# A novel copper(II) coordination at His186 in full-length murine prion protein

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**Abstract**

To explore Cu(II) ion coordination by $His^{186}$ in the C-terminal domain of full-length prion protein (moPrP), we utilized the magnetic dipolar interaction between a paramagnetic metal, Cu(II) ion, and a spin probe introduced in the neighborhood of the postulated binding site by the spin labeling technique (SDSL technique). Six moPrP mutants, moPrP(D143C), moPrP(Y148C), moPrP(E151C), moPrP(Y156C), moPrP(T189C) and moPrP(Y156C,H186A), were reacted with a methane thiosulfonate spin probe and a nitroxide residue (R1) was created in the binding site of each one. Line broadening of the ESR spectra was induced in the presence of Cu(II) ions in moPrP(Y148R1), moPrP(Y151R1), moPrP(Y156R1) and moPrP(T189R1) but not moPrP(D143R1). This line broadening indicated the presence of electron-electron dipolar interaction between Cu(II) and the nitroxide spin probe, suggesting that each interspin distance was within 20 Å. The interspin distance ranges between Cu(II) and the spin probes of moPrP(Y148R1), moPrP(Y151R1), moPrP(Y156R1) and moPrP(T189R1) were estimated to be 12.1 Å, 18.1 Å, 10.7 Å and 8.4 Å, respectively. In moPrP(Y156R1,H186A), line broadening between Cu(II) and the spin probe was not observed. These results suggest that a novel Cu(II) binding site is involved in His186 in the Helix2 region of the C-terminal domain of $moPrP^{C}$.

Key words: prion protein; Cu(II); C-terminal domain; ESR; SDSL; MTSSL; interspin distance; dipolar interaction; histidine residue

## Introduction

Transmissible spongiform encephalopathies (TSEs), or prion diseases, are a group of fatal neurodegenerative disorders including Creutzfeldt-Jacob disease (CJD), Gerstmann-Sträusler-Scheinker (GSS) syndrome, fatal familial insomnia (FFI) and kuru in humans, scrapie in sheep and bovine spongiform encephalopathy (BSE) in cattle [1]. The prion diseases are considered to be caused by the conformational conversion of a normal cellular prion protein ($PrP^{C}$) into the abnormal (scrapie-like and β-sheet-rich) form of prion protein ($PrP^{Sc}$) in endosomes, the main intracellular acidic organelles [2]. The precise mechanism of conversion from $PrP^{C}$ to $PrP^{Sc}$ is still unknown. However, accumulating evidence suggests that Cu(II) ions associated with $PrP^{C}$ play an important role in the conversion process from $PrP^{C}$ to $PrP^{Sc}$ [3,4].

Mammalian $PrP^{C}$ is a ubiquitous glycoprotein attached to the plasma membrane via a glycosyl phosphatidylinositol (GPI) anchor and localized in membrane lipid rafts [5]. Lipid rafts, which are rich in sphingolipids and cholesterol, are associated with endocytosis. The mature prion protein of the mouse (moPrP) consists of 208 amino acids (residues 23-231). The structure of $PrP^{C}$ consists of a tertiary carboxy-terminal domain which contains three α-helices (Helix1, Helix2 and Helix3) and two short anti-parallel β-sheets (Sheet1 and Sheet2) [6]. On the other hand, the N-terminal domain includes an octarepeat (OR) region (residues 61-91) containing multiple copies of the repeat PHGGGWGQ, which has high affinity for binding Cu(II) ions [7,8]. Through this domain does not contribute measurably to the manifestation of the 3D-structure as detected by one-dimensional proton-nuclear magnetic resonance (NMR) [9], some studies using the ESR technique have shown that binding four copper ions induces a unique structure in this region [10-12]. Weiss *et al.* proposed models of the Cu(II) binding structure in the octarepeat region of the full-length

prion protein by using the combination of extended X-ray absorption fine spectroscopy (EXAFS), electron paramagnetic resonance (EPR) and electron nuclear double resonance (ENDOR) [12]. Furthermore, a fifth Cu(II) binding site with higher affinity was identified between 91 and 125 next to the octarepeat motif. The involvement of two histidine residues (H95 and H110) in the Cu(II) binding has been suggested [13-15]. Recent studies have proposed that a novel Cu(II) binding site is included in the C-terminal tertiary regions of $PrP^C$ [16-18]. Brown *et al*. showed that the peptide fragment analogue of PrP (PrP Ac180-193$NH_2$) is the Cu(II) binding motif in the C-terminal region [17]. The binding site location of Cu(II) in the structured region of the protein is suggested to show the involvement of a histidine 187 residue in the human (histidine 186 residue in the mouse). Moreover, when using two shorter peptide fragments (PrP 180-193$NH_2$ and PrP Ac184-188$NH_2$), the affinity of the Helix 2 fragment of PrP was higher than that for the octarepeat and PrP106-126 peptides [18]. However, there is no experimental evidence for the existence of a novel copper binding site in the intact C-terminal domain of the full-length prion protein.

Recently, to obtain information about the pH-induced conformational changes, we employed cysteine-scanning site-directed spin labeling (SDSL) combined with electron spin resonance spectroscopy (ESR), and analyzed the pH-induced mobility changes in one α-helix (Helix1) and two β-sheets (Sheet1 and Sheet2) of mouse $PrP^C$ ($moPrP^C$) [19-22]. Our experimental data using cysteine-scanning spin labeling for 19 cysteine mutants of recombinant $moPrP^C$ clearly demonstrated the presence of three pH-sensitive sites in $moPrP^C$. SDSL-ESR has proven to be a useful technique for protein structural and motional analyses, such as determination of the secondary structure and its orientation, areas of tertiary interactions and domain mobility, which are impossible to obtain by NMR and X-ray crystallographic methods [23,24]. Furthermore, this SDSL-ESR technique is reported to be

useful for characterization of the binding sites of various proteins with paramagnetic metal ions. Originally, this technique was established based on a theory for the distance-dependent relaxation effects on dipolar interactions between metal ions and nitroxide in a rigid lattice system as described by Leigh *et al* [25]. More recently, Hubbell and colleagues have demonstrated that the SDSL technique permits measurement of the distance between a nitroxide spin probe in T7 lysozyme and Cu(II) ion of under physiological conditions [26].

In the present study, to identify a novel Cu(II) ion binding site in the carboxy-terminal domain of full-length moPrP, we examined the distance between Cu(II) ion and a nitroxide spin probe in the amino acid residues neighboring the postulated metal binding site, histidine 186 in the mouse. In this SDSL experiment, the nitroxide side chain (R1) derived from a methane thiosulfonate spin label (MTSSL) was introduced into six mutants of recombinant mouse prion protein, moPrP(D143C), moPrP(Y148C), moPrP(E151C), moPrP(Y156C), moPrP(T189C) and moPrP(Y156C,H186A), which were substituted for a cysteine residue by site-directed mutagenesis (Fig. 1A).

## Materials and Methods

*Construction of moPrP mutants*

cDNA encoding mouse PrP (residues 23-231) was cloned into BamHI/EcoRI sites of pRSETb as described previously [19-22, 27]. In the plasmid encoding moPrP, four amino acid residues, D143, Y148, E151 in the H1 region, Y156 in the loop between H1 and S2 and T189 in the H2 region, were changed to cysteine residues for the labeling site as indicated in Fig. 1A using the PCR-based site-directed mutagenesis method described previously [19-22]. Oligonucleotides used for the mutagenesis were obtained from Sigma Genosys. The changes of these codons were confirmed using a CEQ8800 automated sequencer (Beckman Coulter,

Inc.). Figure 1B shows the 3D structure in the carboxy-terminal domain of moPrP $_{(121-231)}$ as reported by Riek *et al.* (PDB code: 1AG2) [6].

*Expression, Purification and Spin-Labeling of recombinant moPrP mutants*

The expression and purification of recombinant moPrP mutants were carried out as described previously [21-24]. To label the moPrP$^{C}$ mutants with (1-Oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)methane thiosulfonate (MTSSL) (Toronto Research Chemicals, ON, Canada), a 10-fold molar excess of MTSSL was added to each protein and incubated overnight in the dark at 4$^{o}$C. The free MTSSL was removed from the protein using a microdialyzer (Nippon Genetics). The protein concentration was quantified with the Lowry protein assay using BSA as a standard [28]. The final protein purity was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and was at least 98% pure. The positions of D143R1, Y148R1, E151R1, Y156R1, H186 and T189R1 in the 3D structure of the prion C-terminal region are shown in Fig. 1B.

*ESR spectroscopy and distance calculation*

Details of the ESR spectroscopy methods have been published elsewhere [19-22]. The neutralization of the sample solution was carried out by dialysis of the sample against 10 mM HEPES buffer (pH 7.5) and 20% glycerol. ESR spectra were recorded in a quartz flat cell (RST-DVT05; 50 mm x 4.7 mm x 0.3 mm, Radical Research) for spin-labeled samples of 20 μM moPrP$^{C}$ with each concentration of $CuCl_2$ from 0 μM to 160 μM using a JEOL-RE X-band spectrometer (JEOL) with a cylindrical TE011 mode cavity (JEOL). All ESR spectra were obtained at -10$^{o}$C, controlled by a temperature controller (ES-DVT4, JEOL) under the following conditions: 5 mW incident microwave power, 100 kHz modulation frequency, 0.2

mT field modulation amplitude, and 20 mT scan range. In these conditions, containing 20% glycerol, the sample did not freeze. The distance between the nitroxide probe and bound Cu(II) was examined by measuring the intensity decrease of the ESR signal ($M_I$=0, central component) of the spin-labeled PrP on addition of Cu(II) [26-27]. The distance, *r*, was calculated from following equation:

$$r = [(g\beta\mu^2\tau)/(\hbar C)]^{1/6}$$

where the constant *g* is the electronic *g* factor for the nitroxide radical, β is the Bohr magneton and m and $\tau c$ are the magnetic moment and the electron-spin relaxation time of Cu(II), respectively. $\tau_c$ =3 x $10^{-9}$ s was used for the complexed Cu(II) [26]. $\delta H_0$ is the unbroadened natural linewidth in the absence of the Cu(II) obtained from center ($M_I$=0) line of the ESR spectrum and *C* is the dipolar interaction coefficient for the measured spectrum. The value of $C/\delta H_0$ was obtained from the ratio of the broadened to unbroadened amplitude ($I/I_0$) of the ESR spectrum of the spin label in the presence of paramagnetic ion using the graph reported by Voss *et al.* (1995) for labile motion in solution (rigid lattice care) [26].

*Computer simulation*

Molecular modeling was performed using the structure of mouse PrP (PDB code 1AG2) [6] to determine the most probable orientation of the MTSSL spin label. After mutating an amino acid to MTSSL-labeled cystein in silico, the structure was energy-minimized using the CHARMM19 force field in the CHARMM program [29]. Then, a Monte Carlo search was performed to find the lowest energy conformation for the spin label [30]. The topology and force field parameters for the MTSSL spin label were downloaded from the website of Fajer Lab (http://www.sb.fsu.edu/~fajer/Programs/Molecular_Modelling/Molecular_Modelling.htm),

and were used for the calculation.

**Results and Discussion**

The N-terminal domain of prion protein includes an octarepeat (OR) region containing multiple copies of the repeat PHGGGWGQ, which has high affinity for binding four Cu(II) ion [8,9]. Furthermore, a fifth Cu(II) ion binding site was recently identified as a region involved in histidine 95 and histidine 110 in the neighborhood of the OR region. Some studies have suggested that Cu(II) binding to this fifth site is correlated with the conversion into a protease-resistant species and the increase of β-sheet conformation in PrP [13-15]. Recently, a novel sixth Cu(II) binding site in the C-terminal tertiary regions of $PrP^C$ was proposed in the model peptide and the truncated PrP protein [16-18]. To clarify whether the Cu(II) ion is correlated with the C-ternimal region of the full length $moPrP^C$, we introduced a nitroxide spin-probe into the amino acid residues of the neighborhood of the postulated Cu(II) binding site (His186) in recombinant moPrP (rmoPrP) using the SDSL technique. If a Cu(II) ion is situated in the H186 residue and the distance between the Cu(II) ion and spin-probe is distributing within 20 Å, the line broadening of ESR spectrum originating from spin probe is reported to be induced by dipolar-dipolar interaction [26].

At first, we selected the threonine 189 residue for site-directed spin labeling to detect Cu(II), which would be located in histidine 186 residue because T189R1 and H186 postulated Cu(II) binding sites, were located in one pitch different position and the distance between these residues was very close, within about 5 Å. A mouse recombinant protein, moPrP(T189C), was purified, spin labeled, concentrated to 20 μM, and then measured by ESR in the absence or presence of 0, 20, 40, 80, 120 and 160 μM $CuCl_2$. Figure 2(a) shows the effects of $CuCl_2$ on ESR spectra of moPrP(T189C), The intensity of ESR signals was

decreased in a $CuCl_2$ concentration-dependent manner, indicating that line broadening due to dipole-dipole interaction between Cu(II) and the nitroxide probe occurred. To obtain further information about the interaction between Cu(II) and the nitroxide spin probe, the ratios of the peak height (I) of the central ($^{14}N$ hyperfine) component ($M_I$=0) in the ESR spectrum with various concentrations of $CuCl_2$ were plotted against that ($I_0$) without $CuCl_2$ as shown in Fig. 2(b). The relative signal intensity ($I/I_0$ x 100) decreased rapidly until the ratio of [Cu(II)] and [PrP] became 4 and then the relative intensity was maintained at about 20% at [Cu(II)]/[PrP]=6 and [Cu(II)]/[PrP]=8. This indicated that the prion proteins had multiple Cu(II) ion-binding sites and 160 μM $CuCl_2$ ([Cu(II)]/[PrP]=8) was sufficient to estimate the distance between Cu(II) ion coordinated at H186 and the nitroxide side chain.

Next, we examined the effects of $CuCl_2$ on ESR spectra of moPrP(D143R1), moPrP(Y148R1) and moPrP(E151R1), which had the nitroxide probe at α-Helix1, and moPrP(Y156R1), which had the nitroxide probe in the loop region between α-Helix1 and β-Sheet2, as illustrated in Fig. 1(b). The ESR spectra of moPrP(D143R1), moPrP(Y148R1), moPrP(E151R1) and moPrP(Y156R1) without (black line) and with 160 μM $CuCl_2$ (red line) are shown in Fig. 3(a) – (d), respectively. Decreases of the intensity of the ESR spectra due to line broadening were clearly observed in moPrP(Y148R1) moPrP(E151R1) and moPrP(Y156R1), indicating that Cu(II) was close (<20 Å) to sites Y148R1, E151R1 and Y156R1 under physiological conditions. The line broadening was not detected in moPrP(D143R1), indicating a long interspin distance (>20 Å). There was a possibility that Cu(II) ion bonded to the sites other than His186, i.e., the octarepeat region from codon 60 to 90 [11-13] and the region between amino acids 95 and 110 [14,15], were associated with the nitroxide spin probe of these recombinant prion proteins via an unexpected tertiary structure of PrP or intermolecular interaction. Therefore we tested the effect of Cu(II) on

moPrP(Y156R1,H186A), which is a double mutation for disruption of the histidine residue for the Cu(II) binding site. As shown in Fig. 3(e), the reduction of ESR signal intensity was not found in the presence of Cu(II) ions in this double mutation, although the strong line broadening effect of Cu(II) ions was clearly observed in moPrP(Y156R1), as shown in Fig. 3(d). These data allowed us to conclude that a Cu(II) ion coordinated with His186 interacted with the nitroxide probe and produced line broadening of the nitroxide radical by dipole-dipole interaction.

To estimate the distance between the Cu(II) ion and the nitroxide probe, we calculated it based on the Redfield theory developed by Voss *et al.* [26]. Since the spectra of glassy PrP samples at -10$^{o}$C exhibit anisotropic shapes, values of c, the dipolar interaction coefficient, were determined from the plot of I/Io vs. c/δHo for rigid lattice $M_I$=0 line amplitude as described by Voss *et al.* [26]. As listed in Table 1, the distances from Cu(II) estimated for Y148R1, E151R1, Y156R1 and T189R1 ranged from 8.4 Å to 18.1 Å as summarized in Table 1. Figure 4(a)-(e) shows the molecular simulation used to determine the most probable orientation of the spin probe moiety created in each prion mutant and the approximate position of the Cu(II) ion if the histidine 186 residue is important as a novel Cu(II) ion binding site in the prion protein. To compare experimental data and these computer simulation data for the Cu(II) binding structure, we estimated the distance between the oxygen atom of spin-probe and the nitrogen atom, which are the Nδ and Nε positions in the imidazole ring of histidine residue186, respectively (as illustrated by Fig. 4 (f)), in each mutant prion protein. It was clearly demonstrated that each interspin distance was quite similar to the interatom distance shown Table 1. Our data strongly indicated that histidine residue 186 in Helix2 was a novel Cu(II) binding site in the C-terminal core region of prion protein.

A recent study using molecular dynamic (MD) simulation indicated that the break of the salt bridge between Arg156 and Glu196 induced by the protonation of His187 in human prion protein (His186 in mouse prion protein) at acidic pH was the key event underlying the extension of the S2 region and the conversion to β-sheet-rich PrP [31]. Furthermore, His187 in the human is reported to be involved in a pathogenic mutation (H187R) associated with GSS syndrome, which implies a positively charged residue at position 187 [32]. This H187R mutation may induce the break of the salt bridge in a manner similar to that induced by protonation of His187 under acidic pH. In the present study, we demonstrated a novel binding site for Cu(II) ion located in the internal regions of Helix2 and Helix3, as shown in Fig. 4. Langella *et al.* proposed that His187 and Glu196 were the most probable candidates for binding residues of Cu(II) ion according to their computer simulation and that H187R mutation was related to altered Cu(II) binding [32]. These reports and our results suggested that this disruption of the interaction between Cu(II) ion and $PrP^{C}$ by a point mutation of His187 in the human influenced the C-terminal core structure related to induction of prion diseases such as GSS syndrome.

In conclusion, the present cysteine-scanning SDSL-ESR study of full-length recombinant $moPrP^{C}$ provides experimental evidence that a sixth Cu(II) binding site exists in the C-terminal region of $moPrP^{C}$, similarly to a previous study using some peptide fragments. This novel Cu(II) binding site is involved in histidine residue 186 in the Helix2 region of the C-terminal domain of $moPrP^{C}$. The pathogenic mutation at this Cu(II) binding site may be correlated with conversion of $PrP^{C}$ to the pathogenic $PrP^{Sc}$ structure in GSS syndrome

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**Figure Legends**

Figure 1

A schematic diagram of the site-directed spin labeling (SDSL) technique and the mutation site for SDSL-ESR on the 3D structure of moPrP$^{C}$. (a) The chemical reaction of the methanethiosulfonate spin-labeling reagent with the cysteine residue generates the nitroxide side chain (R1) on moPrP$^{C}$. (b) The carboxy-terminal domain of moPrP $_{(121\text{-}231)}$ and five mutation sites, D143, Y148, E151, Y156 and T189, for SDSL-ESR. The side chains of Y156, D143R1, Y148R1, E151R1, Y156R1 and T189R1, were superimposed on the 3D structure of moPrP reported in an NMR study (PDB code: 1AG2, ref. [7]).

Figure 2

The interaction between the Cu(II) ion and nitroxide spin probe on the ESR spectra of full-length moPrP(T189R1) and titration curve of reduction of ESR intensity by Cu(II). (a) The line broadening of ESR spectra originating from the spin probe in T189 due to the presence of various concentrations of Cu(II) (0, 20, 40, 80, 120 and 160 μM). The molar ratio of Cu(II) ions against [PrP] ([Cu(II)]/[PrP]) is shown by the value to the left of each spectrum. (b) The ratios of peak height (I) of the peak-to-peak central component ($M_I$=0) in ESR spectra of Y156R1 and T189R1 in the presence of various concentrations of Cu(II) against those ($I_0$) without Cu(II).

Figure 3

Effects of Cu(II) on ESR spectra of (a) moPrP(D143R1) (b) moPrP(Y148R1), (c) moPrP(Y151R1), (d) moPrP(Y156R1) and (e) moPrP(Y156R1,H186A). Each spin-labeled

moPrP mutant was prepared as a 20 μM concentration in 10mM HEPES buffer (pH 7.5) and 50% glycerol for ESR measurement. The line shapes of spectra were obtained in the absence (black line) and the presence (red line) of 160 μM Cu(II). ([Cu(II)]/[PrP]=8). (f) The distance between the oxygen atom of spin-probe and the nitrogen atom, which are the Nδ and Nε positions in the imidazole ring of histidine residue186, respectively

Figure 4

The computer simulation structures of (a) moPrP(D143R1) (b) moPrP(Y148R1), (c) moPrP(Y151R1), (d) moPrP(Y156R1) and (e) moPrP(T189R1) by energy-minimized calculations using the CHARMM19 force field in the CHARMM program. The orientation of the spin probe was determined by the lowest energy conformation, which was estimated by a Monte Carlo search. From these computer simulation structures, the interatom distance between the oxygen atom of spin-probe and nitrogen atom, which are the Nδ and Nε positions in the imidazole ring of histidine residue186, respectively (as shown in schema as shown in (f)), is presented in Table 1.

**Table1**

Table 1.

Interspin distance between oxygen atom of nitroxide probe and Cu(II) calculated by dipole-dipole interaction and interatomic distance between oxygen atom of nitroxide probe and nitrogen atom of His186 estimated by computer simulation.

| | Interspin distances (Å) | Interatomic distances (Å) | |
|---|---|---|---|
| | r(-NO· – Cu) | r(-NO·-Nδ) | r(-NO·-Nε) |
| D143R1 | > 20 | 23.570 | 21.767 |
| Y148R1 | 12.1 | 11.156 | 8.996 |
| E151R1 | 18.1 | 19.583 | 17.741 |
| Y156R1 | 10.7 | 8.491 | 6.541 |
| T189R1 | 8.4 | 3.016 | 4.989 |

Figure1

Figure 1

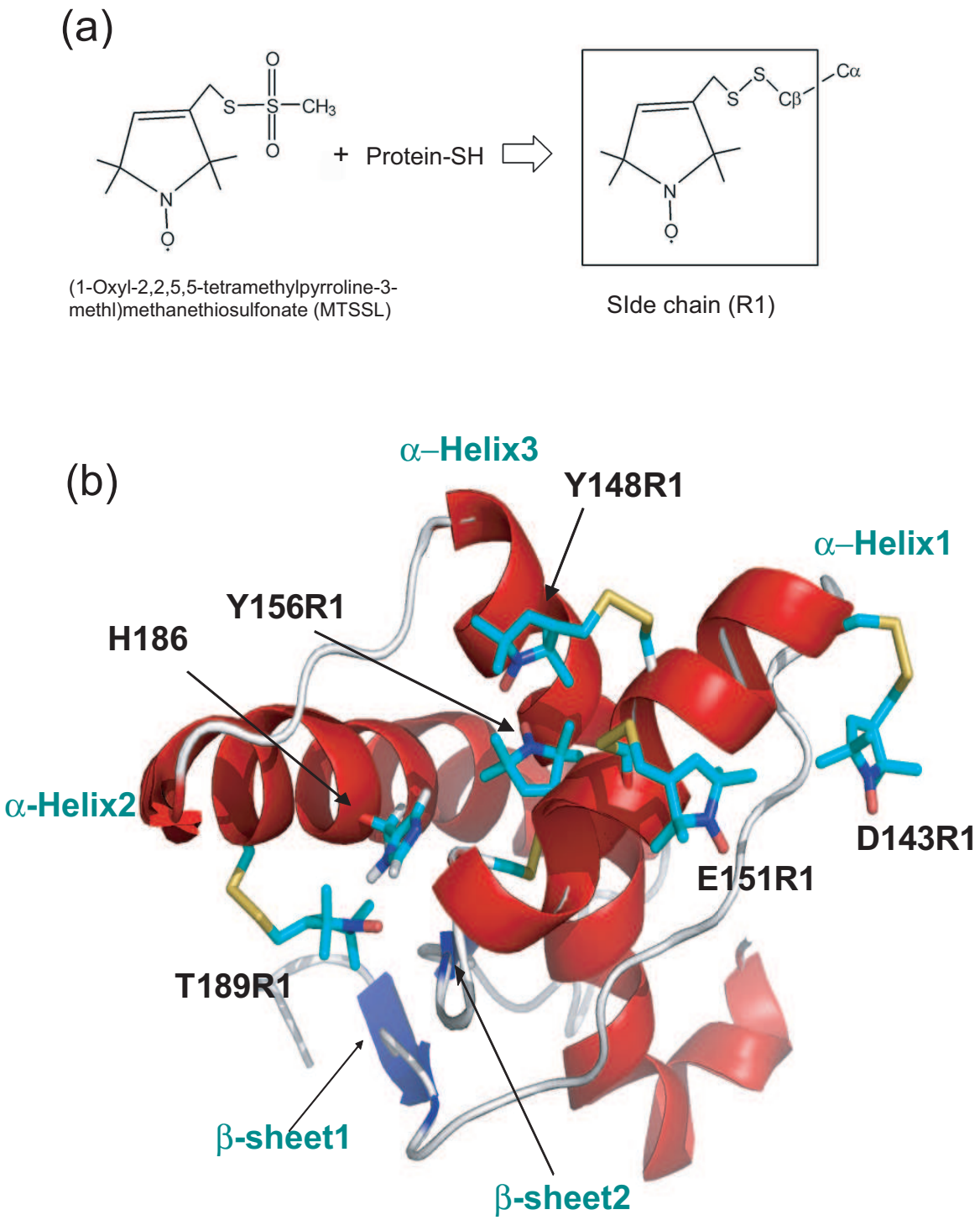

Figure 2

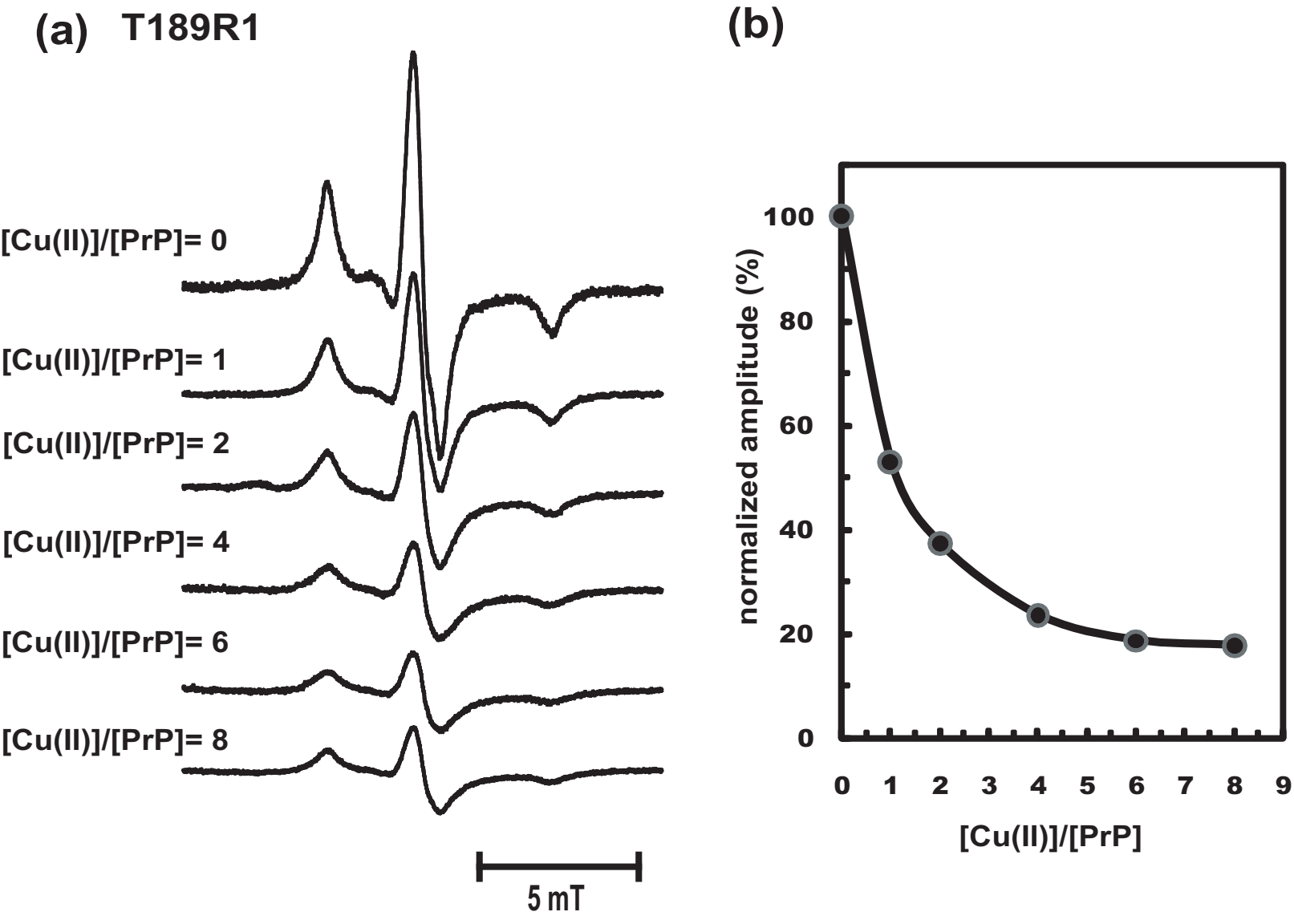
(a) T189R1
[Cu(II)]/[PrP]= 0
[Cu(II)]/[PrP]= 1
[Cu(II)]/[PrP]= 2
[Cu(II)]/[PrP]= 4
[Cu(II)]/[PrP]= 6
[Cu(II)]/[PrP]= 8
5 mT
(b)
normalized amplitude (%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9
[Cu(II)]/[PrP]

Figure3
Figure 3

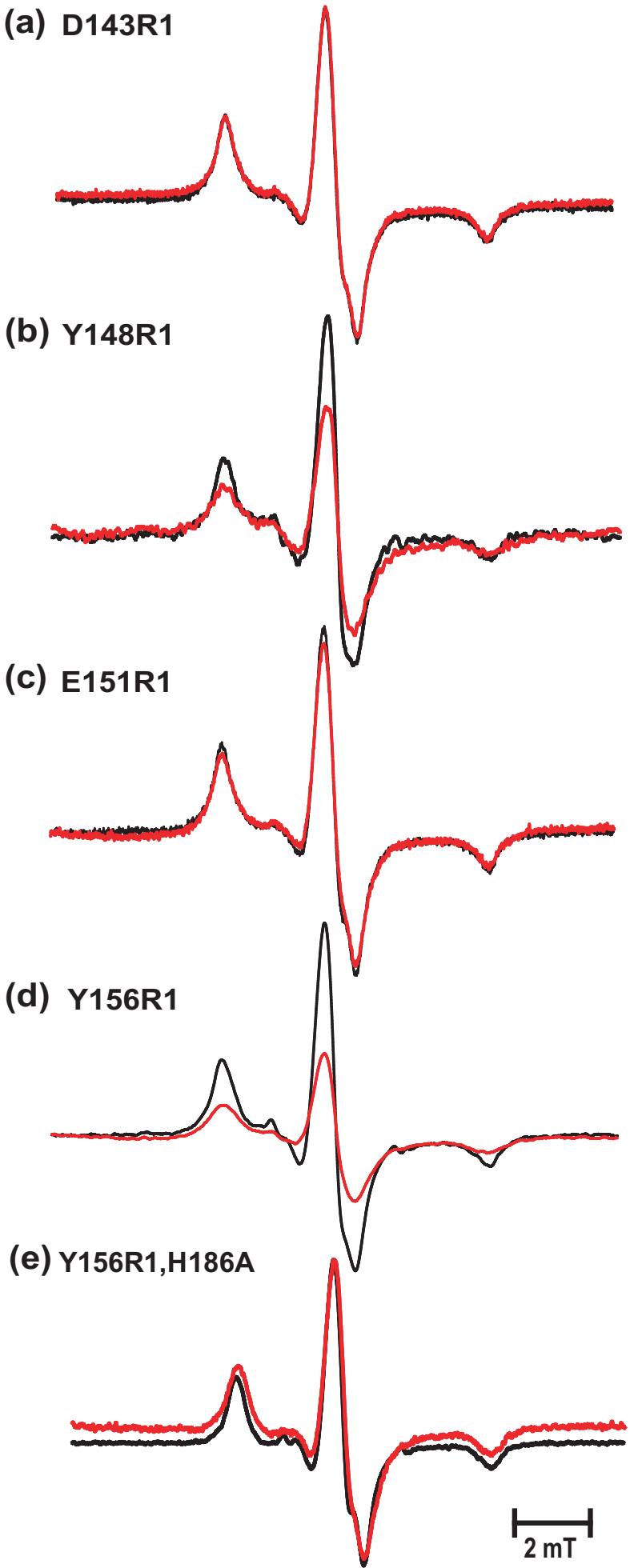

Figure4 Figure 4