Mapping Cu(II) Binding Sites in Prion Proteins by Diethyl Pyrocarbonate Modification and Matrix-assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) Mass Spectrometric Footprinting*

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Although Cu(II) ions bind to the prion protein (PrP), there have been conflicting findings concerning the number and location of binding sites. We have combined diethyl pyrocarbonate (DEPC)-mediated carboxylation, protease digestion, and mass spectrometric analysis of apo-PrP and copper-coordinated mouse PrP23–231 to “footprint” histidine-dependent Cu(II) coordination sites within this molecule. At pH 7.4 Cu(II) protected five histidine residues from DEPC modification. No protection was afforded by Ca(II), Mn(II), or Mg(II) ions, and only one or two residues were protected by Zn(II) or Ni(II) ions. Post-source decay mapping of DEPC-modified histidines pinpointed residues 60, 68, 76, and 84 within the four PHGGGWGWGQ octarepeat units and residue 95 within the related sequence GGTHNQ. Besides defining a copper site within the protease-resistant core of PrP, our findings suggest application of DEPC footprinting methodologies to probe copper occupancy and pathogenesis-associated conformational changes in PrP purified from tissue samples.

Prion diseases are associated with a pathogenic conformational transition in the cellular prion protein (PrPc)† to an infectivity-associated form commonly denoted PrPsc. Although it is clear that PrP gene-ablated (Prnp−/−) mice have no overt phenotype in a laboratory environment, consistent with functional redundancy with other neuronal proteins, it is plausible that the physiological function of PrPsc involves binding of Cu(II) ions. Subsequent to the removal of N- and C-terminal signal peptides, the mature form of mammalian PrPc contains residues 23–231 of a 253-amino acid sequence encoded by a single copy gene, Prnp (1). The C-terminal region of PrPc forms a globular structure comprising three α-helices and two short β-strands (2–4) and, during the process of prion infection, can re-fold into protease-resistant, β-sheet-enriched aggregates (5–9). In contrast, the N-terminal domain of PrP is highly flexible and typically includes five tandem copies of a conserved octapeptide repeat motif (3,4,6). The N-terminal domain of human PrPc contains four identical “octarepeat” sequences (PHGGGWGQ within residues 60–91) and one homologous sequence but lacking a histidine residue (PQGGGWGQ: residues 51–59) (10). Residues 51–91 of mouse PrPc consists of two octarepeat sequences (PHGGGWGQ: residues 59–66 and 83–90), two octarepeat sequences (PHGGGWGQ: residues 67–81), and one homologous sequence lacking a histidine residue (PQGGTWGQ: residues 51–59) (11).

Although the octarepeat motifs within PrP have no sequence homology to classical copper-binding proteins (12), there is now a broad agreement that these motifs bind to copper with a remarkable degree of selectivity; as such they may comprise the prototype of a new class of copper binding motif. Copper binding to the N-terminal octarepeat domain of PrPc was reported in recombinant human PrP23–98 using equilibrium dialysis (13) and in synthesized peptides using mass spectrometry (14,15), fluorescence spectroscopy (14), Raman spectroscopy (16), circular dichroism, proton nuclear magnetic resonance (NMR) spectroscopy (17), electron paramagnetic resonance (EPR), and electron spin-Echo envelope modulation spectroscopy (18). Copper binding has also been reported for nearly full-length forms of Syrian hamster (residues 29–231) (19) and human PrP (residues 91–231) (20) and full-length mouse PrP (residues 23–231) (21). Affinity chromatography using immobilized copper ions has been used to purify mature, glycosylated PrPc isolated from hamster brain (22), while human PrPsc in brain is inferred to be bound to transition metals (including copper) in situ, as assessed by the effects of chelators upon the size of protease-resistant fragments (23). With regard to stoichiometry, the number of Cu(II) binding sites in the N-terminal region of PrPc is reported between 2 and 5.6 being pH-dependent (13, 15, 17–19, 24). At neutral pH, copper binding to the N-terminal domain occurs in the micromolar range with positive cooperativity, with a remarkably close determinations of Hill coefficients by different laboratories: 3.4 (PrP23–98) (13), 3.3 (PrP58–91) (17) and 3.6 (PrP23–98) (21). However, more recently, these stoichiometries and binding constants have been challenged, with the suggestion of two high affinity copper binding sites of $10^{-14}$ and $4 \times 10^{-14}$ M, deduced from the analysis of PrP58–98 and PrP91–231, respectively (20). In this paper, we use chemical modification to map histidine-dependent copper binding sites in native mouse PrP. Our results are discussed in the context of the physiology and pathobiology of PrP.

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‡ The abbreviations used are: PrP, prion protein; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; DEPC, diethyl pyrocarbonate; TOF, time of flight; PSD, post-source decay; ESI, electrospray ionization; NEMO, N-ethylmorpholine.

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Materials and Methods

PrP Proteins—Two recombinant proteins were used for these studies. Purified human PrP23–98 (HuPrP23–98) contains 80 amino acids including four identical octarepeat motifs of the form PHGGGWGQ (13). Full-length mouse PrP (MoPrP23–231) was expressed and purified as described (25, 26). MoPrP23–231 consists of 210 amino acids including four octarepeats in the N-terminal domain (two of the form PHGGGWGQ and two of the form PHGGSWGGQ). All MoPrP23–231 samples were used within six months of preparation to circumvent altered aggregation properties seen in aged samples (26). Fig. 1, B and C show the amino acid sequences and predicted tryptic and chymotryptic peptides of HuPrP23–98 and MoPrP23–231.

Coordination and Modification of HuPrP23–98—Prior to MALDI-MS analysis, HuPrP23–98 (90 μM) was incubated with 10-fold molar excess CuCl2, ZnCl2, NiSO4, MgCl2, MnCl2, or FeSO4 in 25 mM phosphate buffer, pH 7.4, at room temperature for 30 min. In some experiments, after incubation with or without these cations, HuPrP23–98 samples were reacted with 5-fold molar excess DEPC at room temperature for 30 min. The samples were then analyzed by using MALDI-MS without further purification.

Coordination and Modification of MoPrP23–231—5-fold molar excess DEPC was reacted with MoPrP23–231 (90 μM) in NEMO-KCl buffer, pH 7.4, for 30 min at room temperature. In some cases, prior to the DEPC reaction, the protein was incubated with 10-fold molar excess CuCl2 for 30 min. Sequencing grade modified trypsin (Promega) or chymotrypsin (Roche) (the ratio of enzyme:protein was 1:20) was used to digest the intact protein, carboxyethylated protein, and carboxyethylated copper-protein complex for 2 h at 37 °C (trypsin) or at room temperature (chymotrypsin). The samples were analyzed by MALDI-MS without further purification.

Mass Spectrometry and Structural Modeling—MALDI-TOF-MS analyses were carried out using a Perseptive Biosystems Voyager-DE STR mass spectrometer as described previously (27). Post-source decay (PSD) spectra were acquired at DE-reflectron mode. The accelerating voltage was set at 20 kV, grid voltage at 75%, guide wire voltage at 0.024%, and delay time at 100 ns. The timed ion selector was pre-set to the [M+H]+ mass of the peptide. The spectra were acquired in 10–13 segments with mirror ratios 1.0–0.13 and then assembled by the instrument software. Three-dimensional images of PrP121–231 were generated using the program RasMac v2.5 and the NMR co-ordinates from Protein Data Bank file IA02 (2).

Results

Copper Binding Properties of Human PrP23–98 Assessed by MALDI-MS—Studies of PrP/metal interactions commenced with a human PrP N-terminal fragment, HuPrP23–98 (Fig. 1B), previously characterized in an equilibrium dialysis study. In earlier analyses with this recombinant polypeptide fragment, Cu(II) was used in the form of an Cu(II)-glycinex complex. These studies yielded a stoichiometry of 5.6 ± 0.4 Cu(II) ions per peptide at saturation and binding curves indicating that between three and four Cu(II) ions are added to (or charged from) PrP in a highly cooperative fashion (positive Hill coefficient) that between three and four Cu(II) ions are added to (or discharged from) PrP in a highly cooperative fashion (positive cooperativity, Hill coefficient = 3.4) (13). Since the studies presented here involve mass determinations (rather than competition between glycine and peptide molecules for Cu(II) ions), Cu(II) was presented without the intervention of an amino acid chelator. Metal-protein complexes were measured using saturated sinapinic acid in 20% acetonitrile as matrix. The measured mass [M+H]+ of unreacted HuPrP23–98 was 7812.3 Da (calc. 7812.4 Da) (Fig. 2A). After incubation with 10× molar excess of CuCl2 in NEMO-KCl buffer, pH 7.4, at room temperature for 30 min, the Cu(II)-HuPrP23–98 complex was detected by MALDI-MS. In addition to intact protein at m/z 7812.3 Da, peaks indicative of 1 Cu(II), 2 Cu(II), 3 Cu(II), and 4 Cu(II) were observed at m/z 7874.2 Da (calc. 7873.8 Da, assuming a mass of 63.55 for copper and expulsion of two protons from the polypeptide per bound metal ion), 7935.3 Da (calc. 7935.3 Da), 7986.3 Da (calc. 7996.8 Da), and 8061.8 Da (calc. 8058.3 Da) (Fig. 2B). These data are in close accord with other MALDI-MS data (14) and are inferred to represent a stable association with four copper binding sites in the octarepeat repeat region. In independent electrospray ionization MS analyses (ESI-MS), where protein/metal solutions mixtures are introduced into the instrument still in the aqueous phase it is possible to detect two additional, weaker binding sites (i.e. fifth and sixth binding sites) tentatively positioned at the free amino group of the N terminal and involving histidine 95, respectively (15, 21).

Chymotrypsin digestion was used to confirm the location of the four copper binding sites detected by MALDI-MS. A peak at m/z 795.35 Da corresponding to C3, C4, C5, or C6 (GQPHGGGW, calc. 795.35 Da; see Fig. 1B) was observed in the absence of copper with a new peak at m/z 857.35 Da (calc. 857.35 Da) in the presence of copper, indicating that one metal ion bound to His-80, His-86, His-76, or His-84 (data not shown). Lastly, to detect whether other divalent ions bind to the N-terminal region of PrP, HuPrP23–98 (90 μM) was incubated with 10-fold molar excess Zn(II), Ni(II), Mg(II), Ca(II), Mn(II), or Fe(II) in NEMO-KCl buffer, pH 7.4, at room temperature for 30 min before MALDI-MS analysis. No signals corresponding to metal-HuPrP23–98 complexes were detected (Fig. 2, C–H). These results are in accord with other copper-binding experiments using N-terminal PrP peptides (e.g. Ref. 24) and validate the copper-binding behavior and metal selectivity of recombinant PrP23–98.
Copper Protects Five Residues in PrP23–98 from DEPC Modification—The PrP23–98 substrate was used to explore the feasibility of mapping histidine-dependent copper binding sites by protection from DEPC modification. PrP23–98 in NEMO-KCl buffer, pH 7.4, was incubated with or without 10-fold molar excess Cu(II) for 30 min then reacted with 5-fold molar excess DEPC at room temperature for 30 min prior to MALDI-MS analysis. The intact HuPrP23–98 has a mass of 7812.4 Da (Fig. 3A), whereas in the presence of DEPC, species were detected at m/z 7884.8, 7856.8, 8028.4, 8100.3, 8172.5, 8244.4, 8317.1, 8389.3, and 8461.8 Da (Fig. 3B). Based on a predicted mass shift of 72.06 per DEPC adduct, these signals were interpreted as 1 mono- ("1m", calc. 7884.4 Da), 2 mono- ("2m" calc. 7856.4 Da), 3 mono- ("3m" calc. 8028.5 Da), and 4 mono- ("4m", calc. 8100.5 Da), 5 mono- ("5m", calc. 8172.6 Da), 6 mono- ("6m", calc. 8244.7 Da), 7 mono- ("7m", calc. 8316.7 Da), 8 mono- ("8m", calc. 8388.8 Da), and 9 mono-carbethoxylation ("9m", calc. 8460.8 Da), with the largest peak amplitudes corresponding to four and five modifications. The addition of up to nine mono-carbethoxylations per peptide molecule may reflect the involvement of modifications on tyrosine and serine residues and perhaps the free N terminus, in addition to the five histidine residues (Fig. LB).

A distinct mass spectrum was obtained for carbethoxylated HuPrP23–98 formed subsequent to pre-incubation with Cu(II). Here the major species were determined as the starting peptide HuPrP23–98, 1 Cu(II) (7876.5 Da, calc. 7875.9 Da), 2 Cu(II) (7940.1 Da, calc. 7939.5 Da), 3 Cu(II) (8003.5 Da, calc. 8003.3 Da), 3 mono-carbethoxylation (8029.3 Da, calc. 8028.4 Da), 4 Cu(II) (8067.2 Da, calc. 8066.6 Da), 4 mono-carbethoxylation (8089.6 Da, calc. 8100.4 Da), 4 mono-carbethoxylation and 1 Cu(II) (8163.2 Da, calc. 8163.5 Da) (Fig. 3C). Since the maximum number of mono-carbethoxylations observed for HuPrP23–98 is nine (Fig. 3B) while the maximum number of mono-carbethoxylation of pre-formed copper-HuPrP23–98 complexes is four (Fig. 3C), these data imply that five histidine residues within HuPrP23–98 are typically protected by Cu(II) from DEPC modification. Of interest, the largest peak heights in Cu(II)-treated samples corresponded to unmodified peptide or metal ion-peptide complexes with 1–3 copper ions. These results suggest that the 3–4 histidine residues that bind to copper with the greatest avidity are the residues most susceptible to DEPC modification in the naked peptide. Presumably a fraction of the metal-peptide complexes formed during the pre-incubation with Cu(II) are stripped of the metal ions during the MALDI-MS procedure, yielding a peak of free peptide. An alternative (but perhaps less likely) explanation is that PrP23–98 copper complexes with multiple metal ions undergo a conformational change such that all DEPC-reactive residues are shielded from or are less reactive with the chemical modification reagent. Again, dissociation of some metal-peptide complexes during the MALDI-MS procedure could yield the observed peak of free PrP23–98 peptide.

To determine whether any other cations are involved in
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histidine-dependent coordination to HuPrP23–98, the peptide was incubated with 10-fold molar excess of other metals including Ca(II), Mg(II), Mn(II), Zn(II), and Ni(II) in NEMO-KCl buffer, pH 7.4, prior to reaction with DEPC. Peaks of 1–9 mono-carbethoxylation were found in samples incubated with Ca(II), Mg(II), and Mn (Fig. 3, D–F). These data are similar to the MALDI-MS spectra of intact HuPrP23–98 reacted with DEPC (Fig. 3B), except that maximum peak heights were observed at 3- and 4-mono-carboxylations, rather than at 4- and 5-mono-carboxylations. Thus, these three metals provide no significant protection of the HuPrP23–98 peptide. For Zn(II) and Ni(II) the largest peak amplitudes corresponded to yet lesser degrees of mono-carboxylation: 2- and 3-mono with Zn(II), or 1- and 2-mono with Ni(II) (Fig. 3, G and C). Signals deriving from intact peptide (7811.9 Da in Fig. 3G, 7812.8 in Fig. 3H) can also be observed in these analyses. For HuPrP28–98 incubated with Ni(II) or Zn(II), the maximum number of mono-carbethoxylations was seven or eight, respectively. The results suggest that Zn(II) or Ni(II) might partially protect one or two histidine residues from DEPC modification, with a fraction of the metal-peptide complexes dissociating during the course of the MALDI-MS analysis to yield free peptide. While attempts were also made to extend these studies to Fe(II) ions, a surffeit of background noise in the corresponding MS spectra precluded meaningful analysis (not presented; also see Fig. 2H versus Fig. 2, C–G).

Copper Protects Four to Five Residues in PrP23–231 from DEPC Modification—The measured mass ([M+H]+) of the full-length recombinant MoPrP23–231 in NEMO-KCl buffer, pH 7.4, was 23,106.6 Da (calc. 23106.4 Da; the error for this instrument in this analytical mode is less than or equal to 0.05%, i.e., ± 11.55 Da) (Fig. 4A). Since attempts to detect copper adducts by direct MALDI-MS analysis of MoPrP23–231 were not successful, we were prompted to explore the DEPC footprinting technique. In addition to the terminal NH2 group, there are 9 histidine, 13 tyrosine, 12 serine, and 11 arginine residues in this protein. After incubation with DEPC the mass of carboxethoxylated MoPrP23–231 increased, and the peak of carboxethoxylated MoPrP23–231 became wider. The largest peak amplitude was observed m/z of 24,321.6 Da (Fig. 4B) indicating that the most abundant form of the modified MoPrP23–231 had ~17 mono-carboxethoxylations (calc. 24,330.4 Da). The greatest peak amplitude of the carboxethoxylated copper-MoPrP23–231 complex corresponded to m/z 23,972.9 Da (Fig. 4C) indicating that the most abundant form of the modified copper-MoPrP23–231 complex had ~12 mono-carboxethoxylations (calc. 23,972.1 Da). Based on the difference between DEPC modification in the presence and absence of copper we can infer that ~5 histidine residues (i.e. 17 minus 12) are protected by copper coordination. This conclusion was supported by analyses from two additional experiments. The averaged mass difference of 344.9 ± 7.7 Da derived from all three experiments yielded a figure of 4.79 ± 0.11 sites protected by copper per MoPrP23–231 molecule. This stoichiometry is close to that obtained using the N-terminal PrP23–88 fragment.

Five Cu(II)-protected Residues Are Located within an N-terminal Tryptic Fragment of Mouse PrP23–231—To extend the above studies, enzymatic digestion of MoPrP23–231 was used to produce peptide fragments and peptide fragment adducts amenable to more accurate sizing. Accordingly, MoPrP23–231 (90 µM) in NEMO-KCl buffer, pH 7.4, was digested with trypsin. In some cases MoPrP23–231 at the aforementioned concentration was pre-incubated with or without 10-fold molar excess CuCl2 then reacted with 5-fold excess DEPC prior to tryptic digestion (a tryptic peptide map of MoPrP23–231 is presented in Fig. 1C). In the resulting analyses, with the exception of tryptic peptide T5, no copper-dependent changes were apparent in mass spectra of the tryptic peptides. By way of example, a peak at m/z 4987.3 Da (Fig. 5A) corresponds to tryptic peptides T10 and T14 bridged by the disulfide bond between Cys-178 and Cys-213 (calc. 4987.4 Da) (Fig. 1C). This linked peptide contains one histidine residue at residue 176. A signal at m/z 5058.9 Da (calc. 5059.4 Da) was determined in the tryptic digestion of carbethoxylated MoPrP23–231, indicating 1 mono-carboxethoxylate after DEPC treatment (Fig. 5B). Since a similar peak (m/z 5059.7 Da) was detected in the tryptic digestion of carbethoxylated Cu(II)-MoPrP23–231 complex (Fig. 5C), this indicates that Cu(II) does not protect His-176 from DEPC modification. On the other hand, analyses of the N-terminal tryptic peptide T5 revealed a clear protective effect of copper.

Peptide T5 (residues 49–105, Fig. 1C) includes five histidine residues: one histidine per each of the four octarepeats of the form PHGG/G/S/WGQ and one within the degenerate octarepeat related sequence GGTT/HNG. This peptide was represented by a peak at m/z 5876.3 Da (calc. 5876.2 Da) in the tryptic digestion of intact MoPrP23–231 (Fig. 5A). Eight peaks at m/z 6021.3, 6092.9, 6164.2, 6236.0, 6308.2, 6383.7, 6453.2, and 6525.7 Da (Fig. 5B) were detected for the tryptic digestion of carbethoxylated Cu(II)-MoPrP23–231 complex (Fig. 5C), this indicates that Cu(II) does not protect His-176 from DEPC modification. On the other hand, analyses of the N-terminal tryptic peptide T5 revealed a clear protective effect of copper.

**Fig. 4.** Cu(II) protects four to five histidine residues of MoPrP23–231 from carboxethoxylations. MALDI-MS spectra of (A) MoPrP23–231 in NEMO-KCl buffer, pH 7.4, (B) MoPrP23–231 incubated with 5-fold molar excess DEPC, and (C) MoPrP23–231 incubated with 10-fold molar excess Cu(II), then reacted with 5-fold molar excess DEPC. $m = $ mono-carboxethoxylate.
peptide T5 of MoPrP23–231 are protected from DEPC modification by Cu(II) coordination.

Whereas peaks indicative of intact metal-peptide complexes (and free peptide) were noted in the copper-protection experiment involving PrP23–98 (Fig. 3C), peaks of unmodified metal-peptide complexes were not detected in the analysis of the octarepeat containing peptide T5 derived from copper-protected MoPrP23–231 (Fig. 5C). From this observation we infer that additional sequences present in PrP23–98 versus peptide T5 (i.e. residues 23–48), or minor changes within the homologous regions of peptide T5 and PrP23–98 (see Fig. 1, B and C) stabilize copper interactions, such that metal-peptide complexes remain intact for MALDI-MS analyses of HuPrP23–98.

In this regard a tendency for copper-binding constants to increase in affinity in parallel with increasing PrP fragment length has been noted previously (21). Alternatively, extra residues present at the C terminus of peptide T5 versus PrP23–98 (i.e. WNKPPSKPK; Fig. 1B, lower line) may destabilize copper-peptide interactions or desorption such that intact metal-peptide complexes are not detected in the MALDI-MS analysis of peptide T5.

**Delimiting Copper Interacting Areas of MoPrP23–231 by Chymotryptic Mapping**—Chymotryptic mapping was also used to refine the positions of copper binding sites. MoPrP23–231 chymotryptic peptides C3 (residues 57–64) and C6 (residues 81–88) have the same amino acid sequences, GQPHGGGW, while peptides C4 (residues 65–72) and C5 (residues 73–80) share the sequence GQPHGGSW (Fig. 1C).

In the DEPC-treated protein peaks at \( m/z \) 795.32 Da corresponding to peptides C3 and C6 (calc. 795.35 Da), 825.29 Da corresponding to C4 or C5 (calc. 825.36 Da), and 1041.36 Da corresponding to C7 (calc. 1041.45 Da) were found in the mass spectrum of MoPrP23–231 (Figs. 6A and 1C). A peak was also detected at \( m/z \) 1140.57 Da (calc. 1140.45 Da) corresponding to C1, the chymotryptic peptide containing the free N-terminal amino group (Fig. 6A, see also Fig. 1C).

Subsequent to a Cu(II) pre-incubation, a peak at \( m/z \) 795.40 Da was observed and indicated that His-60 and His-84 in chymotryptic peptides C3 and C6, respectively, coordinated to Cu(II) and thereby protected against DEPC modification (Fig. 6C).
where a peak at m/z 825.36 Da indicated that copper protection of His-68 and His-76 (Fig. 6C). In comparisons with Fig. 6B, a peak at m/z 1113.35 Da (1-mono-carbethoxylated C7) disappeared, whereas the peak at m/z 1041.50 Da (unmodified C7) exhibited an amplitude similar to that of the starting material in Fig. 6A. These data indicate that His-95 in full-length PrP is capable of coordinating to Cu(II). In contrast, little change could be observed in the peaks of C1 and carbethoxylated C1 (Fig. 6B and C), indicating that Cu(II) did not coordinate to the C1 peptide. It is also of note that mass spectra presented in Fig. 6 include two peaks corresponding to partial digest products: C18/H11001 C19/H11001 C20 (YRENMY, calc.875.35 Da) and C21/H11001 C22 (RYPNQVYY, calc. 1102.53 Da). These peptides do not contain histidine residues, and their corresponding mass signatures remained invariant throughout the manipulations presented in Fig. 6B and C. This is likely that the tyrosines located within these peptides do not react with DEPC as in all but one case these aromatic residues are disposed toward the hydrophobic interior of the native PrP molecule (2).

Fine Mapping DEPC Modification Sites by Post-source Decay Analysis—Further analysis of the chymotryptic peptides C3/C6, C4/C5, and C7 was attempted with the PSD technique of tandem analysis to verify the inference that the copper-sensitive DEPC modifications resided on histidine residues. In post-source decay experiments, fragmentation of the peptides originates from collisional activation within the field-free region, with the collision-induced peptide fragment ions mostly derived from bond breakage along the peptide backbone (28, 29) and often with transfer of one or two hydrogens to create stable ion structures. In the nomenclature system, the a-, b-, and c-ions all contain the N terminus of the peptide, while the x-, y-, and z-ions all contain the C terminus. The major N terminus-containing ion series is the b-ion series, and the major C terminus-containing ion series is the y-ion series (30). Fig. 7A shows the scheme of nomenclature for peptide PSD fragment ions of C3 or C6 of intact MoPrP23–231. Post-source decay spectra of peptides of C3 or C6 of intact MoPrP23–231 ([M+H]+ 867.35 Da) and their mono-carbethoxylated counterparts ([M+H]+ 875.35 Da) are presented in Fig. 7B and C, respectively. A complete listing of PSD fragment ions is presented in Table I. The largest peak amplitude was obtained for the y6 ion PHGGGW in the unmodified sample, whereas this peak was considerably reduced in the DEPC-modified sample, to be replaced by a species of m/z 682.22 corresponding to y6 plus 72 mass units. Of the other characteristic fragment ions, b4 (sequence GQP) was detected in both intact and carbethoxylated C3 or C6, b4 plus a 72.06-Da adduct was only observed in...
carbethoxylated C3 or C6. These data indicate that histidine residues at positions 60 and 84 were modified by DEPC. The 1 mono-carbethoxylated forms found in all the fragment ions containing histidine residues, but absent from the fragment ions without histidine residues underscore a similar behavior regarding the other variety of octapeptide repeat-derived chymotryptic peptides, namely C4 and C5 (Table I, Fig. 1C). Here the unmodified peptide has a mass of 825.32 Da, Table I, Fig. 6A) and was replaced with a carbethoxylated C4 or C5 species (897.30 Da, Table I, Fig. 7B) while fragment ions of b4 (GQP, 419.86 Da) were replaced by fragment ions of 492.61 Da (corresponding to b4+72 Da; Table I) in the DEPC-treated sample, indicating carbethoxylation on His-68 or His-76. With regard to peptide C7 containing His-95, fragment ions of b4 (GQ) and b6 (GQGGGT) were found in decay spectra arising from both C7 (1041.45 Da, Table I, Fig. 6A) and carbethoxylated C7 (1113.35 Da, Table I, Fig. 6D), while the signal of b4 (GQGGGTH) deriving from the C7 peptide was replaced by a signal of b4+72 Da (Table I). In sum, these PSD data confirm DEPC modification on His-60, His-68, His-76, His-84, and His-95 of the full-length MoPrP23–231.

**TABLE I**

Comparison of post-source decay fragment ions of chymotryptic peptides of intact and carbethoxylated MoPrP23–231

| Fragment | Sequence | Massa | Fragment | Sequence | Massa |
|----------|----------|-------|----------|----------|-------|
| C3 or C6 | GQPHGGGW | 795.32 (795.35) | C3 or C6 | GQPHGGGW+1m | 867.29 (867.35) |
| b2       | GQ       | 185.95 (186.19) | b2       | GQ       | 186.09 (186.19) |
| b3       | GQP      | 283.22 (283.31) | b3       | GQP      | 282.84 (283.31) |
| b4       | GQP      | 420.75 (420.45) | b4+72    | GQPH+1m  | 492.49 (492.45) |
| b5       | GQPHG    | 477.26 (477.50) | b5+72    | GQPHG+1m | 549.06 (549.50) |
| b6       | GQPHGG   | 534.85 (534.55) | b6+72    | GQPHGG+1m| 606.76 (606.55) |
| b7       | GQPHGGG  | 591.44 (591.61) | b7+72    | GQPHGGG+1m| 663.37 (663.61) |
| b17      | GQPHG-NH3| 460.35 (460.47) | a1       | GQPH     | 464.77 (464.44) |
| y1       | W        | 206.78 (206.24) | y1+72    | W        | 206.93 (206.24) |
| y2       | HGGGW    | 513.01 (513.54) | y2       | HGGGW+1m | 585.97 (585.54) |
| y6       | PHGGGW   | 610.33 (610.65) | y6       | PHGGGW+1m| 682.22 (686.65) |
| y9b2     | PH       | 235.03 (235.27) | y9b2     | PH       | 307.24 (307.27) |
| y9b2     | PHG      | 292.10 (292.32) | y9b2     | PHG+1m  | 364.57 (364.22) |
| y9b2-2   | PHGG     | 349.48 (349.37) | y9b2-2   | PHGG+1m | 421.09 (421.37) |

| C4 or C5 | GQPHGGSW | 825.32 (825.36) | C4 or C5 | GQPHGGSW+1m | 897.30 (897.36) |
| b2       | GQ       | 186.01 (186.19) | b2       | GQ       | 186.09 (186.19) |
| b3       | GQP      | 283.24 (283.31) | b3       | GQP      | 282.91 (283.31) |
| b4       | GQP      | 419.86 (420.45) | b4+72    | GQPH+1m  | 492.61 (492.45) |
| b5       | GQPHG    | 477.47 (477.50) | b5+72    | GQPHG+1m | 549.19 (549.50) |
| b6       | GQPHGG   | 534.17 (534.55) | b6+72    | GQPHGG+1m| 606.93 (606.55) |
| b7       | GQPHGGS  | 622.35 (621.63) | b7+72    | GQPHGGS+1m| 693.17 (693.63) |
| y2       | SW       | 292.25 (292.32) | y2       | SW       | 293.72 (292.32) |
| y6       | HGGSW    | 544.09 (543.56) | y6       | HGGSW+1m | 615.86 (615.56) |
| y6b2     | PHGGSW   | 640.31 (640.68) | y6b2     | PHGGSW+1m| 712.16 (712.68) |
| y6b2     | PH       | 235.03 (235.27) | y6b2     | PH       | 307.30 (307.27) |

| C7       | GQGGGTHNQW| 1041.36 (1041.45) | C7       | GQGGGTHNQW+1m | 1113.35 (1113.36) |
| b3       | GQ       | 242.72 (243.24) | b3       | GQ       | 242.82 (243.24) |
| b5       | GQGGGT   | 457.74 (458.45) | b5       | GQGGGT   | 458.25 (458.45) |
| b7       | GQGGGTH  | 595.33 (585.59) | b7+72    | GQGGGTH+1m| 667.40 (667.59) |
| b9       | GQGGGTHN | 709.42 (709.70) | b9+72    | GQGGGTHN+1m| 781.56 (781.70) |
| b17      | GQGGG-NH3| 340.55 (340.32) | b17      | GQGGG-NH3| 339.90 (340.32) |
| a1       | GQGGGT   | 567.53 (567.58) | a1+72    | GQGGGT+1m| 639.57 (639.58) |
| a1-17    | GQGGGTHN-NH3| 664.74 (664.66) | a1-17+72 | GQGGGTHN-NH3+1m| 735.96 (736.66) |
| y9       | HNQW     | 584.35 (584.61) | y9       | HNQW+1m  | 656.54 (656.56) |
| y9       | GTHNQW   | 742.36 (742.77) | y9       | GTHNQW+1m| 814.39 (814.77) |
| y9b2     | HN       | 251.94 (252.25) | y9b2     | HN+1m    | 323.60 (324.25) |

| a       | Measured mass numbers (Da); the calculated mass numbers are in parentheses. m, monocarbethoxylated. |

**DISCUSSION**

**Metal-Protein Binding and Neurodegenerative Disease**

Many proteins associated with neurodegenerative diseases have metal binding properties and/or metal-responsive expression (31). Besides PrP, the ectodomain of the Alzheimer precursor protein APP (32), Aβ peptide (33), and SOD-1 (34) exist in copper-metallated forms, while relationships to Fe metabolism are suggested for frataxin, APP, and huntingtin (35–37). In the documented examples metal binding relates to pathogenesis via an impact on aggregation or production of oxidative damage. Thus defining binding sites and the molecular details of complex formation may provide important and practical insights into pathogenic processes and neuronal biology.

Some metal binding sites are defined by well understood structural criteria and hence amenable to bioinformatic identification. These motifs would include “zinc fingers” (38), and calcium “EF hand” binding sites (39). However proteins with other binding modalities will elude identification by this bioinformatic approach, with PrP being a case in point (6, 24). In this paper we have used a mass spectroscopic-based footprinting technique to position His-dependent metal coordination
sites in PrP. Our approach employs DEPC, a widely used protein modification reagent capable of reacting with histidine residues to produce an N-carbethoxy-histidyl derivative (mono-N-carboethoxhistidine; Fig. 1A). DEPC can also react with other nucleophilic residues including sulfhydryl, arginyl, and tyrosyl residues, as well as with α- and ε-amino groups (40). However, in contrast to arginine, tyrosine, and amino acids with sulfhydryl side chains, the imidazole ring of histidine is known as a copper binding site, and many studies have demonstrated that metal coordination to histidine residues of peptides or proteins protects these amino acids from DEPC modification by copper and the binding constant for an independent copper binding site putatively involving His-95 estimated at around 2 μM.

The data indicate the same stoichiometry. Third, the locations of the histidine residues protected from DEPC modification on PrP are compatible with an oxygen-dependent role in the oxidation of PrP, but not with a role in metal coordination. Furthermore, the data again speak to the issue of selectivity, making it unlikely that low-affinity sites are detected in the DEPC protection studies. The only apparently discordant determination concerns experiments using EPR analysis. Although precise stoichiometries were not emphasized, spectra for two coordination geometries not seen for N-terminal fragments were indicative of two varieties of Cu(II) binding sites specific within PrP121–231. These sites, as present in PrP23–98, PrP200–231, or PrP231–231, have binding affinities estimated in a range from 2.2 × 10⁻⁶ to 10⁻¹⁴ M for the octarepeats and −5 × 10⁻⁶ to 4 × 10⁻¹⁴ M for the site involving His-95 (Table II). In addition to His-110, surface-exposed histidines exist at positions 139, 176, and 186 in the NMR structure of the C-terminal region of PrP (not shown), yet interactions with copper were not detected. There was no copper-dependent alteration for tryptic peptides T10–T14 containing His-176 (T11 and the hydrophobic peptide T7 were not detectable in direct analyses of proteolytic digests), nor was there any increase in overall copper binding stoichiometry when comparing PrP23–98 with MoPrP23–231 (Table II). The failure to detect additional copper sites when proceeding from analyses of PrP23–98 to full-length MoPrP23–231 has been noted previously (21) and argues against the presence of strong copper binding sites in the α-helical C-terminal domain of PrP. These data again speak to the issue of selectivity, making it unlikely that low-affinity sites are detected in the DEPC protection studies. The only apparently discordant determination concerns experiments using EPR analysis. Although precise stoichiometries were not emphasized, spectra for two coordination geometries not seen for N-terminal fragments were indicative of two varieties of Cu(II) binding sites specific within PrP212–231 (50). One type of site was compatible with an oxygen-dependent ligation, perhaps via aspartic or glutamic acid residues and was seen at pH values <7.0 (and therefore potentially

### Table II

| Copper binding to histidine residues | Full-length PrP? | Analytical technique | Reference |
|-------------------------------------|------------------|---------------------|-----------|
| (+)                                 | no               | MALDI-MS            | (24)      |
| (+)                                 | no               | Equilibrium dialysis using glycine chelated copper | (15)      |
| (+)                                 | no               | Proton NMR          | (17)      |
| (+)                                 | no               | Electrospray-MS     | (15)      |
| (+)                                 | no               | Electron spin-echo   | (18)      |
| (+)                                 | yes              | Electrospray-MS, fluorescence spectroscopy | (21)      |
| (+)                                 | no               | fluorescence spectroscopy, MALDI-MS (24) | (20)      |
| (+)                                 | yes              | DEPC footprinting   | This paper |

* Data and numbering scheme refers to residues in mouse PrP23–231 or their equivalents in human or hamster PrP. Histidines located within the octarepeats are shown in italics.

* Determined indirectly; 5.6 copper binding sites were located within PrP23–98, which has a total of five histidine residues.

* Full-length protein precipitated in the presence of copper, precluding Cu(II) binding studies.

* Binding constants of 2.2 and <0.1 μM are assigned for the histidine-containing octarepeat sites.

* Contributions of the histidines to Cu binding documented by substitution of a cyclohexyl ring for the imidazole moiety.

* Binding constants of 4.3−5 μM assigned to this histidine-containing copper binding site.

* Binding constant for four cooperative sites in the octarepeats assigned as 5.5 to 2.2 μM.

* Binding constant for an independent copper binding site putatively involving His-95 estimated at around 2 μM.

* Stoichiometry of one copper coordinated by four histidines and a binding constant of 10⁻¹⁴ M.

* Putatively involving His-96 and His-111 (equivalent to mouse His-95 and His-110) and a binding constant of 4 × 10⁻¹⁴ M.

* ND, not done.
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compatible with our failure to detect His-dependent copper-coordination in the C-terminal domain of PrP when analyzed at pH 7.4. However, the other type of site was speculated to involve a nitrogen ligand. In practice, these are restricted to the aforementioned spatially dispersed histidines 139, 176, and 186. None of these residues are perfectly conserved in vertebrate PrP sequences (51), and our studies failed to detect involvement of His-176. We conclude that further studies will be required to define Cu(II) binding constants of sites putatively involving His-139 and His-186 and address this seeming discrepancy.

Since MALDI-MS and ESI-MS have been used previously to analyze interactions between metal ions and proteins (15, 24, 52) questions arise as to the attributes and/or advantages of the DEPC “footprinting” methodology. For conventional MALDI-MS (53), direct analysis of native metal-peptide complexes is complicated by the required excess of a matrix that is typically strongly acidic and thus favors histidine protonation and protein denaturation. Consequently, MALDI can have a reduced ability to detect low affinity sites when compared with other techniques, although novel sample preparation matrices have now been developed to offset the effect (52). Another approach is to use large excesses of metal ions, although this has a potential to reveal low affinity sites, leading to the problem of discerning of nonspecific interaction metal/peptide interactions. Electrospray MS has different attributes (reviewed by Loo in Ref. 54). These include the sensitive detection of metal-peptide complexes at low metal concentrations, as samples are not desorbed from a matrix but introduced into the instrument while still in an aqueous buffer (although this too is often used at an acidic pH). In the case of the DEPC technique, copper binding sites are revealed indirectly by protection from carboxylation in a chemical reaction performed prior to introduction into the instrument; hence metal-protein complexes need not remain intact for MS analysis, avoiding some technical confounds listed above. These general considerations can be illustrated for the specific case of copper binding to PrP. MALDI-MS analyses failed to reveal binding to full-length protein (not shown) and failed to reveal binding to a “fifth” copper site either in intact PrP23–98 (Fig. 2B) by chymotryptic digests of MoPrP23–231 (not shown). In contrast, this site was detected by DEPC protection (Figs. 3 and 5), by ESI-MS (15, 21), and by other techniques (Table II).

Chemical Modification and Prion Biology—In pioneering experiments to demonstrate the proteinaceous nature of prions (55), DEPC was found to reduce the infectious titer of prion preparation by two to three log units (55). Since these experiments used PrPSc preparations enriched by proteolysis with proteinase K, they indicate that DEPC-reactive amino acid residues modulating prion infectivity must reside in the C-terminal protease-resistant “core” of PrPSc (i.e. PrP27–30) (6). These results, taken together with the footprinting experiments described here, have implications for the nature of studies that might be possible in the future. The first is that DEPC modification need not be restricted to recombinant prion proteins. Instead, DEPC can be reacted with purified mammalian proteins (55) or perhaps even tissue samples. In the latter instance, the modification status of protein samples might be investigated by high-resolution electrophoresis and immunoblotting or by using affinity chromatography to purify samples for MS analysis. In short, because footprinting uniquely records an “imprint” of metal binding, rather than requiring the metal to remain intact during purification or denaturing procedures, future iterations of this technique might provide us with the chemical signatures necessary to identify metallo- or apo-forms of PrPc in tissue samples. A second implication is that mapping DEPC modification sites associated with the drop in infectious titer of PrP27–30 could be of great interest, irrespective of whether metal-binding is involved in modulating the infectious properties of prion proteins (histidines 60, 68, 76, and 84 are absent from PrP27–30 and His-95, putatively involved in Cu(II) binding with very high affinity (20, 49), might be predicted to be coordinated in vivo and hence protected from modification). There is still a paucity of precise structural information about the conversion of PrPc to PrPSc, in major part because PrPSc has poor solubility and is difficult to study by high-resolution biophysical methods. Also, though spectroscopic comparisons between PrPc and PrPSc indicate a profound change in structure to a more β-sheet-rich conformation (8, 9), obtaining antibodies specific to PrPSc neo-epitopes has proven difficult, albeit with one possible exception (56). Rather, in the case of PrPSc, published single chain and monoclonal antibodies define an occlusion (rather than exposure) of epitopes associated with the conversion of PrPc to PrPSc (57–59). We therefore suggest that, since PrP27–30 is clearly reactive with DEPC, comparative mapping of DEPC adducts in PrPc and PrP27–30 by site-specific endoproteolysis and MS may comprise a powerful strategy to define infectivity-associated surface determinants in PrPSc.

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