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Review

Specificity in the Cu^{2+} interactions with prion protein fragments and related His-rich peptides from mammals to fishes

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This paper is dedicated to our great friend Prof. Helmut Sigel from University of Basel on the occasion of his 70th birthday with best wishes for the future challenges.

Contents

Abstract

The prion proteins may play a critical role in copper homeostasis and the antioxidant activity in the brain. This review presents the state of art in the studies on Cu^{2+} prion systems. The proteins discussed are from different species from mammals to fishes. All proteins are His-rich and the research discussed clearly indicates the basic role of imidazole side chains and the adjacent amide nitrogen atoms in metal ion binding. Prions represent the family of proteins with new mode of Cu^{2+} binding which includes the amide nitrogen coordination. The multi-imidazole coordination is also likely and it can play a critical role in the antioxidant activity of the copper–prion complexes. The combination of the imidazole and amide nitrogen atoms to Cu^{2+} ions could also be relevant in histidine-rich peptide antibiotics including demegen. The impact of peptide sequence and His positions on copper binding ability is also discussed.

Keywords: Prion proteins; Binding of Cu^{2+}; His-rich peptides

1. Introduction

Prion diseases are fatal neurodegenerative disorders including spongiform encephalopathies in cattle and sheep, *Kuru* and Creutzfeld-Jacob diseases (CJDs) in humans. Biological functioning of prion protein (PrP) is still not well understood. However, it is generally accepted that prions may play a critical role in the copper homeostasis and the copper-based antioxidant enzymatic activity in the brain [1–3].

The name of prion was proposed in 1982 by Prusiner and his colleagues who purified an abnormal protein from the brains of mice experimentally infected with the scrapie disease [4]. Only by 1990 most people accepted that the cause of the transmissible spongiform encephalopathies was this abnormal form of protein.

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The strong evidence emerged only in 2004 [5]. Recombinant protein injected into mice brain resulted in prion disease that could be transmitted to other mice.

Some forms of prion diseases are inherited like, e.g. Gerstmann–Sträussler–Scheinker syndrome linked to point mutation in the prion gene. The most popular disease became recently described, variant CJD (vCJD) linked to the eating of food contaminated with the bovine spongiform encephalopathy (BSE) agent, although the correlation between contaminated-meat ingestion and vCJD was not fully demonstrated.

Cu binds to cellular form of PrP (PrP^C) *in vivo* [6]. The metal ion coordination occurs in the repeat regions, which are octapeptide repeats in mammals and hexapeptide repeats in birds. The studies with mutant PrP protein lacking one, two or three octarepeats have shown clearly that Cu^{2+} binding is proportional to the number of repeats [7]. Several other authors were suggested that Cu^{2+} binds within the structured C-terminal domain [8,9]. However, there is no real evidence supporting its biological relevance [2,10].

Prion is not only a characteristic protein for mammals. It was also found in many other species including birds and fishes. The studies on non-mammalian proteins are at very early stages and there is no strong experimental support indicating that binding of metal ions (e.g., Cu^{2+}) is biologically relevant. However, these proteins also contain the tandem repeat regions which are rich in His and Gly residues, as it is the case in mammalian prions. This review will present the specific interactions of Cu^{2+} ions with several prion proteins derived from mammals, birds and two types of fishes, fugu and zebra-fish.

2. Binding of Cu^{2+} by the octarepeat units of mammalian prion protein: $(\text{-Pro-His-Gly-Gly-Gly-Trp-Gly-Gln-})_n$

The octapeptide repeat domain is located in the unstructured N-terminus of PrP comprising of about 100 amino acid residues (Fig. 1) [11–13].

The single octapeptide unit – PHGGGWGQ – contains one effective anchoring site for Cu^{2+} ion, an imidazole nitrogen donor [14]. Cu^{2+} anchored at imidazole nitrogen is able to deprotonate amide nitrogen(s) creating very efficient coordination mode $\{N_{imid}, nN^-\}$. The major complex dominating at pH around 7–8 is the species in which metal ion is bound by imidazole and two amide nitrogen atoms (Fig. 2) [15]. This coordination mode was also shown in the solid state complex studied by the X-ray method [16].

The binding pattern of Cu^{2+} to longer repeat units, dimeric or tetrameric octapeptide repeats is distinctly different than that found for the single octapeptide, especially within physiological pH range. The presence of two or four imidazoles within the peptide sequence allow metal ion to form poly-imidazole binding mode, which in the case of octapeptide tetramer, Ac-$(PHGGGWGQ)_4$-NH_2, dominates at pH 7–7.5 (Fig. 3) [17]. In this case four imidazoles derived from peptide tetramer are involved in the metal ion binding. Below pH 6 two species are observed in which Cu^{2+} ion is bound to two (CuH_2L) and three (CuHL) imidazoles, respectively. The binding of one

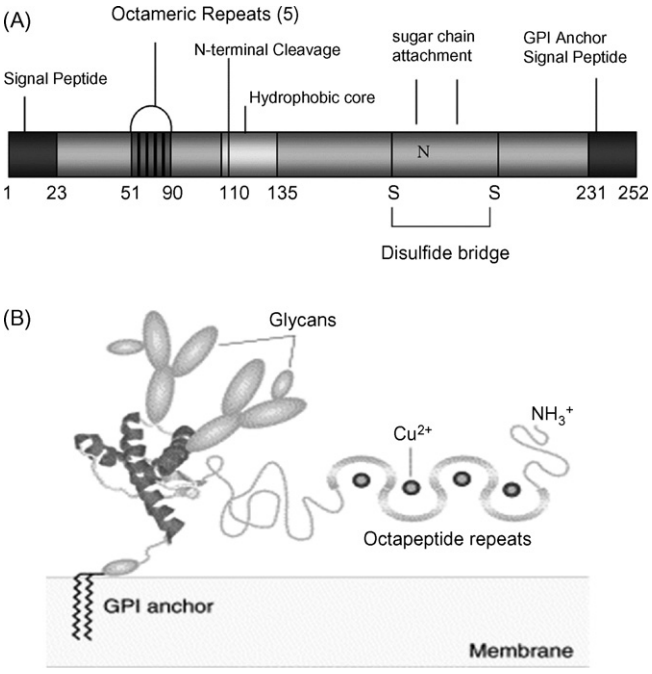

Fig. 1. Schematic representation of PrP. (A) Linear representation of the PrP sequence. Numbers are based on the mouse sequence. (B) Graphic representation showing the secondary structure of the globular C-terminal and N-terminal unstructured domains. Protein is anchored to the cell membrane by a GPI anchor.

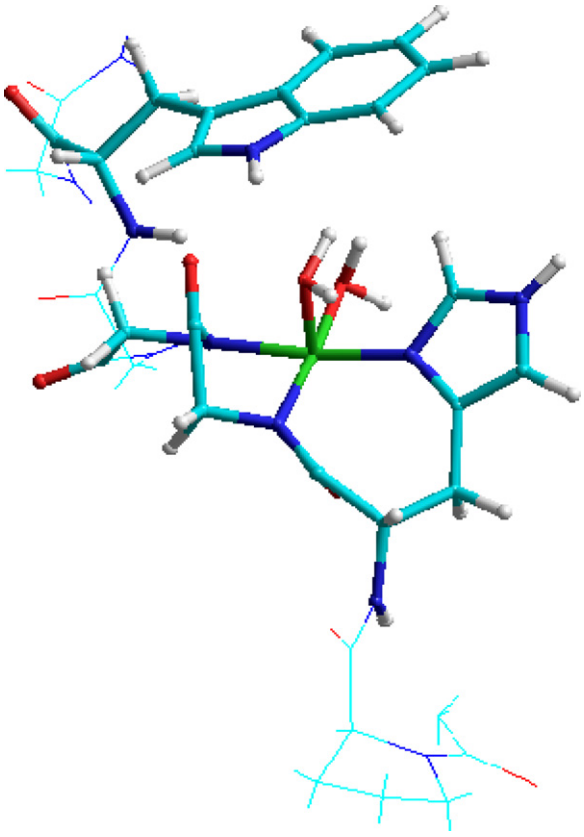

Fig. 2. Molecular structure for the $CuH_{-2}L$ complex in solution for the Ac-PHGGGWG-NH_2 peptide.

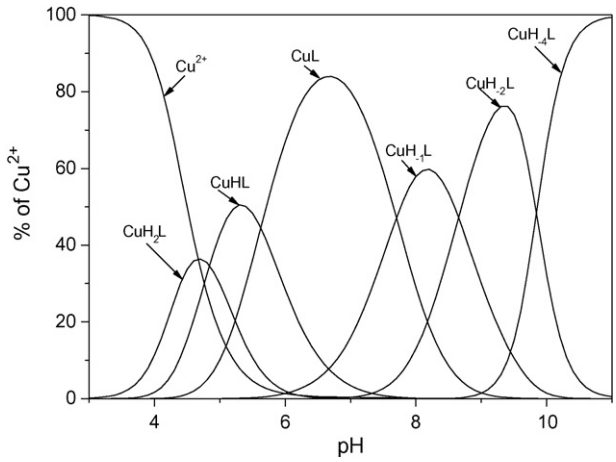

Fig. 3. Species distribution profile for Cu^{2+} complexes of Ac-(PHGGGWGQ)$_4$-NH_2. Metal to ligand ratio 1:1.

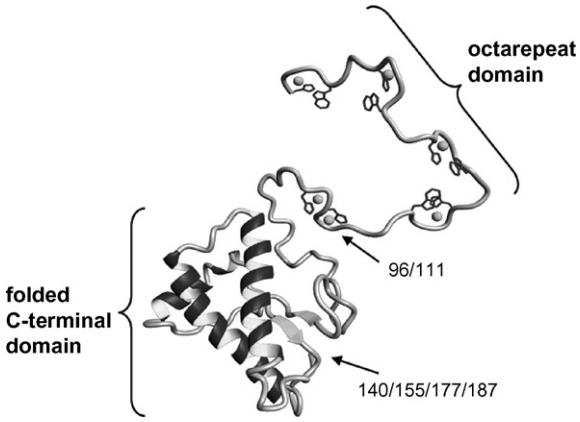

Fig. 4. Models of three-dimensional structure of PrP(61–231) with coppers included. (Reproduced with permission from Ref. [25]).

($CuH_{-1}L$), two ($CuH_{-2}L$) or three ($CuH_{-4}L$) amide nitrogen atoms are observed above pH 7. The $CuH_{-1}L$ species involving poly-imidazole donor set and one amide nitrogen donor coexists with the major CuL species in the physiological pH range (Fig. 3). This coordination pattern dominates for low concentrations of copper. The excess of Cu^{2+} ions makes metal ion binding very similar to that found for the monomeric unit. Each imidazole may act as the anchor for the metal ion and the tetrameric domain may bind up to four Cu^{2+} ions increasing its rigidity and structural features distinctly (Fig. 1 scheme B) [2].

3. Binding of the fifth and sixth copper ion to PrP

According to Brown et al. [6] PrP may bind more than four copper ions *in vivo*. The binding of copper ions within the octarepeat region and its biological relevance seems to be accepted by most of the groups involved in this subject, although some controversy still exists. Much more controversial is the idea and possible biological implications of the binding to fifth and sixth sites in so called the neurotoxic region of PrP [2]. There are two other His residues in the unstructured N-terminal tail towards the C-terminal globular domain. The region comprising PrP106–126 residues was shown to be highly fibrillogenic, resistant to proteinase K and toxic to neurons *in vitro* [18,19] and *in vivo* [20]. PrP106–126 behaves very similarly to scrapie PrP (PrP^{Sc}), a pathologic isomer of PrP and that is why it is very interesting for various studies [21,22]. Jobling et al. [23] have shown that metal ions like Zn^{2+} and Cu^{2+} interacting with PrP106–126 may induce peptide aggregation, which could be abolished by adding chelating agents. Thus, binding of Cu^{2+} ions to this region could have also some biological implications.

In 2001 EXAFS data were published with the suggestion that His-111, Met-109 and His-96 are the coordinating residues [24]. Burns et al. based on the EPR studies suggested that only one His residue (His-96) is involved in the binding of Cu^{2+} [25], while CD data suggested binding of Cu^{2+} ions to His-96 and His-111 simultaneously or independently from each other but with different affinity [26,27]. His-111 site was suggested to have much higher affinity (70%) to bind metal ion than His-96 residue (30%) [27] (Fig. 4).

The PrP106–126 fragment comprising residues: KTNMKHMAGAAAAGAVVGGLG has clearly two distinct domains the N-terminus (KTNMKH) able to bind metal ions using, e.g. imidazole and/or amino (when unprotected) nitrogen anchor and strongly hydrophobic tail starting from Met-112 till C-terminal Gly-126. This hydrophobic sequence is critical for the aggregation process. NMR studies have shown that PrP106–126 with unprotected N-terminal amino group coordinates Cu^{2+} ion *via* amino group and imidazole nitrogen of His-111. This coordination is completed with the amide nitrogen(s) [28]. Although the hydrophobic tail does not coordinate directly to Cu^{2+} ion, the metal ion binding at the N-terminus influence distinctly the structure of the hydrophobic domain (Fig. 5). This induced structure may be more efficient to interact with the other peptide hydrophobic chains. Zn^{2+} ions bind

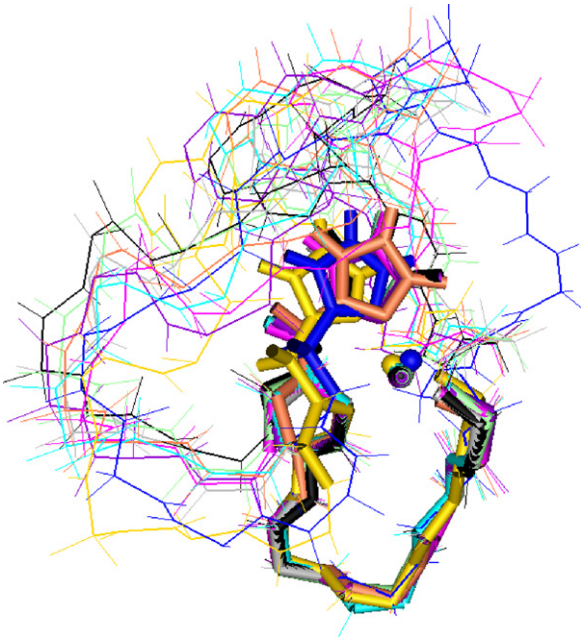

Fig. 5. NMR structure of Cu(II) complex of prion neurotioxic peptide.

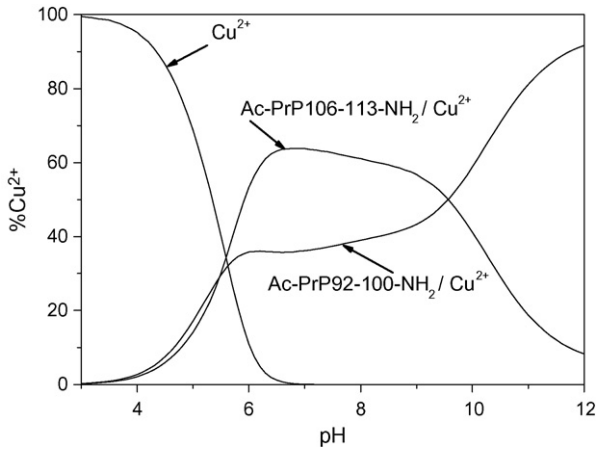

Fig. 6. Competition between Cu(II)-Ac-hPrP$_{106–113}$-NH$_2$ and Cu(II)-Ac-hPrP$_{92–100}$-NH$_2$ complexes.

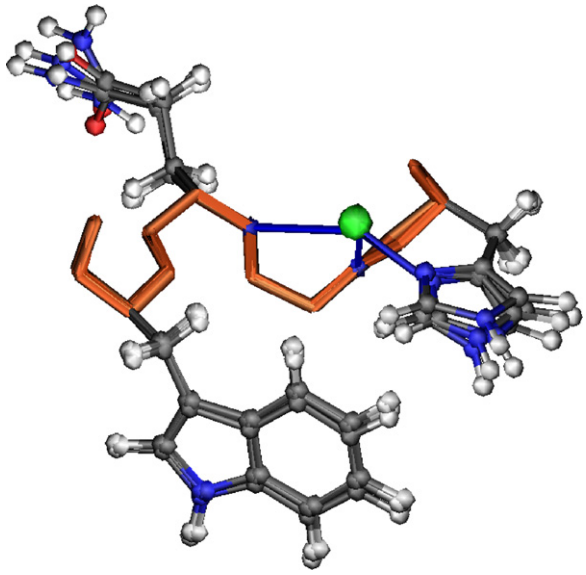

Fig. 8. Structures of the Cu(II)-Ac-hPrP$_{106–113}$-NH$_2$ complex obtained from DYANA simulation.

PrP106–126 much less effectively and their interactions with peptide could not be easily seen in the NMR spectra, while Mn^{2+} ions clearly affected NMR spectra due to the strong interactions with carbonyl groups of Gly-124 and Leu-125 and indirect interactions with His-111 imidazole [28]. These interactions, especially those with carbonyls could be of importance for the neurotoxicity mechanism.

In the direction of N-terminus from His-111 there is another histidyl residue His-96 (human PrP, hPrP). The studies on the hPrP91–120 peptide encompassing both His-96 and His-111 and its subunits including hPrP92–100 and hPrP106–113, have clearly shown that both His residues may act as the binding site for Cu^{2+} ions [29]. The potentiometric and NMR studies at room temperature were consistent with the dominating binding site centered at His-111 (60–70%) when compared to His-96 site (Figs. 6 and 7). The EPR studies performed at liquid nitrogen temperatures have shown just the opposite result. This apparent disagreement was clarified by temperature dependent molecular dynamic calculations [29]. These calculations demonstrated that Met-112 is approaching the metal ion center at room temperature, stabilizing the His-111 binding site through hydrophobic shielding of the copper coordination core. When temperature decreases sulfur of Met-112 drives away from metal ion center allowing the adjacent His-96 site to be more competitive as EPR data have indicated [16,29]. The impact of Met-112 on Cu^{2+} ion binding is quite unusual. Not only it stabilizes the His-111 binding site but also encourages the metal ion binding towards the C-terminus. Cu^{2+} anchored at imidazole nitrogen of His-111 binds amide nitrogen donors of Met-112 and Ala-113 (Fig. 8) [29]. This binding mode was not considered as thermodynamically relevant due to formation of 7-membered chelate ring. However, the Cu^{2+} binding to hPrP octarepeat unit has clearly shown that such binding pattern is quite a realistic one [10].

4. Chicken prion protein

Chicken prion protein (chPrP) was extracted from the brain of domestic fowl as a homolog of human PrP [30]. The amino acid identities of cloned mammalian and avian PrPs agree between 31 and 34%. This relatively high sequence homology leads to very similar protein architectures from both species including the three α-helices and two anti-parallel β-sheets (the common topology core of PrP among vertebrates) and in the case of avian PrP, additionally one short 3_{10} helix (Fig. 9) [12,31]. It is interesting to note that the homology is higher when only the C-terminal regions are compared, due to lower identity within the N-terminal domains. However, despite the differ-

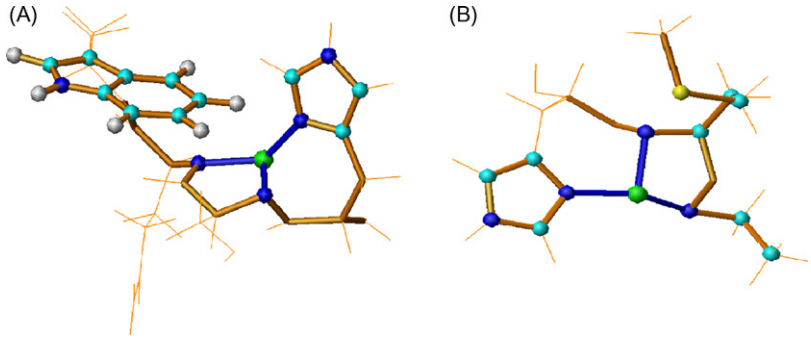

Fig. 7. Structures of (A) Cu(II)-Ac-hPrP$_{92–100}$-HN$_2$ complex and (B) Cu(II)-Ac-hPrP$_{106–113}$-NH$_2$ complex obtained from molecular dynamic simulations and energy minimization.

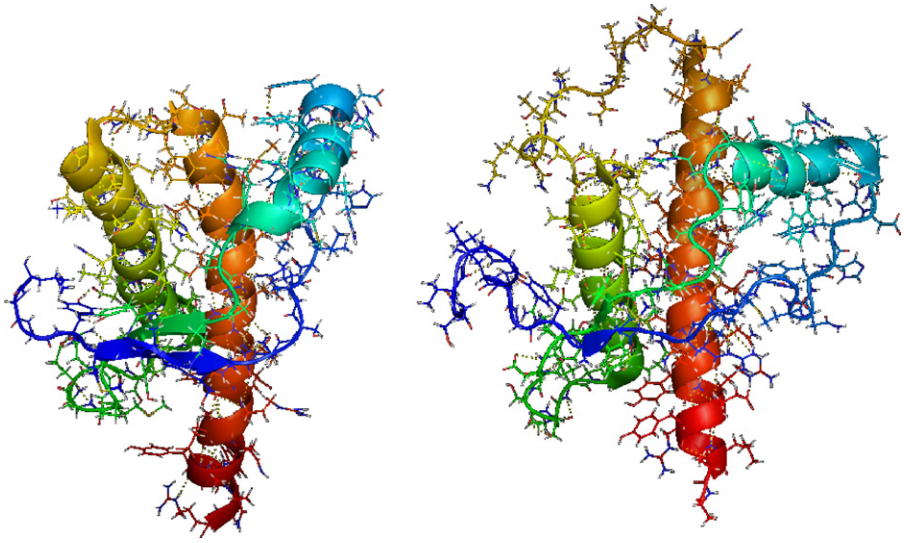

Fig. 9. The similarity of the C terminal regions architecture of human PrP (left picture) and chicken PrP (right picture) [12,30].

ent primary structures of the N-terminal domains, both type of proteins display common features, e.g. disordered conformation, what may implicate their potential physiological function. This can have some relationship to the potential biological activity of the PrP repeat domain like neuronal superoxide dismutase (SOD) [32–35] or PrP induced endocytosis resulting in transport copper from extracellular space to the cellular interior [16,36,37], copper buffering [38] copper sensing [39] and copper-reductase activity [40]. The substantial homology between the avian and mammalian versions of the prion proteins makes it likely that the conclusions concerning the biological functions might be similar to each other [41]. Chicken PrP similarly to mammalian PrP possesses repeated segment, with characteristic hexa-peptide—PHNPGY unit [29]. The coordination pattern of chicken hexapeptide depends strongly on the number of hexapeptide units. Single hexapeptide unit binds copper ion in the physiological pH by $\{N_{im}, N^{-}\}$ donor sets in the main *trans*/*trans* amide bond isomer [42–44]. The involvement of the consecutive amide nitrogen atoms in metal ion chelatation is disrupted by the presence of Pro residue between Asn and Gly residues. Dimeric and tetrameric hexa-peptide units behave differently to its monomeric analog, forming as in the case of human octapeptide region, multi-imidazole bound complexes. These stable complexes involve $\{2N_{im}\}$ and $\{4N_{im}\}$ coordination patterns, respectively (Fig. 10). The comparison of the binding ability of oligomeric hexa-peptide units seems to

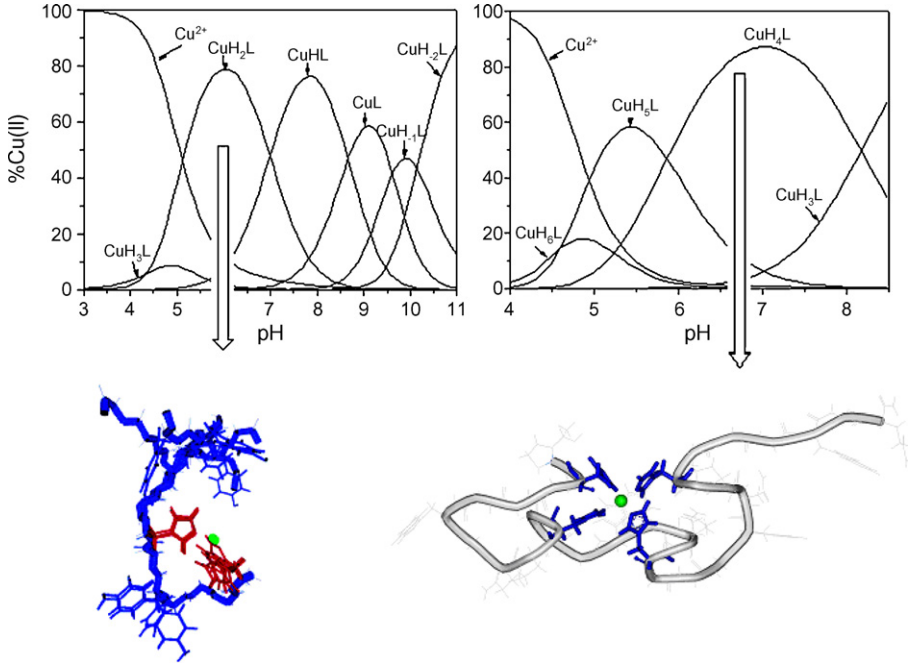

Fig. 10. Species distribution profiles for Cu(II) complexes of Ac-$(HNPGYP)_2$-NH_2 (left picture) and Ac-$(HNPGYP)_4$-NH_2 (right picture) as well as their main complexes structures [41,43].

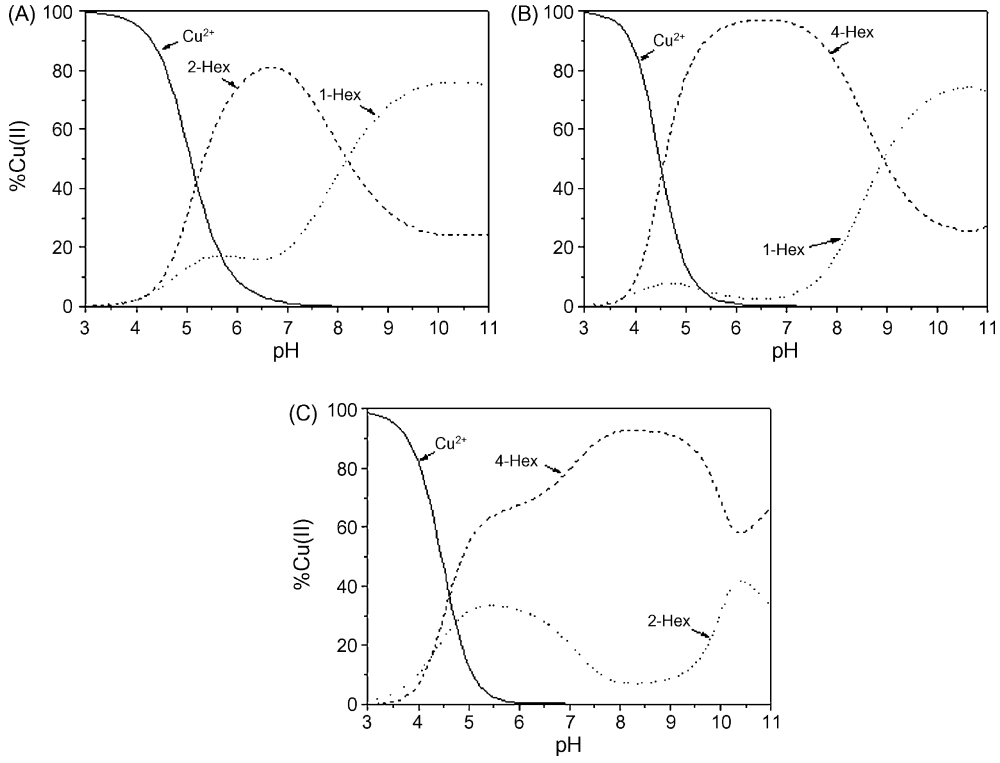

Fig. 11. Distribution profiles of competition between 1-Hex (Ac-HNPGYP-NH_2) and 2-Hex (Ac-$(HNPGYP)_2$-NH_2) (A), 1-Hex (Ac-HNPGYP-NH_2) and 4-Hex (Ac-$(HNPGYP)_4$-NH_2) (B) and 2-Hex and 4 Hex (C), in coordination one copper ion [41].

indicate the cooperative effects when compared (i) to the single hexa-peptide unit (Fig. 11A and B) and (ii) to each other (Fig. 11C).

The multi-imidazole coordination mode might be critical for the biological implications, e.g. for the SOD enzyme activity [45]. The human repeated domain is, however, more effective in binding Cu^{2+} ions than chicken analogous region and also octapeptide single unit is much more effective copper chelator than chicken monomeric hexapeptide (Fig. 12).

5. Fish analogues of prion protein

Several sequences of PrP-like cDNAs have been described for less advanced vertebrate classes, including reptiles, amphibians and fishes [46–50]. Apart from the fact that scrapie infectivity is quickly cleared in tissues of orally infected farm fish (e.g., rainbow trout or turbot) there is not much known about the fish prion proteins [51]. Especially, the interaction of fish prion protein analogues (belonging to superfamily of tetrapod prion proteins) with copper ions is very poorly described. The cloning of cDNAs from Japanese Takifugu (*Fugu rubripes*) have revealed the sequences of two homologues to tetrapod PrP defined as "similar to PrP", st1PrP and st2PrP [48]. The 425 amino acid residue protein st2PrP reveals many features common with the prions family including pI = 9.14, the presence of signal sequence, two typically located cysteine residues, potential glycosylation sites, supposed GPI anchor and irregular repeated domain termed Gly-Pro rich region encompassing residues 96–128. The interactions of Gly-Pro rich region with Cu^{2+} ions display many

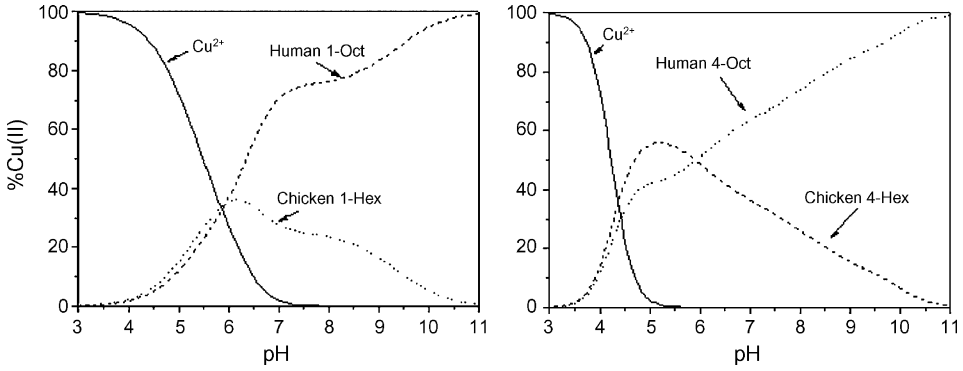

Fig. 12. Distribution profiles of competition between chicken 1-Hex (Ac-HNPGYP-NH_2) and human 1-Oct (Ac-PHGGGWGQ-NH_2) (left panel); chicken 4-Hex (Ac-$(HNPGYP)_4$-NH_2) and human 4-Oct (Ac-$(PHGGGWGQ)_4$-NH_2) (right panel) in coordination one copper ion [41].

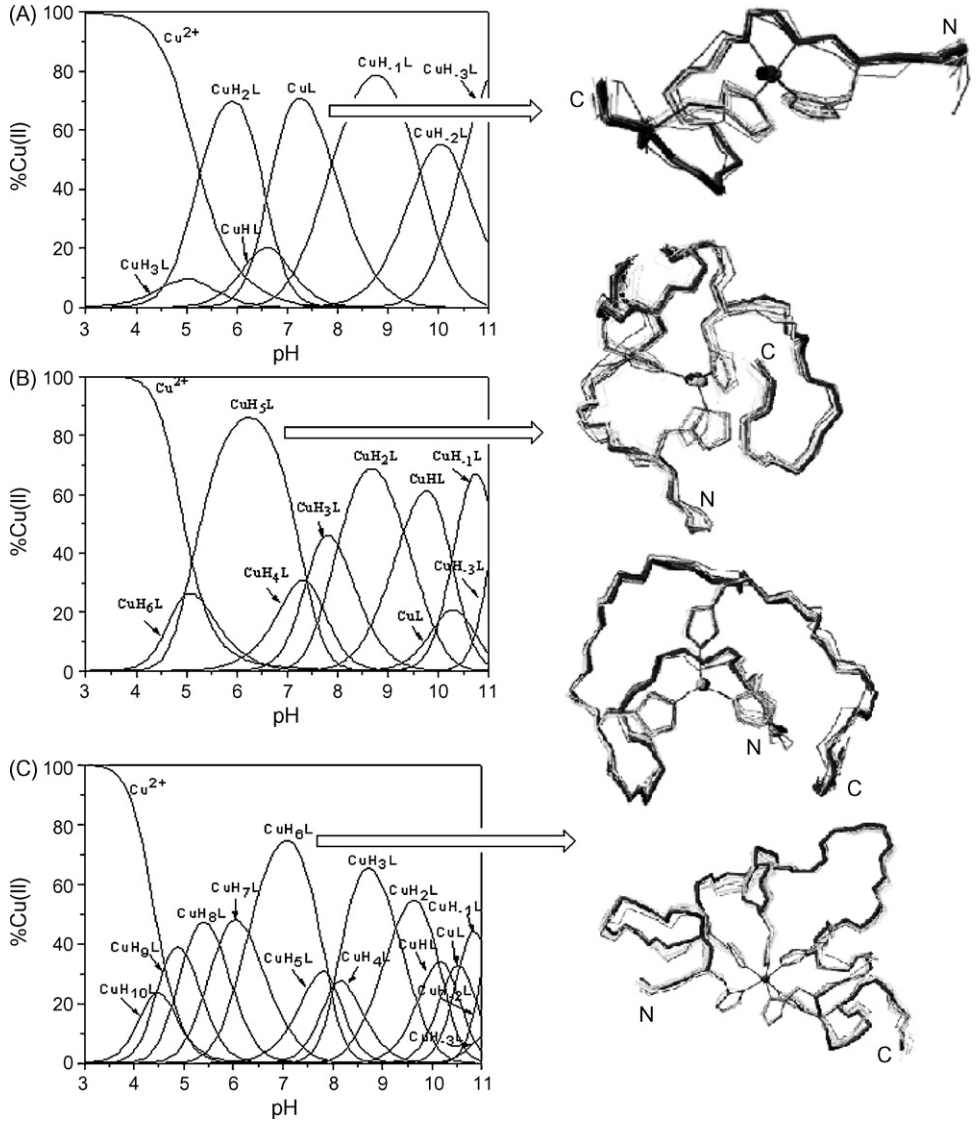

Fig. 13. Species distribution profiles for Cu(II) complexes of st2PrP96–104 (A), st2PrP96–116 (B) and st2PrP96–128 (C) as well as their main complexes structures [51].

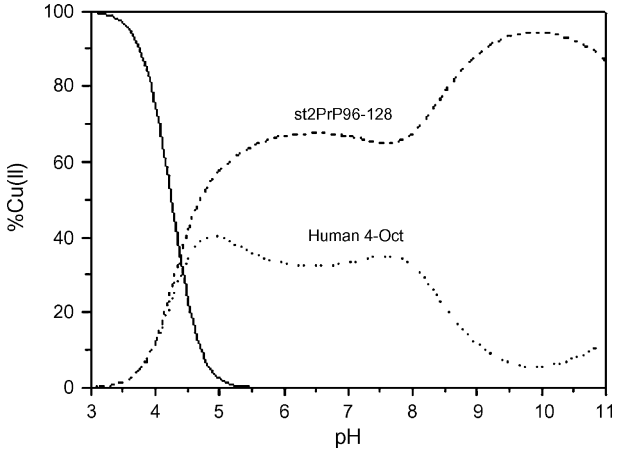

Fig. 14. Distribution profiles of competition between human 4-Oct (Ac-(PHGGGWGQ)$_4$-NH$_2$) and st2PrP96–128 in coordination one copper ion [51].

mutual relationships observed previously in tetrapod PrPs [52]. St2PrP96–104 fragment peptide from peptide-repeat domain binds Cu^{2+} at pH 7.4 *via* $\{2N_{im}, 2N^-\}$ donor set (Fig. 13A), that is much more effective than the binding by human single octapeptide unit, due mostly to the presence of two His residues. Longer fragments (st2PrP96–116 and st2prP96–128) form around physiological pH the multi-imidazole complexes (Fig. 13B and C), as was found in the mammalian and avian PrPs. Such coordination pattern is a favored binding mode for low Cu^{2+} concentrations. Higher concentrations and higher pH favor the involvement of amide nitrogen atoms in the coordination process. The comparison of the binding ability of the whole tandem repeat domain from Takifugu and human PrPs clearly shows that the fish protein binding could be much more effective than the human one (Fig. 14).

The presence of Gly-Pro rich region was found in PrP-like protein from another kind of fish–zebrafish (*Danio rerio*) [53,54]. This 188 amino acid residues protein also possesses

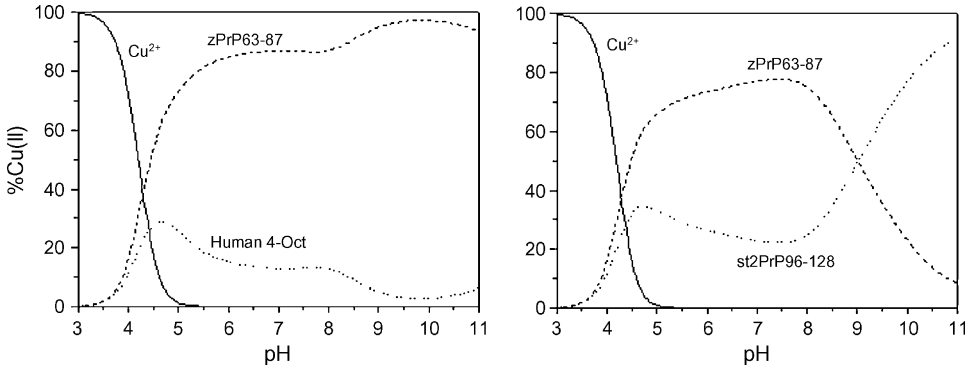

Fig. 15. Distribution profiles of competition between zebrafish PrP Gly-Pro rich domain (zPrP63–87) and human 4-Oct (Ac-(PHGGGWGQ)$_4$-NH$_2$) (left panel) and between zPrP63–87 and st2PrP96–128 (right panel) in coordination one copper ion [54].

a very conservative hydrophobic domain (HD) (similar to fish HD but distinctly differ than mammals and birds), two signal sequences, GPI anchor and one glycosylation site. The impact of copper ion on the structure and complexes stability of zebrafish PrP shows that fish analogues of prion protein have the same features, e.g. are more stable than human or chicken PrPs copper complexes and moreover they form multi-imidazolic complexes in physiological pH range [55]. It is worthy to note, that the binding ability of zebrafish PrPs is even more effective than the fugu protein (Fig. 15).

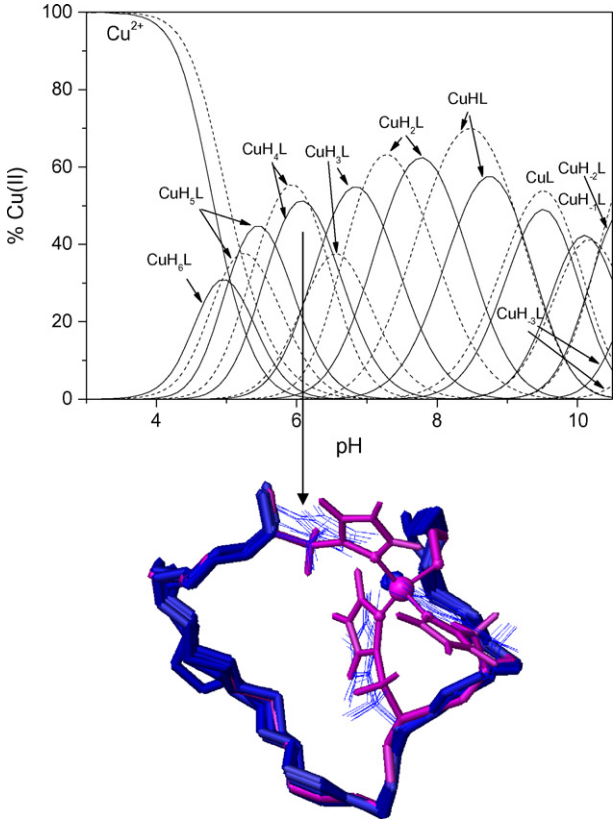

Fig. 16. Species distribution profile for Cu^{2+} complexes of DMG-NH$_2$ (solid line) and Ac-DMG-NH$_2$ (dashed line) and the superimposition of the structures of the multi-imidazolic Cu^{2+}-DMG-NH$_2$ complex.

6. Coordination properties of demegen P-113 towards Cu^{2+} ions

Demegen P-113 (DMG-NH$_2$) belongs to the family of the antimicrobial peptides [56]. It is the smallest fragment of histatin 5 with preserved antifungal activity, especially against *Candida albicans*, *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis* [57,58]. The histatin 5 antimicrobial activities are closely bound to its metal ion binding ability. Dodeca-peptide (DMG-NH$_2$): AKRHHGYKRKFH-NH$_2$ possessing three His residues in its sequence, is also very attractive ligand for metal ions, especially Cu^{2+}. Presence of His-rich domains is similar to those discussed above for prion proteins, therefore the study of the interactions of DMG and its analogues with metal ions can contribute to understanding of the crucial role of poly-imidazole and amide centers in the protein coordination processes.

Thermodynamic and structural studies of the Cu^{2+} complexes of DMG-NH$_2$ and its analogues allowed to determine the participation of imidazole nitrogen atoms in metal binding, especially the role of two adjacent His residues at positions 4 and 5 (Fig. 16) [59]. Comparison of the parent DMG-NH$_2$ with the modified

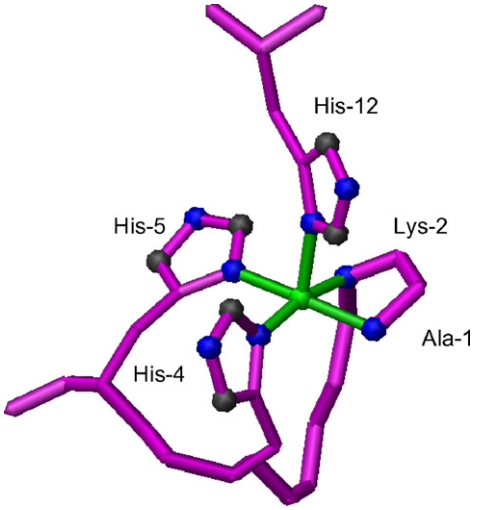

Fig. 17. Energy minimized structure of Cu^{2+}-DMG-NH$_2$ complex showing the $\{3N_{im}, NH_2, N^-\}$ metal donor set.

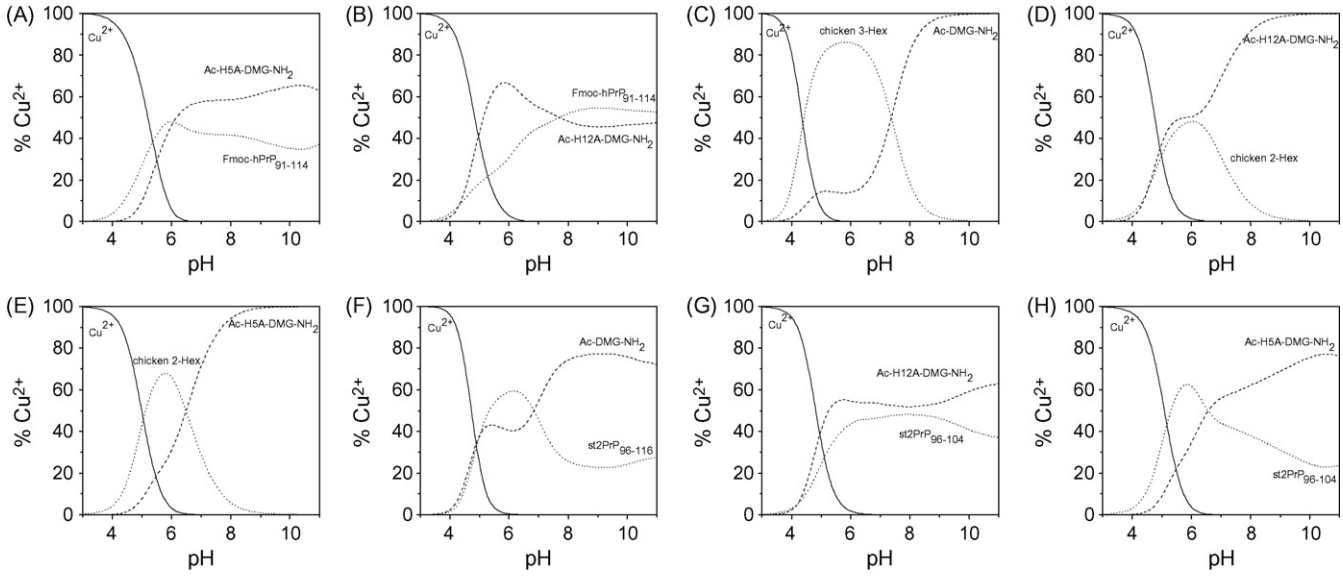

Fig. 18. Distribution profiles of competition between (A) Ac-H5A-DMG-NH_2 and PrP91–114, (B) Ac-H12A-DMG-NH_2 and PrP91–114, (C) Ac-DMG-NH_2 and 3-Hex (Ac-(HNPGYP)$_3$-NH_2, (D) Ac-H12A-DMG-NH_2 and 2-Hex (Ac-(HNPGYP)$_2$-NH_2, (E) Ac-H5A-DMG-NH_2 and 2-Hex (Ac-(HNPGYP)$_2$-NH_2, (F) Ac-DMG-NH_2 and st2PrP96–116, (G) Ac-H12A-DMG-NH_2 and st2PrP96–104, (H) Ac-H5A-DMG-NH_2 and st2PrP96–104 in coordination of Cu^{2+}.

peptides with H5A and H12A mutations shows that His residues are of critical importance during the complex formation, particularly at pH range 4.5–6. Above this pH the amide nitrogen donors are joining the coordination sphere and in the case of the N-unprotected molecules amino nitrogen participates in the metal ion binding as well. When compared with that of DMG-NH_2, the binding ability of H12A-DMG-NH_2 to Cu^{2+} is only slightly less effective. The small differences between demegen and its Ala12 analogue could indicate only a minor role of His-12 in the coordination of Cu^{2+} in the parent peptides. However, NMR data strongly suggest, that imidazole nitrogen of His-12 can complete the donor set in apical position (Fig. 17) [59]. The significance of two adjacent His was proven by the comparison of the binding efficacies of DMG-NH_2 and H5A-DMG-NH_2, where around pH 5–6 very considerable differences in binding ability are seen with the parent peptide being much more effective in metal ion coordination.

The distribution profile of the competition between Ac-H5A-DMG-NH_2 and Fmoc-PrP91-114 fragment in Cu^{2+} coordination shows that both peptides with two His residues have comparable abilities to bind Cu^{2+}, although the PrP neurotoxic fragment binds Cu^{2+} at lower pH range (Fig. 18A). More significant differences are seen between the same fragment of prion protein and Ac-H12A-DMG-NH_2 (Fig. 18B). In the 4–7 pH range this analogue of DMG-NH_2 is more effective ligand indicating that two adjacent His residues are very effective in the coordination. When the stepwise deprotonations result in the involvement of amide nitrogen atoms in the binding mode the efficacies of these peptides become almost identical. Comparison of the binding abilities between demegens and hexapeptides from chicken prion proteins allowed also to make some suggestions (Fig. 18C–E) [42]. Trimeric peptide (3-Hex) at low pH range exhibits the multi-imidazolic coordination mode and forms more stable complexes with Cu^{2+} ion than Ac-DMG-NH_2. Above pH 7, however, demegen becomes more effective ligand than 3-Hex due to the involvement of amide nitrogen atoms in coordination process, while Pro "break points" in 3-Hex prevent the coordination of the successive amide nitrogen.

The binding ability of Ac-DMG-NH_2 and tandem repeat region from fugu fish (st2PrP96–116) are similar to each other at pH below 5 and the donor set involved, $\{2N_{im}\}$, is the same in both peptides (Fig. 18F). Around pH 5–7 fragment of st2PrP is slightly more efficient, because in DMG the third His residue interacts with Cu^{2+} at apical position. Two other peptides: Ac-H12A-DMG-NH_2 and st2PrP96–104 have the same affinity for metal ion in the whole pH range (Fig. 18G), while the competition plot for st2PrP96–104 and Ac-H5A-DMG-NH_2 shows greater differences in the binding abilities of both peptides (Fig. 18H).

7. Conclusion

The prion proteins as well as demegen peptides show new possible paths for Cu^{2+} binding, which is biologically relevant *via* imidazole and amide nitrogen donors. The His-rich protein domains may play a critical role in copper homeostasis, especially in the proteins containing His residues in the very flexible or unstructured domains making these domains similar to much smaller peptides. The binding of four Cu^{2+} ions to the mammalian PrP repeat region may be critical for the metal ion transport inside the cell (internalization) *via* endocytosis mechanism. High pH sensitivity may induce easy Cu^{2+} ion release inside the endosome within the cell. The decrease of pH from 7.4 to 6.0, which is likely inside the endosome, causes release of about 40–50% of prion bound metal ion. The metal ions released may then be reduced to Cu^{+} and transported by chaperon proteins to the specific targets. The Cu^{2+}-PrP^{C} may act also as the SOD enzyme. At lower concentrations of metal ions in the major

species the coordination mode within the physiological pH range involves four imidazole nitrogen atoms. This coordination pattern is similar to that found in CuZn-SOD enzyme. This binding mode facilitates the Cu^{+}/Cu^{2+} redox cycle and dismutation of superoxide radical becomes effective [45]. Thus, the copper transport and the anti-oxidant activity of the Cu–PrP^{C} could be two major biological activities of prion proteins in mammals.

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