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Nickel(II), zinc(II) and cadmium(II) complexes of hexapeptides containing separate histidyl and cysteinyl binding sites†

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Nickel(II), zinc(II) and cadmium(II) complexes of two N-terminally free hexapeptides AAHAAC and AHAAAC containing separate histidyl and cysteinyl residues have been studied by potentiometric and various spectroscopic (UV-Vis, CD, ^{1}H-NMR) techniques. Both peptides have outstanding metal binding ability but the speciation of the systems and the binding sites of peptides reveal a significant specificity. In the nickel(II)-AAHAAC system the amino terminus is the primary nickel(II) binding site in the form of the (NH_2,N^-,N^-,N_{im}) chelate. The C-terminal thiolate function, however, can bind another nickel(II) ion with the involvement of preceding amide nitrogens in metal binding. Zinc(II) and cadmium(II)ions are not able to promote deprotonation and coordination of peptide amide groups of AAHAAC and only mononuclear complexes are formed in which the imidazole-N and thiolate-S^- donors are the primary metal binding sites. In the case of AHAAAC both nickel(II) and zinc(II) ions can induce deprotonation and coordination of the first amide bond in the sequence. This results in the enhanced stability of the corresponding species, [$MH_{-1}L$], containing a tridentate (NH_2,N^-,N_{im}) binding mode at the amino terminus supported by a macrochelate from the distant thiolate group.

Introduction

Peptides are effective complexing agents and are also often used as models for the characterization of the binding sites in metalloproteins. Huge number of papers has already been published in this field in the past few decades and the most important findings are summarized in some general reviews[1-4]. Imidazole-N of histidine and thiolate-S of cysteine are the most common metal binding sites in the metalloproteins. Coordination properties of the histidyl and cysteinyl residues are also frequently studied[2,3,5] and the results reveal a great variety in the complex formation with these moieties. Transition metal complexes of small peptides containing only one of these residues have already been well-characterized but the peptides with multiple binding sites always provide a new challenge for coordination chemists. The biological significance of these molecules is especially high because the development of various forms of neurodegenerative disorders is linked to the abnormal processing of polyhistidine peptides. Among them Alzheimer's disease, Parkinson's disease and prion diseases are probably the best known and most frequently studied. Peptide fragments of prion protein are typical multhistidine peptides containing the histidyl residues in well-separated positions. Their coordination chemistry, especially the copper(II) complexes have been thoroughly studied and the results are collected in specific reviews[6-10]. It is obvious from these compilations that the copper(II)-prion peptide interactions largely depend on the several factors including the number and location of histidyl residues, pH, metal to ligand ratios, etc. Moreover, it was also demonstrated that a series of coordination isomers can exist for almost all possible stoichiometries[11]. The interaction of amyloid-β peptides with metal ions is even more complicated because the presence of other strongly coordinating donors (e.g. terminal amino, carboxylates of Asp and Glu residues, etc.) provides a great structural variety for complex formation[12-14]. Much less number of studies is available for the complex formation of peptides containing thiolate groups. The peptides related to the nickel(II) homeostasis of Helicobacter pylori and various zinc transporters are probably best known examples in this field[5,15-17] but systematic studies on the mutual effects of His and Cys residues have not been published, yet.

In the last few years we launched a program to synthesize N-terminally free oligopeptides containing the specific metal binding sites in well-separated and different locations. The results obtained for the copper(II), nickel(II) and zinc(II) complexes of AHAAAHG and AAHAAAHG peptides made it possible to compare the binding affinities of histidines close to the amino termini and in internal position[18]. A similar study has been performed for the evaluation of the effects of aspartyl and histidyl residues via the complex formation reactions with the peptides ADAAAH and AADAAH[19]. Now in this paper we report the synthesis of two N-terminally free but C-terminally amidated hexapeptides containing Cys and His residues, AHAAAC and AAHAAC. The complexes of a terminally protected tetrapeptide Ac-HAAC were also studied for the

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better understanding of the complex formation processes of the C-terminal part of the hexapeptides. To avoid the possible redox reactions of thiolate residues the nickel(II), zinc(II) and cadmium(II) complexes were studied by the combined application of potentiometric, UV-Vis, CD and NMR spectroscopic methods.

Experimental

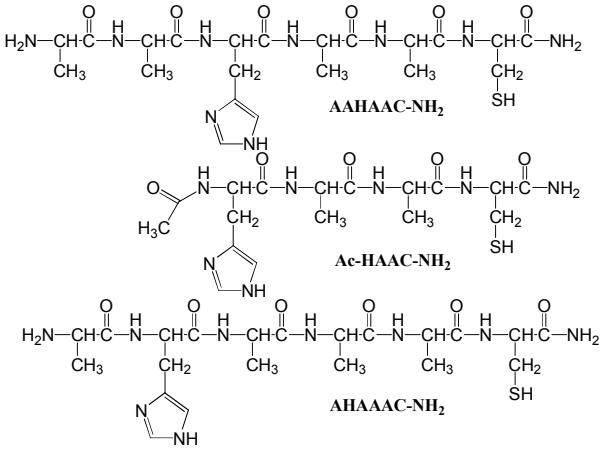
Materials

Chemicals and solvents used for synthetic purposes were purchased from commercial sources in the highest available purity and used without further purification. Rink Amide AM resin, 2-(1-H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and all *N*-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids (Fmoc-Cys(Trt)-OH, Fmoc-His(Trt)-OH and Fmoc-Ala-OH) are Novabiochem (Switzerland) products. 2-methyl-2-butanol, *N*-hydroxybenzotriazole (HOBt), *N*-methyl-pyrrolidone (NMP), 2,2'-(ethylenedioxy)diethane-thiol (DODT) and triisopropylsilane (TIS) were purchased from Sigma-Aldrich Co., while *N,N*-diisopropyl-ethylamine (DIPEA) and trifluoroacetic acid (TFA) were Merck Millipore Co. products. Peptide-synthesis grade *N,N*-dimethylformamide (DMF) and acetic anhydride (Ac_2O) were bought from VWR International, while piperidine, dichloromethane (DCM), diethyl ether (Et_2O), acetic acid (AcOH) and acetonitrile (ACN) from Molar Chemicals Ltd.

Concentrations of the peptide stock solution were determined by potentiometric titrations and the stock solutions of the metal ions were prepared from analytical reagents and their concentrations were checked gravimetrically.

Peptide synthesis and purification

The N-terminally free and protected peptides (Scheme 1) were synthesized by means of microwave-assisted Liberty 1 Peptide Synthesizer (CEM, Matthews, NC) using the Fmoc/tBtu technique and the TBTU/HOBt/DIPEA activation strategy. Cleaving of α-amino protecting group of amino acids and resin



was performed with a 20V/V% piperidine and 0.1 M HOBt·H_2O in DMF at 80 °C with 30 W microwave power for 180 s. Four times excess of amino acids and 30 W microwave power for 300 s were used for coupling at 80 °C in the presence of 0.5 M HOBt and 0.5 M TBTU in DMF as activator and 2 M DIPEA as an activator base. In case of Ac-HAAC the free amino termini was treated with DMF containing 5 V/V% Ac_2O and 6 V/V% DIPEA to perform the acetylated amino group. After finishing the synthesis, the prepared peptide linked to resin was washed with DCM, 96% AcOH, 2-methyl-2-butanol and Et_2O. Fmoc group was removed similarly while the side chain protecting group were cleaved with the mixture of TFA/TIS/H_2O/DODT (94/2.5/2.5/1 V/V%) at room temperature for 2 hours. The resin was filtered from the TFA solution of the peptide which was precipitated in cold diethyl ether. The peptides were washed with cold diethyl ether and dried after separation, redissolved in water and freezing for lyophilization.

Scheme 1. Structural formulae of the peptides

The purity of the synthesized peptides was checked by analytical RP-HPLC analyses using a Jasco instrument equipped with a Jasco MD 2010 plus wavelength detector monitoring the absorbance at 222 nm. The chromatographic conditions were the following: Column: Teknokroma Europa Peptide C18 (250 x 4.6 mm, 120 Å pore size, 5 µm particle size); Elution: gradients elution was carried out using solvent A (0.1 % TFA in water) and solvent B (0.1 % TFA in acetonitrile) at a flow rate of 1 mL/min. From 1 min to 15 min 100% to 85% A, from 15 min to 16 min 85% A and from 16 min to 20 min 85% to 100% A was applied.

Potentiometric measurements

The titrations were accomplished in a 3 mL samples at 2 mM total ligand concentration with the use of carbonate free stock solution (0.2 M) of potassium hydroxide. The metal to ligand ratios were selected as 1:1 and 2:1. During the titrations, argon was bubbled through the samples to ensure the absence of oxygen and carbon dioxide. The samples were stirred by a VELP scientific magnetic stirrer.

All pH-potentiometric measurements were carried out at 298 K and at a constant ionic strength of 0.2 M KCl in the case of nickel(II) and zinc(II), while KNO_3 was used for cadmium(II) to avoid the formation of chlorido complexes.

The pH-potentiometric titrations were performed with a MOLSPIN pH-meter equipped with a 6.0234.100 combination glass electrode (Metrohm) and a MOL-ACS burette controlled by computer. The recorded pH readings were converted to hydrogen ion concentration as described by Irving et al.[20]. Protonation constants of the ligands and overall stability ($\log\beta_{pqr}$) constants of the metal complexes were calculated by means of the general computational programs (PSEQUAD[21] and SUPERQUAD[22]) based on Eqs. (1) and (2).

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

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

Spectroscopic studies

UV-visible spectra of the nickel(II) complexes were recorded from 200 to 1000 nm on a PerkinElmer Lambda 25 scanning

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spectrophotometer in the same concentration range as used for pH-potentiometric titrations.

Circular dichroism spectra of the nickel(II) complexes were registered on a JASCO J-810 spectropolarimeter using 1 mm and/or cm cells in the 200-1000 nm wavelength range at same concentrations as used in pH-potentiometry.

400 MHz ^{1}H-NMR spectra were recorded on a Bruker Avance 400 spectrometer at 298 K. In the case of ^{1}H-NMR spectra chemical shifts were referenced to internal sodium 3-(trimethylsilyl)-1-propane sulfonate (TSP, δ_{TSP}=0 ppm) and D_2O was used as a solvent. In the case of zinc(II) and nickel(II) DCl and KOD, while DNO_3 and KOD were used for cadmium(II) to set the pH of the samples. In the case of TOCSY experiments the samples were prepared in light water using D_2O containing insert capillary for deuterium lock. The spectra were recorded with a standard TOCSY pulse sequence combined with water suppression using 3-9-19 pulse sequence.

Results and discussion

Protonation equilibria of the peptides

Protonation constants of the ligands have been determined by potentiometric titrations and the data are included in Table 1. Both hexapeptides have three protonation sites, the imidazole-N, terminal-NH_2 and side chain thiolate functions. It is clear from Table 1 that the basicities of these sites are close to each other even the numerical values of the stepwise pK values are very similar. pH-dependent ^{1}H-NMR spectra of the ligands have been recorded to determine the basicity order of the protonation sites (see Figures S1 and S2 in the Supplementary information). In principle, these spectra can be used to calculate the protonation microconstants of the peptides. However, it turned out that although the overlap of the formation of the various protonated species is quite significant the overlap of the deprotonation sites is less than 5 % ruling out the exact determination of the microconstants. The evaluation of the NMR spectra, however, reveals that the basicities of the protonation sites increase in the order: imidazole-N < terminal amino < thiolate-S^- and the pK values in Table 1 can be assigned to the specific sites in the same order.

Complex formation with AAHAAC

Stability constants of the nickel(II), zinc(II) and cadmium(II) complexes of AAHAAC are collected in Table 1 and the data suggest a significant difference between the complex formation processes of nickel(II) and the other two metal ions. This is in a good agreement with the expectations because the sequence of the N-terminal domain of this peptide corresponds well to that of albumin with an outstanding copper(II) and nickel(II) binding affinity (ATCUN motif[23]). As a consequence, the speciation of the nickel(II)-AAHAAC system is rather simple, only three species can be detected in the whole pH range. Among them $[NiHL]^{2+}$ is a minor species formed in slightly acidic samples. UV-Vis absorption spectra of the nickel(II) containing systems support that $[NiHL]^{2+}$ is an octahedral complex in which the terminal amino and imidazole-N donors are the metal binding sites. The lack of $[NiL]^+$ complex strongly suggests the cooperative deprotonation of two adjacent amide groups above pH 5.5 resulting in the formation of (NH_2,N^-,N^-,N_{im}) coordinated complex with $[NiH_{-1}L]$ stoichiometry. Absorption spectra clearly indicate that $[NiH_{-1}L]$ contains a square planar nickel(II) ion and the spectral parameters of the absorption maxima (λ_{max} = 425 nm, ε = 157 $M^{-1}cm^{-1}$) corresponds well to those of other albumin-like coordinated nickel(II) complexes[18]. pH-dependent CD spectra of the equimolar samples are plotted in Figure 1 and provide further support for this coordination mode.

It is clear from Figure 1 that nickel(II) ions are present in a single coordination environment above pH 5.5 and the positions and intensities of CD extrema are in agreement with those of other peptide complexes with similar sequences [18,24]. These observations suggest that the species $[NiH_{-1}L]$ and $[NiH_{-2}L]^-$ have the same (NH_2,N^-,N^-,N_{im}) binding mode and they differ only in the protonation stage of the non-coordinated thiol/thiolate side chain. pK value for the deprotonation of the thiol function is 8.77 in the free ligand, while this value is 8.49 in the nickel(II) complex supporting the above-mentioned conclusion. Moreover, recording the ^{1}H-NMR spectra of the free ligand and the equimolar samples at pH 10.0 provided a clear-cut evidence for the presence of non-coordinated thiolate residue in the $[NiH_{-2}L]^-$ species (see Figure S3 in Supplement).

Table 1 Protonation and stability constants (log β_{pqr}) of the metal complexes of the peptides AAHAAC and AHAAAC. T = 298 K, I = 0.2 M (KCl for Ni(II) and Zn(II), KNO_3 for Cd(II)), standard deviations are in parenthesis)

	AAHAAC			AHAAAC		
Species	log β_{pqr}	pK		log β_{pqr}	pK	
$[HL]$	8.77(1)	8.77(1)		8.77(1)	8.77(1)	
$[H_2L]^+$	16.49(1)	7.72(1)		16.48(1)	7.71(1)	
$[H_3L]^{2+}$	22.77(1)	6.28(1)		22.64(1)	6.16(1)	
	Ni(II)	Zn(II)	Cd(II)	Ni(II)	Zn(II)	Cd(II)
$[MHL]^{2+}$	13.10(20)	14.45(2)	15.06(1)	13.08(5)	14.31(3)	14.80(2)
$[ML]^+$		7.11(6)	7.75(3)	7.62(1)	8.09(3)	7.84(5)
$[MH_{-1}L]$	1.79(3)	–0.90(4)	–1.88(4)	1.10(1)	0.85(4)	–0.88(6)
$[MH_{-2}L]^-$	–6.70(10)	–10.76(7)				
$[MH_2L_2]^{2+}$		28.11(4)	28.71(5)			28.10(10)
$[MHL_2]^+$		20.81(8)	21.29(8)			20.90(10)
$[ML_2]$		13.14(7)	13.53(6)			13.17(9)
pK(ML/MLH_{-1})		8.01	9.63	6.52	7.24	7.72

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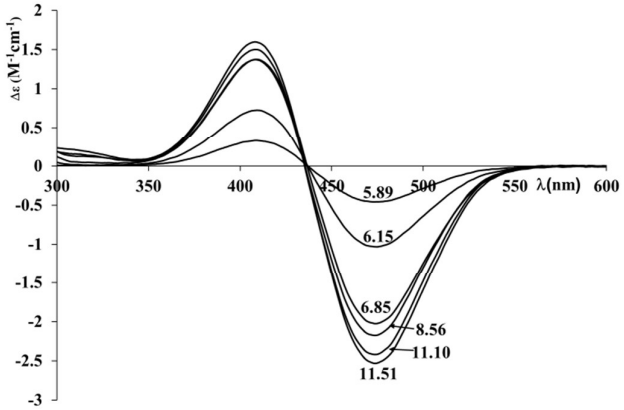

Figure 1. CD spectra of the nickel(II)-AAHAAC system recorded in equimolar samples at different pH values. c_L = 2 mM.

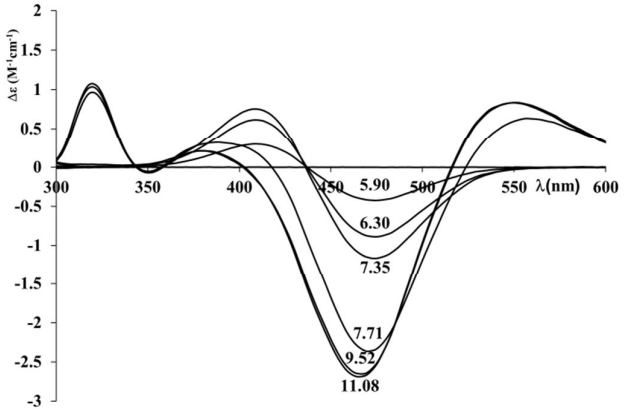

Figure 2 CD spectra of the nickel(II)-AAHAAC = 2:1 system recorded at different pH values. c_L = 2 mM.

All data in the previous paragraph support the presence of a non-coordinated thiolate function in the $[NiH_{-2}L]^-$ complex of AAHAAC. This observation is slightly surprising if one takes into account the high affinity of thiolate-S atoms for nickel(II) binding[5]. However, it should be taken into account that this observation is valid only for the equimolar samples where the albumin-like binding site is favoured over the metal binding at an internal thiolate side chain. Similar observations were obtained for the nickel(II) complexes of the octapeptide AAHAAAHG where the internal histidines were also not able to compete with the albumin site[18]. The presence of this second histidine, however, provided a chance for the binding of another metal ion in the presence of metal ion excess. The similar reaction was detected in the nickel(II)-AAHAAC= 2:1 system, too. Binding of the second metal ions was observed in alkaline samples (above pH 7.7) in parallel with the formation of nickel(II) hydroxide precipitate. This precipitation ruled out the determination of stability constants for the 2:1 species, but the UV-Vis and CD measurements undoubtedly prove the binding of a second nickel(II) ion at the deprotonated thiolate site. A new shoulder starts to develop at λ = 550 nm (d-d transition) and λ = 320 nm (Ni(II)→S^- CT) above pH 7.5 which is characteristic of Ni-S coordinated complexes. The corresponding CD spectra are plotted in Figure 2 and indicate an even more drastic change of CD extrema in the same pH range. The high intensity of the CD spectra of the 2:1 samples can only be explained if the coordination of the preceding amide functions is also considered starting from the thiolate anchoring site.

Table 2 Protonation and stability constants (log β_{pqr}) of the metal complexes of the tetrapeptide Ac-HAAC. T = 298 K, I = 0.2 M (KCl for Ni(II) and Zn(II), KNO_3 for Cd(II)), standard deviations are in parenthesis)

	Ac-HAAC		
Species	log β_{pqr}	pK	
[HL]	8.81(2)	8.81	
$[H_2L]^+$	15.30(3)	6.49	
	Ni(II)	Zn(II)	Cd(II)
$[MHL]^{2+}$	11.23(16)	12.05(17)	12.54(10)
$[ML]^+$	5.17(3)	7.02(3)	7.70(2)
$[MH_{-1}L]$	-	–0.35(7)	–2.35(5)
$[MH_{-2}L]^-$	–10.81(3)	–10.39(6)	–14.21(6)
$[MH_{-3}L]^{2-}$	–21.53(8)	-	-
$[ML_2]$	9.07(10)	13.58(4)	13.34(7)
$[MH_{-1}L_2]^-$	-	3.06(12)	2.27(9)

The results obtained for the metal complexes of the terminally protected tetrapeptide Ac-HAAC provided further evidence for the above mentioned binding mode. Stability constants of the nickel(II), zinc(II) and cadmium(II) complexes of the tetrapeptide are included in Table 2. The speciation curves of the nickel(II)-Ac-HAAC system show that the protonated complex of $[MHL]^{2+}$ is a minor species (see Figure S4 in Supplement) while the parent complexes $[ML]^+$ and $[ML_2]$ predominate below pH 8. The absorption spectra of the samples clearly indicate that all species present above pH 6 have square planar coordination geometry but the CD spectra of the parent and deprotonated complexes are significantly different. Only very low intensity CD extrema can be recorded for the species $[ML]^+$ and $[ML_2]$ suggesting the involvement of the imidazole-N and thiolate-S^- in metal binding. This type of coordination was not observed with the hexapeptide AAHAAC because of the outstanding nickel(II) binding ability of the amino terminus. The CD spectra of the species $[NiH_{-1}L]$ and $[NiH_{-2}L]$ are, however, quite similar to those observed in the nickel(II)-AAHAAC system in the presence of excess nickel(II) ions at high pH values. This observation supports that the C-terminal segment of the hexapeptide can be an independent metal binding site via the coordination of the thiolate-S^- and preceding two amide functions in the form of (N^-,N^-,S^-) fused chelates. This assumption is supported by Figure 3 where the CD spectra of the nickel(II) complexes of the tetra- and hexa-peptides are compared. It can be seen from Figure 3 that the CD spectra of the nickel(II)-AAHAAC= 2:1 samples at high pH well correlate with the sum of the spectra of 1:1 complexes recorded for AAHAAC and Ac-HAAC at the same pH values. The small differences between the recorded and calculated spectra come from the fact that the tetrapeptide Ac-HAAC is not the ideal model because in the case of the tetrapeptide the imidazole-N can also contribute to metal binding starting from

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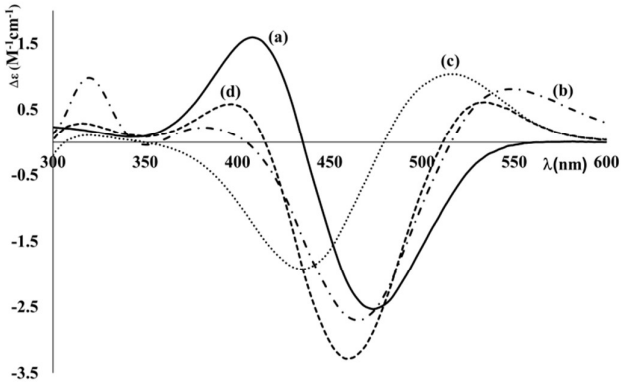

Figure 3 CD spectra of the nickel(II)-AAHAAC = 1:1 (a) and 2:1 (b) systems at pH 11.51 and 11.60, respectively. CD spectra of nickel(II)-Ac-HAAC = 1:1 system at pH 11.02 (c). Spectrum (d) is obtained from the superposition of spectra (a) and (c).

the thiolate function, while it is a major constituent of the N-terminal binding site of the hexapeptide.

A series of previous literature studies support that zinc(II) and cadmium(II) ions are not able to promote the binding of amide nitrogens in the peptides containing the albumin-like sequences [3,18,25]. The data in Table 1 are in agreement with this expectation but also reveal the enhanced thermodynamic stability of the zinc(II) and especially cadmium(II) complexes as compared to those of the corresponding nickel(II) species. This strongly supports that in contrast with the equimolar nickel(II) systems the binding of thiolate residues takes place in all zinc(II) or cadmium(II) containing species. The speciation curves of a multicomponent system provide the first justification of this statement. The concentrations of the various species formed in the zinc(II)-AAHAAC-AAHAAAHG system are plotted in Figure 4.

It is clear from this Figure that the complexation with AAHAAC (ligand A) is favoured over AAHAAAHG (ligand B) in the whole pH range. It is also obvious that only nitrogen donors can be coordinated in the complexes of ligand B, and the enhanced

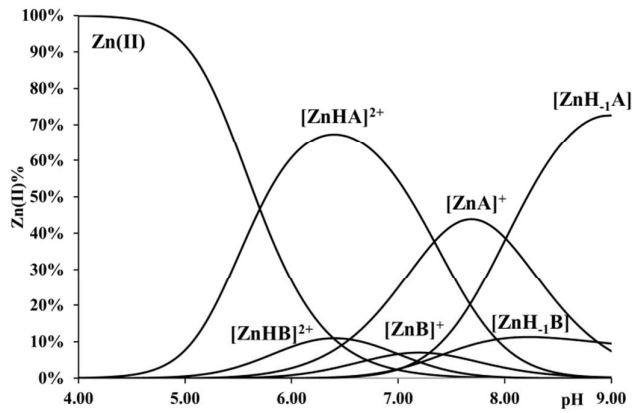

Figure 4 Concentration distribution of the complexes formed in the zinc(II) – AAHAAC (A) -AAHAAAHG (B) = 1:1:1 model system. $c_{Zn(II)}$ = 2 mM.

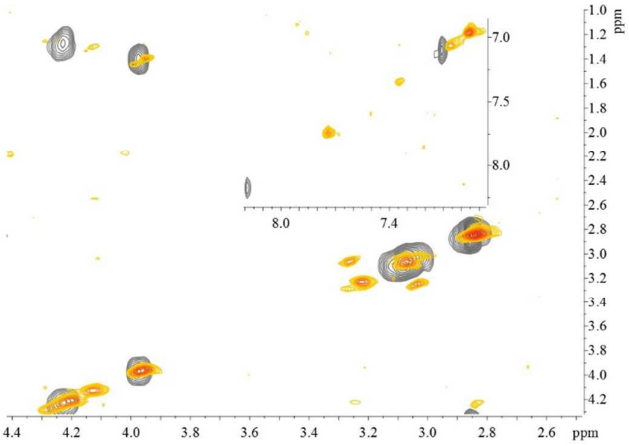

Figure 5 Selected regions of ^{1}H-^{1}H TOCSY spectra of AAHAAC (black) and Zn(II):AAHAAC 1:1 metal to ligand ratio (yellow) at pH 6.0 (c_L = 10 mM)

stability of the complexes of AAHAAC peptide can arise only from the coordination of sulphur atoms. 2D NMR measurements provided a more direct evidence for the occurrence of this binding mode (Figure 5). This spectrum indicates the binding of the imidazole-N and thiolate-S^- donors at pH 6.0 where the species $[ZnHL]^{2+}$ predominates, while the CH-CH_3 signals of the N-terminal Ala remain intact. This (N_{im},S^-) bidentate binding mode in a macrochelate while the amino terminus remains intact is slightly surprising but both imidazole and thiolate donor functions are known as effective ligating sites either for zinc(II) or cadmium(II) ions. On the other hand, the existence of coordination isomers in low concentration cannot be ruled out in which the imidazole or thiolate residues remain protonated. The predominance of the (N_{im},S^-) binding mode is, however, further supported by the results obtained for the zinc(II) and cadmium(II) complexes of Ac-HAAC. The stability constants of the simple $[ML]^+$ complexes of the tetrapeptide are very similar to those reported for the hexapeptide (see Tables 1 and 2). Moreover, this bidentate coordination mode provides the possibility for the formation of bis(ligand) complexes which are present both in the zinc(II) and cadmium(II) containing systems with the AAHAAC and Ac-HAAC ligands.

The comparison of the data obtained for the zinc(II) and cadmium(II) complexes of the peptide AAHAAC suggests very similar complex formation processes and coordination modes of the two metal ions. The enhanced stability of the cadmium(II) complexes is in accordance of the increased affinity of cadmium(II) ions towards the sulfur donor ligands[26]. The only difference is related to the formation of the $[MH_{-1}L]$ species, which is more favoured in the zinc(II) containing systems. This tendency is, however, in accordance with the higher tendency of zinc(II) ion to form hydroxido complexes as compared to that of cadmium(II). A similar tendency of the equilibrium data is valid for the complexes of Ac-HAAC. In other words this means that the deprotonation reactions in the zinc(II)- and cadmium(II)-AAHAAC systems cannot be

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assigned to the amide functions but they belong to hydroxido complex formation with both metal ions.

Complex formation with AHAAAC

Stability constants of the metal complexes of the peptide AHAAAC are collected in Table 1 and in this case the data reveal a similarity in the complex formation processes of the nickel(II) and zinc(II) ions while a significantly different speciation was obtained for the cadmium(II) containing systems. Only 1:1 complexes are formed with nickel(II) and zinc(II) and the formation of zinc(II) complexes is slightly favoured over the corresponding nickel(II) species. This trend unambiguously proves the involvement of thiolate sulphur donor atom in all zinc(II) containing species but the UV-Vis absorption spectra strongly support the occurrence of this binding mode in the corresponding nickel(II) complexes, too. $[NiHL]^{2+}$ is only a minor species in which either the amino or thiolate functions can be protonated. The deprotonation of this site is not accompanied with the development of CD absorption in the visible range below pH 6.0 suggesting that $[NiL]^{+}$ contains a tridentate (NH_2,N_{im},S^-)-coordinated ligand. $[NiH_{-1}L]$ is the major and single species above pH 7.0 and its stoichiometry can be best interpreted by the nickel(II) promoted deprotonation and coordination of the amide-N donor between Ala(1) and His(2) residues. This is a common feature of the nickel(II) complexes with similar sequences. The corresponding pK value is 6.03 in the nickel((II) GlyHis system while pK = 6.52 can be calculated for AHAAAC. The small increase can be explained by the involvement of thiolate residue in nickel(II) binding which enhances the stability of the species $[NiL]^{+}$ and slightly suppresses the amide deprotonation. UV-Vis spectra unambiguously demonstrate that $[NiH_{-1}L]$ contains square planar nickel(II) ion in the (NH_2,N^-,N_{im},S^-) coordination environment. This coordination mode remains intact in a wide pH range but the CD spectra recorded in strongly alkaline samples reveal a reorganization of the coordination sphere of nickel(II) ions. The intensity of the positive Cotton effect of the (NH_2,N^-,N_{im},S^-) coordination mode at 444 nm is continuously decreasing by the increase of pH in parallel with the development of a new negative absorption at 494 nm characteristic of the tetraalanine complexes of nickel(II) ions. The transformation of these coordination modes is not complete even in extremely alkaline conditions (55 % in (NH_2,N^-,N_{im},S^-)- and 45 % in (NH_2,N^-,N^-,N^-)-coordinated forms are present at pH 11.63), so we can conclude that the (NH_2,N^-,N_{im},S^-) binding mode predominates in the biologically relevant pH range, while an equilibrium with 4N coordinated complexes can exist in the strongly alkaline samples.

The CD spectra recorded in the presence of excess of nickel(II) ions and at high pH values reveal the existence of the (N^-,N^-,S^-) coordination mode, too. It is important to note that in contrast with AAHAAC, the peptide AHAAAC cannot keep two equivalents of nickel(II) ions in solution and stability constants for dinuclear species cannot be calculated. This difference can be easily explained by the involvement of the thiolate function of AHAAAC in the mononuclear nickel(II) species, while this site is free in the complexes of AAHAAC. The nickel(II)-hydroxide precipitate, however, slowly dissolves above pH 10 with the concomitant development of the CD absorption of the (N^-,N^-,S^-) coordination mode.

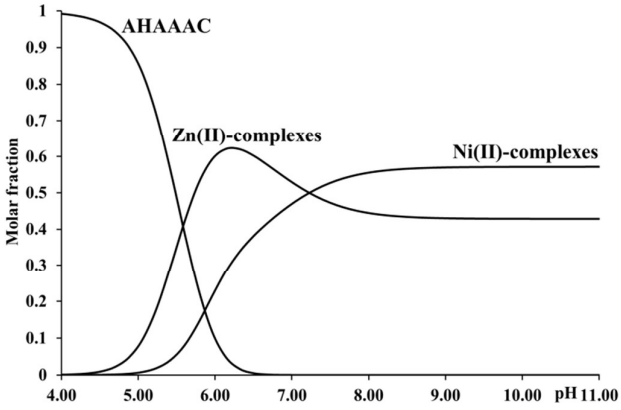

Figure 6 Concentration distribution of the complexes formed in the zinc(II) – nickel(II) - AHAAAC = 1:1:1 system. c = 2 mM.

The metal ion speciation of the zinc(II)- and nickel(II)-AHAAAC systems are compared in Figure 6 reflecting the formation of similar species and the enhanced zinc(II) binding under acidic conditions. In contrast with the nickel(II) containing systems, the protonated complexes are formed in a well detectable concentration in this case and the ^{1}H NMR measurements strongly support the binding of the imidazole-N and thiolate sulphur donors. The slight downfield shift of imidazole-CH and cysteine-CH_2 protons can be observed in acidic pH range while the signals of the alanine residues remain intact. The formation of $[ZnL]^{+}$ significantly overlaps that of the other two species therefore it is difficult to obtain a clear-cut evidence for its binding sites but the (NH_2,N_{im},S^-) tridentate behaviour of the ligand is the most probable similarly to the same species formed with the other hexapeptide AAHAAC. On the other hand, it is also obvious from Figure 6 that the Zn(II) > Ni(II) stability order is reversed by increasing pH when the coordination of amide nitrogens is favoured. This type of reaction is more preferred in nickel(II) peptide complexes but the formation of the species $[ZnH_{-1}L]$ can also be assigned to amide deprotonation and coordination in contrast with the zinc(II)-AAHAAC system where hydroxido complexes were formed in the alkaline samples. 2D NMR spectra of the zinc(II)-AHAAAC = 1:1 system at pH 9.5 is plotted in Figure 7 and the large upfield shift of the His-CH_2 protons is a good indication for the presence of the negatively charged amide-N in the vicinity of the methylene protons. The zinc(II) ion promoted deprotonation of amide groups between the terminal amino and imidazole-N donors of His(2) residues, (NH_2,N^-,N_{im}) coordination, has already been proved in many zinc(II) complexes with peptides containing histidyl residues in position-2 in the sequence[3,18,27]. The corresponding pK value is 7.24 in the zinc(II)-AHAAAC system which is about 0.5 log unit higher than that of the corresponding zinc(II) complexes of GH[28] and AHAAAHG[18] peptides. This is an indirect proof for the

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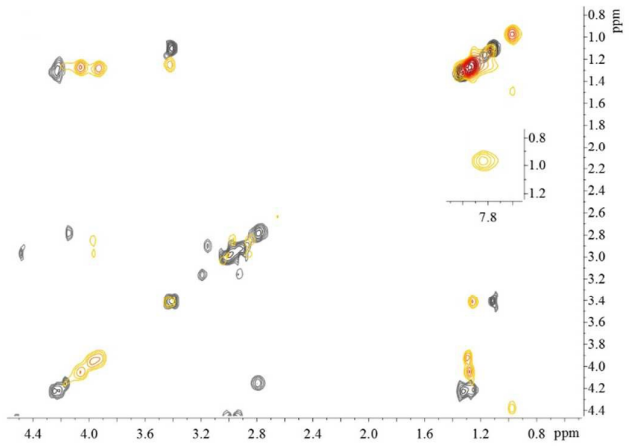

Figure 7 Selected regions of ^{1}H-^{1}H TOCSY spectra of AHAAAC (black) and Zn(II):AHAAAC 1:1 metal to ligand ratio (yellow) at pH 9.2 (c_L=10 mM)

enhanced stability of the zinc(II) complexes of thiolate ligands as compared to the histidine containing analogues. The distribution of zinc(II) ions among the peptides AHAAAC (A-ligand) and AHAAAHG (B-ligand) is shown by Figure S5 in Supplement.

It is clear from this Figure that more than 90 % of zinc(II) ions are bonded to the peptide containing the cysteinyl residue.

In agreement with the results obtained for the metal complexes of the peptide AAHAAC, cadmium(II) ions formed the highest stability complexes with AHAAAC, too. The speciation and the binding modes of several species are, however, different for the zinc(II) and cadmium(II) complexes of AHAAAC. At the same time, there is very high similarity in the cadmium(II) complexes of AAHAAC and AHAAAC ligands (see Table 1). The speciation of the two systems and even the numerical values of the stability constants are quite similar. This observation can be easily explained by the lack of the cadmium(II) promoted amide deprotonation and coordination in the corresponding systems. Up to know, the cadmium(II)-ACSSACS system[29] is the exclusive example for this type of reaction but cadmium(II) promoted amide binding has not been reported for the peptides of histidine, yet. Thus, the species [$CdH_{-1}L$] is a simple hydroxido complex containing a (NH_2,N_{im},S^-) tridentate peptide ligand. 2D NMR measurements give the proof for the hydrolytic reaction (see Figure S6 in Supplement) because the significant upfield shift of the His-CH_2 protons cannot be observed in this case. The existence of coordinative unsaturated cadmium(II) ions makes also possible the formation of bis(ligand) complexes with this peptide, too.

The data of the studied two peptides clearly show that for zinc(II) and cadmium(II) ion the terminal amino and the side chain donor groups serve as the main metal binding sites. Both imidazole and thiolate group is able to coordinate zinc(II) as well cadmium(II) ions. As it is known, however, in the case of zinc(II) and cadmium(II) ions the binding stability of thiolate group generally follows opposite trend (Cd(II) > Zn(II)) to that

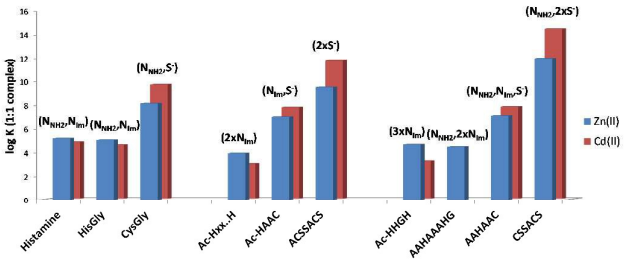

Figure 8 The log K values of zinc(II) and cadmium(II) complexes with 1:1 stoichiometry (Data from refs.: 18,29,30-36)

of histidine (Zn(II) > Cd(II))[26]). The thermodynamic data of these two peptides are agreement with this fact. As a conclusion the substitution of one or two histidyl moieties by cysteinyl residues results in much higher enhancement of stability of cadmium(II) complexes than that of zinc(II) complexes. This effect could be well demonstrated by the Figure 8, where the logK values of Zn(II) and Cd(II) complexes with 1:1 stoichiometry of different peptides containing one or more histidines and/or cysteines are depicted. This tendency supports that the cadmium(II) binding selectivity of this type of peptides could be even raised by insertion of further cysteines in the peptide chains.

Conclusions

Nickel(II), zinc(II) and cadmium(II) binding affinities of two N-terminally free hexapeptides AAHAAC and AHAAAC containing separate histidyl and cysteinyl residues have been compared in this study. Both peptides have outstanding metal binding ability but the speciation of the systems and the binding sites of peptides reveal a significant specificity. The N-terminus of AAHAAC corresponds to albumin-like sequence with enhanced nickel(II) binding affinity. As a consequence, in the equimolar samples of nickel(II) and AAHAAC the amino terminus is the exclusive nickel(II) binding site in the form of the (NH_2,N^-,N^-,N_{im}) chelate. The C-terminal thiolate function, however, can bind another nickel(II) ion with the involvement of preceding amide nitrogens in metal binding. The N-terminally blocked tetrapeptide Ac-HAAC was used to mimic the binding modes of the C-terminal segment of the hexapeptide and the preference for the (N^-,N^-,S^-) binding mode was obtained for this ligand, too. Only mononuclear complexes are formed with zinc(II) and cadmium(II) ions in which the imidazole-N and thiolate-S^- donors are the primary metal binding sites. The thermodynamic stability of this coordination mode follows the Cd(II) > Zn(II) > Ni(II) order which does not correspond to the usual Irving-Williams series, but in neutral and basic media the amide bonded nickel(II) complexes predominate.

In the case of AHAAAC, the imidazole-N and thiolate-S^- donors are the primary metal binding sites in slightly acidic media with the similar enhanced cadmium(II) and/or zinc(II) binding affinity. The major differences in the complex

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formation processes of AAHAAC and AHAAAC are related to the stoichiometry and binding modes of amide coordinated species. Both nickel(II) and zinc(II) ions can induce deprotonation and coordination of the first amide bond in the sequence of AHAAAC. The coordination mode of the corresponding species, [$NiH_{-1}L$], contains tridentate (NH_2,N^-,N_{im}) joined chelates supported by a macrochelate from the distant thiolate group. Neither equilibrium nor NMR spectroscopic measurements indicate cadmium(II) induced deprotonation and coordination of amide groups supporting the same coordination mode of the two peptides in their cadmium complexes.

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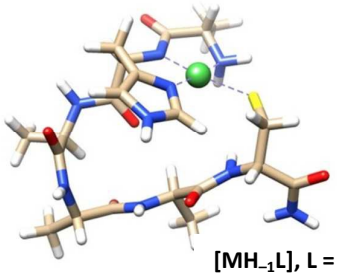
Graphical abstract



[$MH_{-1}L$], L = AHAAAC, M = Ni(II) or Zn(II)

The hexapeptides containing separate histidyl and cysteinyl residues have outstanding metal binding ability but the binding sites of peptides reveal a significant specificity.