Fragment length influences affinity for Cu²⁺ and Ni²⁺ binding to His⁹⁶ or His¹¹¹ of the prion protein and spectroscopic evidence for a multiple histidine binding only at low pH

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The prion protein (PrP) is a Cu²⁺-binding cell-surface glycoprotein. Using various PrP fragments and spectroscopic techniques, we show that two Cu²⁺ ions bind to a region between residues 90 and 126. This region incorporates the neurotoxic portion of PrP, vital for prion propagation in transmissible spongiform encephalopathies. Pentapeptides PrP-(92–96) and PrP-(107–111) represent the minimum motif for Cu²⁺ binding to the PrP-(90–126) fragment. Consequently, we were surprised that the appearance of the visible CD spectra for two fragments of PrP, residues 90–126 and 91–115, are very different. We have shown that these differences do not arise from a change in the co-ordination geometry within the two fragments; rather, there is a change in the relative preference for the two binding sites centred at His¹¹¹ and His⁹⁶.

These preferences are metal-, pH- and chain-length dependent. CD indicates that Cu²⁺ initially fills the site at His¹¹¹ within the PrP-(90–126) fragment. The pH-dependence of the Cu²⁺ co-ordination is studied using EPR, visible CD and absorption spectroscopy. We present evidence that, at low pH (5.5) and sub-stoichiometric amounts of Cu²⁺, a multiple histidine complex forms, but, at neutral pH, Cu²⁺ binds to individual histidine residues. We have shown that changes in pH and levels of extracellular Cu²⁺ will affect the co-ordination mode, which has implications for the affinity, folding and redox properties of Cu-PrP.

Key words: CD, copper binding, EPR, metal co-ordination, prion protein (PrP), spectroscopy.

INTRODUCTION

Transmissible spongiform encephalopathies have been linked to a misfolded form of the prion protein (PrP), PrP^{Sc} (scrapie isoform of PrP). These proteinaceous infectious particles are devoid of genetic material and represent a wholly novel infectious agent [1–3]. Normal cellular prion protein (PrP^C) is a cell-surface glycoprotein, approx. 209 amino acids long, with a glycosylphosphatidylinositol anchor. PrP^C contains two distinct structural regions, including the unstructured N-terminal domain between residues 23 and 126 [4], and the mainly α -helical C-terminal domain between residues 126 and 231 [5]. In the absence of copper, the N-terminal domain exhibits a high degree of flexibility in the main chain [6]. Four octarepeats of the sequence PHGGGWGQ, found between residues 60 and 91 in the unstructured region of PrP, bind up to four Cu²⁺ ions [7–11].

One feature of prion disease is metal imbalance [12]. PrP^{Sc} has been found to be occupied with metal when isolated from diseased brain [13], whereas metal binding to the prion protein is altered in human prion disease [14]. The levels of cellular copper seems to be affected by scrapie infection [15], and copper-catalysed redox damage of PrP [16] is linked to prion disease [17,18]. Furthermore, disease progression in infected mice can be slowed with the use of copper-specific chelation therapy [19].

A direct link between copper binding to the octarepeats and prion disease has been thought unlikely, as studies of mice expressing a truncated version of PrP with the octarepeat region removed were still susceptible to prion infection [20]. However, it has been noted that Cu²⁺ ions bind outside this octarepeat region [11,21–25]. We have shown that Cu²⁺ binds to His⁹⁶ and His¹¹¹ [21,26], in a region considered to be essential for amyloid formation and infectivity in prion disease [27–30]. Using ¹H-NMR and visible CD spectroscopy, we have shown that, like Cu²⁺,

two Ni²⁺ ions bind to His⁹⁶ and His¹¹¹ independently of each other [26]. Cu²⁺ has been implicated in the toxicity of a fragment from this unstructured region, PrP-(106–126), which is neurotoxic in both the soluble and fibrillar states [30–32]. Furthermore, we have shown that Cu²⁺ binding to His⁹⁶ and His¹¹¹ promotes the formation of β -sheet [21], which is consistent with the findings that this unstructured region is key to the misfolding of PrP^C into the β -sheet-rich conformation [33]. Cu²⁺ can also convert the cellular prion protein into a protease-resistant species [34], which is a feature of PrP^{Sc}. The presence, or absence, of Cu²⁺ ions may also cause different strains of prion disease [13,35].

Although the normal physiological role of PrP has yet to be determined, it may have a function in copper homeostasis owing to the ability of PrP^C to bind Cu²⁺ *in vivo* and *in vitro* [7,11]. Copper has been shown to promote the endocytosis of PrP^C [36,37]; however, PrP expression levels do not seem to affect copper delivery [38,39]. PrP^C can act as an antioxidant by sacrificially quenching hydroxyl radicals produced via Cu²⁺/Cu⁺ Fenton's cycling [16], and copper-induced cleavage of the PrP^C main chain has been reported [40,41].

We have previously published data of Cu²⁺ and Ni²⁺ binding to a fragment of PrP, residues 91–115 [21,26]. In these studies, we showed that both Cu²⁺ and Ni²⁺ bind to His⁹⁶ and His¹¹¹ independently of each other, forming a square-planar/tetragonal complex. The metal complex involves co-ordination of main-chain amides preceding the histidine imidazole, as well as the imidazole nitrogen N ϵ . In the present paper, we present data for the larger fragment, PrP-(90–126); this peptide includes additional hydrophobic residues which incorporate the neurotoxic peptide, PrP-(106–126) [30–32]. This region is essential for prion propagation [27–30]. At first observation, the Cu²⁺ visible CD data for Cu₁PrP-(90–126) appear to be very different from that of the slightly shorter fragment, Cu₁PrP-(91–115). One of the aims of the present

Abbreviations used: PrP, prion protein; PrP^C, cellular isoform of PrP; PrP^{Sc}, scrapie isoform of PrP; ROS, reactive oxygen species.

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Table 1 Peptides used in the present study

Residues at positions 96 and 111 are indicated in bold. Note that H96A peptides contain only His¹¹¹; H111A peptides contain only His⁹⁶.

Peptide	Sequence
PrP-(90–126)	GQGGGTHSQWNKPSKPKTNMK HM AGAAAAGAVVGLG
PrP-(91–115)	GQGGGTHSQWNKPSKPKTNMK HM AGA
PrP-(90–126)H111A	GQGGGTHSQWNKPSKPKTNMK AM AGAAAAGAVVGLG
PrP-(90–126)H96A	GQGGGTHSQWNKPSKPKTNMK HM AGAAAAGAVVGLG
PrP-(91–115)H111A	GQGGGTHSQWNKPSKPKTNMK AM AGA
PrP-(91–115)H96A	GQGGGTHSQWNKPSKPKTNMK HM AGA
PrP-(92–96)	GQGGT H
PrP-(107–111)	TNMK H
PrP-(110–115)	K HM AGA

study was to understand the reasons for the differences in the visible CD spectra. We have used a range of peptide fragments and spectroscopic techniques to characterize Cu²⁺ and Ni²⁺ binding to the amyloidogenic fragment of PrP that is essential for prion replication over a range of pH values and metal stoichiometries.

EXPERIMENTAL

Peptide synthesis and purification

Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry was used to synthesize various fragments of PrP, which were N-terminally acetylated and C-terminally amidated in order to mimic this region of PrP within the full-length protein (Advanced Biotechnology Centre, Imperial College London, London, U.K.). The peptides were removed from the resin and deprotected before purification by reverse-phase HPLC. The samples were characterized using MS and ¹H-NMR spectroscopy. Acetylated and amidated peptides are shown in Table 1.

Titration

Small aliquots of fresh aqueous solutions were used to add metal ions (Cu²⁺ as CuCl₂ · 2H₂O, Ni²⁺ as NiCl₂ · 6H₂O). Peptide concentrations were determined using a molecular absorption coefficient at 280 nm of 5690 M⁻¹ · cm⁻¹ multiplied by the number of tryptophan residues in the peptide [42]. In the case of peptides which lacked aromatic residues, the mass was used to approximate the concentration and a 20% water content was assumed. All titrations were carried out in the absence of buffers and the pH was measured before and after acquiring each spectrum, adjusting the pH when necessary using small aliquots of 100 mM NaOH or HCl.

Circular dichroism

CD spectra were recorded on an Applied Photophysics Chirascan instrument at 25 °C, as described previously [8]. A cell with a 1 cm pathlength was used for spectra recorded between 300 and 800 nm, with sampling points every 2 nm. Typically, four scans were recorded, and baseline spectra were subtracted from each spectrum. Data were processed using Applied Photophysics Chirascan Viewer, Microsoft Excel and the KaleidaGraph spreadsheet/graph package. The direct CD measurements (θ , in millidegrees) were converted into molar ellipticity, $\Delta\epsilon$ (M⁻¹ · cm⁻¹), using the relationship $\Delta\epsilon = \theta/33\,000 \cdot c \cdot l$, where c is the concentration and l is the pathlength.

Figure 1 Comparison of visible CD spectra of Cu₁PrP-(91–115) and Cu₁PrP-(90–126)

Both peptides are at pH 7.8 with 1 mol equivalent of Cu²⁺ bound to 0.1 mM peptide.

EPR spectroscopy

X-band EPR spectra were acquired on a Bruker Elexsys E500 spectrometer operating at a microwave frequency of 9.33 GHz. The spectra were acquired over a sweep width of 2500 G (1 G = 10⁻⁴ T), a modulation frequency of 10 G, and a temperature of ~20 K. At least two scans were acquired per sample.

Proton NMR

¹H-NMR spectra were acquired at 296 K on a Varian Unity 600 MHz spectrometer using 5 mm inverse-detection (¹H), triple resonance, z-gradient probes. Spectra were recorded in 100% ²H₂O; a low-power pre-saturation pulse was used to suppress residual water. TOCSY spectra were typically acquired with 2048 (F2) × 512 (F1) complex points, employing a DIPSI2 sequence for isotropic mixing, with a mixing time of ~60 ms. The States-TPPI method was used for quadrature detection in the indirect dimension for two-dimensional spectra. Before Fourier transformation, sine-squared window functions, phase-shifted by 90°, were applied to both dimensions and zero-filled to 2048 real points. Data were processed and analysed using Vnmr (Varian) software running on an SGI O2. Proton resonance assignments of each pentapeptide were obtained using the two-dimensional TOCSY data.

RESULTS

Visible CD of Cu²⁺ and Ni²⁺ binding to PrP fragments containing a single histidine residue at positions 96 and 111

Figure 1 is a comparison of the visible CD spectra of a 1 mol equivalent of Cu²⁺ ions bound to PrP-(91–115) and the longer fragment, PrP-(90–126), at pH 7.8. Initially, we were surprised at the very different appearance of the two spectra as we believe these pentapeptides, PrP-(92–96) and PrP-(107–111), represent the isolated Cu²⁺-binding sites and the Cu²⁺ complex should be unaffected by the presence of residues outside this region. For this reason, we set about comparing the Cu²⁺- or Ni²⁺-loaded visible CD spectra for the PrP-(90–126) analogues containing a single

Figure 2 Visible CD spectra comparing Cu²⁺ or Ni²⁺ bound to PrP fragments containing a single histidine residue at position 96 or 111

(a and b) Spectra for peptides containing only His⁹⁶: PrP-(90–126)H111A and PrP-(91–115)H111A compared with PrP-(92–96). (c and d) Spectra for peptides containing only His¹¹¹: PrP-(90–126)H96A and PrP-(91–115)H96A compared with PrP-(107–111). (a and c) Results are for 1 mol equivalent of Cu²⁺ at pH 7.8. (b and d) Results are for 1 mol equivalent of Ni²⁺ at pH 9.0. All peptide concentrations were 0.1 mM.

histidine residue at position 96 or 111 with similar shorter PrP-(91–115) fragments, and their respective pentapeptides containing His⁹⁶ or His¹¹¹. Figure 2(a) shows the Cu²⁺-bound spectra (1 mol equivalent) for PrP-(90–126)H111A, PrP-(91–115)H111A and PrP-(92–96) at pH 7.8. The three peptides show clear similarities, with a positive CD band at ~500 nm and a negative band at ~600 nm. Similarly, Figure 2(b) compares the same three peptides, but with Ni²⁺ bound (1 mol equivalent) at pH 9.0. Again, there is a positive CD band to shorter wavelengths at ~420 nm and a negative band to longer wavelengths at ~500 nm. There is little variation in the maximal position of the CD bands for both the Cu²⁺ and Ni²⁺ complex for the three peptide analogues, but there is some variation in the intensity. For example, in Figure 2(b), the intensities of the bands at 500 nm for Ni₁PrP-(91–115)H111A, Ni₁PrP-(92–96) and Ni₁PrP-(90–126)H111A are $-1.96 \text{ M}^{-1} \cdot \text{cm}^{-1}$, $2.38 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and $3.28 \text{ M}^{-1} \cdot \text{cm}^{-1}$ respectively, i.e. $2.54 \text{ M}^{-1} \cdot \text{cm}^{-1} \pm 26\%$. At pH ~7.8, the position of the CD bands are relatively insensitive to pH, but the intensities are affected. However, the variations in intensity of the bands between the various fragments ($\pm 26\%$ average intensity) may well be attributed to small variations in pH and concentration. The position of the CD bands is unaffected by fragment length, but there may be subtle effects on the intensity of the CD bands between fragments; this is supported by variations in the appearance of visible CD spectra for Cu₂PrP-(91–115) and Cu₂PrP-(90–126) (see Supplementary Figure S1 at <http://www.BiochemJ.org/bj/404/bj4040393add.htm>).

The Cu²⁺-bound spectra for the other metal-ion-binding site at His¹¹¹ are compared in Figure 2(c). The PrP-(90–126)H96A, PrP-(91–115)H96A and PrP-(107–111) spectra with 1 mol equivalent of Cu²⁺ also give very similar CD spectra, with a negative band at ~500 nm and a positive band at ~620 nm. As with the His⁹⁶-containing peptides, the visible CD spectra of Ni²⁺-bound peptides incorporating the His¹¹¹-binding site echo the Cu²⁺ bind-

ing, with a negative CD band at shorter wavelengths, 420 nm, and a positive band at longer wavelengths, 500 nm.

The visible absorption bands associated with *d-d* electronic transitions have the absorption maximum at ~580 nm at pH 7 (~540 nm at pH 9) for the Cu²⁺ complex with PrP-(90–126) and ~440 nm for the Ni²⁺ complex at pH 8.5, as described previously [26]. There is little difference between the visible absorption bands of Cu²⁺ (or Ni²⁺) binding at His⁹⁶ compared with that at His¹¹¹. The observed molar absorption coefficients and wavelength maxima for Cu²⁺, pH 8, $\epsilon_{540} = 110 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and Ni²⁺, pH 8.5, $\epsilon_{440} = 140 \text{ M}^{-1} \cdot \text{cm}^{-1}$ are typical for a square-planar arrangement of nitrogen and oxygen ligands (Cu-3N1O 580 nm, Cu-4N 540 nm) for both the Cu²⁺ and the Ni²⁺ complexes respectively [43].

It is interesting to note that the CD bands for both the Ni²⁺ and Cu²⁺ complexes incorporating His⁹⁶ are positive at shorter wavelengths of the absorption maximum (~540 nm for Cu²⁺ and ~440 nm for Ni²⁺) and negative at longer wavelengths. The maximal position of the CD bands do not correspond to the absorption maxima, as the absorption band is derived from three overlapping *d-d* transitions [44]. In contrast, the CD bands for both Cu²⁺ and Ni²⁺ complexes at His¹¹¹ produce negative CD bands at shorter wavelengths and positive at longer wavelengths. Thus the complexes at His⁹⁶ and His¹¹¹ give CD spectra that are almost mirror images of each other. We have recently developed a rationale for the striking differences in visible CD spectra and have proposed some empirical rules for predicting the appearance of Cu²⁺ and Ni²⁺ square-planar complexes involving histidine side-chain and amide main-chain co-ordination [44a]. In summary, the spectra in Figure 2 indicate that the two pentapeptides contain all the residues necessary to form the complex at His⁹⁶ or His¹¹¹. The nature of the two complexes does not appear to be influenced by additional residues present in the 91–115 or 90–126 peptide fragments.

Visible CD of PrP-(90–126)

Cu²⁺

Now that we have established that the appearance of the visible CD spectra for PrP fragments contain a single histidine residue at position 96 or 111, we can compare these spectra to those of PrP-(90–126) and PrP-(91–115) in which both histidine residues are present. These comparisons are to determine whether the Cu²⁺ and Ni²⁺ complexes formed are isolated from each other at the two histidine sites, or whether a single metal ion will bind to both His⁹⁶ and His¹¹¹ simultaneously. Figure 3(a) shows visible CD data with 1 and 2 mol equivalents of Cu²⁺ loaded on to PrP-(90–126) at pH 7.8; similar spectra are observed at pH 9.0 (shown in Supplementary Figure 1). Figure 3(c) presents simulated data created by combining CD spectra of the two analogues, PrP-(90–126)H111A and PrP-(90–126)H96A, each loaded with 1 mol equivalent of Cu²⁺ at various percentage ratios.

When the spectra for the one mol equivalent of Cu²⁺-bound PrP-(90–126) analogues are added together (H111A+H96A), they overlay almost identically with the two Cu²⁺-bound PrP-(90–126) spectra, suggesting that Cu²⁺ ions bind independently to either His⁹⁶ or His¹¹¹. The spectra of 1 mol equivalent of Cu²⁺ bound to PrP-(90–126) look very different: the simulations show that when only 1 mol equivalent of Cu²⁺ is added to PrP-(90–126), the appearance of the spectra is most readily simulated using a 95%:5% ratio of H96A/H111A (note that H96A contains His¹¹¹ only). Similar behaviour is observed at pH 9.0 (see Supplementary Figure S1). On the basis of these simulations, we can therefore conclude that the majority of the Cu²⁺ binds to the site at

Figure 3 Cu^{2+} and Ni^{2+} binding to PrP-(90–126) and compared with simulations of H111A+H96A analogues

(a and c) Cu^{2+} binding to PrP-(90–126). (b and d) Equivalent Ni^{2+} binding. (a) Visible CD spectra of 1 (red) and 2 (blue) mol equivalents of Cu^{2+} bound to PrP-(90–126) at pH 7.8. (b) Equivalent Ni^{2+} spectra at pH 9.0. Overlaid on the spectrum of Cu₁PrP-(90–126) (red) is a comparable spectrum of a 95:5 mixture of Cu₁PrP-(90–126)H96A/Cu₁PrP-(90–126)H111A at pH 7.8 (orange). A 30%:70% combination at pH 9.0 is overlaid with Ni₁PrP-(90–126). Cu₂PrP-(90–126) and Ni₂PrP-(90–126) (blue) are similar to spectra obtained from a 1:1 addition (light blue) of the analogue spectra bound to 1 mol equivalent of either Cu^{2+} or Ni^{2+} . (c) Accompanying spectra of Cu₁PrP-(90–126)H111A (blue) and Cu₁PrP-(90–126)H96A (red) and mixtures of: i, 75%:25%; ii, 50%:50%; and iii, 25%:75% Cu₁PrP-(90–126)H111A/Cu₁PrP-(90–126)H96A at pH 7.8. (d) Equivalent Ni^{2+} mixtures at pH 9.0. All peptide concentrations were 0.1 mM.

His¹¹¹ within the PrP-(90–126) fragment at 1 mol equivalent of Cu^{2+} .

It is now clear that, as shown previously for PrP-(91–115) [26], PrP-(90–126) binds both Cu^{2+} ions at individual histidine residues. The reason for the difference in the appearance of the spectra (Figure 1) is not in fact a change in co-ordination geometry for the two peptides, but rather a change in the relative affinity for Cu^{2+} binding to His⁹⁶ or His¹¹¹. We have shown previously [26] that Cu₁PrP-(91–115) can be very closely simulated by the addition of H96A/H111A at 72%:28% ratios (see Supplemental Figure 1). This suggests that the addition of the hydrophobic tail of 11 residues results in the metal-binding site at His¹¹¹ exhibiting a higher affinity for Cu^{2+} relative to His⁹⁶, rather than a change in co-ordination geometry. We have summarized the changes in the relative affinity for the two binding sites as shown in the presence of 1 mol equivalent of Cu^{2+} at various pH values for both PrP-(91–115) and PrP-(90–126), as seen in Figure 4.

Ni^{2+}

Figure 3(b) shows visible CD data for Ni^{2+} at 1 and 2 mol equivalents added on to PrP-(90–126) at pH 9.0. Also shown in Figure 3(d) are Ni^{2+} -loaded mixtures of PrP-(90–126)H111A and PrP-(90–126)H96A, simulated by the addition of the two spectra at various ratios, at pH 9.0. When the analogues are added together (50%:50%), they overlay almost identically with the 2 Ni^{2+} -bound wild-type spectrum. To effectively simulate the spectrum for 1 mol equivalent of Ni^{2+} in PrP-(90–126), it is clear that different ratios of H96A and H111A are required. In the case of Ni^{2+} , 30% H96A and 70% H111A produces the closest

Figure 4 Cu^{2+} - and Ni^{2+} -binding preference for His⁹⁶ and His¹¹¹ showing pH- and fragment-length-dependence

Different percentage ratios of Cu₁PrP-(90–126)H111A and Cu₁PrP-(90–126)H96A required to simulate the spectrum of Cu₁PrP-(90–126) at pH 6.3, 7.8 and 9.0. The same data for PrP-(91–115) are also included, as are the ratios for Ni^{2+} bound to PrP-(90–126) and PrP-(91–115) at pH 9.0.

simulation. This implies that Ni^{2+} has a preference for binding to the His⁹⁶ site over His¹¹¹. The preference for metal ion binding at His¹¹¹ or His⁹⁶ is quite subtle, as Cu^{2+} has a preference for His¹¹¹ and Ni^{2+} has a slight preference for His⁹⁶ in the PrP-(90–126) fragment (see Figure 4).

Figure 5 pH-dependence of Cu-PrP-(90-126) EPR spectra

(a) pH titration of Cu₁PrP-(90-126), indicating the hyperfine-splitting patterns of the three components present. (b) Cu₂PrP-(90-126) compared with the simulated EPR spectrum of the addition of Cu₁PrP-(90-126)H111A with Cu₁PrP-(90-126)H96A at pH 7.8 (both analogue spectra are inset). Peptide concentrations were 0.1 mM, spectra were recorded at 20 K.

Continuous wave EPR of PrP-(90-126) and its analogues and pentapeptides

X-band EPR was used to investigate the ligands involved in the coordination geometry of the Cu²⁺ PrP complexes. EPR spectra of PrP-(90-126) bound to 1 mol equivalent of Cu²⁺ between pH 5.5 and 9 are shown in Figure 5(a). Comparison of PrP-(90-126) and PrP-(91-115), as might be expected, reveal very similar spectra with similar pH-dependence (results not shown). The spectra are consistent with a Type II axial geometry, suggesting a square-planar/tetragonal arrangement. At pH 6.0, a set of signals with $A_{||}$ and $g_{||}$ values of 15.3 mK and 2.29 are present (component 2). A Peisach-Blumberg plot [45] of these values suggests an equatorial co-ordination of 3N1O (or 2N2O). As the pH is raised, the $g_{||}$ and hyperfine splittings shift slightly. At pH 8.0 and 9.0, the $A_{||}$ and $g_{||}$ values of 16.5 mK and 2.27 are observed, suggesting a ~4N (or 3N1O) co-ordination of the Cu²⁺ ion (component 1). The differences in the $A_{||}$ and $g_{||}$ values between components 1 and 2 are not sufficient to resolve two sets of signals: there is simply a shift to higher field of the signals as the pH is raised. These two components represent a change in co-ordinating ligands of Cu²⁺ bound to a single histidine residue (His⁹⁶ or His¹¹¹). The two most probable complexes are indicated in Figure 6. The Cu-PrP-(90-126) EPR spectrum obtained at pH 5.5 has $A_{||}$ and $g_{||}$ of 13.4 mK and 2.35, typical of a Type II 2N2O complex. At this low pH, amide deprotonation even in the presence of Cu²⁺ is unfavourable and the carboxylate co-ordination is favoured.

EPR of peptides containing a single histidine residue, PrP-(90-126)H111A and PrP-(90-126)H96A, give similar spectra, Figure 5(b). There is a shoulder on the hyperfine splitting to high field which is more apparent in the H111A spectrum. At present we have not assigned this feature. As with the visible CD spectra (Figure 3), the EPR spectra at pH 6.0 and above for the Cu²⁺-loaded PrP-(90-126) fragments are readily simulated by the addition of H96A and H111A spectra, as shown in Figure 5(b).

Figure 6 Square-planar metal-binding sites at His⁹⁶ and His¹¹¹ in PrP-(90-126)

The black circle represents either a Cu²⁺ or a Ni²⁺ ion. Zzz represents Thr⁹⁵ or Lys¹¹⁰; Xxx represents Gly⁹⁴ or Met¹⁰⁹. Component 1 is a 4N complex that dominates at pH ~7.5 and above; component 2 is a 3N1O complex found at pH ~6; component 3 may form at pH 5.5 and is consistent with a 2N2O multiple histidine complex. The inset indicates the possible ring puckering of the C β of the six-membered chelate ring between the histidine N α and N δ .

Interestingly, however, it is not possible to simulate the 2N2O spectra at pH 5.5 of PrP-(90-126) using PrP-(90-126)H96A and PrP-(90-126)H111A EPR spectra. The $A_{||}$ and $g_{||}$ of H96A spectra at pH 5.5 are more typical of a 4O spectrum than the 2N2O spectrum observed for PrP-(90-126) at the same pH. This suggests that the pH 5.5 complex may well contain the two histidine residues co-ordinating a single Cu²⁺ ion shown as component 3 in Figure 6.

The EPR spectra of the two pentapeptides were also obtained; the spectra of the two peptides are strikingly similar, with both having Type-II axial geometry. At pH 7.8 and 9, the $A_{||}$ and $g_{||}$ values are very similar to that of component 1 (the 4N species), with $A_{||}$ and $g_{||}$ values of 16.5 mK and 2.27. At pH 6, the $g_{||}$ value shifts to higher field and the hyperfine splitting reduces, with $A_{||}$ and $g_{||}$ values of 15.3 mK and 2.29, component 2 (the 3N1O species).

Finally, the possibility of a coupled Cu²⁺ system with a single bridged histidine residue was investigated. The spectra are not affected by increases in temperature from 20 to 120 K, which suggests that copper is not coupled and there is no histidine bridging between the two Cu²⁺ ions.

pH-dependence of binding

To complement the pH-dependence studies by EPR, Figure 7 shows the pH-dependence of the visible CD spectra of the two pentapeptides, PrP-(107-111) and PrP-(92-96). There is a clear transition in the spectra at ~pH 7. Although, at pH 7.8 and above, the two pentapeptides give characteristic mirror images of each

Figure 7 Visible CD spectra of pH-dependence of pentapeptides

(a) Cu₁PrP-(92–96). (b) Cu₁PrP-(107–111). Peptide concentrations were 0.1 mM.

other. We note that, at pH 6.0, the spectra of the two pentapeptides have a good deal of similarity, with both peptides giving a weaker positive CD band at ~530 nm. The change in co-ordination mode is most apparent from the PrP-(107–111) spectra (Figure 7b). However, the PrP-(92–96) spectra also change from a strong positive band at 500 nm and a negative band at 600 nm to a single weaker band at 530 nm. In agreement with the EPR data (Figure 5), the change in the spectra between pH 7.8 and 7.0 for the single-histidine-containing analogues suggests a change in co-ordination geometry between predominately 4N at pH 7.8 and above and a 3N1O complex at pH 7.0.

Cu²⁺ binding to multiple histidine residues at low pH

It is clear that, at pH 7.8 and pH 9, the visible CD spectra of PrP-(90–126) containing both His⁹⁶ and His¹¹¹ can be readily simulated using spectra of a single-histidine-containing PrP-(90–126) H96A and H111A analogue as described previously (Figure 3 and Supplementary Figure S1). It is also possible to simulate the low-pH (6.5) CD spectra of PrP-(90–126) from the H96A and H111A analogues, suggesting that, even at low pH, single histidine binds to each Cu²⁺ ion as shown in Figure 8(a). We do note, however, that, while the spectra at pH 6.5 (and pH 6.2, Supplementary Figure S1) simulate the trend of the wild-type spectra, the spectra are not a very good fit. The positive CD signal at 530 nm is consistently weaker in the simulated spectra for both the PrP-(90–126) and PrP-(91–115) fragments. A proportion of another component with a positive CD signal at ~530 nm must also be present.

We were interested in probing the effect of low stoichiometric amounts of Cu²⁺ at low pH on the mode of co-ordination. Figure 8(b) shows visible CD spectra of PrP-(90–126) at pH 6.5 with a titration of 0.1 mol equivalent additions of Cu²⁺ ions. A binding curve at 530 nm is shown as an inset in Figure 8(b) and reveals a slightly sigmoidal shape. In particular, with 0.1 and 0.2 mol equivalent of Cu²⁺ added to PrP-(90–126), the visible CD

gives almost no signal. We note that the visible CD of the multiple His complex (component 3, Figure 6) would produce a very weak visible CD spectrum, as amide chelation which produces the vicinal effect seen in visible CD spectra is reduced or absent in this complex [46,47]. The sigmoidal curve at pH 6.5 hints at the presence of low amounts of component 3 with two histidine residues co-ordinating a single Cu²⁺ ion.

We then decided to look more closely at the visible CD and visible absorption spectra of PrP at below the stoichiometric amounts of Cu²⁺. Figures 8(c) and 8(d) show visible CD and the associated absorption spectra over a range of pH values with 0.4 mol equivalent of Cu²⁺ present. Comparison of the visible CD signal at 530 nm (Figure 8c), observed at low pH, with that of a visible absorption band at 590 nm (Figure 8d) reveals some important differences in their appearance. In particular, at pH 5.5, there is no detectable visible CD, but visible absorption spectra at pH 5.5 are apparent with an ϵ_{590} of 35 M⁻¹·cm⁻¹, typical of a 2N2O tetragonal complex [43]. A visible CD signal is not apparent until the pH is raised to 6.0, where a weak positive band at 530 nm is observed (assigned to a 3N1O single histidine complex at His⁹⁶ or His¹¹¹; component 2). Again, at pH 5.5, a multiple histidine complex (component 3) is suggested because the visible CD spectrum of a multiple histidine complex between pH 5.0 and 6.0 is unlikely to generate a visible CD signal, whereas, in contrast, the visible absorption spectrum is observed clearly.

¹H-NMR studies of Ni²⁺ binding to PrP pentapeptides: measuring χ_1 coupling of histidine side chain

To investigate further the nature of the metal complex at His⁹⁶ and His¹¹¹, ¹H-NMR of Ni²⁺-bound PrP fragments was undertaken. Cu²⁺-bound PrP has square-planar geometry, but its paramagnetic properties make ¹H-NMR spectra difficult to interpret. Therefore Ni²⁺ was used as a probe of the Cu²⁺-PrP complex, as Ni²⁺ often mimics Cu²⁺ binding and, when in square-planar low-spin geometry, will produce a diamagnetic complex [48,49].

¹H-NMR studies of Ni²⁺ binding to PrP-(91–115) have been described in detail previously by ourselves [26]. Ni²⁺ titration for both PrP-(92–96) and PrP-(107–111) pentapeptides at pH 9 show a single complex formed in slow exchange with a 1:1 stoichiometry. Analysis of the ¹H-NMR spectra for the two pentapeptides enabled the observation of changes in chemical shifts when Ni²⁺ is bound. Characteristic changes in chemical shift upon Ni²⁺ co-ordination indicate the ligands involved in co-ordination. In particular, H α chemical shifts of Gly⁹⁴, Thr⁹⁵, His⁹⁶, Met¹⁰⁹, Lys¹¹⁰ and His¹¹¹ are the most perturbed upon Ni²⁺ co-ordination. The co-ordination shifts are characteristic of the adjacent main-chain amides being involved directly in chelating the Ni²⁺ ion in PrP-(92–96) and PrP-(107–111).

The predicted 4N square-planar geometry of these two bivalent metal-ion-binding sites at His⁹⁶ and His¹¹¹ are shown in Figure 6. When modelling these complexes, we were aware that the complex could have two different conformations that yet retain their square-planar geometry and ligands. The differences could be in the six-membered chelate ring between the histidine N δ and N α . The imidazole ring remains in the plane of the square-planar arrangement, but the position of the histidine C β can be above or below the plane. This ring pucker is analogous to 'chair' and 'boat' conformations in hexane rings, and is also shown in Figure 6. We were interested in the possibility that the source of the difference in the visible CD for the binding at His¹¹¹ and His⁹⁶ might arise from differences in the ring pucker.

Scalar *J*-coupling in NMR is very sensitive to the torsion angle between coupled protons, and the H α –H β coupling of His⁹⁶ and

Figure 8 Visible CD and absorption spectra of PrP-(90-126) at low pH and different Cu²⁺ stoichiometry

(a) Visible CD spectra of 1 (black solid line) and 2 (grey solid line) mol equivalents of Cu²⁺ bound to PrP-(90-126) at pH 6.5. Overlaid with these spectra are comparable simulated spectra of a 72%:28% combination at pH 6.5 of Cu₁PrP-(90-126)H96A and Cu₁PrP-(90-126)H111A (black broken line). Cu₂PrP-(90-126) is similar to spectra obtained from a 1:1 addition (grey broken line) of the analogue spectra bound to 1 mol equivalent of Cu²⁺. (b) Cu²⁺ titration of PrP-(90-126) at pH 6.5, 0.1 mol equivalent additions up to 2 equivalents. The inset shows a binding curve of the titration at 530 nm. (c and d) pH titration of 0.4 mol equivalent Cu-PrP-(90-126) by visible CD and absorption spectroscopy respectively. Insets show their respective binding curves at 530 and 590 nm respectively. All peptide concentrations are 0.1 mM.

Figure 9 ¹H-NMR spectra of Ni²⁺-bound pentapeptides showing histidine β -resonances

PrP-(107-111) (a) and PrP-(92-96) (b). The histidine H β and H β' resonances are shown with J -coupling values (Hz) indicated. Peptide concentrations were 0.5 mM, 0.9 mol equivalent of Ni²⁺ at pH 9.0.

His¹¹¹ is related to the φ_1 angle [50]. Figure 9 shows the H β and H β' of the histidine residues for these two pentapeptides. The expected two sets of doublets of doublets are well resolved for the Ni₁PrP-(107-111) complex and the ¹H J -coupling is readily measured from the one-dimensional spectra (Figure 9). The $\alpha\beta$ and $\alpha\beta'$ ³ J -couplings give values of 6.0 Hz and 7.2 Hz. J -coupling $\sim$ 6 Hz for both $\alpha\beta$ and $\alpha\beta'$ in the Ni²⁺-bound spectra indicate a χ angle of 60° [50]. In the case of the PrP-(92-96) spectrum, the H β and H β' chemical shifts are almost degenerate: the two sets of

doublets of doublets have coalesced into a single group of overlaid peaks upon Ni²⁺ addition (Figure 9b). However, even from this spectrum it is clear that all J -coupling values are < 7 Hz, thus the χ angle must also be 60° [50]. Alternative conformers (-60° and 180°) can be ruled out, as J -values of 12–14 Hz for one or other of the ³ $J_{H\alpha-H\beta}$ values are required. Thus the differences in the CD spectra (shown in Figure 2) are not due to ring puckering, as both complexes have the same χ angle. The spectra were also acquired at 800 MHz, and the same J -coupling values were observed at the higher field strength.

DISCUSSION

Previously we have shown that Cu²⁺ and Ni²⁺ bind to both His⁹⁶ and His¹¹¹ independently in the PrP-(91-115) fragment [26]. Pentapeptides PrP-(92-96) and PrP-(107-111) contain all the residues necessary for the two binding sites. We were therefore surprised to observe very different visible CD spectra for PrP-(90-126) and PrP-(91-115). By using H111A and H96A analogues of PrP-(90-126) and PrP-(91-115) containing a single histidine residue, we have shown that the mode of Cu²⁺ and Ni²⁺ coordination has not changed for the larger fragment. The reason for the significant difference between the visible CD spectra of PrP-(91-115) and PrP-(90-126) is the relative affinity of the two sites. For example, upon the addition of 1 mol equivalent of Cu²⁺ to PrP-(91-115) (pH 7.8), we observed $\sim 70\%$ of Cu²⁺ binding to His¹¹¹ and $\sim 30\%$ to His⁹⁶. In contrast, when the longer fragment, PrP-(90-126), is used, $\sim 95\%$ of Cu²⁺ binds to His¹¹¹ and only 5% to His⁹⁶. This has the effect of dramatically altering the appearance of the spectra.

The relative preference for His⁹⁶ or His¹¹¹ is interesting and varies considerably depending on which metal ion is co-ordinating, the pH at which co-ordination takes place and the presence of a hydrophobic tail (residues 116–126). The complex centred at His¹¹¹ has a higher affinity for Cu²⁺ than His⁹⁶. The opposite might be expected, as Gly⁹⁴ and Gly⁹³ main-chain co-ordination for the His⁹⁶ complex might allow less steric strain in the complex. It appears that the hydrophobic residues increase the relative affinity of Cu²⁺ for His¹¹¹. In contrast, the reverse effect is observed for the Ni²⁺ complex, as His⁹⁶ has a slightly higher affinity. EPR does not support the presence of an axial ligand stabilizing the His¹¹¹ complex, as Cu-PrP-(92–96) is indistinguishable from Cu-PrP-(107–111) by EPR, although this possibility is not ruled out, as axial ligands can have only subtle effects on Type-II EPR spectra.

A recent study has suggested Cu²⁺ co-ordinates C-terminally from the His⁹⁶ and His¹¹¹ as well as N-terminally [51]. It is clear from Figure 2 and ¹H-NMR of the Ni²⁺ complexes that removal of residues to the C-terminus of His⁹⁶ or His¹¹¹ for the pentapeptides do not have any substantial effect on the co-ordination. Furthermore, the CD spectra with amide co-ordination to the N-terminus has a very different appearance as is indicated for the hexapeptide PrP-(110–115) (see Supplementary Figure 2 at <http://www.BiochemJ.org/bj/404/bj4040393add.htm>) and for the octarepeat peptides [7,8]. The study was based on the effect of paramagnetic broadening of the Cu²⁺ ion in ¹H-NMR spectra [51]. The 'through-space' paramagnetic broadening to the C-terminus of the Cu²⁺-binding site could be caused by the inherent flexibility of the peptide sampling transient conformations in which these residues become close to the paramagnetic ion for brief periods of time.

The visible CD spectra of PrP-(90–126)H111A and PrP-(90–126)H96A analogues containing a single histidine residue are relatively unaffected by pH between ~7.5 and 9, suggesting that 4N complexes dominate at physiological pH and above. In contrast, there is a profound change in the visible CD spectra between pH ~6 and ~7.5, suggesting a change in co-ordinating ligands. Our EPR work also indicates that Cu²⁺ co-ordination is pH-dependent, with a probable 3N1O complex dominating at pH ~6.5 and a 4N complex at pH 7.5. This agrees with a potentiometric study of PrP-(92–96) and PrP-(103–113) fragments which indicates the same transition from 3N1O to 4N, with the 4N dominating above pH 7.6 [52,53]. These two co-ordination modes have recently been modelled using *ab initio* electronic structure calculations [54].

The complexes, components 1 and 2 (Figure 6), require deprotonation of a number of amides. We postulate that, at low pH, a multiple histidine complex, component 3 is favoured. The presence of a visible absorption band and EPR spectra at pH 5.5 coupled with the absence of visible CD bands at the identical pH supports this assertion. This type of complex favoured at low pH and low Cu²⁺ stoichiometry has been shown to be present in multiple octarepeats of PrP [10,55–57]. Metal ion complexes are often described in terms of a single co-ordination geometry. In the case of PrP^C, it is more appropriate to describe the complex formed as an equilibrium between a number of complexes. The dominant species will be influenced by pH and the stoichiometric levels of Cu²⁺ ions present. A multiple histidine complex at sub-stoichiometric Cu²⁺ seems only to be present in appreciable amounts close to pH 5.5. At pH 7.4, the complex with a single histidine and amide co-ordination dominates. *In vivo* PrP will experience very different pHs as it is trafficked between the cell surface and the endosome, where the pH is lower. Clearly, the pH will affect the affinity for Cu²⁺ ions of PrP and the co-ordination mode, which will in turn affect the Cu²⁺-induced folding or misfolding of PrP.

Full-length PrP^C binds between five and six Cu²⁺ ions [11,22]: four Cu²⁺ ions in the octarepeat region and further Cu²⁺ binding at His⁹⁶/His¹¹¹. The N-terminal region, residues 23–126, is unstructured in the absence of Cu²⁺, thus fragments of PrP from this region have been used as models for Cu²⁺ co-ordination to full-length PrP to circumvent solubility problems for full-length PrP at physiological pH with Cu²⁺ ions present. Electron spin echo envelope modulation EPR studies [25] suggest that only the Cu²⁺ complex centred at His⁹⁶ has an appreciable affinity with 4N square-planar co-ordination geometry. Alternatively, it has been proposed that His⁹⁶ and His¹¹¹ could co-ordinate a single Cu²⁺ ion [23,24]. The present study has shown that Cu²⁺ binds to His¹¹¹ independently of His⁹⁶.

The relative preference for binding at His⁹⁶ and His¹¹¹ is affected by fragment length; it remains to be established if the presence of the structured C-terminal domain of PrP will affect the binding of Cu²⁺ further. Two sets of fragments can be generated *in vivo*, in addition to full-length PrP. The so-called α -cleavage at residue 110/111 creates a metal-binding fragment, 23–110, which contains the His⁹⁶-binding site and the octarepeats. ROS (reactive oxygen species)-mediated β -cleavage occurs within, or adjacent to, the octarepeats to produce a fragment that contains both His⁹⁶ and His¹¹¹, but lacks most or all of the octarepeats [41,42]. This *in vivo* fragment created relates to our model fragment, PrP-(90–126), but contains the structured C-terminal domain as well. The Cu²⁺- and Ni²⁺-co-ordination sites described here involve metal binding to an unstructured protein. Unlike preformed sites observed for many metalloproteins, the main chain must be highly flexible before ion co-ordination, in order that it may 'wrap' around the metal ion in a square-planar geometry. PrP^C is unusual in that half of its main chain is natively unstructured, facilitating the co-ordination of five to six Cu²⁺ ions.

The affinity of Cu²⁺ for PrP is still a matter of hot debate in the literature [22,55,58,59]. Two recent studies of Cu²⁺ binding to the four-octarepeats fragment suggests that, at sub-stoichiometric amounts of Cu²⁺, a single Cu²⁺ ion will bind to multiple histidine residues with nanomolar affinity [55,57]. Perhaps counterintuitively, simulations suggest that this high-affinity site is out-competed by a lower-affinity binding mode of four Cu²⁺ ions with micromolar affinity [55]. We have shown that Cu²⁺ binding to the PrP-(91–115) fragment preferentially binds Cu²⁺ over the octarepeat fragment with nanomolar affinity [21]. Kramer et al. [22] have suggested micromolar affinity for full-length PrP, while others have suggested femtomolar affinity for the full-length protein [23,58] when a copper-glycine chelate was used.

A role for PrP^C could be as a sensor of elevated Cu²⁺ levels, as Cu²⁺ levels can be as high as 0.1 mM in the synaptic cleft [60], compared with typical extracellular Cu²⁺ levels of 10 nM [61]. PrP may act as a neuroprotectant during fluxes of Cu²⁺ produced at the synapse, as knockout mice are more sensitive to oxidative stress. Cu²⁺ binding causes folding up of PrP, resulting in endocytosis [36,37]; however, PrP expression does not seem to significantly affect intracellular copper levels [38,39].

Oxidative damage is a key feature of prion disease pathology and binding of up to six Cu²⁺ ions to PrP^C implies that PrP may localize the production of ROS via Fenton's cycling of Cu²⁺/Cu⁺. PrP can act as a sacrificial antioxidant [16], resulting in chemical modification of PrP^C by ROS which could influence PrP misfolding [17,62]. The mode of Cu²⁺ co-ordination is influenced by Cu²⁺ stoichiometry and pH, which in turn will have a profound influence on the structuring of the N-terminal tail and biophysical properties of PrP^C. The Cu²⁺-loaded state of PrP should certainly be considered when investigating its folding and fibrillization properties. Cu²⁺ co-ordination causes significant loss of solubility

[7] and an increased protease resistance [34]. Cu²⁺ binding in the amyloidogenic region (residues 90–126) promotes β -like extended structure [21], which suggests a role for Cu²⁺ ions in promoting extended β -sheet-like structures found in protofibrils. This is supported by the observation that prion disease strain type can be conferred by Cu²⁺ ions [13]. In the present study, we have shown that the copper-co-ordination mode is highly sensitive to pH, stoichiometry and PrP fragment length. This in turn will affect the affinity, redox properties and Cu²⁺-induced folding of the PrP main chain. Thus changes in pH or levels of Cu²⁺ ions may trigger the hydroxyl radical production or amyloidogenicity of PrP observed in prion disease.

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