Monatsh Chem (2011) 142:411–419
DOI 10.1007/s00706-010-0413-2

ORIGINAL PAPER

The effect of point mutations on copper(II) complexes with peptide fragments encompassing the 106–114 region of human prion protein

Csilla Kállay · Ildikó Turi · Sarolta Timári · Zoltán Nagy · Daniele Sanna · Giuseppe Pappalardo · Paolo de Bona · Enrico Rizzarelli · Imre Sóvágó

Received: 6 October 2010 / Accepted: 30 October 2010 / Published online: 2 December 2010
© Springer-Verlag 2010

Abstract The tetrapeptides Ac-SKHM-NH_2, Ac-TKHM-NH_2, Ac-MKHS-NH_2, Ac-S(OMe)KHM-NH_2, and Ac-MKHS(OMe)-NH_2 and the nonapeptides Ac-KTNSKHMAG-NH_2 and Ac-KTNMKHSAG-NH_2 were synthesized and their copper(II) complexes were studied by potentiometric, UV–Vis, circular dichroism (CD), and electron paramagnetic resonance (EPR) spectroscopic methods. These peptides mimic the 109–112 and 106–114 residues of the sequence of human prion protein. The imidazole-N donor atoms of histidyl residues were found to be the primary metal binding sites of all peptide fragments. This binding mode provides a good possibility for the cooperative deprotonation and metal ion coordination of two amide functions preceding histidine. The (N_{im},N^-,N^-)-bonded species predominate in the pH range 5.5–7.0 and the free coordination sites of these species make possible the metal binding of weakly coordinating side chains. The comparison of the potentiometric and spectroscopic results revealed the stabilizing role of the oxygen donors of seryl, threonyl, or methoxyseryl residues of Ac-SKHM-NH_2, Ac-TKHM-NH_2, Ac-S(OMe)KHM-NH_2, and Ac-KTNSKHMAG-NH_2 containing the mutations in position 109. These interactions were, however, not observed in the peptides containing the specific amino acids in other locations of the peptide sequence.

Keywords Bioinorganic chemistry · Metal complexes · Peptides · Prion proteins

C. Kállay · I. Turi · S. Timári · I. Sóvágó (✉)
Department of Inorganic and Analytical Chemistry,
University of Debrecen, Debrecen 4010, Hungary
e-mail: sovago@delfin.unideb.hu

Z. Nagy
Department of Colloid Chemistry,
University of Debrecen, Debrecen 4010, Hungary

D. Sanna
CNR Institute of Biomolecular Chemistry, Traversa La Crucca
3, 07040 Baldinca-Li Punti (Sassari), Italy

G. Pappalardo · E. Rizzarelli
CNR Institute of Biostructures and Bioimagining,
V.le A. Doria 6, 95125 Catania, Italy

P. de Bona · E. Rizzarelli
Department of Chemical Sciences, University of Catania,
V.le A. Doria 6, 95125 Catania, Italy

Introduction

Peptides are versatile and effective ligands and their coordination chemistry has been thoroughly reviewed [1–4]. It is clear from these compilations that the anchoring role of the terminal amino and/or histidyl residues is the most common characteristic of the complex formation processes of small peptides. The proteins responsible for the development of various forms of neurodegenerative disorders are generally rich in histidyl residues and this fact gave a big impetus to studies on the complex-forming ability of the peptide fragments of these proteins [5, 6]. The side chain imidazole nitrogen atoms proved to be the primary ligating sites of the terminally protected peptides containing internal histidyl residues. We reported the copper(II) binding affinity of the peptide fragments of human prion protein (HuPrP) containing histidyl residues outside the octarepeat domain (His96, His111) [7–11]. The predominance of 3N complexes with (N_{im},N^-,N^-) coordination mode was observed in the pH range 6–8 with all peptides studied. In the case of the peptide fragments containing His111, the -MKHM- sequence of the ligands made possible a weak interaction of the thioether

function of Met109 residues in a narrow pH range. Slight increase of pH, however, resulted in the deprotonation and metal ion coordination of the third amide nitrogen and 4N-coordinated complexes were formed with all peptides.

Thioether sulfur atoms of methionine are generally the primary metal binding sites for soft metal ions, e.g., platinum(II) [12] or palladium(II) [13], but their interaction is very weak or negligible with the 3d transition elements [14]. However, both potentiometric and spectroscopic data reported for the tetrapeptide fragments of human and chicken prion protein, HuPrP109–112 (Ac-MKHM-NH_2) and ChPrP122–125 (Ac-FKHV-NH_2), respectively, provided an indication for the existence of a weak Cu–S(thioether) bond in the 3N complexes of Ac-MKHM-NH_2. Similar data were obtained for the corresponding nonapeptides, HuPrP106–114 (Ac-KTNMKHMAG-NH_2) and ChPrP119–127 (Ac-KTNFKHVAG-NH_2), supporting the assumption that the simultaneous metal binding of Met109 and His111 sites (or the XaaMetXaaHisXaa sequences) provide a specific arrangement for metal ion coordination (where Xaa is any amino acid without coordinating side chain).

These results suggest that the presence of other amino acids with weakly coordinating side chains in the same position as Met109 may also enhance the stability or alter the structure of peptide complexes. Alcoholic –OH groups of seryl and threonyl residues are also considered as low affinity metal binding sites [15]. In the case of dipeptides with C-terminal seryl residues, a slight stability enhancement of complexes was only observed and explained by the indirect effect of the –OH group via a bridging water molecule [16, 17]. The stabilizing role of N-terminal seryl residues was reported to be more effective [18] than those of the C-terminal ones and on the basis of electron paramagnetic resonance (EPR) measurements even the metal ion promoted deprotonation and coordination of alcoholic –OH groups were suggested to occur in bis(ligand) and dinuclear complexes above pH 8–9 [19]. The highest degree of stability enhancement from –OH groups was obtained for peptide derivatives containing the α-hydroxymethyl-L-seryl (HmS) residues. In this case, the extra stabilization was observed in the copper(II), nickel(II), and zinc(II) complexes too, and especially effective ligands were obtained if both HmS and His residues were present in the peptide sequence [20–23].

In this paper we report the synthesis and studies of the copper complexes of the serine mutants of human prion peptide fragments, including the nonapeptides Ac-KTNSKHMAG-NH_2 and KTNMKHSAG-NH_2 and the corresponding tetrapeptides Ac-SKHM-NH_2 and Ac-MKHS-NH_2. For the unambiguous clarification of the role of alcoholic –OH groups the methoxyserine (S-OMe) and threonine derivatives of the tetrapeptides, Ac-S(OMe)KHM-NH_2, Ac-MKHS(OMe)-NH_2, and Ac-TKHM-NH_2, were also synthesized and studied.

Results and discussion

All tetrapeptides studied in this work have two protonation sites and the corresponding pK values are included in Table 1. The protonation reactions of the imidazole-N atoms of histidyl and the ε-amino groups of lysyl residues are well separated and take place in the pH ranges 5.5–7.0 and 9.5–11.0, respectively. It is also clear from Table 1 that the differences in the pK values of the various tetrapeptides are very small, suggesting that the replacement of one amino acid with another without coordinating side chain does not significantly affect the acid–base properties of the peptides. In the case of nonapeptides two lysyl residues are present in the sequence, but their protonations occur under similar conditions, as is reported for the tetrapeptides. Both lysyl residues are, however, present in their protonated

Table 1 pK values and stability constants of the copper(II) complexes of the terminally protected tetrapeptides (Ac-$[X]_4$-NH_2)

Ligand $[X]_4$	S(OMe)KHM	SKHM	TKHM	MKHS(OMe)	MKHS	MKHM (HuPrP109–112) [7]	FKHV (ChPrP122–125) [7]
pK(Im)	6.27(1)	6.23(1)	6.18(2)	6.30(1)	6.29(1)	6.22	6.29
pK(Lys)	10.31(1)	10.33(2)	10.25(2)	10.33(1)	10.29(2)	10.28	10.28
$[CuHL]^{3+}$	13.70(2)	13.52(2)	14.29(4)	13.96(7)	13.84(3)	13.98	13.88
$[CuH_{-1}L]^{+}$	2.29(1)	3.34(2)	3.65(2)	2.53(4)	2.34(1)	2.70	2.02
$[CuH_{-2}L]$	−6.37(1)	−4.41(3)	−4.65(2)	−6.58(6)	−6.73(2)	−6.26	−6.50
$[CuH_{-3}L]^{-}$	−16.87(1)	−15.19(4)	−15.32(3)	−16.73(7)	−16.89(2)	−16.32	−16.69
pK(12)	5.71	5.09	5.32	5.72	5.75	5.64	5.93
pK(3)	8.66	7.75	8.30	9.11	9.07	8.96	8.52
pK(4)	10.50	10.78	10.67	10.15	10.16	10.06	10.19
log K(Cu + HL)	3.39	3.19	4.04	3.63	3.55	3.70	3.60

$I = 0.2$ mol dm^{-3} KCl, $T = 298$ K, standard deviations are in parentheses

forms in most of the copper(II) complexes, resulting in different stoichiometries and charges of the same coordination modes formed with tetra- and nonapeptides. As a consequence, the equilibrium data obtained for the nonapeptides and their complexes are included in a separate table (Table 2).

The equilibrium and structural characterization of the copper(II) complexes of the tetra- and nonapeptide fragments of human and chicken prion proteins have been published by us elsewhere [7, 8]. It is clear from Tables 1 and 2 that the same species were formed with the mutants containing seryl or methoxyseryl residues instead of Met109 or Met112. The same effect was obtained for the peptide Ac-TKHM-NH_2 containing a threonyl residue instead of Met109. Even the pH-dependent metal ion speciation of the systems is very similar to those reported for the native peptide fragments [7, 8]. This is further supported by Fig. 1, where the metal ion speciation of the copper(II) complexes of Ac-SKHM-NH_2 and Ac-MKHS-NH_2 is compared.

Three major binding modes of the ligands can be identified in all systems and defined in terms of the number of coordinated nitrogen donor atoms. The $[CuHL]^{3+}$ complexes of tetrapeptides and $[CuH_2L]^{4+}$ complexes of nonapeptides are formed in rather low concentrations in all cases and their stoichiometries can be best explained by the monodentate binding of side chain imidazole-N donor atoms. The EPR spectroscopic parameters (see Table 3) and the lack of circular dichroism (CD) activity of these complexes are in good agreement with the Cu–N_{im}

Table 2 pK values and stability constants of the copper(II) complexes of the terminally protected nonapeptides (Ac-$[X]_9$-NH_2)

Ligand $[X]_9$	KTNSKHMAG (M109S)	KTNMKHSAG (M112S)	KTNMKHMAG HuPrP(106–114) [8]	KTNFKHVAG ChPrP(119–127) [8]
pK(Im)	6.07(1)	6.18(1)	6.24	6.22
pK(Lys1)	9.83(2)	9.88(2)	9.91	9.89
pK(Lys2)	10.69(2)	10.62(2)	10.54	10.62
$[CuH_2L]^{4+}$	23.75(4)	23.57(3)	23.54	24.19
$[CuL]^{2+}$	13.58(2)	12.27(1)	12.39	12.39
$[CuH_{-1}L]^{+}$	6.10(2)	4.73(2)	4.56	5.34
$[CuH_{-2}L]$	−3.91(2)	−5.38(3)	−5.45	−4.65
$[CuH_{-3}L]^{-}$	−14.60(3)	−15.88(4)	−16.11	−15.25
pK(12)	5.09	5.65	5.58	5.90
pK(3)	7.48	7.54	7.83	7.05
pK(4)	10.01	10.11	10.01	9.99
pK(5)	10.69	10.50	10.66	10.60
log K(Cu + H_2L)	3.23	3.07	3.09	3.68

$I = 0.2$ mol dm^{-3} KCl, $T = 298$ K, standard deviations are in parentheses

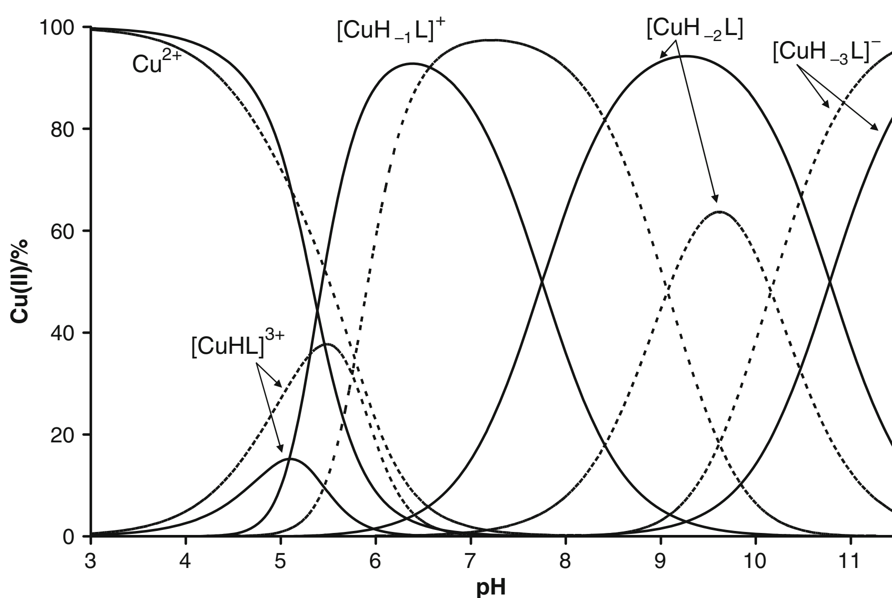

Fig. 1 Species distribution of the complexes formed in the copper(II)–Ac-SKHM-NH_2 (*solid line*) and copper(II)–Ac-MKHS-NH_2 system (*dotted line*) ($c_{Cu(II)} = c_L = 3 \times 10^{-3}$ mol dm^{-3}) as a function of pH

Table 3 EPR spectroscopic parameters of the tetra- and nonapeptide fragments of prion protein

Ligand	Species	$\lvert A_{\parallel}\rvert$ (10^{-4} cm^{-1})	$g_{\parallel}$
KTNFKHVAG [8]	1N	135	2.366
	3N	169	2.231
	4N	194	2.201
KTNMKHMAG [8]	1N	133	2.366
	3N	174	2.220
	4N	198	2.198
KTNSKHMAG (M109S)	1N	141	2.364
	3N	193	2.220
	4N	204	2.204
KTNMKHSAG (M112S)	1N	141	2.363
	3N	167	2.228
	4N	199	2.196
S(OMe)KHM	1N	–	–
	3N	175	2.229
	4N	197	2.198
MKHS(OMe)	1N	–	–
	3N	169	2.223
	4N	195	2.190
TKHM	1N	141	2.368
	3N	188	2.227
	4N	199	2.218

coordination mode. The log K values in the last rows of Tables 1 and 2 provide further support for this assumption.

In the case of tetrapeptides, the species $[CuH_{-1}L]^+$ predominates in the pH range 5.0–7.0 and it is formed in a cooperative process when the loss of two amide protons results in the formation of a 3N (N_{im},N^-,N^-)-coordinated complex. The species $[CuL]^{2+}$ with (N_{im},N^-) binding sites can also be present in very low concentrations, but its formation overlaps very much with those of $[CuHL]^{3+}$ and $[CuH_{-1}L]^+$ and neither the equilibrium nor the spectroscopic parameters can be unambiguously determined for $[CuL]^{2+}$ complexes of tetrapeptides. According to a literature survey the cooperative deprotonation of the first two amide nitrogens seems to be a common feature of peptides containing histidyl residues in internal positions [4].

UV–Vis and CD spectra of copper(II) complexes have been recorded at many different pH values and the use of the PSEQUAD program made it possible to calculate the spectra of all individual species. These data are collected in Tables 4 and 5.

Figure 2 is used to compare the CD spectra of the species $[CuH_{-1}L]^+$, $[CuH_{-2}L]$, and $[CuH_{-3}L]^-$ of the Ser-mutated peptides Ac-SKHM-NH_2 (a) and Ac-MKHS-NH_2 (b), whereas Fig. 3 shows the same spectra of their methoxyserine counterparts Ac-S(OMe)KHM-NH_2 (a) and Ac-MKHS(OMe)-NH_2 (b).

Several important conclusions can be drawn from the comparison of the four sets of CD spectra shown in Figs. 2 and 3. First, it is evident that the CD spectra of Ac-SKHM-NH_2 are completely different from those of the other three ligands. On the other hand, the parameters of the absorption and CD spectra of the copper(II)–Ac-SKHM-NH_2 and copper(II)–Ac-TKHM-NH_2 systems are almost the same (Table 4). Moreover, the CD spectra of the 4N complexes (species $[CuH_{-3}L]^-$) of Ac-MKHS-NH_2 and Ac-MKHS(OMe)-NH_2 are also very similar to each other. A comparison with the literature data on similar peptides reveals that the last two spectra correspond well to those of any other histidine peptide with the same (N_{im},N^-,N^-,N^-) coordination mode in the form of (6,5,5)-membered chelate rings [7–11, 24]. The unusual CD spectra of the copper(II)–Ac-SKHM-NH_2 and copper(II)–Ac-TKHM-NH_2 systems suggest the existence of a different binding mode in their copper(II) complexes. The residue Met109 is replaced by serine or threonine in these peptides and this can provide a good chance for a weak binding of the protonated alcoholic –OH group. In accordance with this expectation, slightly enhanced stability constants can be calculated for the $[CuH_{-1}L]^+$ species of Ac-SKHM-NH_2 and Ac-TKHM-

Table 4 UV–Vis and circular dichroism spectral parameters of the 3N- and 4N-coordinated copper(II) complexes of tetrapeptides

Ligand $[X]_4$	Binding sites	S(OMe)KHM	SKHM	TKHM	MKHS(OMe)	MKHS	MKHM HuPrP(109–112) [7]	FKHV ChPrP(122–125) [7]
UV–Vis, λ_{max} (nm)/ε (dm^3 mol^{-1} cm^{-1})	N_{im},N^-,N^-	610/89	592/68	591/57	630/117	628/94	629/123	606/98
	N_{im},N^-,N^-,N^-	520/104	554/80	551/83	525/112	522/92	521/127	525/142
CD, λ_{max} (nm)/Δε (dm^3 mol^{-1} cm^{-1})	N_{im},N^-,N^-	635/−0.28	605/−0.70	592/−0.66	665/+0.13	655/+0.12	660/+0.15	605/−0.35
		525/+0.29			535/+0.36	537/+0.40	530/+0.33	510/+0.40
	N_{im},N^-,N^-,N^-	665/+0.32	550/−0.54	558/−0.57	640/+0.92	638/+1.08	652/+0.92	650/+1.00
		570/−0.50sh	480/+0.19	479/+0.24	495/−1.42	495/−1.56	494/−1.56	500/−1.33
		495/−0.66						

Table 5 UV–Vis and circular dichroism spectral parameters of the 3N- and 4N-coordinated copper(II) complexes of nonapeptides

	Ligand $[X]_9$				
	Binding sites	KTNSKHMAG (M109S)	KTNMKHSAG (M112S)	KTNMKHMAG HuPrP(106–114) [8]	KTNFKHVAG ChPrP(119–127) [8]
UV–Vis, λ_{max} (nm)/ε (dm^3 mol^{-1} cm^{-1})	N_{im},N^-,N^-	592/64	616/107	616/77	610/72
	N_{im},N^-,N^-,N^-	562/95	532/120	532/108	538/117
CD, λ_{max} (nm)/Δε (dm^3 mol^{-1} cm^{-1})	N_{im},N^-,N^-	602/−0.91	552/+0.30	760/−0.22	615/−0.19
		332/+0.69	475/−0.20	535/+0.39	525/+0.42
		254/+3.20	371/+0.32	385/+0.11	350/−0.77
			321/−0.58	330/−0.61	248/+7.21
			251/+5.47	249/+8.29	224/−7.69
	N_{im},N^-,N^-,N^-	558/−0.51	624/+0.82	631/+1.05	645/+1.27
		486/+0.08	498/−1.14	495/−1.22	500/−1.35
		266/+2.32	355/−0.14	317/+1.27	325/+0.97
		223/−15.21	316/+0.94	292/+0.16	293/−0.75
			257/+6.03	256/+7.82	260/+7.14
					224/+25.4

NH_2. The increase of stability is also reflected in the low values of the deprotonation of the first two amide functions (see pK(12) values in Table 1) and in the slightly reduced pH range of complexation as shown by Fig. 1.

Both potentiometric and spectroscopic data support the notion that the deprotonation reactions of the Met109Ser and Met109Thr mutated peptides are different from all the others. In the case of Ac-SKHM-NH_2 and Ac-TKHM-NH_2 the species $[CuH_{-2}L]$ and $[CuH_{-3}L]^-$ have the same CD and absorption spectra, whereas that of $[CuH_{-1}L]^+$ is different from any of the other tetrapeptides (see Table 4). The similarities in the spectra of $[CuH_{-2}L]$ and $[CuH_{-3}L]^-$ suggest that the deprotonations of the non-coordinated lysyl side chains are the major processes during the transformation of $[CuH_{-2}L]$ to $[CuH_{-3}L]^-$. As a consequence, the low pK(3) value and the different CD spectra of $[CuH_{-1}L]^+$ can be best explained by the deprotonation and metal ion coordination of the third amide functions of the peptides in the pH range of 7.0–8.0. On the contrary, both Fig. 1 and spectroscopic data suggest the overlap of amide and lysyl deprotonation reactions in the copper(II) complexes of the other tetrapeptides. The low pK values of amide deprotonation reactions of Ac-SKHM-NH_2 and Ac-TKHM-NH_2 strongly suggest that the weak axial interaction of the alcoholic –OH groups of serine and threonine facilitates the metal binding of the third amide nitrogen. Alternately, the conformational change caused by a hydrogen bond between the Ser/Thr–OH and Lys–NH_2 groups may also be responsible for the promotion of amide binding.

The results obtained for the nonapeptides provide further support for these assumptions. It was demonstrated in our previous publication [8] that the complex formation processes of the nonapeptide fragments of prion protein are very similar to those of the short tetrapeptide fragments. The presence of two uncoordinated lysyl side chains, however, results in the different stoichiometries of the same coordination modes; e.g., the ($N_{im}N^-$,N^-)-bonded species corresponds to the stoichiometries $[CuH_{-1}L]^+$ and $[CuL]^{2+}$ for Ac-FKHV-NH_2 and Ac-KTNFKHVAG-NH_2, respectively. Another difference between the copper(II) complexes of tetra- and nonapeptides is reflected in the pK(3) values. All of these data in Table 2 are in the range of 7–8, supporting the complete separation of amide and lysyl ammonium deprotonation reactions. In agreement with this expectation the last two deprotonation reactions are not accompanied by any spectral changes and the spectroscopic parameters are the same for the species $[CuH_{-1}L]^+$, $[CuH_{-2}L]$, and $[CuH_{-3}L]^-$ having the same 4N coordination modes. This is best illustrated by CD and EPR spectra of the nonapeptides depicted in Figs. 4 and 5. The comparison of Figs. 2b and 4b reveals the high similarity in the coordination modes of Ac-MKHS-NH_2 and Ac-KTNMKHSAG-NH_2. In the case of the nonapeptide, the (N_{im},N^-,N^-,N^-) coordination mode exists in the species $[CuH_{-1}L]^+$ and the further two deprotonation reactions are connected to the lysyl side chains without any change in coordination sphere of the metal ion. This is also supported by Fig. 5 in which the EPR spectra of the copper(II)–Ac-KTNMKHSAG-NH_2 system are plotted at four different pH values. The species $[CuL]^{2+}$ predominates at

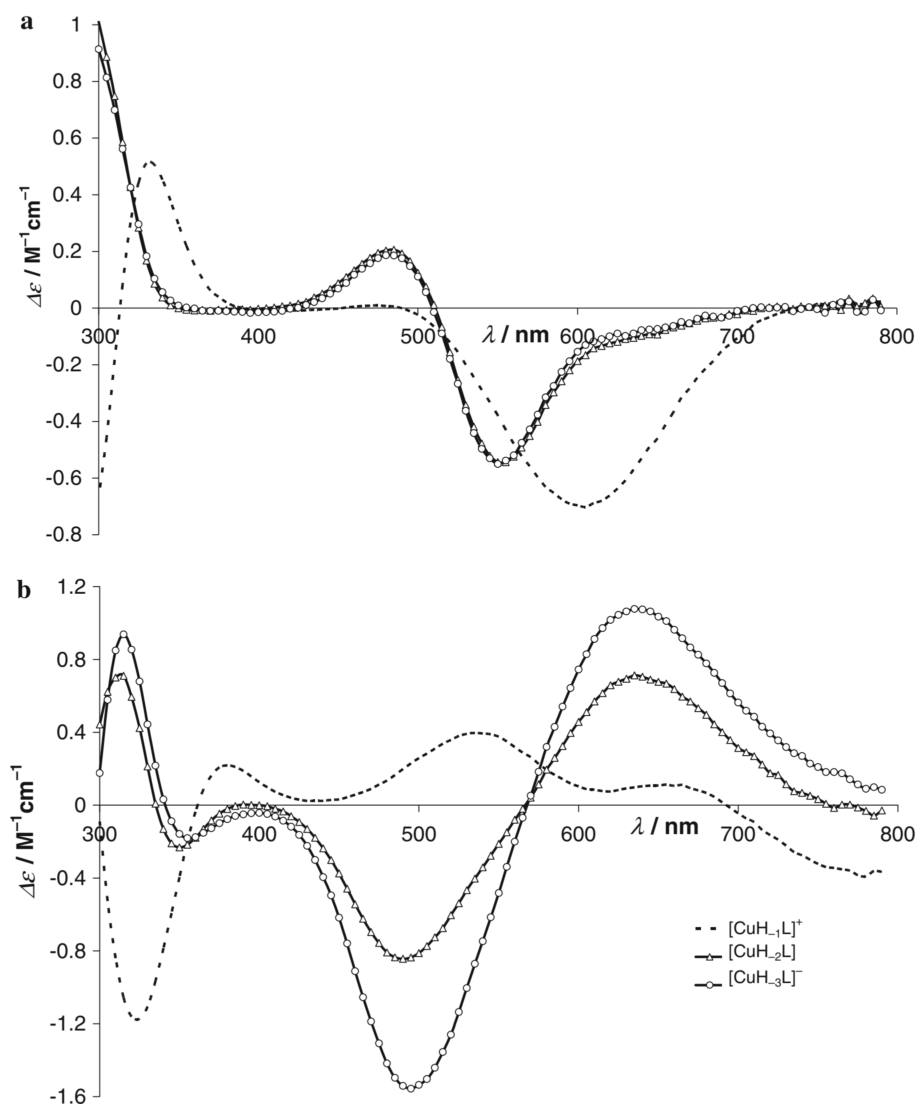

Fig. 2 Circular dichroism spectra of the complexes formed in the copper(II)–Ac-SKHM-NH_2 (**a**) and copper(II)–Ac-MKHS-NH_2 (**b**) systems

pH 6.55 and its spectral parameters correspond well to those of the common (N_{im},N^-,N^-)-coordinated species. This spectrum is completely changed by pH 9.05, supporting the involvement of the third amide group in metal binding. Further increase of pH, however, does not affect the EPR and CD parameters. On the other hand, the comparison of Fig. 4a, b reveals a big difference in the conformation of the two nonapeptides containing the Ser residues at positions 109 and 112, respectively. There is, however, an almost complete agreement in the spectroscopic data of the corresponding tetra- and nonapeptides Ac-SKHM-NH_2 and Ac-KTNSKHMAG-NH_2, supporting the involvement of the seryl–OH group in metal binding for both Met109Ser mutated peptides. The metal ion coordination of the hydroxyl groups will not change the number of coordinated nitrogen donor atoms around the metal ions; thus, the EPR spectroscopic parameters for the $[CuH_{-1}L]^+$ to $[CuH_{-3}L]^-$ species of the two nonapeptides are not much different. The major difference is reflected in the parameters of $[CuL]^{2+}$ where the common (N_{im},N^-,N^-) coordination mode is changed to (N_{im},N^-,N^-,OH). The $g_{\parallel}$ values of the unsaturated (N_{im},N^-,N^-)-bonded species in Table 3 correspond well to the metal ion coordination of three nitrogen donors in all peptides, but the $|A_{\parallel}|$ values are generally in the range 160–170 × 10^{-4} cm^{-1}. In the case of Ac-KTNSKHMAG-NH_2 this value is, however, much higher, $|A_{\parallel}| = 193 \times 10^{-4}$ cm^{-1}. It is a common feature of EPR spectra that the increase in the number of coordinated donor atoms is accompanied by an increase of the hyperfine splitting constant [25] and, as a consequence, these data support the presence of a seryl–OH donor function in the equatorial plane.

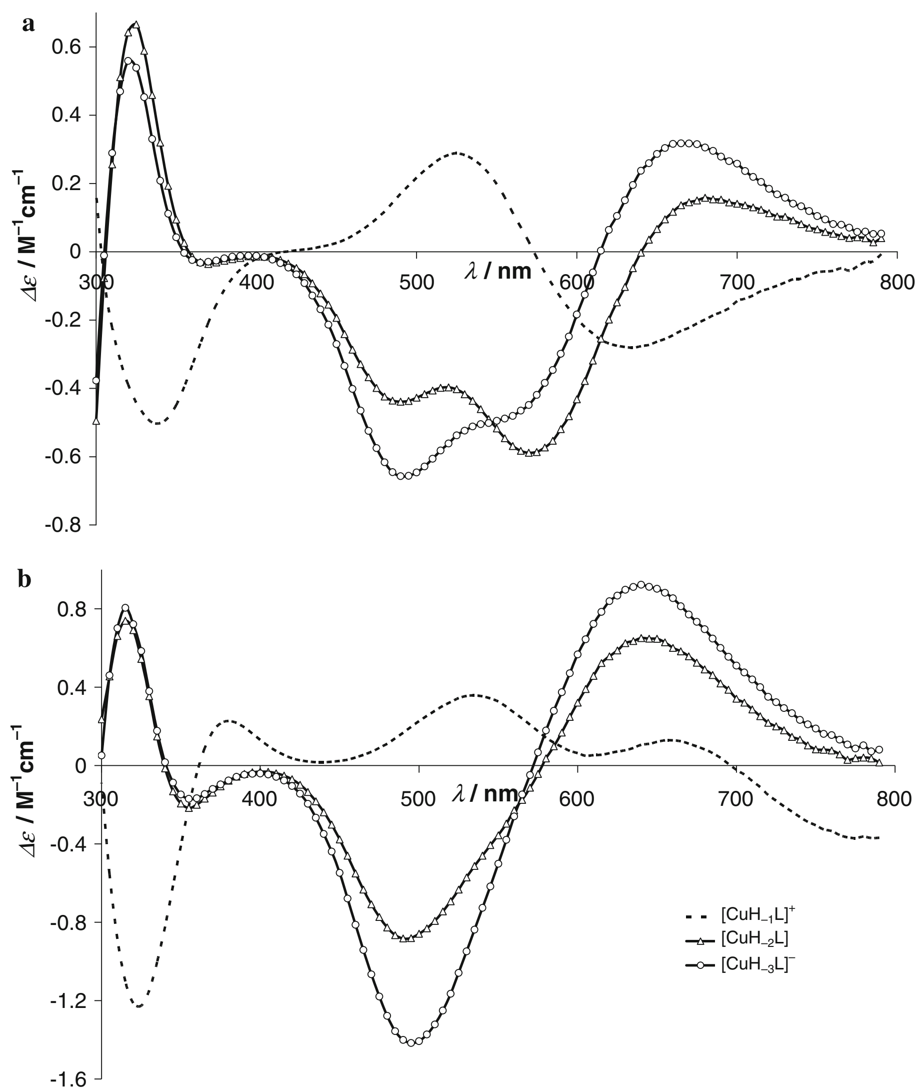

Fig. 3 Circular dichroism spectra of the complexes formed in the copper(II)–Ac-S(OMe)KHM-NH_2 (**a**) and copper(II)–Ac-MKHS(OMe)-NH_2 (**b**) systems

Experimental

Materials

Stock solutions of copper(II) ions were prepared from analytical grade reagents ($CuCl_2 \cdot 2H_2O$) and their concentrations were checked gravimetrically via the precipitation of oxinate. The other solutions (KOH, HCl, KCl, potassium hydrogen phthalate) were also prepared from analytical grade reagents.

Synthesis of prion fragments and mutants

The tetra- and nonapeptide fragments of human and chicken prion proteins were prepared by solid-phase peptide synthesis and details of the procedure and purification of the peptides are described in our previous publications [7–10]. A similar procedure was used for the synthesis of the peptides containing the Ser or Ser(OMe) and Thr residues.

Potentiometric measurements

The pH-potentiometric titrations in the pH range 2.5–11.0 were performed on 3-cm^3 samples in the concentration range 1×10^{-3}–4×10^{-3} mol dm^{-3} at metal ion to ligand ratios between 1:1 and 1:2. The measurements were made with a MOLSPIN pH meter equipped with a 6.0234.100 combined electrode (Metrohm) and a MOLACS microburette controlled by computer.

The titrations were performed with carbonate-free stock solution of potassium hydroxide of known concentration. During the titration argon was bubbled through the samples to ensure the absence of oxygen and carbon dioxide and for stirring of the solutions. All pH-potentiometric measurements were carried out at a constant ionic strength of 0.2 M

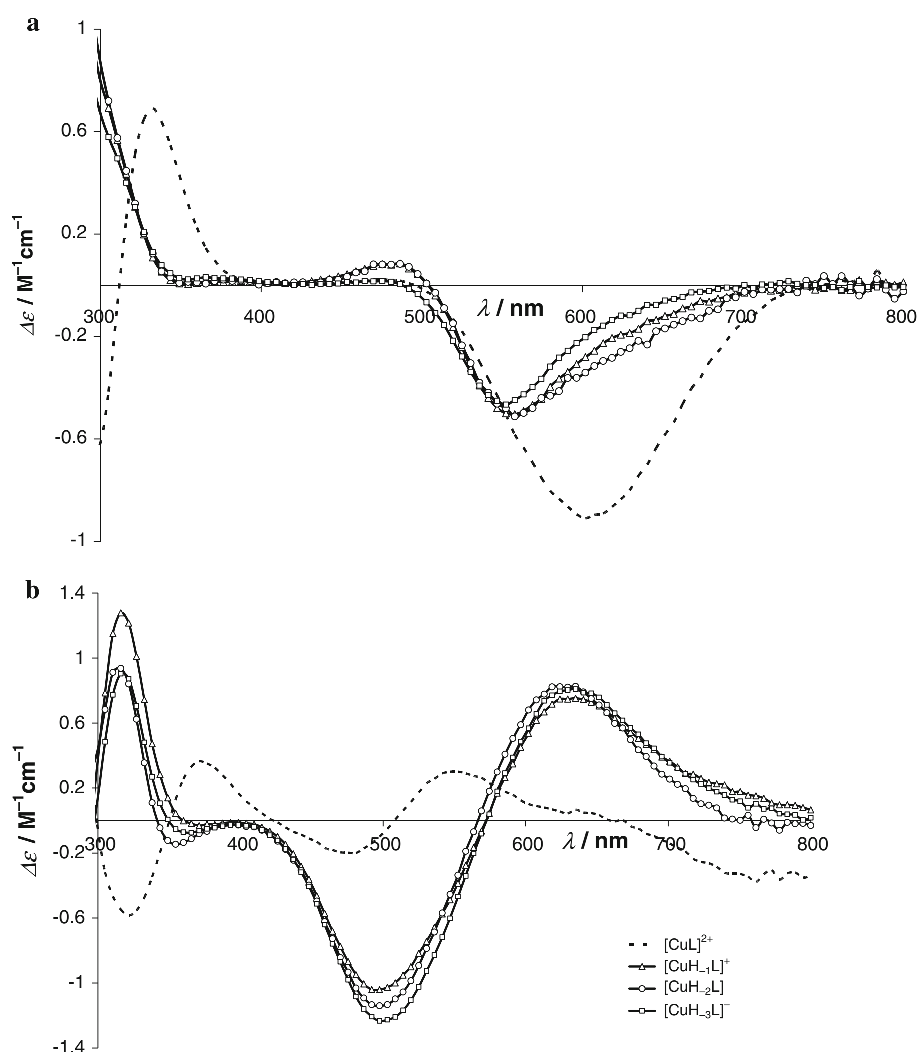

Fig. 4 Circular dichroism spectra of the complexes formed in the copper(II)–Ac-KTNSKHMAG-NH_2 (**a**) and copper(II)–Ac-KTNMKHSAG-NH_2 (**b**) systems

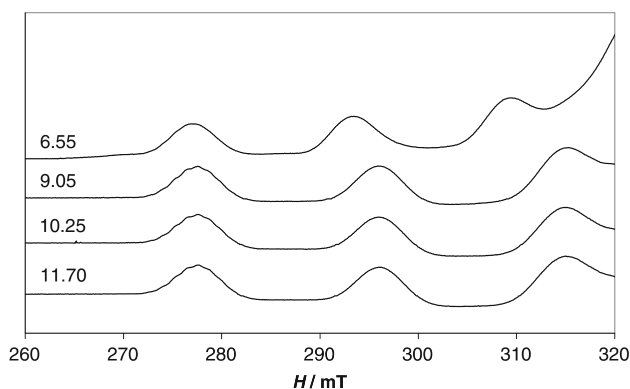

Fig. 5 EPR spectra of the copper(II)–Ac-KTNMKHSAG-NH_2 system at 1:1 metal ion to ligand ratio ($[Cu^{2+}] = 2 \times 10^{-3}$ mol dm^{-3}) at four different pH values

KCl and at constant temperature (298 K). The number of experimental points was around 50–70 data (cm^3, pH) for each titration curve. The pH readings were converted into hydrogen ion concentration as described earlier [26].

Protonation constants of the ligands and the overall stability constants ($\log \beta_{pqr}$) of the complexes were calculated by means of general computational programs, PSEQUAD [27] and SUPERQUAD [28] using Eqs. 1 and 2.

$$pM + qH + rL \rightleftharpoons M_pH_qL_r \quad (1)$$

$$\beta_{pqr} = \frac{[M_pH_qL_r]}{[M]^p[H]^q[L]^r} \quad (2)$$

Spectroscopic studies

UV–Vis spectra of the copper(II) complexes were recorded on a Perkin-Elmer Lambda 25 double beam spectrophotometer in the same concentration range as used for pH-potentiometry.

The EPR continuous wave spectra were recorded at the X-band at 120 K, using a Bruker EMX spectrometer. Copper(II) stock solution was prepared from $CuSO_4 \cdot 5H_2O$ enriched with ^{63}Cu to get better resolution of EPR spectra.

Metallic copper (99.3% ^{63}Cu and 0.7% ^{65}Cu) was purchased from JV Isoflex (Moscow, Russia) for this purpose and converted into the sulfate.

CD spectra of copper(II) complexes were recorded on a JASCO J-810 spectropolarimeter using 1- or 10-mm cells in the 200- to 800-nm range in the same concentration range as used for potentiometry. CD spectra of the individual species were calculated by the same general program (PSEQUAD) as used for the evaluation of potentiometric measurements.

Acknowledgments This work was supported by the MTA(Hungary)-CNR(Italy) bilateral program and the research grant OTKA-NKTH 77586, OTKA 72956 and PD 72321 and TAMOP 4.2.1/B-09/1/KONV-2010-0007 (Hungary).

References

1. Sigel H, Martin RB (1982) Chem Rev 82:385
2. Sóvágó I (1990) In: Burger K (ed) Biocoordination chemistry, metal complexes of peptides and derivatives. Ellis Horwood, Chichester, p 135
3. Kozlowski H, Bal W, Dyba M, Kowalik-Jankowska T (1999) Coord Chem Rev 184:319
4. Sóvágó I, Ősz K (2006) Dalton Trans 3841
5. Brown DR, Kozlowski H (2004) Dalton Trans 1907
6. Millhauser GL (2004) Acc Chem Res 37:79
7. Jószai V, Nagy Z, Ősz K, Sanna D, Di Natale G, La Mendola D, Pappalardo G, Rizzarelli E, Sóvágó I (2006) J Inorg Biochem 100:1399
8. Di Natale G, Grasso G, Impellizzeri G, La Mendola D, Micera G, Mihala N, Nagy Z, Ősz K, Pappalardo G, Rigó V, Rizzarelli E, Sanna D, Sóvágó I (2005) Inorg Chem 44:7214
9. Grasso D, Grasso G, Guantieri V, Impellizzeri G, La Rosa C, Milardi D, Micera G, Ősz K, Pappalardo G, Rizzarelli E, Sanna D, Sóvágó I (2006) Chem Eur J 12:537
10. Ősz K, Nagy Z, Pappalardo G, Di Natale G, Sanna D, Micera G, Rizzarelli E, Sóvágó I (2007) Chem Eur J 13:7129
11. Di Natale G, Ősz K, Nagy Z, Sanna D, Micera G, Pappalardo G, Sóvágó I, Rizzerelli E (2009) Inorg Chem 48:4239
12. Hahn M, Wolters D, Sheldrick WS, Hulsbergen FB, Reedijk J (1999) J Biol Inorg Chem 4:412
13. Bóka B, Nagy Z, Várnagy K, Sóvágó I (2001) J Inorg Biochem 83:77
14. Várnagy K, Bóka B, Sóvágó I, Sanna D, Marras P, Micera G (1998) Inorg Chim Acta 275–276:440
15. Pettit LD, Robbins RA (1995) In: Berthon G (ed) Metal-peptide complex formation, in handbook of metal–ligand interactions in biological fluids, vol 1. Marcel Dekker, New York, p 636
16. Gergely A, Farkas E (1982) J Chem Soc Dalton Trans 381
17. Bühl M (2000) J Inorg Biochem 80:137
18. Farkas E, Kiss T (1989) Polyhedron 8:2463
19. Szabó-Plánka T, Árkosi Z, Rockenbauer A, Korecz L (2001) Polyhedron 20:995
20. Kowalik-Jankowska T, Stasiak M, Leplawy MT, Kozlowski H (1997) J Inorg Biochem 66:193
21. Mlynarz P, Gaggelli N, Panek J, Stasiak M, Valensin G, Kowalik-Jankowska T, Leplawy ML, Latajka Z, Kozlowski H (2000) J Chem Soc Dalton Trans 1033
22. Mlynarz P, Kowalik-Jankowska T, Stasiak M, Leplawy ML, Kozlowski H (1999) J Chem Soc Dalton Trans 3673
23. Mlynarz P, Bal W, Kowalik-Jankowska T, Stasiak M, Leplawy ML, Kozlowski H (1999) J Chem Soc Dalton Trans 109
24. Kállay C, Várnagy K, Malandrinos G, Hadjiliadis N, Sanna D, Sóvágó I (2006) Dalton Trans 4545
25. Nishida Y, Hayashida K, Kida S (1980) J Coord Chem 10:101
26. Irving H, Miles G, Pettit LD (1967) Anal Chim Acta 38:475
27. Zékány L, Nagypál I (1985) In: Leggett D (ed) Computational methods for the determination of formation constants. Plenum, New York, p 291
28. Gans P, Sabatini A, Vacca A (1985) J Chem Soc Dalton Trans 1195