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Short Communication

Fluorescence measurements revealed two distinct modes of metal binding by histidine-containing motifs in prion-derived peptides

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ABSTRACT

Prion proteins are infectious agents causing transmissible spongiform encephalopathies in a misfolded protease-resistant form of protein. Human PrP possesses 7 potential copper-binding sites. Notably, four of putative copper-binding sites are located in the octarepeat region (PrP 60-91). Recent studies have shown that peptides derived from human PrP effectively bind Cu^{2+} to form the Cu-centered catalytic complex required for generation of superoxide by coupling the oxidation of neurotransmitters and their analogues. In this study, we have studied the minimal motifs required for binding of metals within human PrP, by assessing (1) the peptide-dependent quenching of Tb^{3+} fluorescence and (2) the Cu^{2+}-dependent quenching of intrinsic fluorescence in human PrP octarepeat-derived peptides. Assays with peptide-dependent quenching of Tb fluorescence supported the positive role of the His-ended X-X-H motif (in this case Q-P-H tripeptide sequence) rather than His-started H-G-G-G-W motif, as metal chelating motifs in short peptides. Controversially, the role of combination of Pro and His residues was supported by the Cu-dependent peptide fluorescence quenching assay. Above data suggested that there are two distinct modes of metal binding to His residues in the octarepeat regions in PrP, possibly by co-ordinations of His-started and His-ended motifs around the target metals depending on the conditions given.

KEYWORDS: Cu-binding, fluorescence, octarepeat, peptide, prion

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INTRODUCTION

Prion proteins (PrP) are infectious agents causing transmissible spongiform encephalopathies (TSE) in a misfolded protease-resistant form known as PrP^{res} [1]. In general, the protease-sensitive form known as PrP^{sen} represents the intrinsic cellular PrP (PrP^{C}) and PrP^{res} represents the infectious scrapie isoform of PrP (PrP^{Sc}).

Studies have shown that PrPs form a group of copper-binding proteins [2, 3]. For an instance, human PrP has 7 potential copper-binding sites (Fig. 1). In the so-called "octarepeat" region (PrP 60-91) in human PrP, in which amino acid sequence "P-H-G-G-G-W-G-Q" repeatedly appears in tandem, each repeat unit possibly binds single Cu^{2+} at physiological neutral and basic range of pH [4-6]. Similarly, bovine PrP sequence contains six octarepeats thus possessing at least six putative copper-binding sites with high affinity [3, 7]. In chicken, the copper-binding sites analogous to the octarepeats are known as hexa-repeats with each repeat consisting of H-N-P-G-Y-P sequence and here again His residues play a key role in anchoring of copper [8].

Morante *et al.* [7] showed that partial occupancy of copper on bovine PrP is manifested by binding of

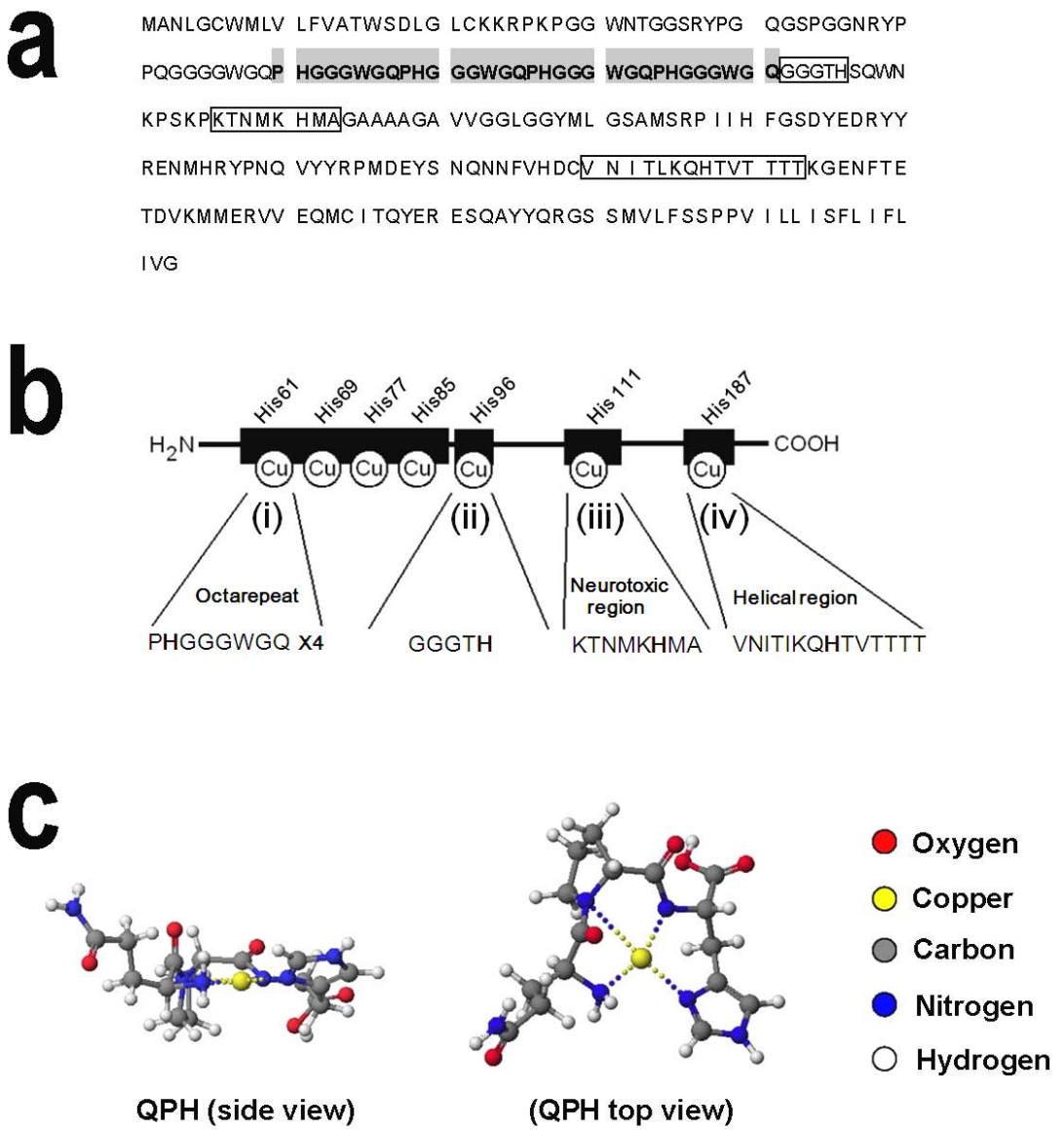

Fig. 1. The Cu-binding site and Cu-binding model of PrP. (a) Cu-binding site in PrP. (b) Amino acid sequence of octarepeat peptide. (c) Likely structure of peptide (QPH)-Cu complex.

copper to PrP in the intermolecular or inter-octarepeat orientations while total occupancy of copper is manifested by intra-repeat binding of copper to the octarepeat region. *In vitro* studies have shown that the actual least motif in the octarepeats necessarily required for binding of copper consists of 4 amino acids H-G-G-G [4] or 5 amino acids H-G-G-G-W [3].

There are additional Cu-binding sites on PrP such as amino acid regions 92-96 (G-G-G-T-H) [3], 124-126 (K-H-M) [9] and 180-193 (V-N-I-T-K-Q-H-T-V-T-T-T-T) [10]. Importantly, all studies suggested that His residue in each region (or each repeat unit) plays a key role in anchoring the copper (Fig. 1b).

Many metalloproteins behave both as oxidants and as antioxidants in biological systems [11-15]. PrP-derived Cu-binding small peptides were shown to be active in both the enhancement of oxidative reactions targeting the cells and the protection of biological components from the oxidative stress, both due to the metal-binding nature of the peptides, depending on the conditions given [16-23].

Therefore, the interaction between Cu^{2+} and PrP (binding of Cu^{2+}) should be carefully monitored for further studying the modes of actions of PrP behaving as a metalloprotein.

Accordingly [1, 24], several diagnostic approaches for detection of human prion disease based on the conformation-dependent immunoassay (CDI assay) were developed. These approaches aim to determine both the proteinase K-sensitive and resistant forms of PrP without the use of proteolytic digestion while all conventional immunoassays for PrP^{Sc} rely on the limited proteolysis to eliminate PrP^{C}. Such CDI technique with enhanced sensitivity is based on the detection of PrP bound on microtitre plates using lanthanide-conjugated monoclonal antibodies [24, 25]. The dissociation-enhanced lanthanide fluoro-immunoassay designated as DELFIA, utilizes the intrinsic fluorescence of specific lanthanides such as europium (Eu), samarium (Sm) and terbium (Tb). In DELFIA, the fluorescent signals for lanthanides are designed to be enhanced in the presence of the fluorescence-enhancing chelates.

In some cases, the fluorescence of lanthanides could be largely altered without such chelates when reacting with metal-binding proteins or peptides. Thus, apart from DELFIA, fluorescent nature of lanthanides, chiefly of Tb^{3+}, has been used for assessing the mode of metal binding to proteins and peptides since such ions of lanthanides mimic the behavior of copper, zinc, manganese and magnesium, and binds to proteins [26].

In this study, we have studied the minimal motifs required for binding of metals (chiefly copper) to the human PrP by assessing (1) the peptide-dependent quenching of Tb^{3+} fluorescence and (2) the copper-dependent quenching of intrinsic fluorescence in human PrP octarepeat-derived peptides.

MATERIALS AND METHODS

Peptides and chemicals

Peptides corresponding to Cu-binding sequence in PrP protein were synthesized to examine the behavior of such Cu-binding domains. The peptides were obtained from the custom peptide service department of Sigma Genosys Japan, Ishikari, Hokkaido, Japan. The amino acid sequences of the peptides chemically synthesized were purified on high pressure liquid chromatography prior to the experimental use.

Other chemicals including terbium (III) chloride hexahydrate, used in this study were of reagent grade purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Fluorometric analysis

Terbium (Tb)-dependent florescence and intrinsic fluorescence from the peptides and from amino acids were detected in phosphate buffered saline using a fluorescence spectrophotometer (F-4500 Hitachi High-Technol. Co., Tokyo) as described [16]. The three-dimensional (3D) spectral measurement of fluorescence was carried out at the excitation wavelength between 200 and 600 nm with 5 nm intervals and emission wavelength between 200 and 600 nm with 5 nm intervals.

In the phosphate-buffered medium, the actual concentrations of free Tb^{3+} after addition of mM orders of $TbCl_3$ were expected to be as low as µM order due to interaction between phosphate and Tb ion. The observed Tb-fluorescence is likely due to free Tb^{3+}, and thus quenching of Tb-fluorescence can be achieved by µM ranges of peptides or chelaters as previously described [16]. Here, quenching of Tb-fluorescence by PrP-derived peptides was performed with 1 mM $TbCl_3$ dissolved in 50 mM potassium phosphate buffer (pH. 7.0) supplemented with and without peptides of interest (up to 30 µM). Intrinsic fluorescence in PrP-derived peptides and free amino acids up to 30 µM dissolved in 50 mM potassium phosphate buffer (pH. 7.0) were also assessed with a fluorescence spectrophotometer in the presence and absence of $CuSO_4$.

RESULTS AND DISCUSSION

Quenching of Tb-fluorescence by octarepeat peptides

As previously reported [16], free Tb^{3+} showed the peaks of fluorescence with excitation wavelength at 224 nm and emission wavelengths at 545 nm and 585 nm (Fig. 2a). The Tb-dependent fluorescence was drastically lowered in the presence of peptides of octarepeat series confirming the metal binding nature of the peptides used (Fig. 2b-d).

Due to fluorescent nature and similarity to behavior of Cu ion, Tb ion has been used as a model for

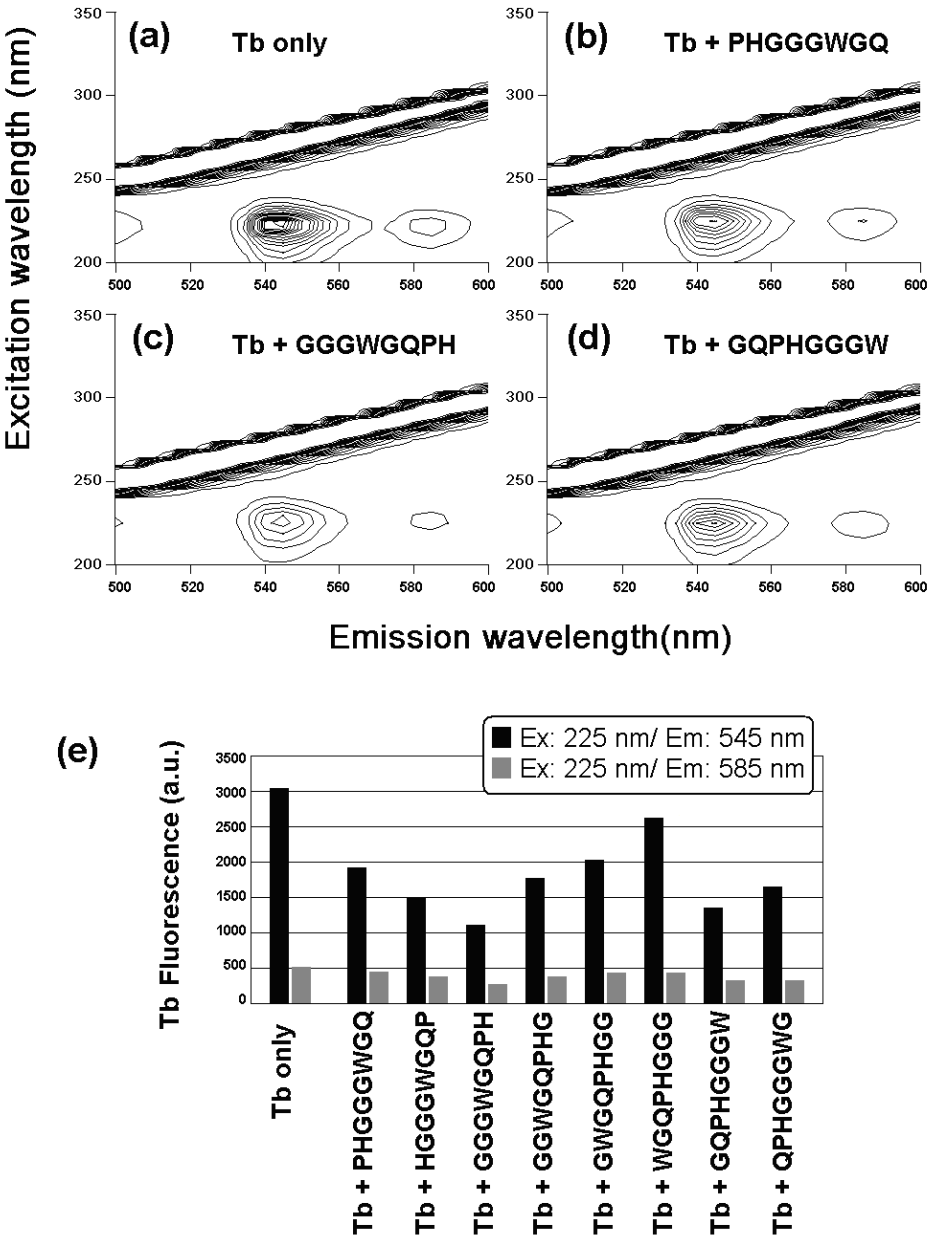

Fig. 2. PrP-dependet quenching of fluorescence in Tb solution. Typical 3D representations (contour plot) of the Tb-fluorescence spectra in the presence and absence of PrP-derived octapeptides are shown in (a) Tb alone, (b) Tb and PHGGGWGQ, (c) Tb and GGGWGQPH, and (d) Tb and GQPHGGGW. (e) Effects of eight different peptide sequences found in PrP octarepeat region on quenching of Tb-fluorescence are compared.

studying the kinetic analysis of metallo-complex formation by short Cu-binding peptides such as a 17 amino acid-sized conatntokin-G [26]. In the present study, the peptide ending with His residue (GGGWGQPH) showed highest activity for quenching of the Tb-fluorescence among eight different octapeptides tested (Fig. 2e).

Peptides showed intrinsic fluorescence in the absence of Tb

In the absence of fluorescent REE ions such as Tb^{3+}, the PrP-derived peptides tested here showed intrinsic fluorescence (Fig. 3a). The fluorescent nature of octapeptide could be attributed to the presence of Trp residue. Among amino acids

constituting the octarepeat, only tryptophan showed major (excitation at 280 nm, emission at 365 nm) and minor (excitation at 230 nm, emission at 365 nm) peaks of intrinsic fluorescence (Fig. 3b-g). Comparison of fluorescence between the Trp-containing peptides and free Trp suggested that fluorescence by Trp is largely lower by conjugation with other amino acids.

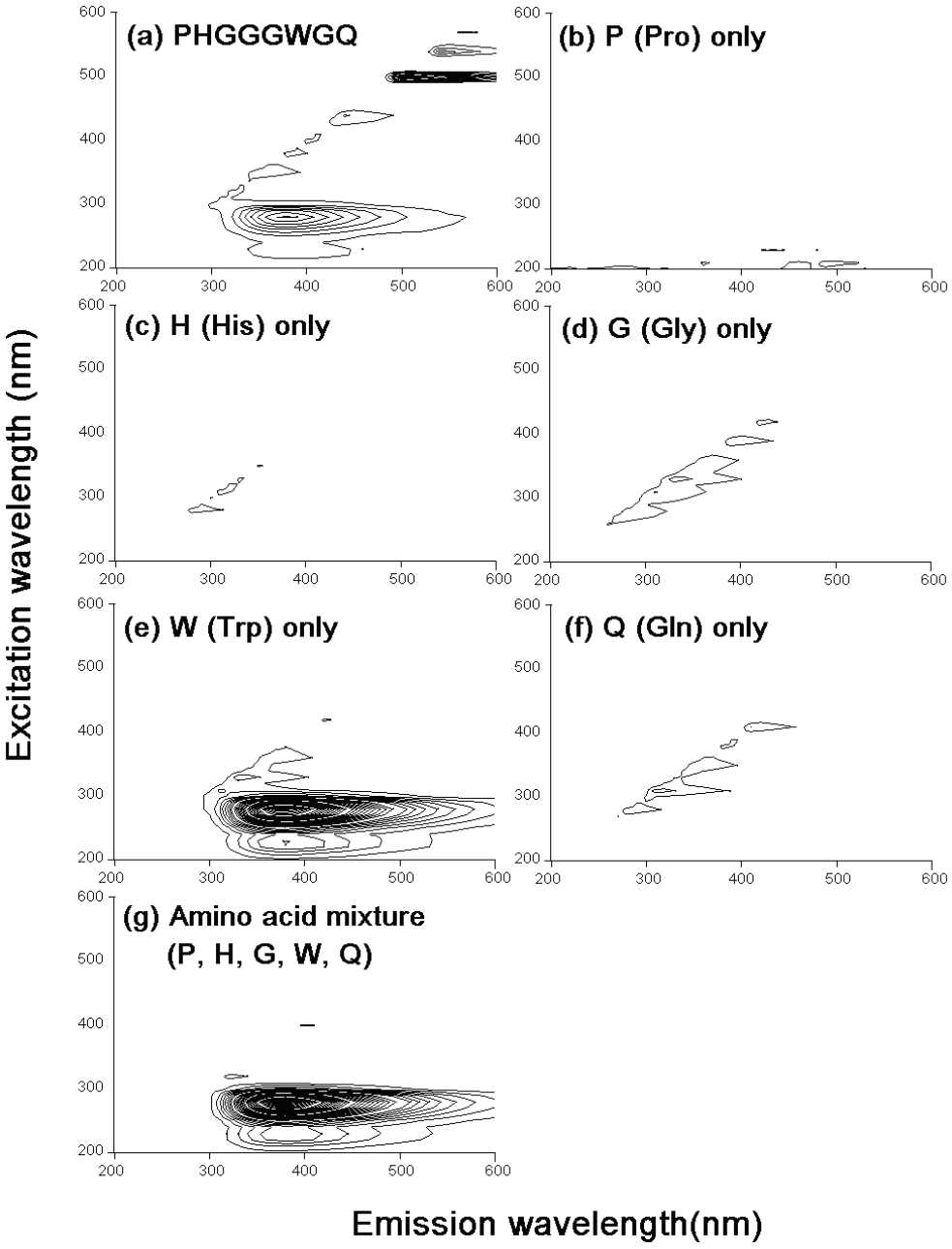

Fig. 3. Intrinsic fluorescence in octarepeat peptide and free amino acids. Typical 3D-representations (contour plot) of the Peptide-FL and W-FL are shown. Typical results for octapeptide PHGGGWGQ (a), free proline (b), free histidine (c), free glycine (d), free tryptophane (e), free glutamine (f), and mixture of five amino acids (g). Concentration of the octapeptide and free proline, histidine, tryptophane, and glutamine were 30 µM. Concentrations of free glycine was 30 µM (d) or 120 µM (g).

As expected, the PrP octarepeat-derived short peptides lacking Trp residue showed no fluorescence, further confirming the role of Trp residue for fluorescence (Fig. 4). Again, data suggested that Trp-residue is required for the fluorescent nature of the octarepeat peptides and that conjugation of longer chains to tryptophan lowers the intrinsic fluorescence.

Quenching of peptide fluorescence by copper

As the characteristics of fluorescence which is intrinsic to the human PrP-derived octarepeat peptides were spectroscopically determined above, the impact of Cu-binding to the peptide on the yield of fluorescence was tested (Fig. 5). The intrinsic fluorescence in the octarepeat sequence-derived peptides and free tryptophan was shown to be quenched in the presence of excess of $CuSO_4$. The presence of Cu^{2+} in the range of molar ratios (Cu^{2+}/peptide) between *ca.* 0.2 and 1.0 showed linear decrease in fluorescence from the most peptides tested, suggesting that the mode of interaction between the PrP-derived peptide and metal ions can be optically monitored.

Kinetic analysis revealed that fluorescence from the PrP octarepeat-derived octapeptides showed higher sensitivity to the presence of low concentration of Cu ion (Fig. 5). Among the octapeptide sequences tested, PHGGGWGQ peptide was shown to be most sensitive to the low Cu concentration although this sequence lack the presence of metal-binding motif X-X-H. In turn, the role or significance of another motif, namely, H-G-G-G-W was suggested. It must be noted that our discussion does not cover the behavior of shorter Cu binding motif H-G-G-G reported by Bonomo *et al.* [4]. Since fluorometric analysis is based on the Trp-dependent fluorescence, Cu-mediated in the tetrapeptide HGGG could not be assessed by our approach.

Two distinct metal-binding motifs overlaid in the PrP octarepeat region

According to earlier studies, at least single His residue is required for binding of copper on PrP-derived peptides [16, 17], and the catalytically active copper-binding motif within PrP-derived peptides was determined to be X-X-H (where X can be any

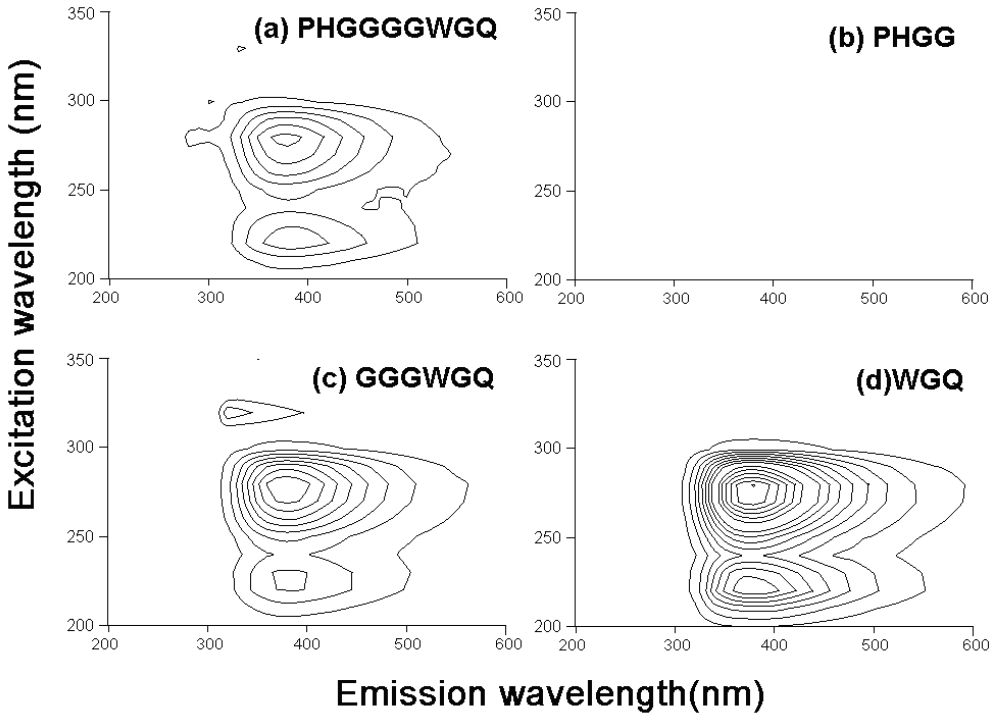

Fig. 4. Comparison of intrinsic fluorescence in PrP octarepeat-derived short peptides. (a) Octapeptide PHGGGWGQ, (b) tetrapeptide PHGG, (c) hexapeptide GGGWGQ, (d) tripeptide WGQ.

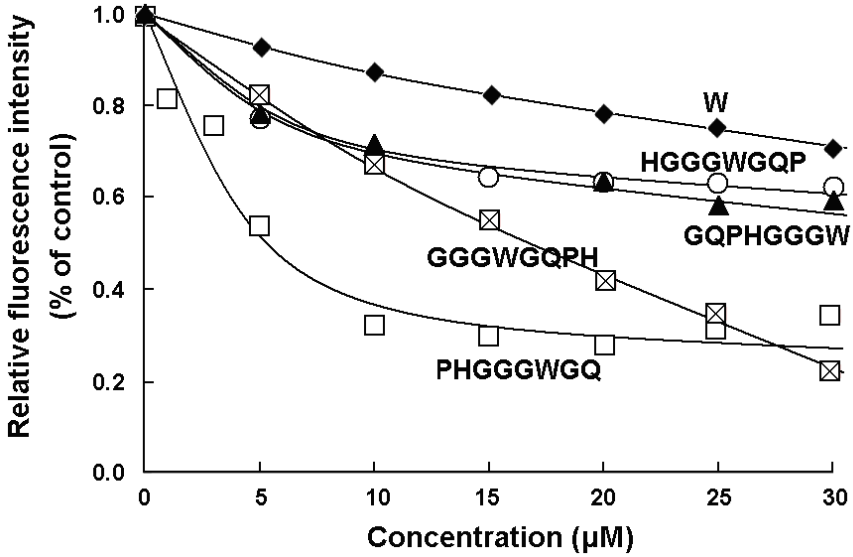

Fig. 5. In the presence of 30 μM peptides or tryptophan, fluorescence quenching action of copper at various concentrations (up to 30 μM) was examined.

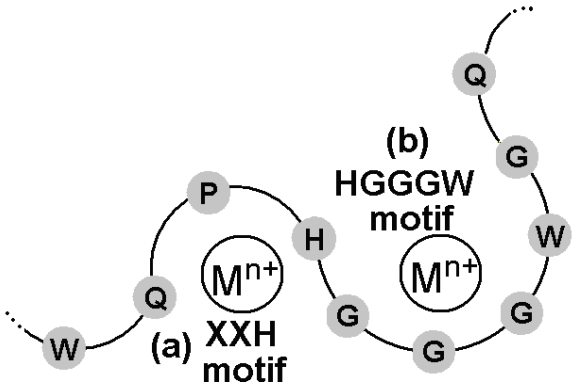

Fig. 6. Proposed involvement of overlapped two distinct motifs in the octarepeat region in binding to metal cations. (a) X-X-H motif [15, 16, 19] suggested to bind Tb in the Tb-fluorescence assay. (b) H-G-G-G-W motif [3] suggested to bind Cu in the peptide-fluorescence assay. M^{n+}, metal cations.

amino acids followed by His) [15, 19]. Furthermore, our demonstrations have shown that the PrP-derived and related short peptides ended with X-X-H motif, effectively bind Cu in the biological media and thus prevent the damaging impacts of Cu ions against DNA degradation [23] and living plant cells [19]. Okobira *et al.* [27] has demonstrated that Cu-binding peptides with the X-X-H motif conjugated to organic materials could form a novel class of bioengineering tools. Accordingly, a tripeptide, glycyl-glycyl-histidine (G-G-H, one of X-X-H motif derivatives) as Cu-binding domain, was introduced onto the glycidyl methacrylate-grafted porous hollow fiber membrane made on the polyethylene platform by radiation-induced graft polymerization.

On the other hand, *N*-terminal His-started oligo-peptides were also used as model for Cu-binding in earlier *in vitro* studies describing that the actual least motif in the octarepeats necessarily required for binding of Cu consists of 5 amino acids H-G-G-G-W [3] or 4 amino acids H-G-G-G [4]. Therefore, in the mammalian PrP octarepeat regions, in which PHGGGWGQ is repeated for four (human) to six (bovine) times, both metal binding motifs could be overlaid by sharing common His residue and thus co-existed.

Our fluorescent assay with Tb-fluorescence quenching supported the positive role of His-ended peptides. The obtained data clearly supported the view that the peptides sharing an intact X-X-H motif (in this case PQH motif) located at *C*-termini rather than N-terminal H-G-G-G-W motif, is desirable as metal chelating peptides.

Controversially, the role of Pro-started motif followed by (H-G-G-G-W) was supported by the Cu-dependent peptide fluorescence quenching assay.

Among the octapeptide sequences examined, the PHGGGWGQ peptide was shown to be the most sensitive to the low Cu concentration although this sequence lacks the presence of intact metal-binding X-X-H motif.

The present study suggested that there are two distinct modes of metal binding to His residues in the octarepeat regions in mammalian PrP, possibly by co-ordinations of His-ended motif (Fig. 6a) or His-started motif (Fig. 6b) around the target metals depending on the conditions given. The proposed two-motif model must be further testified by future experiments.

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