Identification of the Cu(II) Coordinating Residues in the Prion Protein by Metal-Catalyzed Oxidation Mass Spectrometry: Evidence for Multiple Isomers at Low Cu(II) Loadings[†]

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Abstract

While the Cu(II) binding sites of the prion protein have been well studied under Cu-saturation conditions, the identity of the residues involved in coordinating Cu(II) at low stoichiometries and the order in which the binding sites load with Cu(II), remain unresolved. In this study, we have used two mass spectrometry based methods to gather insight into Cu(II)-prion binding under different stoichiometric loadings of Cu(II). The first method uses metal-catalyzed oxidation reactions to site specifically modify the residues bound to Cu(II) in solution, and the second method determines Cu binding sites based on the protection of His from modification by diethyl pyrocarbonate when this residue binds Cu(II) in solution. For both methods, the residues that are labeled by these reactions can then be unambiguously identified using tandem mass spectrometry. Upon applying these two complementary methods to a construct of the prion protein that contains residues 23-28 and 57-98, several noteworthy observations are made. Coordination of Cu(II) by multiple His imidazoles is found at 1:1 and 1:2 PrP:Cu(II) ratios. Notably, there appear to be four to seven isomers of this multiple histidine coordination mode in the 1:1 complex. Furthermore, our data clearly show that His96 is the dominant Cu(II) binding ligand, as in every isomer His96 is bound to Cu(II). The individual octarepeat binding sites begin to fill at ratios of 1:3 PrP:Cu(II) with no clear preference for the order in which they load with Cu(II), although the His77 octarepeat appears to saturate last. The existence of several 'degenerate' Cu binding modes at low PrP:Cu(II) ratios may allow it to more readily accept additional Cu(II) ions, thus allowing PrP to transition from a singly Cu(II) bound state to a multiply Cu(II) bound state as a function of cellular Cu(II) concentration.

The normal cellular form of the prion protein, denoted PrP^C, is expressed in the central nervous systems of mammals and avian species (1,2). The precise physiological function of PrP is not known, although its role in neurodegenerative diseases, specifically the transmissible spongiform encephalopathies, has been unequivocally established (1,3). Most hypotheses as to the normal, cellular function of PrP focus on the fact that it is capable of binding divalent copper with moderate to high affinity (4-9). There is also growing evidence that PrP may interact with zinc (10). Cellular studies show that both copper and zinc induce endocytosis of PrP, thus linking metal binding to a physiological response (5,11).

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Given PrP's connection to these two metals, significant efforts have been made to characterize the metal binding. Most progress has been made on characterizing PrP's Cu(II)-binding modes (7,12-15), whereas to our knowledge, few molecular details on the zinc binding coordination sphere(s) have been published (16). The metal binding domain of PrP resides in the flexible N-terminal domain, spanning residues 60-96 (human sequence) and has been shown to bind approximately five Cu(II) ions (12,17,18) (Figure 1). This region contains four repeats of the highly conserved octapeptide PHGGGWGW sequence (residues 60-91) followed by GTH (residues 94-96). Recent studies also indicate that another binding site centered on His111 exists (14,15,19). When fully Cu(II)-saturated, the HGGGW segment of each octarepeat coordinates a Cu(II) ion using a single histidine (His) imidazole, two deprotonated glycine (Gly) amides, and a glycine carbonyl oxygen (7). The tryptophan (Trp) indole ring is held in close proximity to the Cu(II) center, possibly via a hydrogen bonding interaction to an axially bound water molecule. The GTH sequence, often referred to as the 'fifth binding site', coordinates Cu(II) using deprotonated amide nitrogens from the Gly, Thr and His residues as well as the imidazole ring of His (12).

Although the coordination spheres for the Cu(II) binding sites populated under Cu(II)-saturation conditions are known with precise atomic detail, there is controversy over the Cu(II) coordination modes populated at intermediate and low Cu(II) occupancies. Both Chattopadhyay et al. and Wells et al. find evidence for a coordination mode involving multiple histidine imidazole groups at low Cu(II) occupancy (13,14,19). In contrast, Klewpatinond et al. find that Cu(II) ions are initially bound by sites located at His96 and His111 before the HGGGW segments of the octarepeat region begin binding (15). Some of these discrepancies may arise due to the fact that these researchers use different models systems, conditions and techniques.

In this study, the copper binding sites of PrP(23-28, 57-98) at different stoichiometric loadings of Cu(II) were examined using two mass spectrometry (MS)-based methods. The region spanning residues 57-98 contains the histidine-centered Cu(II) binding sites identified by studies on the full prion protein (20,21). The goals are to identify the residues involved in coordinating Cu(II) at low stoichiometries and to determine the order in which the binding sites load with Cu(II). The MS-based methods have two notable advantages over the spectroscopic methods, such as EPR or CD, previously used to study the Cu(II) binding of prion protein fragments. The MS-based methods can identify the specific amino acids bound to Cu(II) without having to resort to isotopic labeling, and the presence of multiple Cu-protein isoforms can become apparent. The first MS-based method was recently developed in our group and is based on metal catalyzed oxidation (MCO) reactions and MS (22-26). We have demonstrated that the MCO/MS method can identify the metal binding residues of peptides and proteins. The method relies on the generation of reactive oxygen species at a redox-active metal center such as Cu that then oxidize nearby amino acids in a given peptide or protein (Scheme 1). If the appropriate reaction conditions are chosen, oxidation is limited to the side chains of the amino acids bound to the metal. The oxidatively modified sites are then identified using tandem MS (MS/MS). A second complementary technique is a covalent labeling method that relies on diethyl pyrocarbonate (DEPC) to modify histidine residues and MS to identify these modified sites. Reactions with DEPC can be used to identify Cu(II) binding sites because histidine's reactivity with DEPC is significantly decreased when this residue is bound to Cu (20,27). Qin et al. applied a similar approach to probe metal binding sites in the PrP(23-231) protein using a 10-fold molar excess of Cu(II) (20). The present work differs in two respects: (1) we have used DEPC to probe metal binding under different stoichiometric loadings of Cu(II), and (2) confidence in the assigned Cu(II) binding sites by this approach is greater because we have used electron transfer dissociation (ETD) MS/MS to determine the extent to which individual His residues are bound at different Cu(II) stoichiometries. ETD has been found to be less

sensitive to side-chain modifications and often provides better sequence information for modified peptides than collision-induced dissociation (CID) (28,29).

In accordance with previous work, we find evidence for coordination of Cu(II) by multiple His imidazoles at low PrP:Cu(II) ratios (13,14,19). Most notably, there appear to be several possible isomers of this multiple histidine coordination mode, four to seven by our current estimations. Our data clearly show that His96 is the dominant Cu(II) binding ligand, as in every isomer His96 is bound to Cu(II). Further, Cu(II)'s preference for His residues follows: His96 > His61 ~ His 69 > His77 ~ His85. The individual octarepeat binding sites begin to fill at ratios of 1:3 PrP:Cu(II) with no clear preference for the order in which they load with Cu(II), although the His77 octarepeat appears to saturate last.

MATERIAL AND METHODS

Peptide Synthesis

All peptides (Table 1) were prepared by solid-phase synthesis on an automated PS3 synthesizer (Protein Technologies, Inc.) using standard fluorenylmethoxycarbonyl (Fmoc) methods. Protected amino acids and coupling reagents were purchased from Protein Technologies, Inc. (Tucson, AZ). The peptides were acetylated at the N-terminus and amidated at the C-terminus. Peptides were cleaved from a Rink Amide MBHA resin, obtained from Novabiochem (Gibbstown, NJ), over a 2 hour period using 95% trifluoroacetic acid/ 2.5% water/ 2.5% triisopropylsilane solution (v/v/v). Finally, all peptides were purified by reverse-phase HPLC, using a C18 column, and characterized by electrospray ionization mass spectrometry (ESI-MS) on a Micromass Q-ToF micro.

Materials

Hydrogen peroxide (30%), formic acid, tris(hydroxymethyl)-aminomethane (Tris), and tris (hydroxymethyl)aminomethane hydrochloride (Tris-HCl) were obtained from EM Science (Gladstone, NJ). Diethyl pyrocarbonate (DEPC), imidazole, sodium ascorbate, ascorbic acid, copper(II) sulfate, and ammonium acetate were purchased from the Sigma-Aldrich Corporation (St. Louis, MO). Acetic acid and HPLC-grade methanol were obtained from Fisher Scientific (Fair Lawn, NJ). Centricon molecular weight cutoff (MWCO) filters were obtained from Millipore (Burlington, MA). All reagents were used as provided. Distilled, deionized water was generated with a Millipore (Burlington, MA) Simplicity 185 water purification system.

Metal Catalyzed Oxidation (MCO) Reactions

Metal catalyzed oxidation reactions of all the peptides were performed at least three times at room temperature in aqueous solutions containing 100 μM peptide, 100-500 μM $CuSO_4$, 10 mM ascorbate, 2 mM H_2O_2, and 50 mM Tris-HCl/Tris, buffered to a pH of 7.4. Control reactions were performed by incubating peptides with ascorbate and oxidant in the absence of any metal ions. In all cases the reactions were initiated by the addition of ascorbate, H_2O_2, or both. Reactions were stopped after 30 minutes by the addition of 1% (by volume) of glacial acetic acid. Samples of PrP(23-28, 57-98) were then immediately desalted using 3,000 Da molecular weight Centricon filters (Millipore, Bedford, MA) and frozen until ready for further analysis. The other peptide samples were immediately frozen until ready for further analysis.

Diethyl Pyrocarbonate (DEPC) Modification Reactions

DEPC modification reactions of all the peptides were performed at least three times in 50 mM ammonium acetate buffer solutions (pH 7.4) containing 100 μM peptide and 100-500 μM $CuSO_4$. Before reaction with DEPC, the Cu-peptide complexes were incubated for 30 min to ensure complex formation. Samples were then reacted with 5-fold excess DEPC for 5 min.

Control experiments were performed under the same conditions without CuSO$_4$. In all cases reactions were stopped by the addition of 10 % (by volume) of a 1.0 M imidazole solution, which effectively acts to quench any further reactions of DEPC. Samples of PrP(23-28, 57-98) were then immediately desalted using 3,000 Da molecular weight Centricon filters (Millipore, Bedford, MA) and frozen until ready for further analysis. The other peptide samples were immediately frozen until ready for further analysis.

Instrumentation

Mass spectral analyses were performed on either a Bruker (Billerica, MA) Esquire LC quadrupole ion trap mass spectrometer or a Bruker HCTultra PTM Discovery system quadrupole ion trap mass spectrometer. On both mass spectrometers the source conditions were chosen to maximize the ion signal of the desired ions. For direct injection experiments on the Esquire LC, the sample was delivered at 1 μL/min using a syringe pump. HPLC-MS analyses on this mass spectrometer were conducted using an HP1100 (Agilent, Wilmington DE) system with a Discovery C18 column (2.1 × 150 mm; Supelco). The LC effluent was split in a 1:4 ratio with the smaller outlet being fed into the electrospray ionization (ESI) source of the quadrupole ion trap mass spectrometer. For separation of the oxidized isomers of some peptides, a binary gradient was used. The two mobile phases that were used were (A) water with 0.1% formic acid and (B) methanol with 0.1% formic acid. During the separation of the oxidized isomers, the percentage increases of mobile phase B were: 0-4 min, hold at 10%; 4-10 min, 10-30%; 10-24 min, hold at 30%; 24-30 min 30-100%.

Tandem mass spectrometry (MS/MS) experiments using electron transfer dissociation (ETD) were carried out on a Bruker HCTultra PTM Discovery System quadrupole ion trap mass spectrometer. Samples were infused at a flow rate of 1 to 2 μL/min using a Cole Palmer syringe pump (Vernon Hills, IL). ETD was performed using total reaction times of 50 to 200 ms and low mass cutoffs ranging from m/z 30 to 200; these values were chosen to maximize the dissociation efficiency. Accumulation times for the fluoranthene radical anion, which was used as the ETD reagent, were optimized for each experiment but were typically between 3 ms and 5 ms.

RESULTS

MCO reactions of a single octarepeat, PrP(60-67)

Metal catalyzed oxidation (MCO) reactions of the octarepeat (PHGGGWGQ) were performed to determine the oxidation pattern that should result when the octarepeat Cu binding site is filled. MCO reactions of a 1:1 complex of Cu:Ac-PHGGGWGQ-NH$_2$ result in the incorporation of a single oxygen atom and the formation of two oxidized isomers. The oxidized isomers can be separated by LC (Figure 2a inset), and the residues oxidized in each isomer can be identified by MS/MS. The tandem mass spectrum (Figure 2a) of the first oxidized isomer, corresponding to the first LC peak, shows unmodified b_2, b_3, b_4, and b_5 product ions that are identical to the m/z ratios of the product ions in the tandem mass spectrum of the unoxidized peptide (data not shown). Mass shifts of 16 Da for the a_6, b_6 and b7 product ions indicate that Trp65 is the oxidized residue in this first isomer. In contrast, the tandem mass spectrum (Figure 2b) of the second isomer, corresponding to the third LC peak, shows oxidized product ions in the series from b_2 to b_7, which confirms oxidation occurs at His61. We find that about 75% of the peptide is oxidized at Hi61, while the remaining 25% is oxidized at Trp65. Oxidation of the His and Trp residues is consistent with the model for Cu binding to the octarepeat (7) — His is bound to Cu(II), while Trp is in close proximity to the Cu(II) binding site. Given its expected proximity to Cu(II) and the ease with which it can be oxidized, Trp oxidation is expected. No evidence is found for oxidation of any of the Gly residues that contribute backbone amides to the Cu(II) binding site. This is not surprising as Gly lacks a side chain that

can be oxidized. We have demonstrated previously that backbone cleavage can occur during the MCO reactions of Cu-protein complexes (23), but no definitive evidence for this is found in the current study. We conclude that His and Trp oxidation are MCO/MS indicators of Cu binding to the octarepeat.

DEPC reactions of a single octarepeat, PrP(60-67)

DEPC reactions of the octarepeat-Cu(II) complex were also done to serve as a complementary indicator of Cu binding to the octarepeat. DEPC can react selectively with His residues, but this reaction is significantly slowed when His residues are bound to metal ions. As a control experiment, DEPC was reacted with the octarepeat in the absence of Cu, and the mass spectrum of the reaction products shows abundant ions corresponding to the DEPC-modified peptide (Figure 3a). When DEPC reacts with the 1:1 Cu(II) complex of the peptide, the relative abundance of modified peptide drops threefold, which is consistent with Cu binding inhibiting the reaction of DEPC with His (Figure 3b). MS/MS of the ion corresponding to the DEPC modified peak after reaction with either the metal-bound or metal-free peptide confirms that modification occurs at His only (data not shown). This result indicates that Cu(II) simply drops the reactivity of His toward DEPC. In this peptide sequence, other amino acids are not modified by DEPC when Cu(II) is bound.

MCO reactions of PrP(89-98)

MCO reactions of the so-called fifth binding site, as modeled by WGQGGGTHNQ, were also done to determine the oxidation pattern that should result when this binding site is filled. MCO reactions of the 1:1 complex of Cu:Ac-WGQGGGTHNQ-NH$_2$ lead to the incorporation of a single oxygen atom and the formation of two oxidized isomers. The oxidized isomers can be separated by LC (data not shown), and the modification sites can be confirmed by MS/MS in a manner similar to that described above for the octarepeat. The MS/MS data indicate that Trp89 is the oxidized residue in one of the isomers, and His96 is the oxidized residue in the other isomer (data not shown). Oxidation at Trp89 accounts for 30% of the oxidized peptide, while oxidation at His96 accounts for the remaining 70%. Previous EPR studies of Cu(II)'s binding to this peptide indicate that His96 is one of the ligands (12), so oxidation of this residue is expected. The previous EPR studies are not clear about the proximity of Trp89 to Cu(II), but our MCO/MS results suggest that Trp may be close to Cu(II). Taken together, oxidation of His96 and Trp89 are MCO/MS indicators of Cu(II) binding to PrP(89-98).

DEPC reactions of PrP(89-98)

Complementary information about the Cu(II) binding site of this peptide was obtained from DEPC reactions of the Cu(II) complex of Ac-WGQGGGTHNQ-NH$_2$. In contrast to the results from the DEPC reaction of the complex of the octarepeat, the reactions of the Cu-bound form of WGQGGGTHNQ lead to modification exclusively at Thr95, while DEPC reactions of the non-Cu bound form of this peptide result in modification at His96 as indicated by MS/MS data (not shown). Furthermore, in the presence of Cu, the peptide's reactivity with DEPC drops by about a factor 2. DEPC is known to react with other residues that have nucleophilic side chains such as Tyr and Lys residues (30-32) and recently, the reactivity of DEPC with Thr residues has also been reported (33). Overall, the reactivity of the Cu-bound form of the peptide is reduced about 2-fold when compared to the non-Cu bound form. The observation that His modification is replaced by Thr modification is a useful indicator of Cu binding to the GTH binding site.

MCO reactions of PrP(23-28, 57-98)

With the appropriate indicators of Cu(II) binding to both PrP(60-67) and PrP(89-98) determined, the MCO reactions of PrP(23-28, 57-98) were performed to explore its Cu(II)

binding site(s) under different stoichiometric loadings of Cu(II). The mass spectra resulting from the MCO reactions of complexes with stoichiometries ranging from 1:1 to 1:5 of PrP (23-28, 57-98):Cu(II) indicate that singly, doubly, and triply oxidized ions are observed (spectra not shown). As the Cu(II) concentration is increased the degree of oxidation, as measured by the weighted average number of oxidative modifications, increases linearly. The increased amount of oxidation is fully expected as the higher stoichiometries of Cu(II) cause more peptide binding sites to be filled. To determine the amino acids that comprise the binding sites at the different stoichiometric loadings, we performed MS/MS experiments only on the singly-oxidized peptides. MS/MS experiments on the doubly- and triply-oxidized peptides could lead to incorrect conclusions because the first oxidation event could disrupt the peptide structure and cause a change in Cu(II) binding sites. In such a case, the second and third oxidation events might not then be representative of the original Cu(II) binding mode.

Unlike the MS/MS experiments with PrP(60-67) and PrP(89-98), ETD rather than CID was used to sequence PrP(23-28, 57-98) because ETD has been found to give more accurate and readily interpretable spectra and more extensive sequence information for oxidized peptides, especially large peptides (29). As an example of the type of data that is obtained after the MCO reactions of PrP(23-28, 57-98), the ETD spectrum of the singly-oxidized peptide ion from the MCO reaction of the 1:1 peptide:Cu complex is shown (Figure 4). This spectrum indicates that all of the His residues in the peptide are oxidized but to varying extents (Figure 4 and Table 2). The oxidation extent of each His residue is determined by comparing the oxidation percentages of the appropriate product ion in the ETD spectrum. For example, Figure 4b shows expanded regions of the z_2, z_3, z_{10}, and z_{14} product ions and, where applicable, their oxidized versions. The percentage oxidized is obtained by dividing the ion abundance of the oxidized product ion by the sum of the ion abundances of the oxidized and unoxidized product ions. ETD product ion formation has been shown to be relatively insensitive to side chain modifications (29), so we feel that the percent oxidation from the ETD spectra are reliable indicators of the real degree of oxidation. From the data in Figure 4b, we conclude that the oxidation extent for His96 is about 32%. We arrive at this value by subtracting the oxidation percentage for the z_2 ion (0%) from the oxidation percentage of the z_3 ion (32%). In a similar manner the oxidation percentage for Trp89 is determined to be 0% by subtracting the oxidation percentage of the z_6 (or z3) ion (32%) from the oxidation percentage of the z_{10} (32%) ion. As a final example, the oxidation percentage of His85 is determined to be 12% by subtracting the oxidation percentage of the z_{10} ion (32%) from the oxidation percentage of the z_{14} (44%) ion. A similar type of accounting can be done for the other His residues oxidized in the 1:1 complex and also can be done for the residues oxidized during the MCO reactions of the complexes with higher stoichiometric loadings of Cu(II) (Scheme 2 and Table 2). In general, the errors in the oxidation percentages range from ± 2 to ± 8%.

The data for the MCO reactions of the 1:1 and 1:2 complexes (Table 2) show that His residues are oxidized but no Trp residues are oxidized. These results are noteworthy for three reasons. First, the failure to observe oxidation of Trp residues probably indicates that only His residues comprise the Cu(II) binding sites and that the coordinating ligands around Cu(II) are not provided by a single octarepeat sequence in either the 1:1 or 1:2 complex. This is consistent with previous studies in which low stoichiometric loadings of Cu resulted in complexes bound only by His residues (13,14). Second, all of the His residues are found to be oxidized. Our previous MCO/MS work has indicated that under appropriate reaction conditions Cu(II) binding is necessary for oxidation to occur at a given residue (22-26). With this in mind, oxidation of each His residues indicates that Cu can bind to each of the His residues in the 1:1 and 1:2 complexes. It is unlikely, however, that all 5 His residues are simultaneously bound to Cu(II). Indeed, previous Cu(II)-binding studies on a PrP construct with 4 octarepeats suggests that only three or four His residues are simultaneously bound at low stoichiometric loadings of Cu(II) (13). Thus, in both the 1:1 and 1:2 complexes there are likely a mixture of

binding modes that differ only in the His residues that are bound to Cu. Third, the trend in the oxidation extent (i.e. His96 > His61, His69 > His77, His85) might suggest that 1:1 Cu:peptide isoforms with His96 bound to Cu(II) are the most abundant, while the isoforms with His77 and His85 bound to Cu(II) are the least abundant.

An alternate explanation for the trend in oxidation extent is the possibility that different Cu (II)-binding isoforms make different Cu(I) sites during the initial reduction step of the MCO cycle (Scheme 1). Two-coordinate Cu(I) sites are known to be much less reactive with O_2 than three-coordinate Cu(I) sites (34). So, if the different isoforms are reduced to different Cu(I) coordination numbers, then the trend in oxidation may not reflect Cu(II) occupancies but instead might simply reflect different rates of ROS production. While we cannot rule out this possibility in the current data set, in our previous MCO/MS work we observed that all residues known to bind the Cu(II) center are oxidized to similar extents. We feel that this observation would be unlikely if, upon reduction to Cu(I), the ligand set around the metal ever dropped to two-coordinate. A more compelling reason why we do not favor the formation of Cu(I) sites with different coordination numbers is the very good consistency of the MCO data with the DEPC data (see below).

The data for the MCO/MS analysis of the higher stoichiometric loadings of Cu (1:3 to 1:5) provide additional insight into Cu(II) binding to PrP(23-28, 57-98) (Scheme 2 and Table 2). Two observations are noteworthy. First, in contrast to the 1:1 and 1:2 complexes, the MCO reactions of the 1:3, 1:4, and 1:5 complexes result in the oxidation of Trp residues. These data indicate that all the octarepeat binding sites begin to be filled at stoichiometries of 1:3 and higher, whereas they are not filled at lower stoichiometries. This observation is also consistent with the recent report by Millhauser and co-workers (13), which indicates a different coordination environment at higher stoichiometric loadings of Cu(II). There is not a clear preference for which octarepeat segment is filled first. Second, the 1:3, 1:4, and 1:5 binding stoichiometries result in essentially the same trend in oxidation extent as the 1:1 and 1:2 stoichiometries: His96 > His61, His69 > His77, His85. This trend suggests the importance of His96 binding site at all stoichiometric loadings. As an aside, the accuracy of the MCO method is evidenced by the fact that Trp57 is not oxidized even at higher Cu loadings (Scheme 1 and Table 2). Trp57 is not part of any octarepeat, so it is not expected to be oxidized, and indeed this is what is observed.

DEPC reactions of PrP(23-28, 57-98)

As a complement to the MCO/MS data, DEPC modification reactions on PrP(23-28, 57-98) with different stoichiometric loadings of Cu were also examined. Mass spectra obtained after DEPC reactions of Cu bound and non-Cu bound PrP(23-28, 57-98) indicate that the presence of Cu reduces the reactivity of DEPC with the peptide in a manner similar to that described above for the octarepeat (spectra not shown). Furthermore, the extent of modification decreases in an approximately linear fashion as the stoichiometry of Cu(II) increases. The ETD spectrum obtained after the DEPC reaction of the Cu(II)-free form of PrP(23-28, 57-98) indicates that all five His residues in the peptide are modified but to varying extents (Scheme 3 and Table 3). Just as in the MCO/MS experiments, MS/MS was performed only on the singly-modified peptide ions to avoid potentially misleading results due to disruption of the peptide structure upon modification. As an example, the ETD spectrum of the singly-modified peptide ion obtained after the DEPC reaction of the 1:1 complex is shown in Figure 5. The modification extent of each residue is determined by comparing the modification percentages of the appropriate product ion in the ETD spectrum. For example, Figure 5b shows expanded regions of the z_3, z_4, c_8, and c_{16} product ions and, where applicable, their modified versions. From the data in Figure 5b, we conclude that there is no modification at His96, but modification of Thr95 is observed at 9%. We arrive at this value using the same accounting method for determining

the degree of oxidation, as discussed in the previous section. A similar type of accounting can be done for the other residues modified in the 1:1 complex and also can be done for the residues modified during the DEPC reactions of the complexes with higher stoichiometric loadings of Cu(II) (Scheme 3 and Table 3). For the 1:1 complex, the results indicate that His96 is the least reactive, His85 is the most reactive, and His61, His69, and His77 have similar reactivities. If one recalls that lower DEPC reactivity corresponds to Cu(II) binding, then it is clear that this reactivity trend is qualitatively similar to that observed in the MCO/MS experiments. The clearest conclusion from the two data sets (i.e. MCO and DEPC) for the 1:1 complex is that His96 is the dominant Cu(II) binding site.

When the data from the DEPC reactions of all the complexes with different stoichiometric loadings of Cu are considered as a whole, three observations are noteworthy. First, the failure of His96 to react with DEPC whenever Cu is present suggests that this residue is a Cu(II) binding site in all of the complexes, at both low and high stoichiometric loadings of Cu. Second, His77 is the last His site filled as the Cu(II) concentration is increased. At stoichiometric loadings of 1:2, 1:3, and 1:4, this residue remains the most reactive, indicating that this residue is less likely to be bound by Cu at these stoichiometric loadings than the other His residues. This observation is very consistent with the MCO/MS data, which shows this residue to be the least reactive. Third, all the His residues, and probably all the octarepeats, are filled at a stoichiometric loading of 1:5 because none of the His residues react with DEPC under this condition.

DISCUSSION

Collectively, the data presented here demonstrate that Cu(II) ions are bound by PrP's metal binding domain using different coordination modes that are a function of Cu(II) concentration. Since the Cu(II)-coordination spheres are known for the case where the metal binding domain is fully saturated, we were able to develop MCO/MS and DEPC/MS indicators for these binding modes. For a single octarepeat, oxidation of both the His and Trp residues is indicative of the coordination sphere observed in the X-ray structure of the HGGGW-Cu(II) complex (7) (Figure 6a). This involves equatorial coordination of Cu(II) by the His imidazole nitrogen, deprotonated amide nitrogens from the following two Gly residues and the carbonyl oxygen of the second Gly. The Trp indole ring is held in close proximity to the Cu(II) center, possibly via a hydrogen bonding interaction to an axially bound water molecule. A complementary indicator is the protection of His from DEPC modification when it binds Cu(II), although this is less structurally informative than the MCO/MS indicator.

In a similar manner, oxidation of the His and Trp residues in the Cu(II):Ac-WGQGGGTHNQ-NH$_2$ complex is indicative of the coordination sphere revealed by numerous spectroscopic studies (12,35,36) (Figure 6b). Here, Cu(II) is coordinated by deprotonated amide nitrogens from the Gly, Thr and His residues as well as the imidazole ring of His. Interestingly, the DEPC modification is much more informative for this site as His is protected from modification, but the Thr side-chain becomes readily functionalized. When the PrP(23-28, 57-98) peptide was fully Cu(II)-loaded (i.e. exposed to 5 eq. of Cu(II)), both MCO/MS and DEPC/MS indicators were observed. Thus, we conclude these methods are reliable reporters of the local Cu(II)-coordination spheres.

The MCO/MS and DEPC/MS monitored Cu(II) titration of PrP(23-28, 57-98) reveal the existence of coordination mode(s) different than those exhibited by a single octarepeat (PrP 60-67) or single '5th binding site' (PrP 89-98). At 1:1 and 1:2 PrP(23-28, 57-98):Cu(II) ratios, all of the His residues were subject to oxidation, although to varying extents, whereas no Trp residues were oxidized. In addition, all His residues were afforded protection from DEPC modification, again to varying extents, plus Thr95 became increasingly DEPC modified.

Together, these data indicate that the coordination spheres exhibited by the octarepeat-Cu(II) or WGQGGGTHNQ-Cu(II) complexes are not populated in PrP(23-28, 57-98) at these Cu(II) stoichiometries. The simplest explanation for these observations is the existence of a binding mode or modes that involve multiple His coordination of a single Cu(II) ion. Further, the different extents to which the His residues are oxidized or protected from DEPC modification suggest that isomers of this binding mode exist.

Other research groups have found evidence for the coordination of a single Cu(II) ion by multiple His imidazoles at low peptide:Cu(II) ratios, and our findings here support this. Using peptide models of only the octarepeat region (residues 57-91) and a suite of EPR techniques, Chattopadhyay et al. propose a coordination sphere where Cu(II) is equatorially ligated by three or four His imidazoles (13). Wells et al. performed a variety of spectroscopic studies on an octarepeat peptide spanning residues 57-91 plus a construct encompassing residues 91-115 (14). Their findings corroborate those of Chattopadhyay et al. with regard to multiple His binding by the octarepeat region, and they highlight the existence of two binding sites C-terminal to this region. They also examined recombinant, full length human PrP (residues 23-231) at pH 5.5 by 2D NMR and found that six His residues (the four from the octarepeat region, His96 and His111) coordinated two Cu(II) ions (19). The lower pH conditions used in their study favor formation of the multiple His coordination sphere at low Cu(II) occupancy. Based on their observation that the signal heights for all six N-terminal His residues (i.e. those contained between residues 57-111) plus the N-terminal amine were significantly reduced upon the addition of 1 equivalent of Cu(II), they conclude that at least seven functional groups contribute to coordinating a single Cu(II) ion. Since it is unfeasible that this number of ligands would simultaneously coordinate a single Cu(II), they propose there must be an ensemble of different coordination spheres that involve different combinations of these ligands, yet have comparable stabilities.

Our data are clear on the fact that His96 is a dominant Cu(II) binding ligand in the PrP(23-28, 57-98) peptide. At every PrP:Cu(II) ratio, i.e. from 1:1 to 1:5, His96 was preferentially oxidized and, conversely, preferentially provided protection from DEPC modification. This piece of information allows us to put an upper limit on the number of possible isomers that can exists at 1:1 PrP(23-28, 57-98):Cu(II) ratios. If it is assumed that Cu(II) is bound by four His imidazoles then there are four possible isomers, and if it is assumed that Cu(II) is bound by three His imidazoles then there are seven possible isomers. Currently, our measurements are done on the mixture of these isomers, but we cannot definitely comment on exactly how many isomers are present.

In agreement with the work by Millhauser and coworkers, plus Wells et al., our data show that the individual octarepeat binding sites begin to be filled at stoichiometries of 1:3 and higher as indicated by the oxidation of the Trp residues. There is not a clear preference for the order in which the octarepeat segments load with Cu(II), although the His77 octarepeat appears to saturate last.

As mentioned above, Wells at al. also indicate that His111 is involved in Cu(II) coordination as one of several His ligands. Klewpatinond et al. instead argue that the residues spanning 107-111, TNMKH (human sequence), constitute a high affinity binding site where three deprotonated amides and the His111 side chain coordinate the Cu(II) ion (15). Since our peptide only extends to residue 98, and thus lacks His111 plus the adjacent residues, we are unable to comment on this in much depth. However, given that the binding site at His96 (GTH) appears to be of higher affinity than that centered at His111 (TNMKH) (12,15) and the fact that at low Cu(II) stoichiometries His96 is involved in a multiple His binding mode, it is likely that His111 would behave in a similar manner as suggested by the NMR study by Wells et al. Nevertheless,

it is clear that in order to obtain a complete picture of the Cu(II) binding process, systems must include the region encompassing His111.

The ability of PrP to bind Cu(II) using multiple His ligands, as observed at low Cu(II):PrP stoichiometries, is interesting for a number of reasons. Firstly, this is the highest affinity binding mode and thus very likely populated under physiological conditions where the Cu(II) concentration is low and there are numerous competitors. Secondly, as Cu(II) binding by PrP is very pH sensitive this mode may predominate at lower pH values (e.g. 5.5) and thus might also occur in the acidic environment of the endosome. Thirdly, as suggested by others, the participation of several His residues in the binding of a single Cu(II) offers a potential mechanism by which PrP molecules can self-associate (19,37,38). In such a self-association mode, a single Cu(II) ion would be coordinated by His residues from at least two different PrP molecules. Lastly, a multiple His binding mode will clearly cause compaction of the 60-98 region (possibly up to 111), as the His residues are spread throughout it, and thus a significant change in the shape of the protein. It will most certainly change the orientation of the N-terminus with respect to the C-terminus.

It is curious that nature would design a copper-binding protein with a Cu(II) ion held in an ensemble of different coordination spheres even though the coordinating species are all essentially equivalent, i.e. His imidazoles. This leads us to speculate that 1:1 PrP:Cu complex may bear some of the hallmarks of a 'natively unfolded' protein. Two major features of this class of proteins include the ability to bind several different targets and precise thermodynamic control over binding processes (39,40). No matter what conformation apo-PrP exists in before binding, it should readily be able to coordinate a Cu(II) ion without needing significant energy to reorganize. Additionally, if there is exchange between the several 'degenerate' Cu-binding modes, the PrP:Cu complex may possess enough 'plasticity' to readily accept additional Cu (II) ions. If PrP had a single, very stable binding site then there might be a significant barrier for the protein to change its conformation in order to accept additional copper. Consideration of these findings makes it difficult to envision an enzymatic function for PrP, as the different isomeric Cu(II) binding modes would be expected to reduce the specificity of substrate binding.

Abbreviations

PrP, prion protein
PrP^C, cellular isoform of PrP
PrP(23-28, 57-98), residues 23 through 28 and 57 through 98 of PrP
MCO, metal catalyzed oxidation
MS, mass spectrometry
MS/MS, tandem mass spectrometry
DEPC, diethyl pyrocarbonate
ETD, electron transfer dissociation
CID, collision-induced dissociation
LC, liquid chromatography
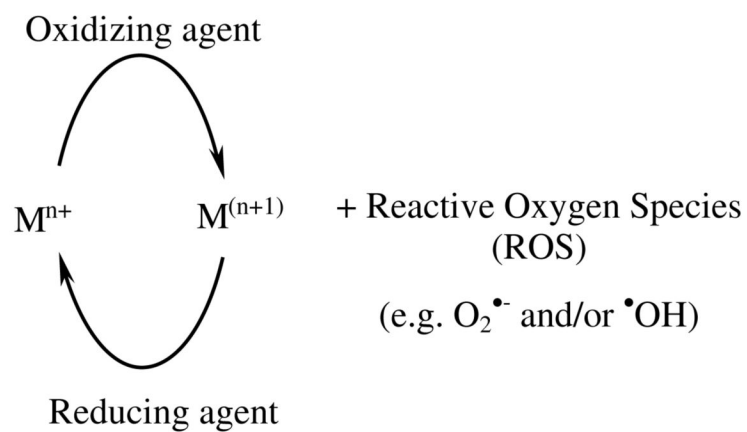NMR, nuclear magnetic resonance.

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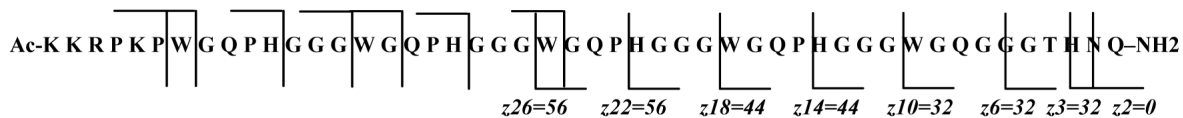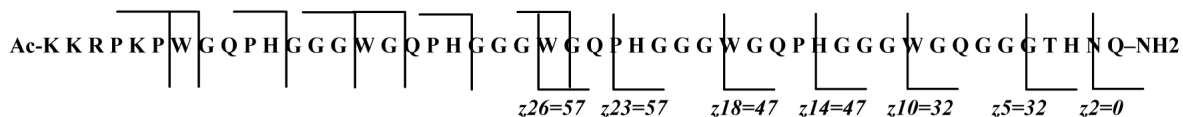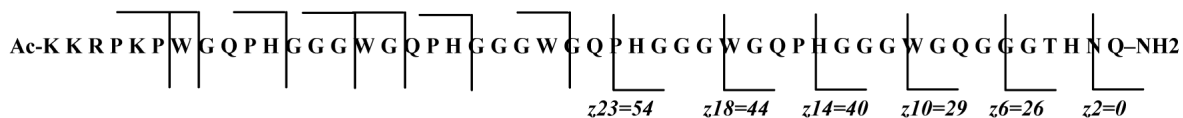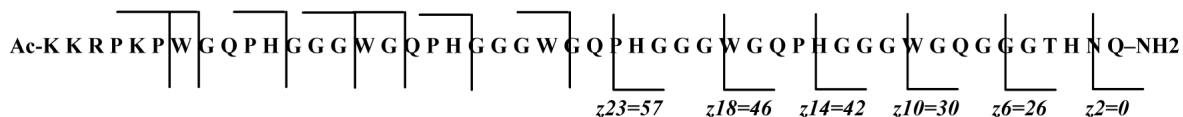
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Oxidizing agent

$$M^{n+} \qquad M^{(n+1)} \quad + \text{Reactive Oxygen Species}$$
$$\text{(ROS)}$$
$$\text{(e.g. } O_2^{\bullet -} \text{ and/or } {}^{\bullet}OH)$$

Reducing agent

Scheme 1.
Generation of reactive oxygen species during MCO reactions.

1: 1 PrP: Cu ratio MCO data

c6=0 c7=0   c11=23 c14=23 c16=23 c19=44 c23=44

Ac-K K R P K P W G Q P H G G G W G Q P H G G G W G Q P H G G G W G Q P H G G G W G Q G G G T H N Q–NH2

z26=56   z22=56   z18=44   z14=44   z10=32   z6=32 z3=32 z2=0

1: 2 PrP: Cu ratio MCO data

c6=0   c7=0   c11=23 c14=23 c16=23 c19=43 c23=43

Ac-K K R P K P W G Q P H G G G W G Q P H G G G W G Q P H G G G W G Q P H G G G W G Q G G G T H N Q–NH2

z26=57 z23=57   z18=47   z14=47   z10=32   z5=32   z2=0

1: 3 PrP: Cu ratio MCO data

c6=0 c7=0 c11=18   c15=22   c19=35   c24=39

Ac-K K R P K P W G Q P H G G G W G Q P H G G G W G Q P H G G G W G Q P H G G G W G Q G G G T H N Q–NH2

z25=61 z23=61   z18=51   z14=47   z10=35   z5=30 z3=30 z2=0

1: 4 PrP: Cu ratio MCO data

c6=0 c7=0   c11=18 c14=18 c16=23 c19=41 c23=46

Ac-K K R P K P W G Q P H G G G W G Q P H G G G W G Q P H G G G W G Q P H G G G W G Q G G G T H N Q–NH2

z23=54   z18=44   z14=40   z10=29   z6=26   z2=0

1: 5 PrP: Cu ratio MCO data

c6=0 c7=0   c11=18 c14=18 c16=24 c19=38 c23=43

Ac-K K R P K P W G Q P H G G G W G Q P H G G G W G Q P H G G G W G Q P H G G G W G Q G G G T H N Q–NH2

z23=57   z18=46   z14=42   z10=30   z6=26   z2=0

Scheme 2.
Oxidation percentages obtained after ETD analysis of MCO reacted PrP(23-28, 57-98) with various copper stoichiometries.

1: 0 PrP: Cu ratio DEPC data

$c8=0$ $c10=0$ $c14=16$ $c16=16$ $c20=39$ $c24=39$

Ac-K K R P K P W G Q P H G G G W G Q P H G G G W G Q P H G G W G Q P H G G W G Q G G G T H N Q–NH2

$z24=61$ $z20=45$ $z17=45$ $z12=18$ $z10=18$ $z3=18$ $z2=0$

1: 1 PrP: Cu ratio DEPC data

$c6=20$ $c8=20$ $c10=20$ $c11=37$ $c16=37$ $c19=52$ $c24=52$

Ac-K K R P K P W G Q P H G G G W G Q P H G G G W G Q P H G G G W G Q P H G G G W G Q G G G T H N Q–NH2

$z29=48$ $z22=48$ $z21=30$ $z16=30$ $z13=9$ $z12=9$ $z8=9$ $z4=9$ $z3=0$

1: 2 PrP: Cu ratio DEPC data

$c8=23$ $c10=23$ $c14=33$ $c16=33$ $c20=44$ $c24=44$

Ac-K K R P K P W G Q P H G G G W G Q P H G G G W G Q P H G G G W G Q P H G G G W G Q G G G T H N Q–NH2

$z24=56$ $z20=43$ $z17=43$ $z12=32$ $z10=32$ $z4=32$ $z3=0$

1: 3 PrP: Cu ratio DEPC data

$c8=39$ $c14=40$ $c16=40$ $c19=43$ $c21=43$

Ac-K K R P K P W G Q P H G G G W G Q P H G G G W G Q P H G G G W G Q P H G G G W G Q G G G T H N Q–NH2

$z24=57$ $z20=45$ $z17=45$ $z12=41$ $z10=41$ $z4=41$ $z3=0$
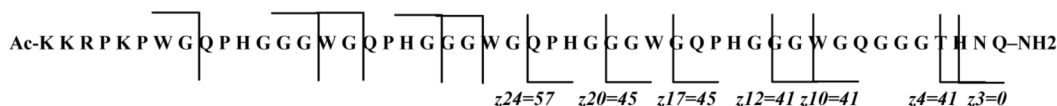
1: 4 PrP: Cu ratio DEPC data

$c8=48$ $c10=48$ $c14=48$ $c16=48$ $c20=48$

Ac-K K R P K P W G Q P H G G G W G Q P H G G G W G Q P H G G G W G Q P H G G G W G Q G G G T H N Q–NH2

$z24=52$ $z20=50$ $z17=50$ $z12=50$ $z10=50$ $z4=50$ $z3=0$
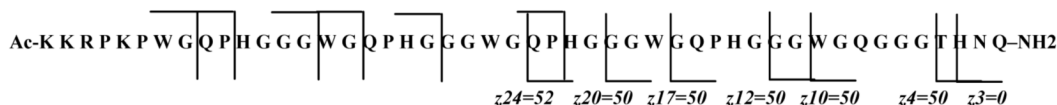
1: 5 PrP: Cu ratio DEPC data

$c8=50$ $c10=50$ $c14=50$ $c16=50$ $c20=50$ $c26=50$

Ac-K K R P K P W G Q P H G G G W G Q P H G G G W G Q P H G G G W G Q P H G G G W G Q G G G T H N Q–NH2

$z24=50$ $z20=50$ $z17=50$ $z12=50$ $z10=50$ $z4=50$ $z3=0$

Scheme 3.
Oxidation percentages obtained after ETD analysis of DEPC reacted PrP(23-28, 57-98) with various copper stoichiometries.
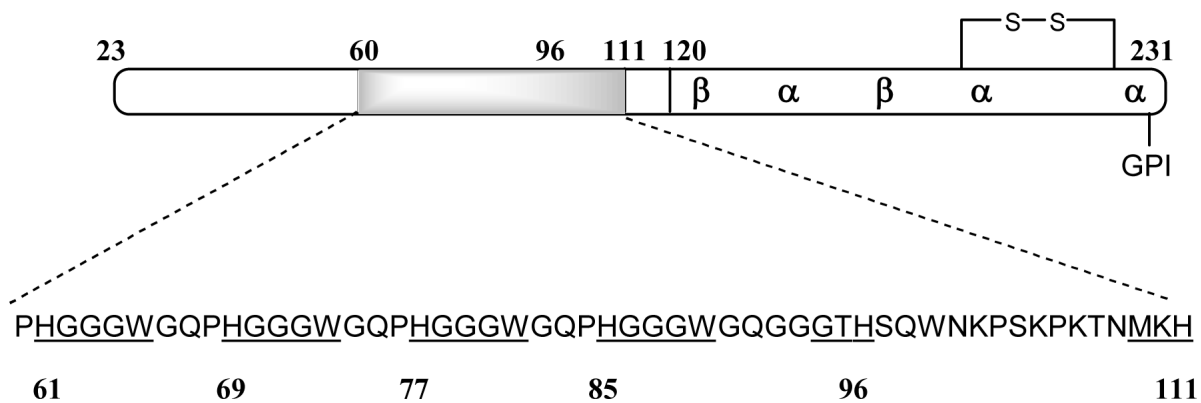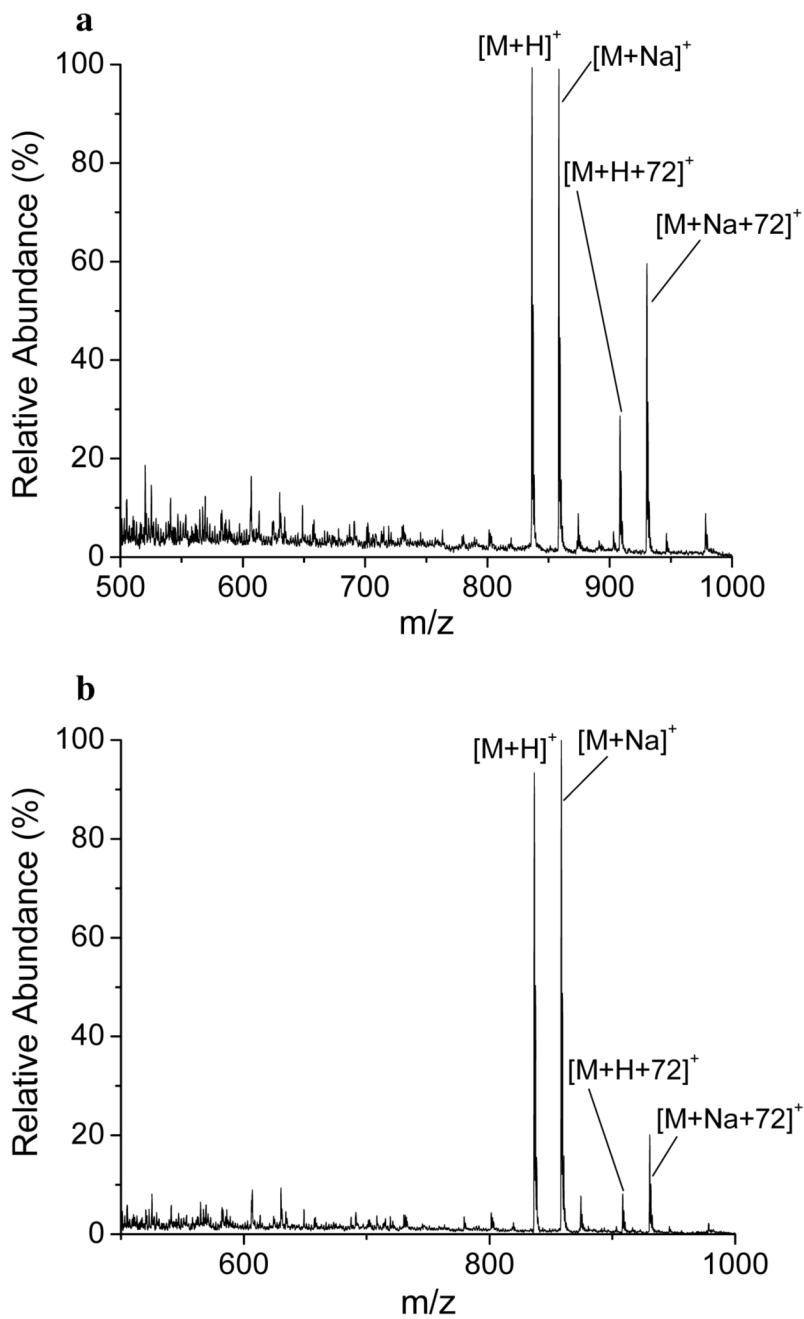
Figure 1.
Sequence information and structural details for human PrP. The metal binding region resides in the flexible N-terminal domain and the five main Cu(II) binding sites are located in the region of residues 60-96. The underlined residues constitute binding sites under Cu(II)-saturating conditions.
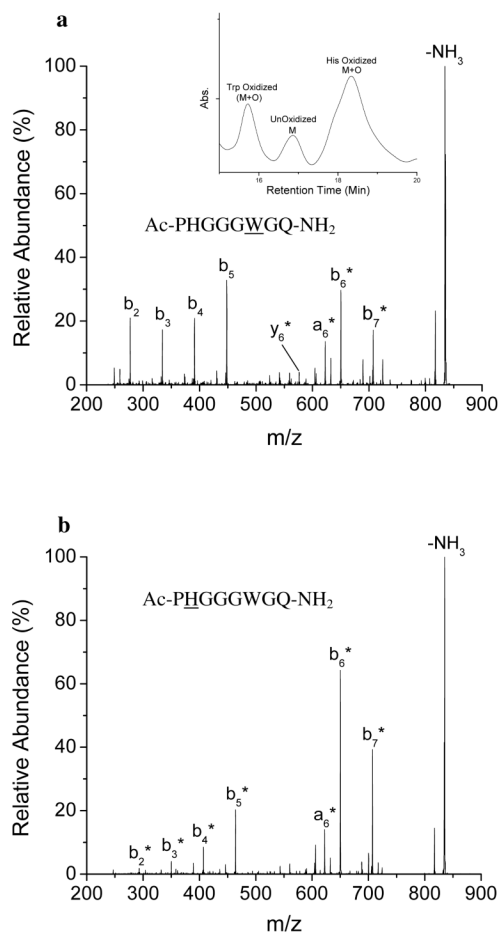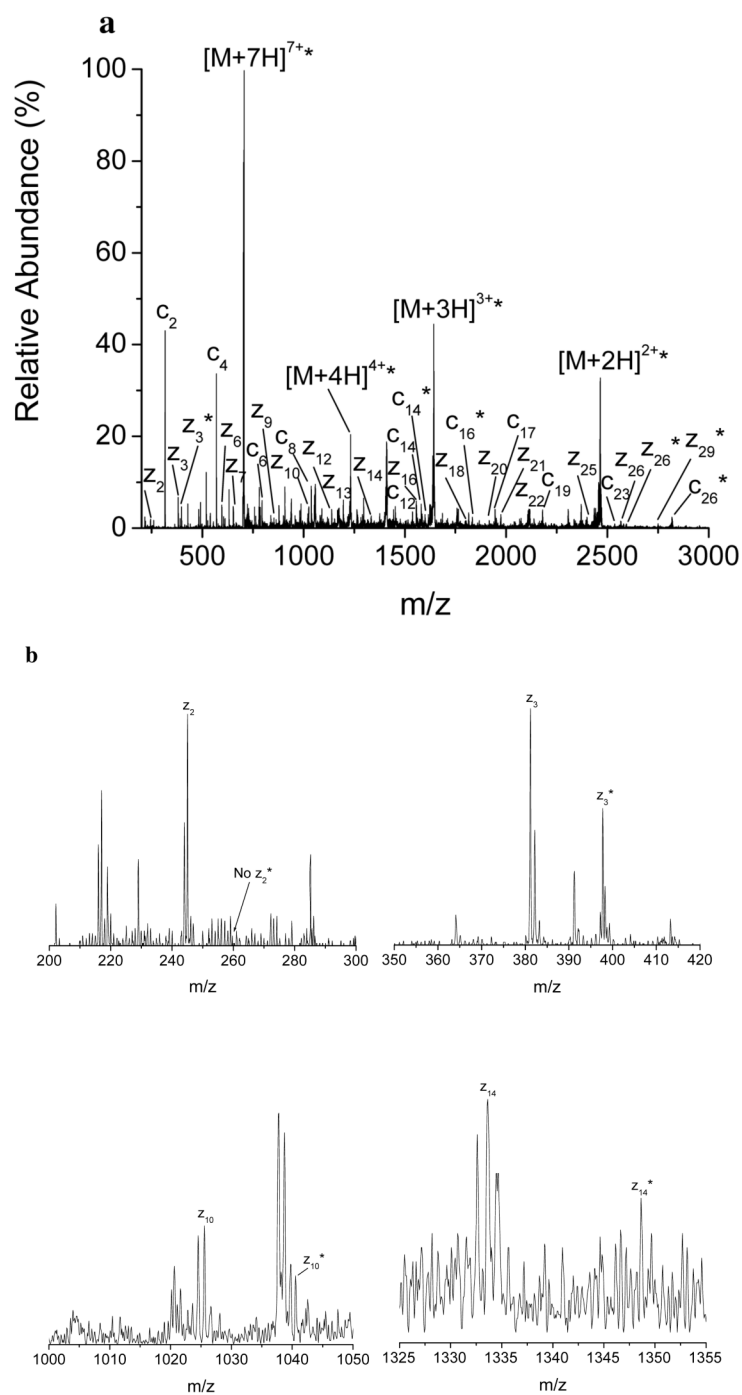
Figure 2.
CID spectra of the oxidized single octarepeat PrP(60-67) peptide. (a) Product ion spectrum of the Trp65-oxidized PrP(60-67) peptide $(M+O+H)^+$. (b) Product ion spectrum of the His61-oxidized PrP(60-67) peptide $(M+O+H)^+$. The ions labeled with asterisks are oxidized product ions. The individual oxidized Trp65 and oxidized His61 were obtained by separating them by HPLC. Inset figure is the HPLC chromatogram of oxidized sample of PrP(60-67).
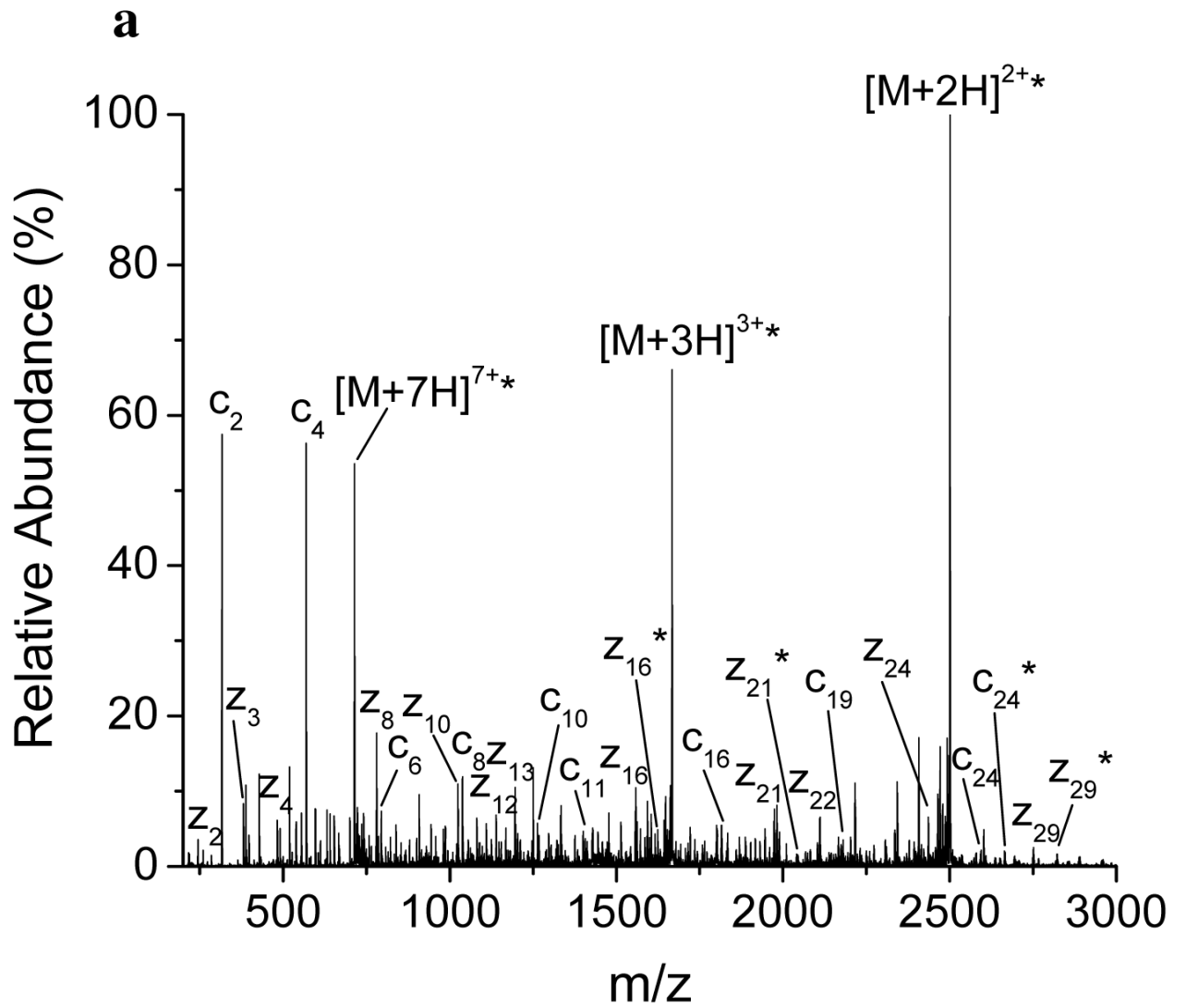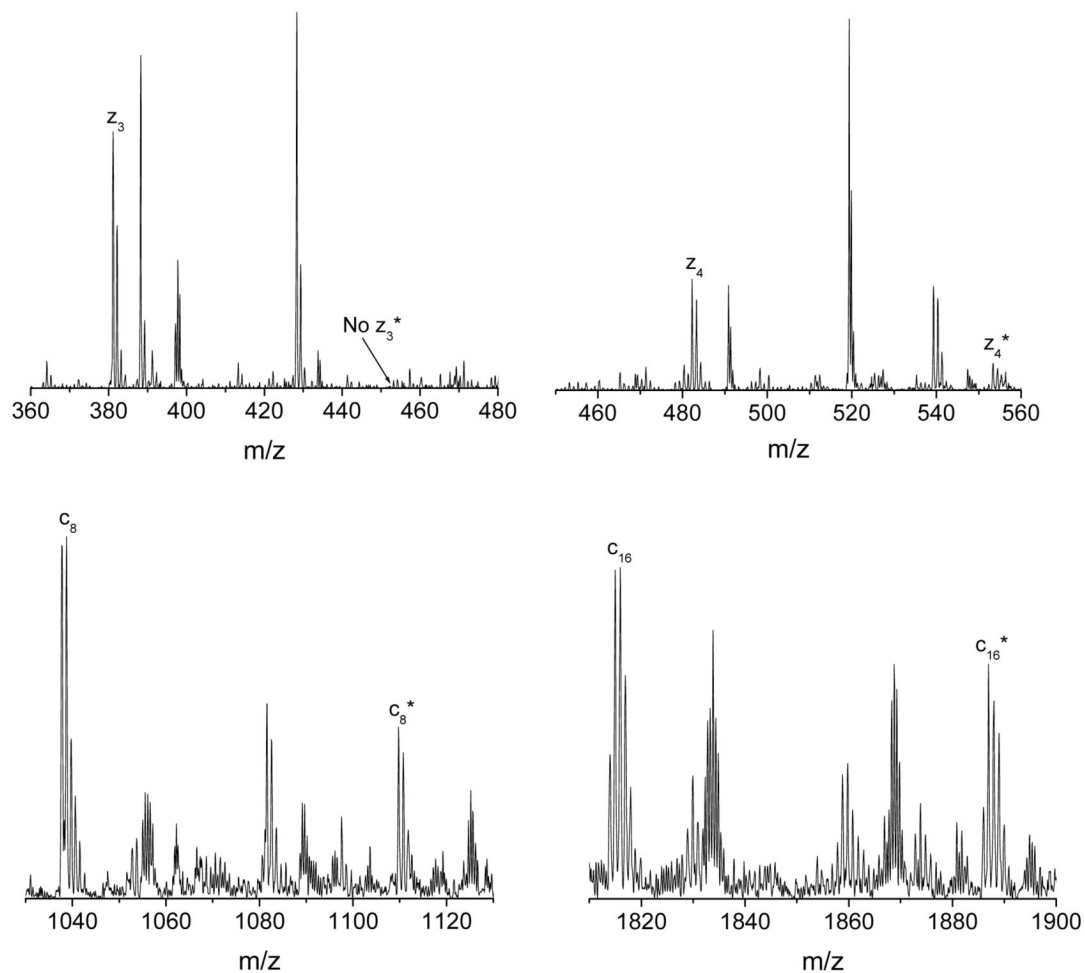
Figure 3.
Mass spectrum of the DEPC modified PrP(60-67) peptide after reaction (a) in the absence of Cu and (b) in the presence of Cu. A mass increase of +72 Da is observed upon reaction with DEPC.
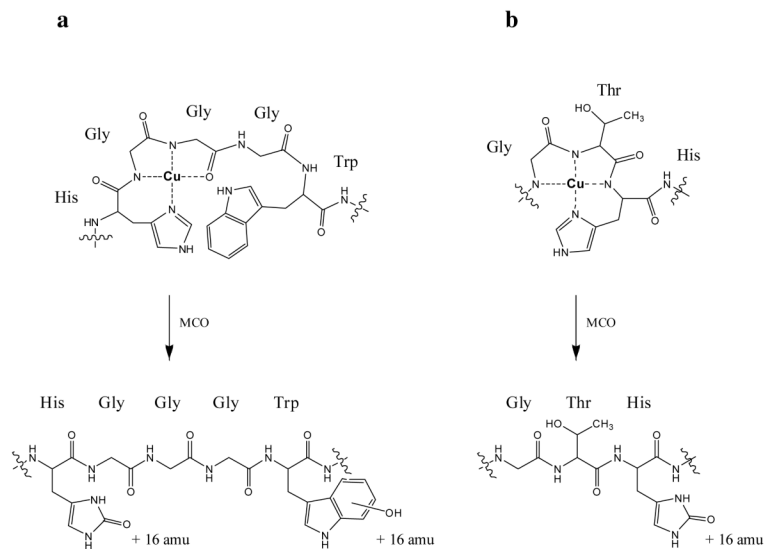
Figure 4.
(a) ETD spectrum of the singly-oxidized peptide ion from the MCO reaction of the 1:1 peptide:Cu complex. (b) Expanded regions of the z_2, z_3, z_{10}, and z_{14} product ions and their oxidized versions. The ions labeled with asterisks are oxidized product ions.

a

b

Figure 5.
(a) ETD spectrum of the singly-modified peptide ion obtained after the DEPC reaction of the 1:1 peptide:Cu complex. (b) Expanded regions of the z_3, z_4, c_8, and c_{16} product ions and their modified versions. The ions labeled with asterisks are DEPC-modified product ions.

Figure 6.
(a) The structure of a single octarepeat binding site was determined from crystallographic and spectroscopic data. The metal catalyzed oxidation of this site leads to oxidation of both the His and Trp residues. (b) The coordination sphere for the GTH site, residues 94-96, is a model based on spectroscopic data. The metal catalyzed oxidation of this site leads to oxidation of the His residue.

Table 1

PrP-Derived Peptide Sequences

PrP(60-67)	Ac-PHGGGWGQ-NH$_2$
PrP(89-98)	Ac-WGQGGGTHNQ-NH$_2$
PrP(23-28,57-98)	Ac-KKRPKPWGQ(PHGGGWGQ)$_4$GGGTHNQ-NH$_2$

Table 2

Oxidation percentages obtained by MCO reactions of **PrP**(23-28, 57-98) with various copper concentrations.

	H-61	H-69	H-77	H-85	H-96	W-57	W-65	W-73	W-81	W-89
1:0(P:Cu)	0	0	0	0	0	0	0	0	0	0
1:1(P:Cu)	23	21	12	12	32	0	0	0	0	0
1:2(P:Cu)	23	20	10	15	32	0	0	0	0	0
1:3(P:Cu)	18	13	10	12	30	0	4	4	4	5
1:4(P:Cu)	18	18	10	11	26	0	5	5	4	3
1:5(P:Cu)	18	14	11	12	26	0	6	5	4	4

Table 3

DEPC modification percentages obtained by DEPC reactions of **PrP**(23-28, 57-98) with various copper concentrations.

	H-61	H-69	H-77	H-85	H-96	T-95	K-23,24,27[a]
1:0(P:Cu)	16	23	16	27	18	0	0
1:1(P:Cu)	17	15	18	21	0	9	20
1:2(P:Cu)	10	11	13	11	0	32	23
1:3(P:Cu)	1	3	12	4	0	41	39
1:4(P:Cu)	0	0	2	0	0	50	48
1:5(P:Cu)	0	0	0	0	0	50	50

[a]Total percentage of three Lys residues modified