two remaining histidines, all five basic sites were occupied by the two Cu^{2+} ions; no protons could be accommodated and the maximum charge attainable was four.

Amines are known to bind metal ions (Hughes, 1981). To elucidate further any role that the N-terminal amine might play, SHaPrP(57–91) was blocked by acetylation. The acetylated peptide SHaPrP(Ac57–91) was infused into the mass spectrometer. In the absence of Cu^{2+}, the acetylated peptide was soluble and gave mass spectral peaks of normal intensity, but with 10 μM Cu^{2+} the peptide became insoluble, causing reduced signal response and clogged capillary lines. To obtain an adequate spectrum of the peptide in the presence of Cu^{2+} required 30% methanol instead of the 10% used for the other spectra. In a control experiment, this concentration of methanol did not adversely affect the binding of Cu^{2+} to SHaPrP(57–91) and, therefore, would not be expected to inhibit binding to SHaPrP(Ac57–91). However, an approximately fourfold reduction in the signal response was observed for the acetylated peptide compared with the nonacetylated species. Thus, in this instance quantitative measurements were considered to be unreliable and an evaluation of the binding strength was not possible. Addition of more Cu^{2+} further reduced the solubility of the complex.

By contrast, the longer, more hydrophilic sequence prepared with an N-terminal acetyl group, designated SHaPrP(Ac23–98), was soluble in the presence of Cu^{2+}. Comparison of the Cu^{2+} binding properties of N-terminally free and acetylated peptides at both pH 6.0 and 7.4 showed that the free N-terminus played little part in Cu^{2+} binding at low Cu^{2+} concentrations. However, at higher Cu^{2+} concentrations SHaPrP(Ac23–98) showed a substantial reduction in Cu^{2+} binding compared to the unacetylated peptide (Fig. 5). Thus, SHaPrP(Ac23–98) at pH 6 in the presence of excess Cu^{2+} showed strong binding of only one Cu^{2+} ion, substantially weaker binding of a second Cu^{2+}, and virtually no binding of three Cu^{2+} ions. At pH 7.4 the addition of 25 μM Cu^{2+} caused the binding of an average of 2 Cu^{2+} ions per peptide, i.e., 5 μM Cu^{2+} remained free in solution, whereas with SHaPrP(23–98), no measurable free Cu^{2+} remained. Also at pH 7.4, SHaPrP(Ac23–98) with 50 μM Cu^{2+} solution bound only 60% of the available Cu; 20 μM remaining unbound compared with only 4.5 μM for SHaPrP(23–98) under the same conditions.

To clarify further the role of the PrP N-terminus, we prepared a peptide SHaPrP(23–60) containing no complete octarepeats and devoid of histidine but possessing a free amino terminus. This was observed to bind a single Cu^{2+} ion at both pH 6 and pH 7.4. Cu^{2+} binding by this peptide at pH 7.4 is shown in Figure 6, plotted in two different ways. In trace (1), the data are presented in the same manner as in Figures 2 and 4; i.e., the ratio of bound Cu^{2+} is plotted vs. the total concentration of Cu^{2+} added. However, in (2) it is shown as a saturation curve in which the ratio of bound Cu^{2+} to total peptide is plotted against the calculated free Cu^{2+}

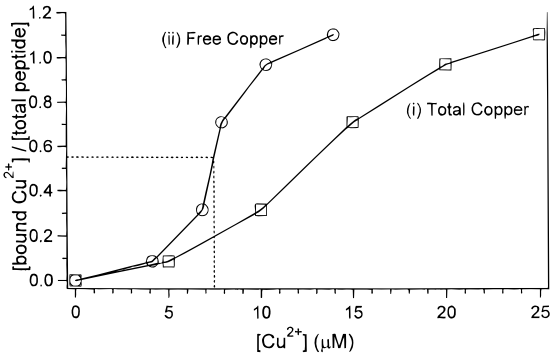

Fig. 6. The binding of Cu^{2+} ions to 10 μM SHaPrP(23–60) at pH 7.4, (i) as a function of the concentration of total Cu^{2+} added and (ii) as a function of the concentration of free Cu^{2+}, calculated as the difference between added Cu^{2+} (0–25 μM) and the complexed Cu^{2+} observed in the ESI mass spectra. $K_D1 = 7.5$ μM can be derived from the half-maximal value in curve (ii).

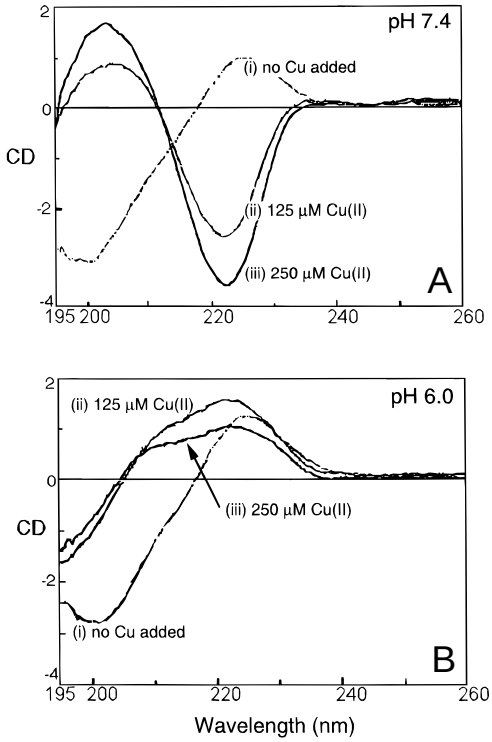

Fig. 7. CD spectra showing ellipticity (millidegrees) vs. wavelength (nm) for 50 μM SHaPrP(57–91) in 20 mM ammonium acetate solution, recorded in a 1.0 mm path length cell, at (**B**) pH 6.0 or (**A**) pH 7.4. The concentration of Cu^{2+} was either (i) 0, (ii) 125 μM, or (iii) 250 μM.

concentration in solution. This latter curve is sigmoidal and trends to a maximum of approximately one Cu^{2+} binding per peptide molecule, with K_D derived from the half-maximal concentration of 7.5 μM. Although this falls within the physiological Cu^{2+} concentration, it is questionable whether this weaker binding would allow this site to compete effectively for physiological Cu. Multiple nonspecific Cu^{2+} ion addition to this peptide was observed at higher Cu^{2+} concentrations.

Control peptides

The binding of Cu^{2+} to other PrP peptides of comparable size was monitored using Cu^{2+}:peptide molar ratios of 1:1 and 2.5:1. Both SHaPrP(90–144) and MousePrP(177–230) showed only weak, apparently nonspecific binding of Cu^{2+}, even though both peptides contain histidine and both have free N-terminal amino groups. SHaPrP(142–177), which contains one histidine, did show weak Cu^{2+} binding but it became largely insoluble and could not be monitored effectively by ESI-MS. Copper binding was monitored for all peptides listed in Table 2 under the carefully controlled conditions described here. Other peptides from the PrP sequence did not exhibit avid Cu^{2+} binding under a variety of experimental conditions. The PrP octarepeat region was clearly unique in its ability to bind multiple Cu^{2+} ions with high affinity.

Conformational effects observed by CD

The far-UV CD spectrum of SHaPrP(57-91) was compared at pH 6.0 and 7.4 in the absence and the presence of Cu^{2+} ions (Fig. 7). In the absence of Cu^{2+}, the spectrum was the same as had been reported previously for PrP octarepeat peptides (Hornshaw et al., 1995b; Smith et al., 1997; Viles et al., 1999). In one case, this pattern has been interpreted as corresponding to a nonrandom, extended conformation with properties similar to the poly-L-proline type II left-handed helix (Smith et al., 1997). This conclusion is not consistent with the NMR studies, perhaps because this peptide is rich in tryptophan, which can contribute substantially to CD spectra in the far UV (Freskgard et al., 1994). In any event, the spectrum was unaffected by pH [compare Figs. 7A(i) and 7B(i)]. However, addition of a 2.5- or fivefold molar ratio of Cu^{2+} revealed a pH-dependent structural change. At pH 6 only modest spectral changes were induced by binding of Cu^{2+} ions; the maximum at ~224 nm showed a blue shift of ~2 nm and the minimum at ~202 showed a corresponding shift of ~7 nm (Fig. 7B). In contrast, Cu^{2+} binding at pH 7.4 induced a major conformational change; the CD spectrum was dominated by a new minimum at 222 nm and a new maximum at 204 nm (Fig. 7A). These results are consistent with CD data in a recent study in which two of us participated (F.E. Cohen & S.B. Prusiner) (Viles et al., 1999). However, in that study the lack of a Cu^{2+}-induced change in the CD spectrum at pH 6 was interpreted as an indication that Cu^{2+} did not bind at this pH, whereas ESI-MS shows quite clearly that Cu^{2+} does bind to the four-octarepeat peptide at this lower pH with high avidity but with reduced stoichiometry, and almost certainly with a completely different structure.

The effects of desolvation conditions

Experiments were carried out to establish that the Cu^{2+} binding observed in ESI-MS was not significantly influenced by the desolvation conditions. The temperature of the first orifice encountered by the spray in the electrospray interface of the mass spectrometer was reduced from its normal setting of 150 °C down to 75 °C. Under these more gentle desolvation conditions, the overall sensitivity decreased and the amount of Na^+, K^+, and NH_4^+ adduct formation increased relative to protonated species. However, at a 1:1 ratio of peptide to Cu^{2+}, the ratio measured between the amount of free and complexed peptide was unaffected. De-

creasing the nebulizer gas flow rate had similar effects to decreasing the temperature. Thus, at least for strong binding such as occurred between SHaPrP(57–91) and a single Cu^{2+} ion, the relatively harsh desolvation conditions used throughout the experiment did not affect the degree of binding observed. On the other hand, milder conditions resulted in an increase in apparent nonspecific binding, suggesting that weak metal binding sites could be underestimated by ESI-MS measurements under stringent conditions, such as were used throughout these experiments. In practice, suppression of weak binding sites is probably advantageous; ESI-MS showed that nickel and zinc bind weakly to SHaPrP(57–91), but tryptophan fluorescence spectroscopy indicated that these ions induce no structural change in the protein (Stöckel et al., 1998).

Discussion

Copper is essential for life and plays a central role in many biological process, mainly due to the Cu^{2+}/Cu^{+} redox couple that is fundamental to numerous oxidative/reductive pathways and electron transfer reactions. It is readily complexed by a variety of nitrogen-, oxygen-, and sulfur-containing ligands. Furthermore, its concentration is relatively high in the brain (23 μg/g dry weight) compared with other tissues. Therefore, the numerous independent reports that Cu^{2+} binds to PrP with micromolar dissociation constants and induces structure in an otherwise flexible domain implies some functional significance.

Strong support for the use of ESI-MS to study Cu^{2+} binding to PrP comes from the stoichiometries and dissociation constants measured in this study compared with those obtained by more conventional methods, summarized in Table 1. Fluorescence quenching gave an overall dissociation constant of 6.7 μM at pH 7.4 for the octarepeat peptide PrP(60–91) (Hornshaw et al., 1995b). Equilibrium dialysis showed that for the binding of 5.6 Cu^{2+} ions to recombinant HuPrP(23–98) at pH 7.4, half-maximal binding occurred at 5.9 μM Cu^{2+} (Brown et al., 1997a). In another study carried out at pH 6 using fluorescence quenching and equilibrium dialysis, half-maximal binding was achieved at 14 μM Cu^{2+} for recombinant SHaPrP(29–231) and at ~9 μM for SHaPrP(57–91) (Stöckel et al., 1998). Thus, dissociation constants measured previously for Cu^{2+} binding have been in the low micromolar range. The stepwise $K_D n$'s measured in the present study for the process $\text{Peptide}\cdot(n-1)Cu^{2+} \leftrightarrow \text{Peptide}\cdot nCu^{2+}$ are in the low micromolar range for K_D1 and K_D2. Although these are not directly comparable to earlier reported values for the overall binding, they are of the same order of magnitude. K_D's measured in this study by ESI-MS suggest rather tighter binding than reported previously, particularly for the cooperative binding of the third and fourth Cu^{2+} ions to the extended peptides incorporating the fifth histidine at residue 96, K_D's for which were found to be nanomolar.

We have established that ESI-MS is able to monitor specific interactions between PrP peptides and Cu^{2+} ions and to distinguish these from weak, seemingly nonspecific associations with other metals. Measuring an equilibrium constant by ESI necessitates some assumptions. The first assumption is that the relative amounts of free and complexed peptide can be measured by assuming equal signal responses for both. The second and perhaps more important is that a measurement of ion current in the gas phase quantitatively represents the solution equilibrium. We know that in the process of desolvation, the charged droplets shrink until they reach the Rayleigh limit and break apart (Kebarle & Tang, 1993). Shrinking droplets are small isolated chambers whose contents become progressively more concentrated, compared with the bulk solution from which they came. As they pass through the interface, the droplets are heated by collisions with hot gas but cooled by evaporation, causing their temperature to fall to ~10 °C below ambient, and the analyte is completely desolvated within ~450 μs (Kebarle & Tang, 1993). From this point on the analyte exists in the form of isolated ions that are stable on the timescale of the mass spectrometric separation and detection, thus the sample is effectively "frozen." Ultimately, the validity of this technique for monitoring complex formation is dependent upon competition between the rate of any changes in equilibrium caused by the effects outlined above vs. the rate of evaporation. Only for the fastest solution reactions is this likely to present a problem, and there is increasing acceptance of the use of ESI-MS for such studies of noncovalent interactions.

The octarepeat region of PrP is a paradoxical element in the story of prion diseases. Not only is this feature highly conserved across all mammalian species, but avian PrP such as chicken, with only 30% homology, also has multiple repeats close to the N-terminus. In this case the repeating unit is the six residue sequence PGYPHN (Harris et al., 1991; Gabriel et al., 1992). Thus, both mammals and birds share a repeating, histidine-containing motif that can be represented as GXPH or PHXXG. Mammalian PrP has been described as containing five octarepeats but there are only four true repeats containing histidine (Oesch et al., 1985). Similarly, chicken PrP has been described as containing nine hexarepeats, but these are not all true repeats and only six contain histidine (Harris et al., 1991; Gabriel et al., 1992). After treatment of PrP^{Sc} with proteinase K, the protease-resistant core (PrP 27–30) retains full infectivity, despite the loss of the N-terminal region 23–89, including the octarepeats. Furthermore, transgenic mice that express a truncated protein of only 106 amino acids, devoid of the region 23–88, can develop and transmit a prion disease (Muramoto et al., 1996; Supattapone et al., 1999). Thus, the octarepeats are not essential for infectivity or the propagation of prion disease, and yet the only pathogenic mutation to the PrP gene identified outside of the region 90–231 corresponds to insertion of additional copies of the octarepeat (Owen et al., 1989), a mutation that could cause a further increase in Cu^{2+} retention.

Our data are the first to implicate the N-terminus in binding copper to the octarepeats, even though binding to the free amino terminal peptide SHaPrP(23–60) was relatively weak. The combined effect of Cu^{2+} binding to nonacetylated SHaPrP(23–98) was greater than the sum of binding to the N-terminus of SHaPrP(23–60) and the octarepeats of SHaPrP(Ac23–98). Consequently, the roles of the amino terminus and the octarepeat region in binding Cu^{2+} ions are cooperative, both in terms of the maximum stoichiometry for Cu^{2+} ions bound and the binding affinities. However, it was noted that the entire N-terminal region corresponding to SHaPrP(23–98) showed weaker binding for a single Cu^{2+} ion than either of the N-terminally truncated peptides SHaPrP(57–91) or SHaPrP(57–98), whereas the binding of the second and subsequent Cu^{2+} ions was stronger. Close proximity of the N-terminus in the shorter octarepeat peptides apparently encourages participation of the free amino group in binding the first Cu^{2+}; in the longer peptide with the N-terminus remote from the octarepeats, this cooperative effect occurs only after the first Cu^{2+} ion has bound.

It is likely that a normal function of PrP relates to its Cu^{2+} binding properties. At least for Cu^{2+} binding to the octarepeats, there is a structural change, as observed by quenching of the tryptophan fluorescence and changes in CD spectra for both synthetic

peptides and recombinant protein. The pH dependence of Cu^{2+} binding suggests that the histidine residues are involved. The pK_a of the histidine imidazole is 6–7 when histidine is exposed to aqueous solution. The fact that significant binding of the first Cu^{2+} ion is observed in the pH range 4–5 would indicate that, in the presence of Cu^{2+}, the histidine imidazole is in an environment which excludes it from the solvent. Fluorescence quenching by Cu^{2+} of synthetic octarepeat peptides (Hornshaw et al., 1995b; Stöckel et al., 1998) and recombinant SHaPrP(29–231) (Stöckel et al., 1998) indicates that some or all of the tryptophan residues have also shifted to a more hydrophobic environment, i.e., Cu^{2+} induces a change in conformation. This is consistent with near-UV CD of recombinant SHaPrP(29–231) that showed a change in the tertiary environment of the aromatic residues in the presence of Cu^{2+} (Stöckel et al., 1998). Quenching of tryptophan fluorescence when Cu^{2+} bound to recombinant SHaPrP(29–231) showed a similar pH dependence; at pH 4 the fluorescence was not quenched, but a significant quench of the signal occurred at pH 5, which increased at higher pH (Stöckel et al., 1998).

It has been proposed that the 1:1 stoichiometry observed at pH 7.4 for Cu^{2+} binding per octarepeat is achieved by each histidine acting as a bidentate ligand, binding to two Cu^{2+} ions to form a unique planar structure (Viles et al., 1999). Although this requires ionization of the Nϵ2 NH, the pK_a of which is normally 14, there are precedents such as the binding of both copper and zinc in superoxide dismutase (Parge et al., 1992). This suggests that the binding of the first metal ion to Nδ1 can lower the second pK_a significantly. In the current study, ESI-MS has confirmed that the binding of four Cu^{2+} ions to the four-octarepeat peptide does indeed involve the elimination of four protons. Loss of these protons from four tryptophan residues is less likely as these normally have a pK_a of ~18. An alternative proposed structure for the 1:1 Cu/octarepeat complex at pH 7.4 involving one histidine and two glycyl amides per octarepeat does not explain the cooperativity of binding to the four-octarepeat species (Miura et al., 1999).

Stöckel et al. (1998) showed that recombinant SHaPrP(29–231) binds two Cu^{2+} ions per protein molecule at pH 6. Unfortunately, these experiments on recombinant PrP were limited to pH 6 as this species proved to be insoluble at higher pH when complexed with Cu^{2+}. Other studies on peptides at higher pH suggested a binding ratio of one Cu^{2+} ion per octarepeat. Here, using peptides, we have demonstrated by ESI-MS that the binding of Cu^{2+} is highly pH-sensitive, e.g., although SHaPrP(57–91) can bind two Cu^{2+} ions at pH 6 with high affinity, it binds between three and four Cu^{2+} ions at pH 7.4. Similarly, SHaPrP(57–98) and SHaPrP(23–98), both of which contain four octarepeats plus a fifth histidine, showed a dramatic increase in Cu^{2+} binding between pH 6 and 7.4, when the number of Cu^{2+} ions bound per peptide molecule increased from three to between four and five. Clearly, the fifth Cu^{2+} ion is binding to a site common to the C-termini of the peptides terminating at 98, probably involving His96. Thus, the present study is in broad agreement with the earlier reports that at pH 7.4, peptides consisting of four octarepeats bind four Cu^{2+} ions and recombinant HuPrP(23–98) binds between five and six Cu^{2+} ions (Table 1).

The pH-dependent differences in Cu^{2+} binding provide a mechanism whereby PrP might play an important role in transporting Cu^{2+} from the extracellular medium into the cell. PrP^C is concentrated in caveolae-like domains before being endocytosed to endosomes and secondary lysosomes (Taraboulos et al., 1992, 1995; Vey et al., 1996), and chicken PrP has been observed to cycle between endosomes and the cell surface (Shyng et al., 1993). For part of their existence, caveolae are cell surface invaginations, open to the extracellular environment and consequently, likely to be at physiological pH 7.4. At this time, PrP might efficiently extract and bind Cu^{2+} from the extracellular medium. Then the caveolae could become sealed off and the pH reduced by an ATPase proton pump (Mineo & Anderson, 1996). Endocytosis could then transport PrP^C into the cell in endosomes and secondary lysosomes, where lowered pH would cause much of the PrP-bound Cu^{2+} to be released. Such a mechanism for the sequestration and transport of ions and small molecules into the cell by caveolae has been described as potocytosis (Anderson et al., 1992). Interestingly, it has been reported that Cu^{2+} itself rapidly and reversibly stimulates the endocytosis of PrP in neuroblastoma cells (Pauly & Harris, 1998), suggesting that Cu^{2+} may actively participate in its own transport. Any absence of PrP would be expected to result in lower levels of copper, consistent with the report that some subcellular brain fractions from *Prnp*$^{0/0}$ mice have levels of this element only 10% of those found in wild-type mice (Brown et al., 1997a). Furthermore, membrane fractions from wild-type cultured cerebellar cells treated with the PrP-releasing enzyme phosphatidylinositol-specific phospholipase C (PIPLC) showed a reduction in Cu^{2+} levels. By contrast, PIPLC had no effect on the already lowered Cu^{2+} levels in PrP-deficient cells (Brown et al., 1997a), consistent with the observation that copper/zinc superoxide dismutase activity is depressed in PrP-deficient cells (Brown et al., 1997b).

Copper and oxidation may be implicated in other more common neurodegenerative diseases; the amyloid precursor protein can bind Cu^{2+} (Multhaup et al., 1996) and displays pH-dependent, Cu^{2+}-induced aggregation (Atwood et al., 1998). Mutations in the superoxide dismutase gene are responsible for a substantial proportion of familial cases of amyotrophic lateral sclerosis (Cudkowicz & Brown, 1996). Interestingly, a prion disease-like pathology can be induced in mice by treatment with the Cu^{2+} chelating compound cuprizone (Pattison & Jebbett, 1971; Kimberlin et al., 1974). However, although Cu^{2+} appears to be involved in the normal function of PrP^C , there is no direct evidence that it plays any role in the development, transmission, or pathology of prion diseases.

Materials and methods

Peptides were synthesized by standard stepwise solid phase methods, either on a Perkin Elmer-ABI model 432 or a Millipore model 950 synthesizer using Fmoc chemistry. After cleavage from the resin, the crude products were purified by semi-preparative, reversed-phase gradient high-performance liquid chromatography (HPLC) with a Vydac C-4 or C-18 column using a gradient of 0.03% trifluoroacetic acid (TFA) and 0.024% TFA/acetonitrile. Fractions were monitored by ESI-MS and those fractions containing pure peptides were pooled and lyophilized. Any remaining TFA was removed by repeated resolubilization and lyophilization. Dry materials were weighed on a microbalance and solutions were made up in HPLC grade water and methanol (Fisher, Pittsburgh, Pennsylvania). Copper(II)sulfate and nickel(II)sulfate (Aldrich, Milwaukee, Wisconsin) were prepared as aqueous solutions without purification. Zinc(II)sulfate was purchased as a 0.05 M solution from Aldrich (Milwaukee, Wisconsin). Final solutions were made up in 10% methanol and contained 10 μM peptide and metal ions at the stated concentrations. Unless stated otherwise, they were buffered to pH 6.0 or pH 7.4 with 1 mM ammonium acetate (Fluka, Milwaukee, Wisconsin)/ammonium hydroxide (Mallinck-

rodt, Paris, Kentucky). The pH was recorded after the addition of metal ions and peptides. To establish complete equilibration before recording any data, solutions were infused into the mass spectrometer for not less than 40 min. Mass spectra were then accumulated over a period of 5 min.

All mass spectra were collected with an orthogonal acceleration time-of-flight mass spectrometer (oaToFMS) (PE Biosystems Mariner, Framingham, Massachusetts) equipped with an ESI source and an ion mirror (reflectron). A syringe pump (Harvard Apparatus, Holliston, Massachusetts) provided a stable liquid flow at 3 μL/min to the fused silica spray capillary. This capillary protruded a short distance from the end of a stainless steel tube, through which nitrogen gas was passed to assist in desolvation. The spray assembly was held at a potential of 3 kV to induce charging of the liquid droplets, which were directed through a series of heated apertures into a vacuum chamber. Here they were desolvated to form isolated ions, which were directed by lenses into the high vacuum region of the time-of-flight mass analyzer. All m/z values reported here correspond to the lowest mass ions within each isotopic cluster, and all calculated ion masses are based on the monoisotopic rather than average atomic weights. Except where state otherwise, data presented in graphical plots and histograms were derived from the mass spectra by addition of the signal intensities from all of the individual charge states [e.g., 3+, 4+, and 5+ for SHaPrP(57–91)].

CD spectroscopy was carried out at room temperature (~23 °C) in a spectropolarimeter (Jasco 715, Easton, Maryland) continuously flushed with dry nitrogen, using a circular quartz cell of path length 1 mm. The peptide concentration [SHaPrP(57–91)] was 50 μM in 20 mM ammonium acetate solution, at pH 6.0 or 7.4. The concentration of copper(II)sulfate was either 0, 125, or 250 μM, i.e., a Cu^{2+}:peptide molar ratio of 0, 2.5, or 5. The wavelength range studied was restricted to ≥195 nm due to excessive absorption by the buffer at lower wavelengths. Data are presented from single scans with subtraction of buffer spectra, without mathematical smoothing.

Acknowledgments

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