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ARTICLE

Zinc(II) and cadmium(II) complexes of N-terminally free peptides containing two separate cysteinyl binding sites

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The hexa- and hepta-peptides CSSACS-NH_2 and ACSSACS-NH_2 have been synthesized by solid phase peptide synthesis and their zinc(II) and cadmium(II) complexes studied by potentiometric, NMR spectroscopic and ESI MS techniques. Both peptides have outstanding zinc(II) and cadmium(II) binding affinity but their coordination chemistry is different. In the case of the hexapeptide, the amino terminus is the primary metal binding site in the form of a stable (NH_2,S^-) 5-membered chelate supported by macrochelation via the distant cysteinyl residue. The heptapeptide ACSSACS-NH_2 is a slightly less effective metal binder but its coordination chemistry is more versatile. The thiolate groups are the primary binding sites for both metal ions and an 18-membered (S^-,S^-) macrochelate is the favored coordination mode of the peptide. In slightly basic samples the deprotonated amino group can also contribute to metal binding. Moreover, the interaction of the terminal amino-N and the thiolate-S^- of Cys(2) moiety can promote the deprotonation and metal ion coordination of the amide group between these residues. This reaction results in the formation of the (NH_2,N^-,S^-) fused chelates supported by the thiolate of the distant cysteinyl residue. Zinc(II) induced deprotonation and coordination of amide groups have already been described in various peptides of histidine, but in the case of cadmium(II) this is the first example for the formation of a Cd-N(peptide amide) bond.

Introduction

The imidazole-N of histidyl and thiolate-S donor atoms of cysteinyl residues are the most common metal binding sites of proteins. Metal complexes of peptides of histidine have been widely studied in the last few decades and the most important findings are summarized in several reviews[1-4]. Multihistidine peptides can easily form both homo- and hetero-nuclear complexes[5] and the studies on this subject are especially promoted by the possible involvement of these peptides in neurodegeneration[6,7]. The biological importance of peptides of cysteine is also outstanding; the blue copper proteins, zinc finger proteins, iron-sulfur proteins and metallothioneins represent probably the most common examples. The synthesis and studies of the peptide complexes containing cysteinyl residues are, however, rather complicated because of the parallel acid-base and redox reactions with many metal ions. These redox reactions cannot occur in the systems containing nickel(II), zinc(II), cadmium(II) or bismuth(III) ions and, as a consequence, these are probably the most studied metal ions by the peptides of cysteine[8-10]. It is a common feature of these peptides that the presence of thiolate side chains significantly enhances the metal binding ability towards the borderline and soft metal ions but the specific coordination modes highly depend on the nature of the metal ions. Nickel(II) ion was reported to promote binding of both thiolate and deprotonated amide residues[11,12], while the formation of stable (S^-,NH_2) or (S^-,COO^-) chelates was characteristic of the zinc(II) and cadmium(II) containing systems[13]. Thiolate bridged polynuclear complexes can be formed with any metal ions but these species are especially important in the cadmium(II) complexes[14,15].

Studies on the zinc(II) complexes of polythiolate peptides were first promoted by the structural characterization of zinc finger proteins. The results obtained for the related small peptides containing one or two cysteinyl residues revealed a high structural variety of these species[16-19]. More recently, the discovery of various human zinc transporter proteins and the peptides related to helicobacter pylori containing both histidyl and cysteinyl binding sites gave further impetus to the studies on the zinc(II) complexes of polythiolate ligands[20-23]. These studies provide further examples on the structural variety of the corresponding zinc(II) complexes. Moreover, it is another important conclusion from these works that the metal ion affinity, especially the nickel(II) over zinc(II) preference, highly

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depends on the sequence of polythiolate ligands. The studies on the cadmium(II) complexes of thiol containing amino acids and peptides have been reviewed recently[24]. In agreement with the expectations, the results reveal a high similarity in the structure and stoichiometry of the corresponding zinc(II) and cadmium(II) complexes. On the other hand, it is also clear from these studies that cysteine is the only amino acid residue which is able to change the zinc(II) over cadmium(II) preference of peptides and the formation of polynuclear clusters is much favored with cadmium(II) ions.

The previous literature studies on the zinc(II) and cadmium(II) complexes of polythiolate peptides reveal the determining role of the amino acid sequences of peptides in complex formation. The better understanding of this role requires further systematic studies on the model peptides containing cysteinyl residues in specific positions. A similar study has already been performed for the corresponding peptides of histidine containing this moiety in both N-terminal and internal positions[25]. Now in this paper we report the synthesis and the results obtained for the zinc(II) and cadmium(II) complexes of two N-terminally free peptides containing two separate cysteinyl residues in different environments. The hexapeptide CSSACS-NH_2 contains one cysteine at the N-terminus providing a chance for the formation of a stable (S^-,NH_2) 5-membered chelate, while the other cysteine can work as an independent metal binding site. Alcoholic-OH side chains of serine is generally not a metal binding site for these metal ions but the presence of polar side chains significantly enhances the solubility of peptides and their complexes as compared to those of the alanine counterparts. In the heptapeptide ACSSACS-NH_2 the first cysteine may promote amide binding or the two separate cysteines and the terminal amino group can form various macrochelates.

Experimental

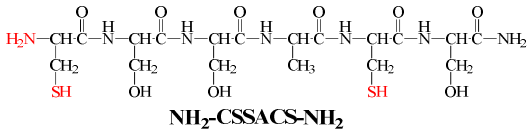
Peptide synthesis and other materials

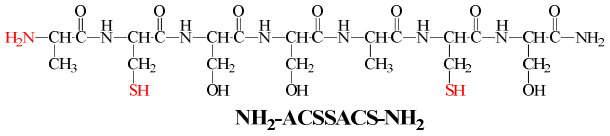
Both terminally free peptides CSSACS-NH_2 and ACSSACS-NH_2 (see Scheme 1 and assignments in the Supplement Figure S1 and Figure S2) were synthesized by solid phase peptide synthesis using a microwave-assisted Liberty 1 Peptide Synthesizer (CEM, Matthews, NC). Fmoc-protected amino acid derivatives were introduced according to the Fmoc/*t*Bu technique and the TBTU/HOBt/DIEA strategy. Cleaving on the α-amino protecting group of amino acids and resin was performed by 30 Watts microwave power for 180 s at 80 °C using 20 V/V% piperidine and 0.1 M HOBt·H_2O in DMF. Four times excess of amino acids and 30 Watts 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 in NMP as activator base. After building up the peptide sequences, Fmoc group was removed similarly while the side chain protecting groups were cleaved with the treatment of TFA/TIS/H_2O/ 2,2'-(ethylenedioxy)diethanethiol (94/2.5/2.5/1 V/V) mixture, simultaneously with the removal of the peptide from the resin, at room temperature for 2 h. The resin was filtered from the trifluoracetic acid solution of the peptide which was precipitated in cold diethyl ether. The crude product was also washed using this solvent and dried after separation followed by dissolution in water and freezing for lyophilization.

The purity of the prepared products was checked by analytical RP-HPLC using a Jasco instrument equipped with a Jasco MD-2010 plus multiwavelength detector monitoring the absorbance at 222 nm.

Gradient elution was carried out using solvent A (0.1 V/V% TFA in acetonitrile) and solvent B (0.1 V/V% TFA in water) at a flow rate of 0.8 ml/min. From 1 min to 15 min 0 to 25% of A, from 15 min to 16 min 25% of A and from 16 min to 20 min 25 to 0% of A was applied and a Teknokroma Europa Peptide C18 chromatographic column (250 x 4.6 mm, 120 Å pore size, 5 µm particle size) was used.

Chemicals and solvents used for synthetic purpose 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-Ser(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 (DIEA) 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 Ltd and piperidine, while dichloromethane (DCM), diethyl ether (Et_2O), acetic acid (AcOH) and acetonitrile (ACN) from Molar Chemicals Ltd.





Scheme 1. Structural formulae of ligands. The donor atoms are represented in red.

Potentiometric measurements

The pH-potentiometric titrations were performed in 3 mL samples at 2 mM ligand concentration with the use of carbonate-free stock solution (0.2 M) of potassium hydroxide. The metal ion to ligand ratios were selected as 1:1, 1:2 and 1:3. During the titration, 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. The ionic strength was adjusted to 0.2 M with KCl in the case

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of zinc(II) complexes, while KNO_3 was used for cadmium(II) to avoid the formation of chlorido complexes. pH measurements were made with a MOLSPIN pH-meter equipped with a 6.0234.100 combination glass electrode (Metrohm) and a MOL-ACS microburette controlled by a computer. The recorded pH readings were converted to hydrogen ion concentration. Protonation constants of the ligands and overall stability constants (lg β_{pqrs}) of the metal complexes were calculated by means of the general computational programs, PSEQUAD and SUPERQUAD as described in our previous publications[24,25]. The equilibrium constants were defined by equations (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 measurements

400 MHz ^{1}H and 88.8 MHz ^{113}Cd 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) DCl and KOD, while DNO_3 and KOD were used for cadmium(II) to set the pH of the samples. ^{1}H-^{1}H COSY experiments were carried out with standard pulse sequences. TOCSY spectra were registered in aqueous solution using a D_2O capillary. The experiments were carried out with a standard pulse sequence combined with water suppression using 3-9-19 pulse sequence with gradients.

For diffusion measurements stimulated spin echo sequence (PGSE) was used with longitudinal eddy-current delay (LED) and bipolar gradient pulses (BIPLED) by the standard program provided with the spectrometer[28]. In the experiments we used a constant diffusion time (Δ), typically 50–80 ms, and gradient pulse length (δ), 2 ms. The pulsed gradient strength (G) increased with 32 square distant steps from 0 to approximately 50 Gauss cm^{-1}. The diffusion coefficient was determined from the attenuation of echo signals by increasing the pulsed gradient strength (G) as shown in the SI according to eqn. The samples were prepared in light water using D_2O containing insert capillary for deuterium lock. No water suppression was used in order to avoid peak distortion. The integrated intensity of methyl peaks at about 1 ppm was used for the evaluation. The calculation of the real numeric values of diffusion coefficients (D calibration) was performed using the known diffusion coefficient of H_2O at the given temperature[29]. From the diffusion coefficients the hydrodynamic radii (R_H) of the species can be calculated by the Stokes–Einstein equation.

^{113}Cd NMR spectra were recorded in DMSO-d^6 solution. The spectra were externally referenced to 0.1 M $Cd(NO_3)_2$ in DMSO-d^6. Acquisition parameters were the following: pulse length 15 µs (60°), pulse repetition time 10 s, spectral width 42 kHz, data point 65536 and total number of collected scans 16000. Spectra were evaluated by Bruker TopSpin™ and MestReNova software.

ESI-TOF-MS measurements were carried out on a Bruker micrOTOF-Q 9 ESI-TOF instrument in the negative mode. Temperature of drying gas (N_2) was 453 K. The pressure of the nebulizing gas (N_2) was 0.3 bar. The capillary voltage applied was 4000 V. The spectra were accumulated and recorded by a digitalizer at sampling rate of 2 GHz. The samples were performed in water at c_L= 0.1 mM at different metal to ligand ratio. KOH was added to the samples to adjust the pH.

Results and discussion

Protonation equilibria of the peptides

Both peptides have three protonation sites, the terminal amino and the two thiolate groups. Their pK values have been determined by potentiometric titrations and the data are included in Table 1. It is clear from Table 1 that protonation reactions of the three sites significantly overlap especially in the case of the heptapeptide. As a consequence, the pK values cannot be unambiguously assigned to specific donor functions.

Tab.1. Protonation constants of the peptides (T = 298 K, I = 0.2 M, KCl for zinc(II) and KNO_3 for cadmium(II) complexes, standard deviations are in parenthesis)

Species	CSSACS-NH_2		ACSSACS-NH_2	
	logβ	pK	logβ	pK
$[HL]^-$	9.21(3)	9.21	9.01(1)	9.01
$[H_2L]$	17.56(1)	8.35	17.21(1)	8.20
$[H_3L]^+$	24.06(2)	6.50	24.69(1)	7.48

pH-dependent ^{1}H NMR spectra have been recorded for both peptides (see Figure S3 and S4 in Supplementary information). The significant overlap of the signals of the -CH-CH_2- protons of cysteinyl residues hinders the exact calculation of protonation microconstants but the relative basicity of the functional groups can be estimated from these spectra. NMR peaks of the methyl group of alanine, can be easily assigned and reflect the pH range for the deprotonation of the terminal ammonium group. In the case of ACSSACS-NH_2 this process takes place from pH 7.0 to 9.0 suggesting that the lowest pK value belongs mainly to an ammonium function, while the deprotonation of the two thiol groups overlaps. Comparison of the pK values with the micro- and macro-constants reported for L-cysteine and its simple dipeptides (GlyCys and CysGly[13]) strongly supports that the more acidic thiol group is the one close to the amino terminus. In the case of CSSACS-NH_2 the first pK value (pK_1=6.50) is lower than the first dissociation constant of ACSSACS-NH_2 (pK_1=7.48) that can mean the formation of intramolecular hydrogen bond between the thiolate group and the N-terminal NH_2-group. This assumption was proved by ^{1}H-NMR spectroscopy.

Zinc(II) and cadmium(II) complexes of the hexapeptide CSSACS-NH_2

Stability constants of the zinc(II) and cadmium(II) complexes of CSSACS-NH_2 have been determined by potentiometric

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titrations and the data are included in Table 2. It is clear from Table 2 that the speciation of the two systems is very similar and quite simple; only mononuclear 1:1 complexes are formed in measurable concentration. Moreover, in the physiological pH range [ML] exists as an exclusive species for both metal ions (Figure 1.).

Tab.2. Stability constants of the zinc(II) and cadmium(II) complexes of CSSACS-NH_2 (T = 298 K, I = 0.2 M, KCl for Zn(II) and KNO_3 for Cd(II), standard deviations are in parenthesis)

Species	Zn(II)	Cd(II)
$[MHL]^+$	16.79(2)	18.87(4)
[ML]	12.02(3)	14.38(3)
$[MH_{-1}L]^-$	2.99(9)	3.20(3)
pK(MHL/ML)	4.77	4.49
pK(ML/MLH_{-1})	9.03	11.18

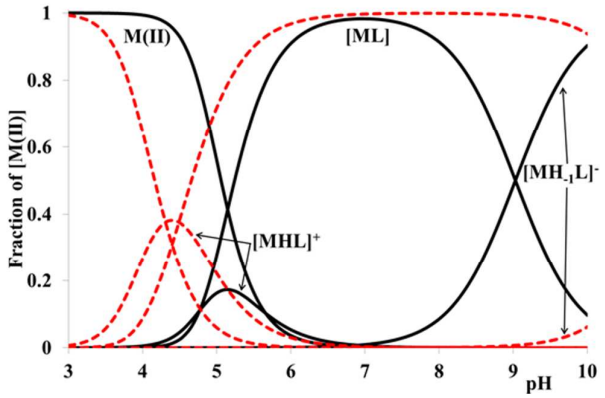

Fig.1. Concentration distribution curves of the complexes formed in the zinc(II) (solid lines) and cadmium(II) (dashed lines)–CSSACS-NH_2 system at 1:1 ratio (c_L = 1 mM)

It is also obvious from the data in Table 2 that the stability constants of these peptide complexes are much higher than those reported for simple peptides suggesting the governing role of the N-terminal cysteinyl residue in complex formation via the stable (NH_2,S^-) 5-membered chelate. Equilibrium constants cannot give the answer for the coordination mode of the peptide but a comparison of data with those reported for L-cysteine or D-penicillamine and CysGly can help to answer this question. These ligands have the same N-terminal binding sites but the corresponding stability constants for the species [ZnL] are 8.20 and 8.15 for L-cysteine[30] and CysGly[13], respectively, while in the case of [CdL] 11.53 and 9.84 were reported for D-penicillamine and CysGly[14], respectively. These values are significantly lower than those obtained for the complexes of the hexapeptide suggesting the involvement of all three binding sites in zinc(II) and cadmium(II) binding. Another difference in the complex formation processes of the hexapeptide and CysGly is the lack of the bis(ligand) complexes with the former ligand. This supports the tridentate coordination of CSSACS-NH_2, which consists of the (NH_2,S^-) 5-membered chelate supported by an 18-membered macrochelate created by the internal cysteinyl residue. Moreover, the lack of bis(ligand) complexes suggests a tetrahedral coordination geometry for both metal ions, which is characteristic of polythiol ligands.

The reliability of the speciation model and the suggested binding modes of complexes were further supported by NMR and ESI MS measurements, too. The ^{1}H NMR spectra of the free peptide (Figure 2.a) and those of the corresponding zinc(II) (2.b) and cadmium (2.c) containing systems at pD 3.60 are shown in Figure 2. The NMR spectra of the free ligand and the zinc(II) containing system are the same under this condition suggesting the presence of free metal ion and ligand in the sample (Figure 2.a and b). The lack of complexation is in a good agreement with the speciation model (Figure 1). The significant line broadening in the cadmium(II) containing system, however, supports complex formation by this pH (Figure 2.c). Moreover, this finding supports the higher thermodynamic stability of the cadmium(II) complexes.

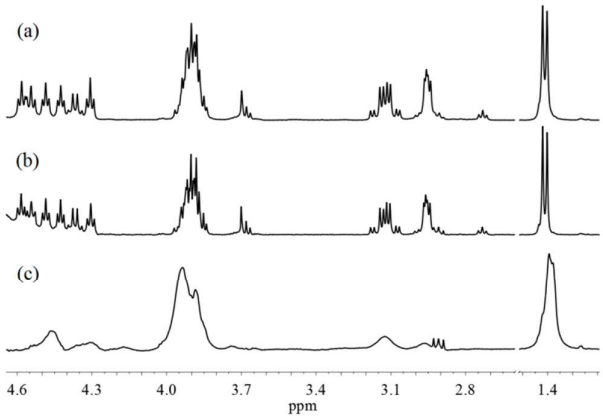

Fig.2. ^{1}H NMR spectra of CSSACS-NH_2 (a) and the corresponding zinc(II) (b) and cadmium(II) (c) containing systems at 1:1 metal to ligand ratios at pD 3.60.

The similar broadening of NMR signals can be observed in the corresponding zinc(II) complexes above pH 4.5 justifying the equilibrium model but ruling out the exact assignment of the various peaks and the determination of the metal binding sites. On the other hand, there is no any further change in the NMR spectra in the pH range 5-10 supporting the predominance of a single species in both the zinc(II) and cadmium(II) containing systems. The ESI MS results provide further support for this statement. ESI MS spectra of the cadmium(II) complex of CSSACS-NH_2 has been recorded at pH 7.3 and shown in Figure 3. The good agreement of the calculated and measured spectra can be used as a direct proof for the existence of the [CdL] complex at this pH. Mass spectra of the corresponding zinc(II) complexes are shown in Figure S5 in the Supplement. Moreover, ^{113}Cd-NMR spectra of the cadmium(II)– CSSACS-NH_2 system have also been recorded at 1:2.5 ratio in the presence of two equivalents of KOH. DMSO was used as a solvent in this experiment because of the low solubility of [CdL] species in aqueous media. A single peak was detected at 458 ppm and according to literature data[31] it corresponds well to the (S^-,S^-,NH_2) coordination mode.

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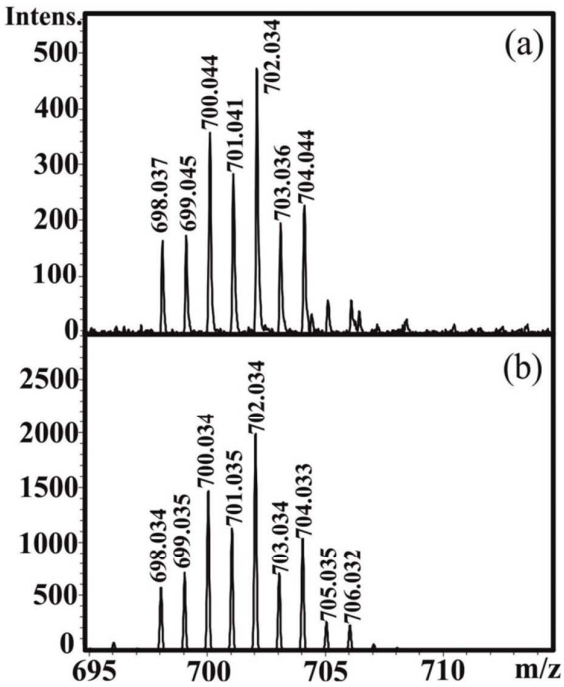

Fig.3. ESI MS spectra recorded in the cadmium(II)–CSSACS-NH_2 system at pH 7.3 (a) and calculated spectra for the species $[CdLCl]^-$=$[Cd(C_{18}H_{31}N_7O_9S_2)Cl^-]$(b).

Zinc(II) and cadmium(II) complexes of the heptapeptide ACSSACS-NH_2

Stability constants of the metal complexes of the heptapeptide have been calculated from the potentiometric titration curves and the data are collected in Table 3. It is clear from Table 3 and also from the speciation curves (Figure 4) that the complex formation processes of the two metal ions are quite similar.

Tab.3. Stability constants of the zinc(II) and cadmium(II) complexes of ACSSACS-NH_2
(T = 298 K, I = 0.2 M, KCl for Zn(II) and KNO_3 for Cd(II), standard deviations are in parenthesis)

Species	Zn(II)	Cd(II)
$[MHL]^+$	17.00(4)	19.23(2)
[ML]	9.88(7)	11.98(5)
$[MH_{-1}L]^-$	1.92(6)	3.41(5)
$[MH_2L_2]$	32.68(4)	34.35(9)
$[MHL_2]^-$	24.94(5)	26.40(10)
$[ML_2]^{2-}$	16.80(4)	17.29(8)
log K_1/K_2	2.96	6.67
log K_1/K_2(H)	1.32	4.11
pK (MHL/ML)	7.12	7.25
pK (ML/$MH_{-1}L$)	7.96	8.57
pK (MH_2L_2/MHL_2)	7.74	7.95
pK (MHL_2/ML_2)	8.14	9.11

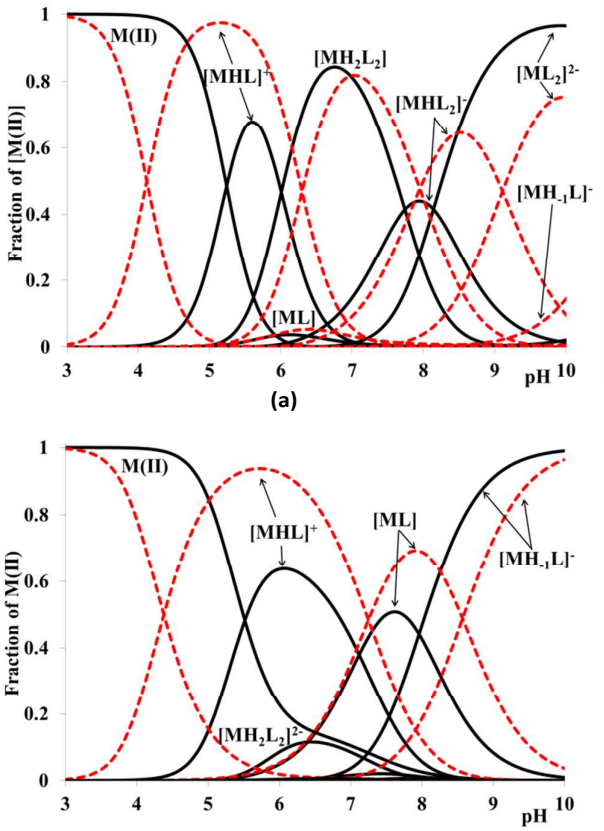

Fig.4. Concentration distribution curves of the complexes formed in the zinc(II) (solid lines) and cadmium(II) (dashed lines)–ACSSACS-NH_2 system at 1:3 ratio (a) and 1:1 ratio (b) (c_L = 2 mM)

The protonated complexes $[MH_2L_2]$ and $[MHL]^+$ predominate in the slightly acidic pH range indicating the involvement of only two functional groups in metal binding in these species. There is no chance for the formation of a stable 5- or 6-membered chelate with this ligand thus either one of the thiols or the amino group can be protonated. pK values for the deprotonation of the complexes are also included in Table 3 but these values are rather similar to those of the free ligand and do not help to assign the protonated sites. ^{1}H NMR spectra of the zinc(II) and cadmium(II) containing systems, however, helped to answer this question. pH-dependent NMR spectra of ACSSACS-NH_2 have already been plotted in Figure S4 and the NMR peaks of the two Ala residues can be easily assigned in these spectra. NMR peaks of the methyl group of the terminal Ala residue are shifted from 1.55 ppm to 1.26 ppm in the pH range 6-10, while the signals of the other Ala side chain remain intact in the whole pH range. In the presence of zinc(II) or cadmium(II) ions there is no any measurable change in the positions of the signals of the terminal Ala residue, although the extent of complexation is almost 100% by this pH. This observation unambiguously proves that the amino group of terminal Ala residue remains protonated in the species $[MH_2L_2]$ and $[MHL]^+$, while the two thiolate residues are the major metal binding sites in the form of an 18-membered macrochelate. Further increase of pH results in the upfield shift of the methyl protons but this occurs in parallel with the

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free ligand suggesting that the terminal amino group does not have a significant contribution to metal binding.

The difference in the preferred binding modes of the hexa- and heptapeptides is reflected in the possibility of the formation of bis(ligand) complexes, too. The stable tridentate coordination of CSSACS-NH_2 hindered the formation of any bis(ligand) complexes, while these species predominate with the heptapeptide in the presence of excess of ligand. ESI MS measurements have also been performed to prove the formation of bis(ligand) complexes. Mass spectra of the cadmium(II) complex of ACSSACS-NH_2 have been recorded at 1:2 metal to ligand ratio at pH 9.34. The good agreement of the experimental and calculated spectra (Figure 5) provides a direct evidence for the existence of $[CdL_2]^{2-}$ species in this sample.The data described in the previous paragraph seem to support the same speciation and a high similarity in the binding modes of ACSSACS-NH_2 in the zinc(II) and cadmium(II) complexes. The preference for cadmium(II) binding over zinc(II) can also be observed although its extent is smaller than for the hexapeptide. The ratios of stepwise stability constants, however, reveal some important differences in the complex formation processes of the two peptides. These values can be calculated for both the protonated ($logK_1/K_2(H)$) and the parent complexes ($\log K_1/K_2$) and the data are also included in Table 3. It is clear from these data that the enhanced stability of the cadmium(II) complexes is mainly reflected in the data obtained for the 1:1 species, $[CdHL]^+$ and [CdL]. As a consequence, the ratio of stepwise stability constants is unusually high for the cadmium(II) complexes suggesting some difference in the coordination geometry of the two metal ions in the corresponding complexes. Moreover, the comparison of the ($logK_1/K_2(H)$) and ($\log K_1/K_2$) values reveals that the deprotonation of the protonated complexes results in a further increase of the ratio of stepwise constants supporting the contribution of the terminal amino group to metal binding. This statement may contradict with the NMR spectra discussed in the previous paragraph where the high similarity of the NMR peaks of the methyl protons of terminal Ala residue was detected for the free and coordinated ligand. However, it should be considered that the significant line broadening and the fast ligand exchange reactions make it difficult to distinguish between the NMR signals of free and weakly coordinated ligands.

The most interesting finding of this study is probably connected to the presence and especially the binding mode of the species $[MH_{-1}L]^-$. This species was formed with both metal ions and predominates above pH 9.0 but only in equimolar samples (see Figure 4.b). The same species were also detected for the hexapeptide and the extra deprotonations were explained by hydroxido complex formation. The unsaturated coordination sphere of the metal ions supported this assumption. The same interpretation may be applied for the complexes of ACSSACS-NH_2, too but the stability contstants, as well as ESI MS and ^{1}H NMR measurements led to a different conclusion.

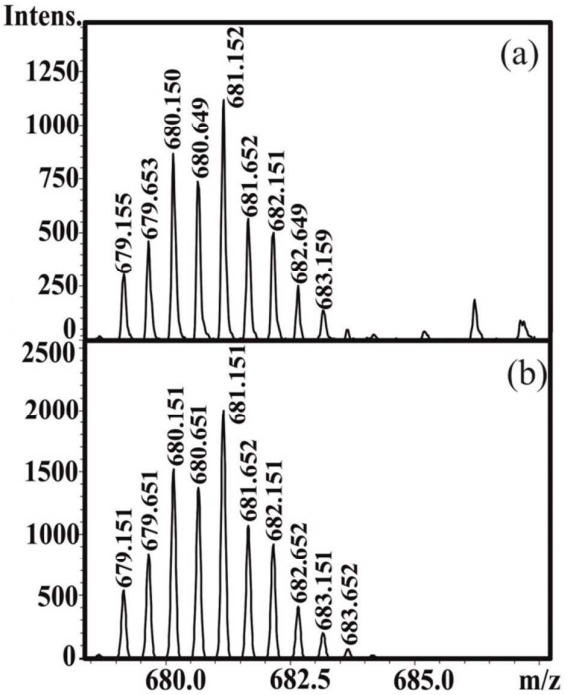

Fig.5. ESI MS spectra recorded in the cadmium(II) –ACSSACS-NH_2 system at pH 9.34 (a) and calculated spectra for the bis(ligand) complex $[CdL_2]^{2-}$=$[Cd(C_{42}H_{72}N_{16}O_{20}S_4)^{2-}]$(b).

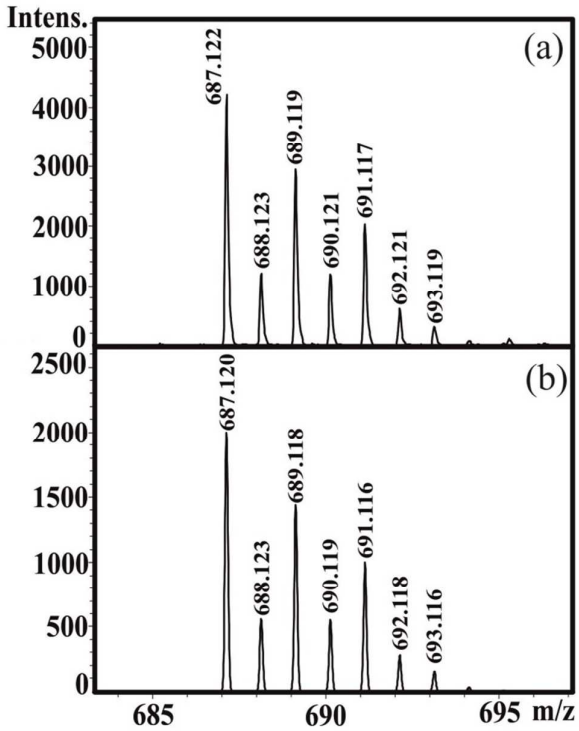

Fig.6. ESI MS spectra recorded in the zinc(II) –ACSSACS-NH_2 system at pH 9.84 (a) and calculated spectra for the species $[ZnH_{-1}L]^-$=$[Zn(C_{21}H_{35}N_8O_{10}S_2)^-]$ containing deprotonated amide nitrogen (b)

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The pK values characterized these deprotonation processes are different in the case of ACSSACS-NH_2 from those of CSSACS-NH_2 or other cysteine containing peptides with free carboxylate group on C-termini[14,15] or terminally protected multicysteine peptides with Ac-Xaa-Cys-……..-NH_2 sequences[32, 33,34]. For example the pK(ZnL/ZnH_{-1}L)=7.95 for ACSSACS-NH_2, while 9.05 for CSSACS-NH_2 and 8.65 for Ac-SCHGDQGSDCSI-NH_2[34]. In the case of AlaCys and AlaAlaCys the formation of $[ZnH_{-1}L]^-$ (and $[CdH_{-1}L]^-$) complexes were not detected, at the same time the pK(ZnL_2/$ZnH_{-1}L_2$) are 10.18 for AlaCys and 9.26 for AlaAlaCys, respectively. Larger differences can be observed in the case of cadmium(II) complexes. pK(CdL/CdH_{-1}L)=8.57 for ACSSACS-NH_2, and 11.18 for CSSACS-NH_2, while the pK(CdL_2/$CdH_{-1}L_2$) are 10.83 for AlaCys and 10.61 for AlaAlaCys, respectively. These parameters raise the possibility of metal ion induced deprotonation of amide nitrogen of ACSSACS-NH_2.
ESI MS spectra of the 1:1 samples have been recorded for both metal ions at pH 9.84 and are shown in Figure 6 for zinc(II) and in Figure S6 in the Supplement for cadmium(II). The agreement of the measured and calculated spectra can be obtained if the $[MH_{-1}L]^-$ stoichiometry is not a hydroxido complex but belongs to the deprotonation and coordination of an amide nitrogen. The sequence of the peptide strongly supports that it should be the amide between the terminal Ala and subsequent Cys residues in the form of the (NH_2,N^-,S^-) fused chelate supported by a macrochelate from the distant thiolate group.
This assumption was confirmed with homonuclear ^{1}H NMR experiments. TOCSY spectra of zinc(II) and cadmium(II) $[MH_{-1}L]^-$ were compared with the ligand ACSSACS-NH_2 (Figure 7). In particular, the presence of the metal ion caused a chemical shifts variations especially on Ala(1)-CH_3 and Cys(2)-CH_2 resonances. These resonances are the most sensitive for the deprotonation and coordination of amide bond of the heptapeptide. The more rigid structure of the complexes decrease the probability of the presence *trans axial* conformation of CH_2-CH motives resulting in smaller vicinal, ^3J, scalar coupling between protons in these moieties especially when they are in the neighborhood of coordinated donor groups. Determination of the coupling constant could only be possible of (S)-CH_2-CH moiety in the free ligand as 6.3 Hz, however in the complex these peaks do not show fine structure because of the line broadening. Since in COSY (TOCSY) experiments the intensity of cross peaks is proportional to the values of the coupling constants we can obtain at least qualitative information about the ^3J value. In Figure 7 it is seen that we have very weak cross peaks between (S)-CH_2-CH protons in the complexes indicating the probability of smaller dihedral angle because of the more rigid structure. If it was OH^- coordination then the lack of fused chelates would allow more flexibility leading to larger vicinal coupling constant (Scheme 2.).

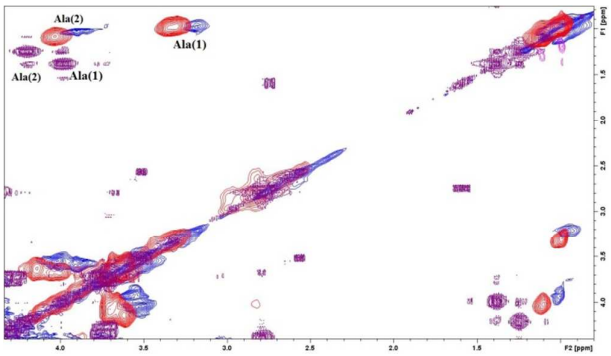
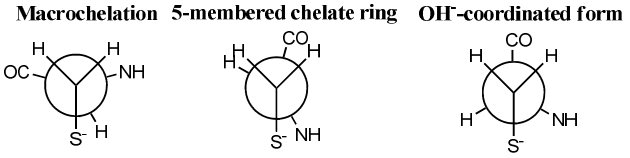


Figure 7. Selected region of COSY and TOCSY spectra of the free ligand (purple) and cadmium(II) (red) and zinc(II) (blue) $[MH_{-1}L]^-$ complex



Scheme 2. Newman projection of $[MH_{-1}L]^-$ with different types of coordination

Comparing to the ^{1}H NMR spectra of both metal complexes to that of the free ligand the most striking difference is the broadened peaks of CH_2 and CH protons in the presence of the diamagnetic metal ions. It means acceleration of transverse relaxation. One of the sources of this increase in relaxation rate can be the presence of conformers and exchange between them. However, if we compare the ^{1}H NMR at room temperature and at 0 ^oC only very slight changes in the line widths is observed leading us to different conclusion, namely the line broadening is the result of the increased rotation correlation time of the metal ion containing molecules. In the case of free ligand sharp peaks reflect the fast local rotation correlation motions of the peptide backbone resulting in slow relaxation. When the Cd^{2+} or the Zn^{2+} ions coordinate the formed fused chelate rings and the additional macrochelate reduce the rate of the this local rotation motion and the long total rotation correlation time of large complex will determine the relaxation of the protons.
By means of NMR diffusiometry we could determine the translation diffusion coefficient of the free ligand (ACSSACS-NH_2) and zinc(II) and cadmium(II) complexes. The diffusion coefficient of ACSSACS-NH_2 D = 4.5x10^{-10} m^2s^{-1} from which an apparent hydrodynamic radius could be calculated as R_L = 0.6 nm. This relatively small size means that there are already intramolecular hydrogen bonds in this heptapeptide resulting in coil like structure. Coordination cadmium(II) or zinc(II) to ACSSACS-NH_2 caused increase in size. The diffusion coefficient are D_{Zn} = 3.2x10^{-10} m^2s^{-1} and D_{Cd} = 2.8x10^{-10} m^2s^{-1}. These values correspond to hydrodynamic radius R_{Zn} = 0.8 and R_{Cd} 0.9 nm respectively (Figure S7 and S8). This increase in size supports that the coordination removes some hydrogen bonds originally present in the free ligand and cause increase in size. The intramolecular hydrogen bonds are probably substituted with hydrogen bonded water making the complexes more bulky then the free ligand.
In general ^{1}H NMR also confirms that zinc(II) and cadmium(II) complexes of ACSSACS-NH_2 have very similar solution structure supporting the similarity in their thermodynamic behavior.
Zinc(II) ions generally do not induce deprotonation and coordination of amide functions, except in the case of several histidine containing peptides. Among them, the NH_2-Xaa-His… sequences are the best known[25,35,36] in which the imidazole-N

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of histidine is in the same position as the cysteinyl residue in ACSSACS-NH_2. Studies on the zinc(II) complexes of terminally protected multicysteine peptides containing the thiolate functions in similar environments have been reported recently without any indication of zinc(II) induced amide coordination[32,33,34]. The presence of the terminal amino group in our peptide is, however, a significant difference in the potential binding sites of ACSSACS-NH_2 and those of the literature studies.

On the other hand, in the case of cadmium(II) even these peptides of histidine are not able to induce the formation of a peptide complex containing cadmium(II)-amide bond. The ability of this type of metal binding by the NH_2-Xaa-Cys-sequence is, however, strongly supported by the nickel(II) complexes of the terminally free dipeptide GlyCys and AlaCys where the formation of the same species was described with an outstanding thermodynamic stability[12,15]. On the contrary, this reaction was not observed in the corresponding zinc(II) and cadmium(II) complexes[13,14]. However, it must be considered that in the case of the C-terminally free dipeptide the thiolate can easily form a stable (S^-,COO^-) 6-membered chelate and this interaction suppresses the amide binding. In the case of ACSSACS-NH_2 there is no another strongly coordinating donor function in chelating position with the thiolate group. Thus, the high affinity of cadmium(II) ions to thiolate binding and the specific sequence of the peptide including the presence of free amino terminus are mutually responsible for the observation of the cadmium(II) induced amide deprotonation.

Conclusions

The hexa- and hepta-peptides CSSACS-NH_2 and ACSSACS-NH_2 have outstanding zinc(II) and cadmium(II) binding affinity but their coordination chemistry is quite different. In the case of the hexapeptide containing N-terminal Cys residue, the amino terminus is the primary metal binding site in the form of a stable (NH_2,S^-) 5-membered chelate supported by macrochelation of the distant cysteinyl residue. This tridentate coordination mode is especially favored by cadmium(II) and hinders the formation of bis(ligand) complexes. Thus, the speciation of the zinc(II) and cadmium(II)-CSSACS-NH_2 systems is rather simple. The species [ML] predominates around physiological pH, while hydroxido complexes can be present in alkaline samples. The heptapeptide ACSSACS-NH_2 is a slightly less effective metal binder but its coordination chemistry is more versatile. The thiolate groups are the primary binding sites for both metal ions and the formation of the 18-membered (S^-,S^-) macrochelate is the favored coordination mode of the peptide. This bidentate coordination mode provides a good chance for the formation of bis(ligand) complexes, too. In slightly basic samples the deprotonated amino group can also contribute to metal binding. Moreover, the interaction of the terminal amino-N and the thiolate-S of Cys(2) moiety can promote the deprotonation and metal ion coordination of the amide group between these residues. This reaction results in the formation of the (NH_2,N^-,S^-) fused chelates supported by the thiolate of the distant cysteinyl residue. Zinc(II) induced deprotonation and coordination of amide groups have already been described in various peptides of histidine, but in the case of cadmium(II) this is the first example for the formation of a Cd-N(peptide amide) bond.

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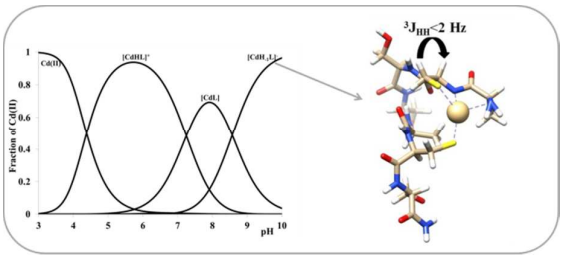
Graphical abstract



The novel synthesized cysteine peptides form stable zinc(II) and cadmium(II) complexes; the specific sequence makes possible metal induced amide deprotonation.