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The binding of Ni(II) ions to terminally blocked hexapeptides derived from the metal binding -ESHH- motif of histone H2A

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The coordination properties of Ni(II) ions towards the terminally blocked (CH_3CONH- and -$CONH_2$) hexapeptides -TESHHK-, -TASHHK-, -TEAHHK-, -TESAHK- and -TESHAK- were studied by using potentiometric and spectroscopic techniques (UV/Vis, CD, NMR). The peptides were chosen in such a way as to compare the effect of Glu, Ser and His residues on the stability, the coordination and hydrolytic abilities of the complexes formed. All peptides bind to Ni(II) ions initially through one or two imidazole nitrogens in weakly acidic and neutral solutions forming slightly distorted octahedral complexes. At higher pH values, a series of square-planar complexes are formed, where Ni(II) ions bind simultaneously through an imidazole and three amide nitrogens in an equatorial plane. This proposed conformation includes the participation of only one imidazole nitrogen, in the case of all peptides, in the coordination sphere of Ni(II) ions. In basic solutions, the peptides -TASHHK- and -TESAHK- were hydrolyzed in a Ni(II)-assisted fashion. No hydrolytic processes were noticed in peptides -TEAHHK- and -TESHAK- where the Ser or His-5 residues are replaced with the Ala residue. The Ni(II)-assisted hydrolysis of the analogues of -TESHHK- may provide an insight into the novel mechanism of genotoxicity, combining the damage to the nucleosome with the generation of further toxic Ni(II) species.

Introduction

Although nickel is recognized to be an essential element for bacteria, plants, animals and humans,[1,2] nickel compounds are known as human carcinogens.[3] The molecular mechanisms by which nickel carcinogenicity is exerted are not fully understood, but it is believed to involve promutagenic DNA damage, epigenetic effects in chromatin and impairment of DNA repair from nickel binding inside the cell nucleus.[4–8] For these reasons, it is very important to detect and study the specific Ni(II) binding sites inside the cell nucleus. It is known that Ni(II) ions bind to DNA only weakly,[9] leaving the proteins to play a critical role in coordination to toxic nickel and formation of the active species. The abundance of histones, the basic proteins that provide a scaffold for the DNA double helix, organized in repeating units called nucleosomes,[10] makes them prime candidates for this role.

Studies on Ni(II) interactions with peptide or protein fragments, especially histones, may help to a better understanding of the mechanisms of carcinogenicity and acute toxicity of this ion. On the other hand, a plethora of studies[11–14] indicate that His and Cys residues are the major coordination sites of Ni(II) and other transition metal ions in protein amino acid sequences. For example, the interaction of Ni(II) ions with the tetrapeptide Ac-Cys-Ala-Ile-His-am (-CAIH-) representing the 110–113 residues of histone H3, located at the center of the octamer structure, indicated that Ni(II) ions form a very strong complex, that could mediate oxidative damage to DNA.[13–15] In addition, the coordination properties of Ni(II) ions towards peptide models containing the C-terminal "tail" -ESHH-, which corresponds to the 121–124 residues of a major variant of mammalian histone H2A, was also described.[16,17] It was found that above pH 7, Ni(II) complexation with the hexapeptide Ac-Thr-Glu-Ser-His-His-Lys-am (-TESHHK-) and the peptide $H2A_{34}$ (-LLGKVTIAQGGVLPNIQAVLLPKKTESHHKAKGK-) was accompanied by hydrolytic cleavage of the -Glu–Ser- peptide bond and formation of a very strong square-planar Ni(II) complex with the resulting -SHHK- sequence.[16,17] Cu(II) ions provide similar hydrolytic activity against the -Glu–Ser- peptide bond with kinetics more than three times slower than those for Ni(II).[17] The complex formed was stable enough and allowed to conclude a possible Ni(II) binding to nucleosome[18] with a subsequent promotion of oxidative DNA damages, via Fenton-like mechanisms.[17] Furthermore, a study with the peptide model Ac-Ala-Lys-Arg-His-Arg-Lys-am (-AKRHRK-) of the N-terminal "tail" of histone H4 and its modifications has also been reported, supporting the hypothesis that this sequence is a potential binding site for Ni(II) and Cu(II) ions.[19,20]

Recently, we studied the interaction of Cu(II) ions with the H2A histone model blocked hexapeptide -TESHHK-[21] and compared the results to those of Ni(II) ions.[16] We found that the Cu(II)-TESHHK- complex was capable of efficient activation of hydrogen peroxide, leading to oxidative damage of 2′-deoxyguanosine (dG).[21] A subsequent systematic study of the interaction of Cu(II) ions with the hexapeptides Ac-Thr-Ala-Ser-His-His-Lys-am (-TASHHK-), Ac-Thr-Glu-Ala-His-His-Lys-am (-TEAHHK-), Ac-Thr-Glu-Ser-Ala-His-Lys-am (-TESAHK-) and Ac-Thr-Glu-Ser-His-Ala-Lys-am (-TESHAK-) indicated that while the replacement of a Ser by an Ala residue leads to the formation of more stable species, the replacement of any of the His residues with Ala reduces the stability of all species.[22]

In this paper we present the results of a study of Ni(II) ion interactions with the blocked peptides -TESHHK-, -TASHHK-, -TEAHHK-, -TESAHK- and -TESHAK-, models of the -ESHH- motif of histone H2A. These systematic changes in the peptide sequence will allow us to locate the amino acid which

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Table 1 ^{1}H-NMR assignment of -TESHHK-, -TASHHK-, -TEAHHK-, -TESAHK- and -TESHAK- at pH* 10.0 (in ppm relative to TSP)

Assignment	-TESHHK-	-TASHHK-	-TEAHHK-	-TESAHK-	-TESHAK-
Acetyl	2.26	2.13	2.25	2.26	2.27
Thr α	4.44	4.27	4.45	4.45	4.44
β	4.39	4.25	4.38	4.38	4.37
γ	1.37	1.27	1.37	1.37	1.37
Glu α	4.48	—	4.47	4.50	4.50
β′	2.17	—	2.18	2.12	2.10
β″	2.20	—	2.20	2.21	2.19
γ	2.43	—	2.43	2.44	2.42
Ser α	4.55	4.41	—	4.56	4.54
β′	3.92	3.84	—	4.00	3.98
β″	3.96	3.88	—	4.02	4.00
Ala α	—	4.42	4.40	4.48	4.48
β	—	1.42	1.49	1.49	1.52
His^4 α	4.60	4.62	4.72	—	4.75
β	3.20	3.09	3.22	—	3.25
C_2	7.85	7.73	7.84	—	7.79
C_4	7.07	6.94	7.06	—	7.08
His^5 α	4.58	4.60	4.72	4.71	—
β	3.23	3.08	3.19	3.22	—
C_2	7.82	7.68	7.81	7.77	—
C_4	7.03	6.91	7.02	7.02	—
Lys α	4.45	4.30	4.43	4.41	4.42
β′	1.96	1.78	1.88	1.87	1.93
β″	2.05	1.89	2.00	1.98	2.03
γ	1.56	1.48	1.54	1.53	1.61
δ	1.85	1.73	1.79	1.77	1.81
ε	3.11	3.07	3.08	3.07	3.09

is responsible for hydrolysis and to obtain an insight into the mechanism of genotoxicity involving histone hydrolysis and subsequent oxidation of DNA bases.[16,17,21]

Experimental

Materials

$Ni(NO_3)_2{\cdot}6H_2O$, $NiCl_2{\cdot}6H_2O$, NaCl, HNO_3, KNO_3, acetonitrile (HPLC grade), dicyclohexylcarbodiimide (DCC), HCl, and NaOH were obtained from E. Merck (Darmstadt, Germany). 1-Hydroxybenzotriazole (1-HOBt), trifluoroacetic acid (TFA), trifluoroethanol (TFE), 3-(trimethylsilyl)propionic acid sodium salt (TSP), anisole, D_2O, NaOD and DCl were purchased from Aldrich Chemical Co. (Milwaukee, WI). Chelex 100 resin was purchased from Sigma Chemical Co. (St. Louis, MO). Isopropanol, dimethylformamide, diethyl ether and dichloromethane were purchased (analytical grade) from Lab-Scan Chemical Co. (Dublin, Ireland). The protected amino acids, Fmoc-His(Mtt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Glu(tBu)-OH, Fmoc-Thr(tBu)-OH and Fmoc-Ala-OH and the resin H-Linker-2-chlorotrityl were purchased from CBL Chemicals Ltd. (Patras, Greece).

Peptide synthesis

The hexapeptides Ac-Thr-Glu-Ser-His-His-Lys-am (-TESHHK-), Ac-Thr-Ala-Ser-His-His-Lys-am (-TASHHK-), Ac-Thr-Glu-Ala-His-His-Lys-am (-TEAHHK-), Ac-Thr-Glu-Ser-Ala-His-Lys-am (-TESAHK-) and Ac-Thr-Glu-Ser-His-Ala-Lys-am (-TESHAK-) were synthesized in the solid phase, as described previously.[22] In addition to ESI-MS used for the characterization of the peptides[22] one- and two-dimensional ^{1}H-NMR techniques (TOCSY and NOESY) were also used (Table 1).

Potentiometry

The protonation and stability constants of Ni(II) complexes of -TESHHK-, -TASHHK-, -TEAHHK-, -TESAHK- and -TESHAK-, in the presence of 0.1 M KNO_3, were determined by using pH-metric titrations over the pH range 2.5–10.5, at 25 °C, with 0.1 M NaOH as titrant (Molspin automatic titrator, Molspin Ltd., Newcastle-upon-Tyne, UK). Changes of pH were monitored with a combined glass–silver chloride electrode calibrated daily in H^+ concentrations by HNO_3 titrations.[23] The time to reach pH-equilibrium during titrations was varied from 1–10 min, depending on the pH value (longer near the equivalent point). The equilibrium state was checked by achieving stable pH values in the third decimal point, for at least 1 min duration. Sample volumes of 1.5 mL and concentrations of 1 mM of the peptides and 0.5–1 mM $Ni(NO_3)_2{\cdot}6H_2O$ were used. The experimental data were analyzed using the SUPERQUAD program.[24] Standard deviations computed by SUPERQUAD refer to random errors.

NMR spectroscopy

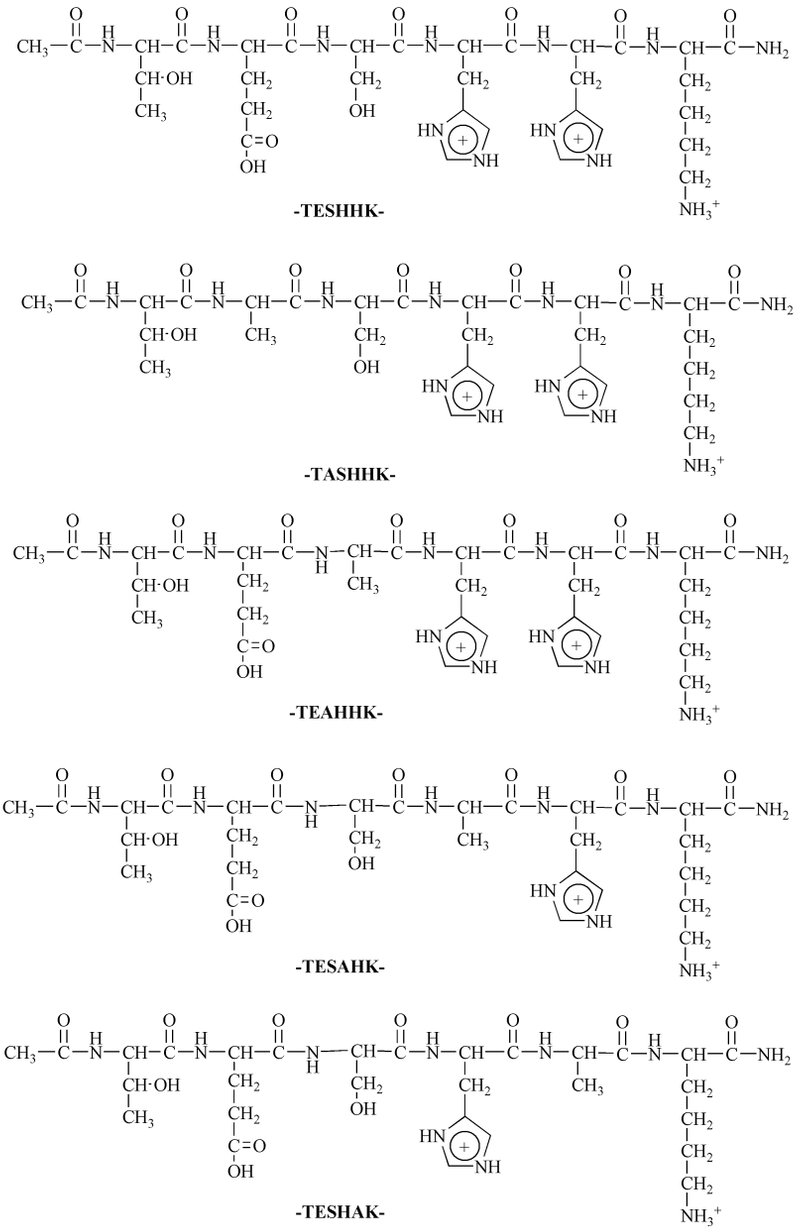
NMR experiments were performed on a Bruker AMX 400 MHz spectrometer. The one dimensional experiments were carried out in 99.9% D_2O solutions at a peptide concentration of 5 mM and peptide : Ni(II) ratio of 1 : 1 at pH* 10.0 at 25 °C.† NMR titrations were carried out for the peptides -TESHHK-, -TASHHK- and -TEAHHK-. A combination of NOESY and TOCSY experiments were used to assign the spectra of both free and Ni(II)-bound peptides (1 : 1 ratio) (c = 15 mM) at the same pH* and temperature.

In a separate experiment, a series of one dimensional spectra of samples containing -TASHHK- or -TEAHHK- with Ni(II) ions in a 1 : 1 ratio (c = 4 mM) (Bruker AMX 400 MHz) and of samples containing -TESAHK- or -TESHAK- with Ni(II) ions in a 1 : 1 ratio (c = 3.4 mM) (Bruker AC 250 MHz), were recorded at pH* 10.8 at 37 °C during the time of incubation, to study the hydrolysis reaction.

UV/Vis spectroscopy

UV/Vis spectra were recorded on a Shimadzu UV-2401PC spectrophotometer, at 25 °C, in the spectral range of 300–900 nm, in 1 cm cuvettes, using Ni(II) concentrations of 1 mM and a Ni(II)-to-peptide ratio of 1 : 1. The samples were titrated with

† The pH* reading of the electrode was not corrected for the isotope effect.



Scheme 1 Fully protonated forms of the peptides used.

NaOH by adding very small amounts of concentrated solutions manually. Changes of pH were monitored with a combined glass–silver chloride electrode over the pH range 6–11.

CD spectroscopy

CD spectra were recorded on a JASCO J-715 spectropolarimeter, in the spectral range of 250–800 nm, in 1 cm cuvettes, using Ni(II) concentrations of 3 mM and Ni(II)-to-peptide ratios of 1 : 1 at pH 6–10.7.

Results and discussion

Characterization of free peptides in solution

The five peptides (Scheme 1) were chosen in such a way as to compare the effect of Glu, Ser and His residues on the coordination properties and hydrolytic abilities of the complexes formed. Both the N- and C-termini of the analogues were blocked by acetylation and amidation, respectively, to make the peptides more realistic models of the -ESHH- motif of histone H2A.

The ^{1}H-NMR spectra were assigned with the use of TOCSY and NOESY experiments. The assignments of free peptides at pH* 10.0 are presented in Table 1. The protonation constants and dissociation macroconstants of the peptides were measured by potentiometry and are presented in Table 2. It must be noticed that these dissociation macroconstants are in good agreement with those of the peptides containing Glu, Lys and His residues.[20,25] The highest values of dissociation macroconstants of each peptide are assigned to the ε-amino group of Lys residues. The lowest values of the peptides, except -TASHHK-, are assigned to the carboxyl group of Glu residues. However, the other values of macroconstants corresponding to deprotonations at His residues, were found to be separated by less than one log unit in the first three peptides. This might suggest a possibility of concurrent deprotonations at these two residues. Thus, a series of one dimensional NMR spectra at various pH* values were recorded and used in the Rabenstein–Sayer method[26] for more realistic calculation of the ionization constants. According to this method the microscopic acid dissociation constants of molecules having two groups of almost similar acidity can be determined by NMR titrations. The change in chemical shift of a unique resonance with changes in pH gives directly the fractional deprotonation of one of the two groups as a function of pH.[26] The values found with this method are in good agreement with the corresponding

Table 2 Protonation constants and dissociation macroconstants of -TESHHK-, -TASHHK-, -TEAHHK-, -TESAHK- and -TESHAK-

	HL		H_2L		H_3L		H_4L	
	$\log\beta^a$	pK_a	$\log\beta^a$	pK_a	$\log\beta^a$	pK_a	$\log\beta^a$	pK_a
-TESHHK-	10.28(1)	10.28	17.06(1)	6.78	22.96(1)	5.90	26.81(1)	3.85
			pK_a* 6.88(1)		pK_a* 6.12(1)			
-TASHHK-	10.48(1)	10.48	17.10(1)	6.62	22.85(1)	5.74	—	
			pK_a* 6.64(1)		pK_a* 6.06(1)			
-TEAHHK-	10.25(1)	10.25	16.93(1)	6.68	22.93(1)	6.00	27.03(1)	4.10
			pK_a* 6.72(1)		pK_a* 6.15(1)			
-TESAHK-	10.37(1)	10.37	16.60(1)	6.23	20.64(1)	4.04	—	
-TESHAK-	10.41(1)	10.41	16.68(1)	6.27	20.71(1)	4.04	—	

pK_a calculated from potentiometric results. pK_a* calculated from ^{1}H-NMR results. [a] $\beta = [H_jL_k]/([H]^j[L]^k)$, standard deviations of the last digit are given in parentheses.

Table 3 Stability constants of Ni(II) complexes with -TESHHK-, -TASHHK-, -TEAHHK-, -TESAHK- and -TESHAK-

	NiHL	NiL		$NiH_{-1}L$		$NiH_{-2}L$		$NiH_{-3}L$		$\log K$*
	$\log\beta^a$	$\log\beta^a$	pK_{a1}	$\log\beta^a$	pK_{a2}	$\log\beta^a$	pK_{a3}	$\log\beta^a$	pK_{a4}	(4N)
-TESHHK-	14.04(2)	—		−2.16(2)		−11.52(1)	9.36	−22.80(5)	11.28	−28.58
-TASHHK-	14.20(1)	5.86(1)	8.34	−1.52(1)	7.38	−10.16(1)	8.64	−20.49(1)	10.33	−27.26
-TEAHHK	14.10(1)	6.00(1)	8.10	−2.62(1)	8.62	−11.48(1)	8.86	−22.34(1)	10.86	−28.41
-TESAHK-	13.02(1)	—		−3.00(1)		−11.58(1)	8.58	−22.67(1)	11.09	−28.18
-TESHAK-	13.61(1)	5.80(1)	7.81	−3.12(1)	8.92	−11.55(1)	8.43	−22.74(1)	11.19	−28.23

[a] $\beta = [Ni_iH_jL_k]/([Ni]^i[H]^j[L]^k)$, standard deviations of the last digit are given in parentheses. $\log K^* = \log\beta\{NiH_{-2}L\} - \log\beta\{H_2L\}$.

values found from potentiometry for the peptides containing two histidine residues. The higher pK_a values correspond to the His residue closer to the C-termini of the hexapeptide, in accord with our NMR results and literature.[27–29]

Coordination properties of Ni(II) complexes

(I) Potentiometric and spectroscopic (UV/Vis–CD) studies. The stability constants of Ni(II) complexes with the five peptides, calculated from pH-metric titrations using the SUPERQUAD program are presented in Table 3. Fig. 1 represents the species distribution diagrams for Ni(II) complexes with the peptides -TESHHK-, -TASHHK-, -TEAHHK-, -TESAHK- and -TESHAK- calculated for a 1 : 1 ratio and $c = 1$ mM like the one used in UV/Vis spectroscopy, using stability constants obtained from potentiometry. The CD spectra and the spectroscopic parameters of Ni(II) complexes with all peptides are presented in Fig. 2 and Table 4, respectively. In addition, the match between CD parameters and the species distribution diagrams for Ni(II) complexes is presented in Fig. 3.

The species distributions in Fig. 1 indicate that Ni(II) ions start binding with all peptides at about pH 5, forming four species with the peptides -TESHHK- and -TESAHK- and five species with -TASHHK-, -TEAHHK-, and -TESHAK-, in the pH range of 5.0–10.5. At acidic pH values the first species formed with all peptides is probably NiHL. The stoichiometry and the d–d transition at 636–685 nm for these complexes (Table 4) indicates the involvement of one or two imidazole nitrogens bonded to Ni(II) ions,[30,31] similar to the analogous Cu(II) complexes.[22] Bal *et al.*[16] showed that the carboxylate oxygen of the Glu residue of -TESHHK- may also participate in the coordination sphere of Ni(II) ions forming a slightly distorted octahedral complex. The d–d bands of NiHL species in the CD spectra are very weak due to the existence of large macrochelate loops.[13]

On increasing the pH, the complexes NiHL with the peptides -TASHHK-, -TEAHHK- and -TESHAK- release a proton with a pK_a of 7.81–8.34 (Table 3), abstracted from the amide nitrogen of one of the His residues, forming a stable six-membered chelate ring. The resultant species, NiL, cannot be detected spectroscopically with satisfactory results due to overlap with other species in the pH range 6.5–9.5 and their low concentration. However, they are characterized on the basis of stoichiometry and the values of the stability constants. The values of the stability constants for the NiL species (Table 3) are comparable to other similar complex species with Ala-Gly-Gly-His (-AGGH-) and its analogues,[32] supporting the coordination of Ni(II) ions through the imidazole nitrogen of one of the His residues and the amide nitrogens of the same histidine.

Above pH 7, $NiH_{-1}L$ and $NiH_{-2}L$ species are associated with a further amide bond deprotonation with a pK_a of 7.38–8.92 and 8.43–9.36 (Table 3), respectively, completing the equatorial plane of the Ni(II) ions. The formation of the stable five-membered chelate rings by consecutive nitrogens is the driving force for this coordination process. From Table 3 it can be seen that in the case of -TASHHK- $pK_{a2} < pK_{a1}$ and for -TESHAK- $pK_{a3} < pK_{a2}$. It must be noticed that this behavior is characteristic of the cooperative coordination process found in peptide complexes with Ni(II).[12,20] Similarly with NiL, the $NiH_{-1}L$ species can not be detected spectroscopically with satisfactory results. The values of the stability constants of these species (Table 3) are similar to other complex species with -AGGH- and its analogues,[32] supporting the 3N coordination mode. The most probable interpretation of these species is the presence of the same donor set as in the NiL complexes but with the additional deprotonation and coordination of a neighboring amide nitrogen atom.

The d–d transitions near 440–450 nm and the CD parameters (Table 4) in the $NiH_{-2}L$ species,[20,25] (Table 4) suggest the simultaneous coordination of an imidazole and three amide nitrogens equatorially, forming a stable square-planar complex. This conformation, including the participation of only one imidazole nitrogen, in the case of all peptides, in the coordination sphere of Ni(II) ions, is supported from the comparable values of $\log K^*$ of the species $NiH_{-2}L$ presented in Table 3. As can be seen the $\log K^*$ values of these complexes are very similar with comparable complexes of Ni(II) with the peptide Ac-AKRHRK-am[19] and Ac-TRSRSHTSEGTRSR-am[25] having $\log K^*$ −28.70 and −28.16, respectively. The last two cases showed that the coordination sphere of Ni(II) ions consists of an imidazole and three amide nitrogens $\{3N^-_{am}, 1N_{im}\}$,

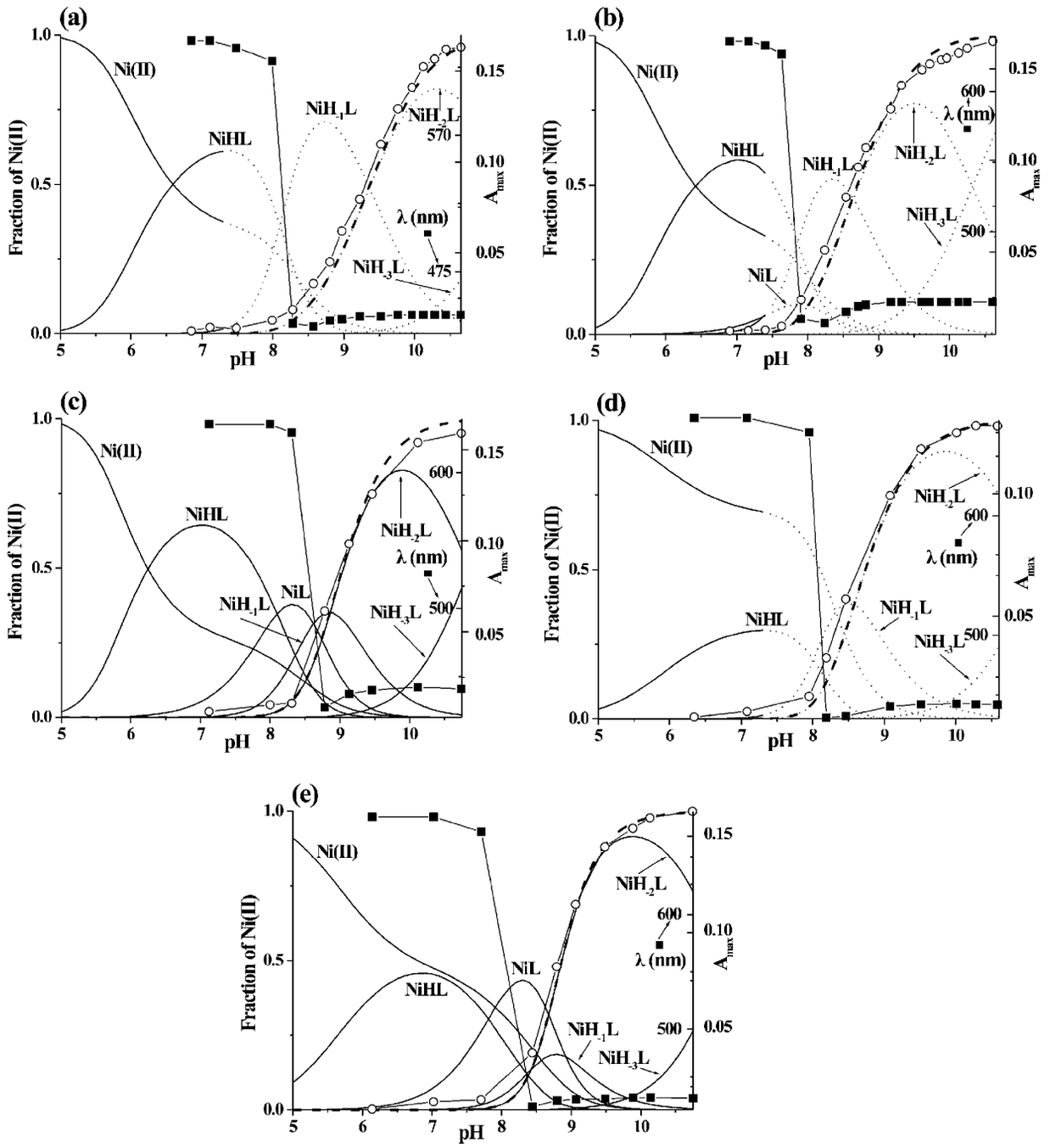


Fig. 1 Species distribution diagrams of Ni(II) complexes with (a) -TESHHK-, (b) -TASHHK-, (c) -TEAHHK-, (d) -TESAHK- and (e) -TESHAK-, calculated for reagent concentrations used in UV/Vis spectroscopy, using stability constants obtained from potentiometry: $c_{Cu} = c_L = 1$ mM. UV/Vis spectroscopic parameters, absorption (○) and λ_{max} (■) are overlaid. The line --- represents the sum $NiH_{-2}L + NiH_{-3}L$. The curves in (a), (b) and (d) are represented with dotted lines because of possible peptide hydrolysis above pH 7.5.

supported from the d–d transitions near 440 nm and the CD parameters near 510 nm (+1.151) and 422 nm (−2.495).[19,25] Thus in the $NiH_{-2}L$ species the same type of coordination with the previous comparable complexes which is typical for -Xaa-Yaa-His- motifs[11,19,20,25,33] may be postulated. The log$K$* of the $NiH_{-2}L$ complex with -TASHHK- is at least one log unit higher than the values of the other peptides, indicating that the absence of a Glu residue is stabilizing this species. In addition, the correlation species distribution diagrams of Ni(II) ions between -TEAHHK-/-TESHAK- and -TEAHHK-/-TESAHK-, presented in Figs. 4a and 4b, respectively, indicate that while in neutral and slightly basic solutions (pH 6–9) Ni(II) ions prefer to bind to the peptides with two histidine residues; in more basic solutions Ni(II) ions may coordinate to both of the peptides with the same selectivity. This is additional evidence that Ni(II) ions in octahedral species NiHL and NiL of all peptides are bound to both histidine residues[16] while in the square-planar $NiH_{-2}L$ species they are bound to only one of them.

Above pH 9 deprotonation of the $NiH_{-2}L$ species takes place with pK_a 10.33–11.28 forming the $NiH_{-3}L$ species. The only side-chain which may undergo deprotonation is the ε-amino group of the lysine residue. These pK_a values do not suggest interaction of the lysine amino group with the metal ion (Table 3). The difference in the acidity of the non-coordinated ε-amino group of the lysine residue (10.33–11.28) may possibly be due to intramolecular hydrogen bonding between this group, the free histidine imidazole nitrogens and the carboxyl group of glutamic acid. Note that there is no significant difference in the pK_a values of the free and bound hexapeptide -TASHHK-, where there is no glutamic acid in the sequence. Spectral parameters of $NiH_{-3}L$ species presented in Table 4 indicate the

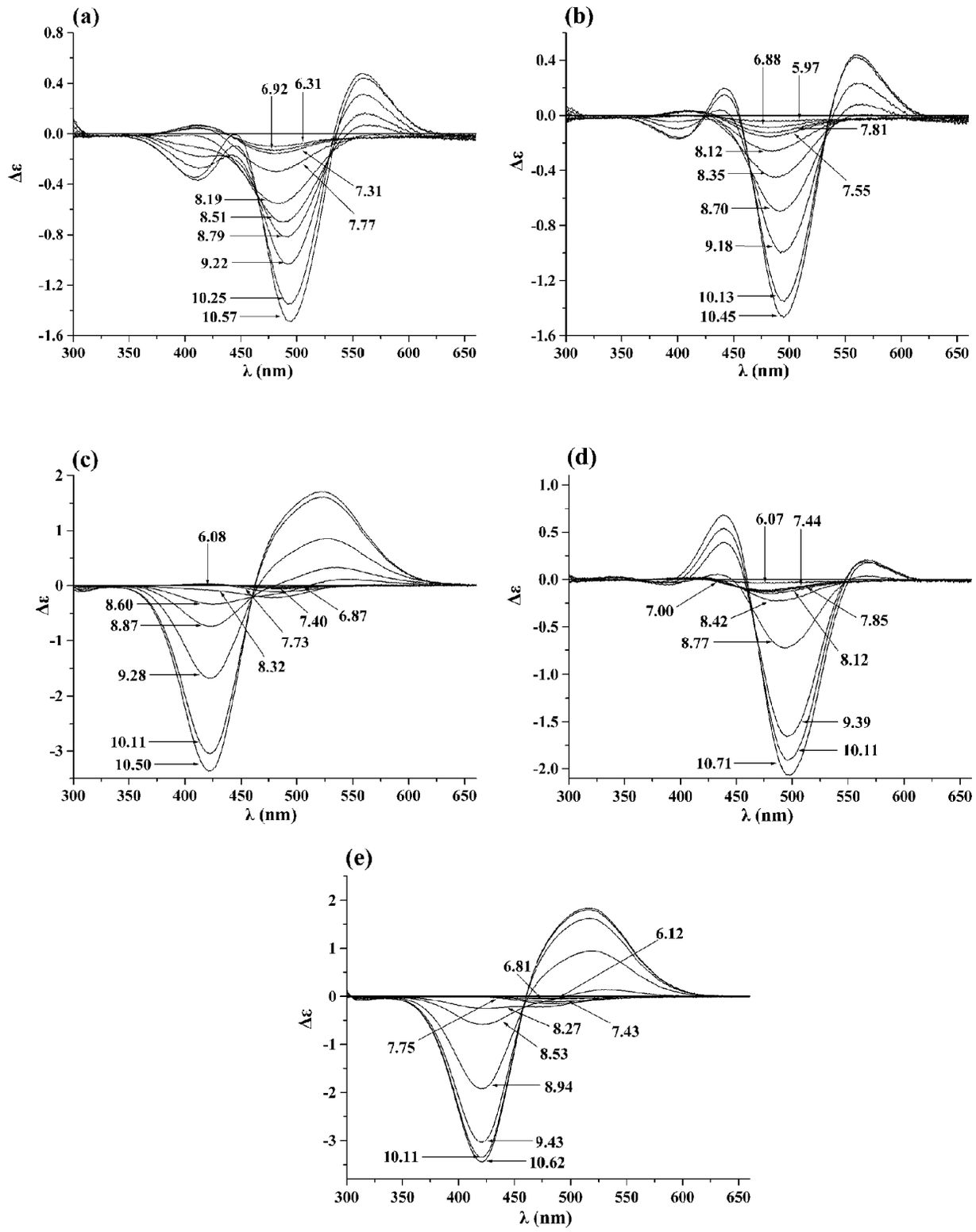


Fig. 2 CD spectra of Ni(II) complexes with (a) -TESHHK-, (b) -TASHHK-, (c) -TEAHHK-, (d) -TESAHK- and (e) -TESHAK- measured at various pH values (indicated on the graph).

same type of coordination of Ni(II) ions as in complex $NiH_{-2}L$. The coordination mode suggested above for the square-planar complexes in basic solution supports the binding of imidazole belonging to His at position 5, while the imidazole of His at position 4 as well as the ε-amino group of Lys remain deprotonated and uncoordinated. This is comparable to the analogous Cu(II) complexes of the same peptide models,[22] -His-His-(-HH-)[29] and -His-Gly-His-Gly- (-HGHG-),[34] where Cu(II) ions were bound equatorially to the His residue at the C-terminus.

(II) NMR studies. One- and two-dimensional (TOCSY) ^{1}H-NMR spectra of the hexapeptides at pH* 10.0 in D_2O solutions were recorded in the presence and absence of Ni(II) ions, to study and identify the proposed complexes above. Despite the overlap and the broadening (due to the paramagnetic character of the minor octahedral Ni(II) complexes) of some signals, especially in the low chemical shift region of the spectra, significant chemical shift changes were observable as a result of nickel complexation.

Table 4 Spectroscopic data of Ni(II) complexes with -TESHHK-, -TASHHK-, -TEAHHK-, -TESAHK- and -TESHAK-

	-TESHHK-		-TESHHK-		-TESHHK-		-TESHHK-		-TESHHK-	
Species	UV/Vis[a]	CD[b]	UV/Vis[a]	CD[b]	UV/Vis[a]	CD[b]	UV/Vis[a]	CD[b]	UV/Vis[a]	CD[b]
NiHL	636 (19) 377 (29)	480 (−0.16) 411 (+0.07)	636 (7) 378 (30)	481 (−0.09) 412 (+0.02) 312 (−0.03)	636 (6) 378 (30)	485 (−0.06) 417 (+0.03)	682 (11) 409 (59)	477 (−0.11) 406 (+0.02) 297 (−0.03)	685 (12) 380 (12)	482 (−0.06) 297 (−0.04)
NiL		—		[c]	630 (10)	479 (−0.22)		—	433 (38)	477 (−0.22) 425 (−0.25) 308 (−0.03)
$NiH_{-1}L$	441 (45)	561 (+0.17) 490 (−0.81) 415 (−0.18) 318 (−0.02)	435 (51)	488 (−0.46)	427 (61)	535 (+0.33) 423 (−0.74) 306 (−0.04)	432 (76)	489 (−0.23) 416 (+0.02) 300 (−0.03)		[c]
$NiH_{-2}L$	445 (157)	559 (+0.48) 493 (−1.35) 412 (−0.37)	450 (150)	560 (+0.24) 494 (−1.00) 439 (+0.04) 400 (−0.10)	442 (154)	522 (+1.60) 422(−3.05) 306 (−0.12)	442 (125)	566 (+0.20) 496 (−1.91) 439 (+0.54) 337 (−0.03)	440 (154)	516 (+1.80) 421 (−3.34) 309 (−0.08)
$NiH_{-3}L$	445 (163)	560 (+0.44) 493 (−1.48) 409 (−0.38)	450 (165)	560 (+0.45) 495 (−1.48) 441 (+0.20) 400 (−0.17)	441 (159)	522 (+1.71) 422 (−3.36) 307 (−0.10)	442 (127)	567 (+0.18) 497 (−2.06) 439 (+0.68) 337 (−0.02)	440 (163)	516 (+1.84) 421 (−3.45)

[a] λ_{max}/nm (ε/M^{-1} cm^{-1}). [b] λ_{max}/nm ($\Delta\varepsilon$). [c] These species could not be detected spectroscopically because of their low concentrations and overlap with others.

In Fig. 5 the high chemical shift region of the ^{1}H NMR spectra of the peptide -TESHAK- in the absence or presence of Ni(II) ions is presented. It is clear that in the presence of Ni(II) ions at pH* 10.0 the signals of imidazole protons are shifted up-field (Table 1) (Im C_2-H 7.67, $\Delta\delta$ −0.12 ppm; Im C_4-H 6.93, $\Delta\delta$ −0.15 ppm) producing a characteristic cross-peak in TOCSY experiments supporting the complexation of Ni(II) ions through the imidazole ring of the His residue. The comparisons of ^{1}H-NMR and TOCSY spectra between the free (Table 1) and bonded -TESHAK- peptide indicate that Thr (α 4.45, $\Delta\delta$ +0.01 ppm; β 4.40, $\Delta\delta$ +0.03 ppm, γ 1.43, $\Delta\delta$ +0.06 ppm), Ala (α 4.48, $\Delta\delta$ 0.00 ppm; β 1.53, $\Delta\delta$ +0.01 ppm) and Lys (α 4.41, $\Delta\delta$ −0.01 ppm; β′ 1.92, $\Delta\delta$ −0.01 ppm; β″ 1.98, $\Delta\delta$ −0.05 ppm; γ 1.63, $\Delta\delta$ +0.02 ppm; δ 1.84, $\Delta\delta$ +0.03 ppm; ε 3.13, $\Delta\delta$ +0.04 ppm) residues remain uncoordinated. In contrast, the larger shifts for protons of Glu (α 4.24, $\Delta\delta$ −0.26 ppm), Ser (α 4.29, $\Delta\delta$ −0.25 ppm) and α and β protons of His (α 4.10, $\Delta\delta$ −0.65 ppm; β 3.83, $\Delta\delta$ +0.58 ppm) residues strongly suggest their participation in the coordination sphere of Ni(II) ions. It is obvious that Ni(II) ions are coordinated through the peptide nitrogen of these residues supporting the proposed coordination mode for the square-planar Ni(II) complexes as established by potentiometric titrations and other techniques (*vide infra*).

Similarly ^{1}H-NMR and TOCSY spectra of the peptide -TESAHK- in the presence of Ni(II) ions at pH* 10.0 supported the coordination of Ni(II) ions through the peptide nitrogens of Ser (α 4.34, $\Delta\delta$ −0.22 ppm), Ala (α 4.25, $\Delta\delta$ −0.23 ppm) and His residues (α 4.23, $\Delta\delta$ −0.48 ppm; β 3.63, $\Delta\delta$ +0.41) and the imidazole ring of the His residue (Im C_2-H 7.55, $\Delta\delta$ −0.22 ppm; Im C_4-H 6.86, $\Delta\delta$ −0.16 ppm) forming a square-planar complex. The positions of the remaining amino acid signals were almost unaffected (about 0.02 ppm), indicating that they were not involved in the coordination mode of the complexes.

In the case of peptides containing two His residues in their sequence the assignment of the ^{1}H-NMR spectra in the presence of Ni(II) ions was more complicated. Nevertheless, significant chemical shifts in the low chemical shift region of the spectra indicated that Ni(II) ions bind to -TEAHHK- hexapeptide through the amide nitrogens of Ala (α 3.58/3.44, $\Delta\delta$ −0.82/−0.96 ppm; β 1.21/1.46, $\Delta\delta$ −0.28/−0.03 ppm) and the two His residues (α 4.24/4.22/4.23, $\Delta\delta$ −0.48/−0.50/−0.49 ppm; β 3.41/3.37/2.99, $\Delta\delta$ +0.19/+0.15/−0.23 ppm). It must be mentioned that signals of histidine protons of free peptide were not detected and this supports the binding of Ni(II) ions through the amide nitrogens of both His residues. The last results indicate that coordination of the imidazole ring of His in position 5 is retained, while His in position 4 remains deprotonated and uncoordinated. The simultaneous coordination of Ni(II) ions to both of the His residues and to the amide nitrogen of His in position 5 excludes the formation of square-planar complexes due to steric hindrance. The two and three sets of signals corresponding to the Ala and His residues, respectively, suggest the existence of two different species in solution, which is also supported from the species distribution plots in Figs. 1 and 3. In addition, in the aromatic protons region of the ^{1}H-NMR spectrum (Fig. 6) of -TEAHHK- in the presence of Ni(II) ions, five peaks were observed producing four cross-peaks (Im C_2-H/Im C_4-H 7.89/7.10, 7.51/7.05, 7.38/7.07 and 7.35/7.02 ppm) in the TOCSY experiment. All sets of signals were shifted (Table 1), supporting that none of them corresponds to the imidazole protons of the free peptide. The two sets of signals at 7.89/7.10 and 7.51/7.05 ppm may belong to the same complex due to the similar intensity of their cross-peaks in the TOCSY experiment. This complex could be the square-planar $NiH_{-2}L$ (see Potentiometric and spectroscopic (UV/Vis–CD) studies section) where the sets at 7.89/7.10 and 7.51/7.05 ppm correspond to the uncoordinated imidazole ring of His in position 4 and to the coordinated imidazole ring of His in position 5, respectively. The other two sets of signals which overlap each other in the ^{1}H-NMR spectrum, at 7.38/7.07 and 7.35/7.02 ppm, may belong to a minor (low intensity signals) octahedral complex where Ni(II) ions are bound with both histidines. In contrast, the available NMR data indicate that Lys, Glu and Thr residues are not coordinated, although the up-field shift of the β protons of the Thr residue at 4.02 ppm ($\Delta\delta$ −0.36 ppm) suggest the participation of its hydroxyl group in a hydrogen bond. In some cases, the two signals observed for the same protons of the last three residues, as confirmed in the TOCSY spectra, support the existence of two different complexes in solution.

Similarly one dimensional ^{1}H-NMR and TOCSY spectra of the peptide -TASHHK- in the presence of Ni(II) ions at pH* 10.0 suggest the same coordination mode of Ni(II) ions in a square-planar arrangement as in the case of the -TEAHHK- hexapeptide. In the high chemical shift region of the ^{1}H-NMR spectrum there are only four signals (Im C_2-H/Im C_4-H 7.68/7.08 and 7.63/6.92 ppm) corresponding to the imidazole protons of two His residues indicating the existence of only one complex in solution unlike the case of -TEAHHK-.

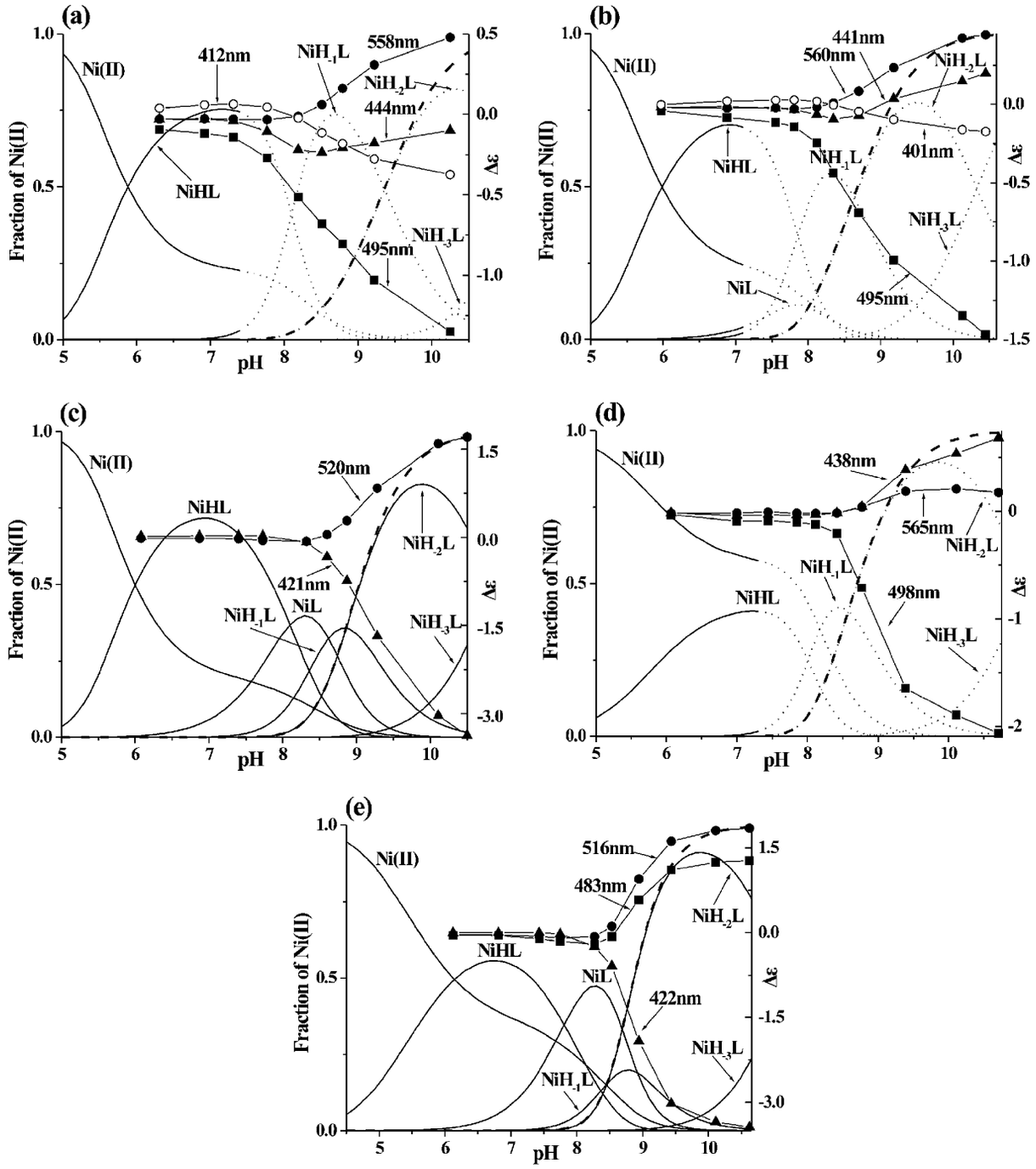


Fig. 3 Correlations between the speciation and the CD parameters. (a) Ni(II)/-TESHHK-, (b) Ni(II)/-TASHHK-, (c) Ni(II)/-TEAHHK-, (d) Ni(II)/-TESAHK- and (e) Ni(II)/-TESHAK-, calculated for reagent concentrations used in CD spectroscopy, using stability constants obtained from potentiometry: $c_{Cu} = c_L = 3$ mM. The line --- represents the sum $NiH_{-2}L + NiH_{-3}L$. The curves in (a), (b) and (d) are represented with dotted lines because of possible peptide hydrolysis above pH 7.5.

A series of one dimensional ^{1}H-NMR spectra of freshly prepared samples containing each one of the hexapeptides with Ni(II) ions in a 1 : 1 ratio were recorded at pH* 10.8 at 37 °C during the time of incubation (about 8 hours). Figs. 7 and 8 represent the ^{1}H-NMR spectra of -TESAHK-/Ni(II) and -TASHHK-/Ni(II), respectively, at different incubation times. It must be noticed that pH* was adjusted before and after recording the spectra to assure its stability. When Ni(II) ions (pH* was adjusted after addition) were added to a solution of -TESAHK- (Fig. 7) at pH* 10.8 the signals of the imidazole protons were shifted up-field indicating coordination of Ni(II) ions, as discussed previously. After 3 hours of incubation at 37 °C two new peaks (Im C_2-H 7.47, Im C_4-H 6.92 ppm) appeared in the region of the imidazole protons supporting the formation of a new complex. While the intensity of the initial peaks at 7.55 and 6.86 ppm were reduced during the time of incubation, the new peaks increased in intensity. Finally, the full disappearance of the initial peaks after 8 h of incubation allowed us to conclude the existence of only one complex in solution. The same situation occurred when Ni(II) ions were added to a solution of -TASHHK- (Fig. 8) at pH* 10.8, producing four new signals in the region of imidazole protons (two His residues) after several hours of incubation at 37 °C.

It is noticed that in samples containing -TESHAK- or -TEAHHK- and Ni(II) ions in a 1 : 1 ratio new peaks were not produced in the region of imidazole protons after the same incubation time. Obviously, Ni(II) complexation with the peptides -TESAHK- and -TASHHK- is accompanied by a

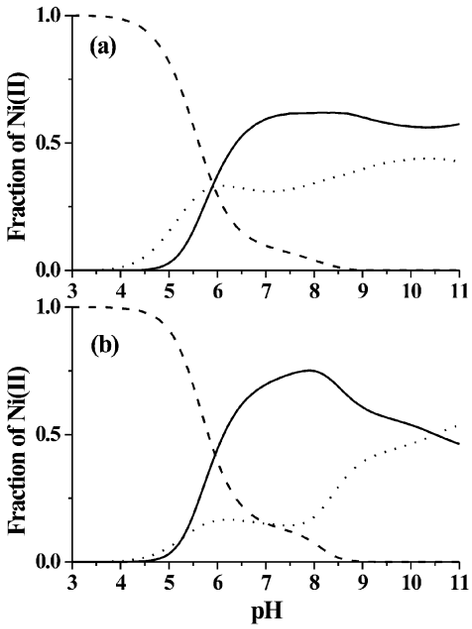


Fig. 4 The correlation species distribution diagrams of (a) Ni(II)/-TEAHHK-/-TESHAK- and (b) Ni(II)/-TEAHHK-/-TESAHK-, in aqueous solution with a ratio 1 : 1 : 1 (c = 3 mM). (---) Ni(II), (——) Ni(II) species with -TEAHHK- and (• • •) Ni(II) species with -TESAHK- or -TESHAK-.

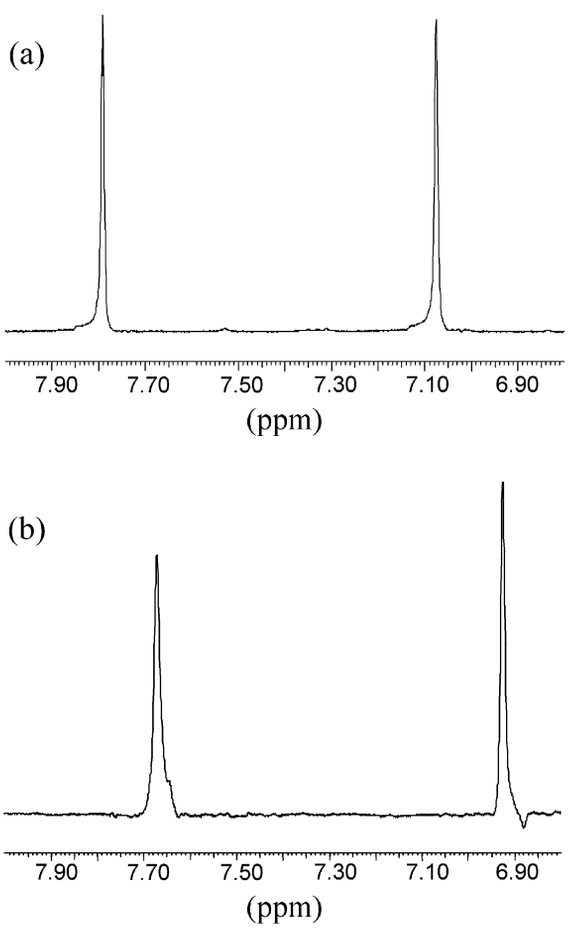


Fig. 5 High chemical shift region of ^{1}H-NMR spectra of -TESHAK- at pH* 10.0 in 99.9% D_2O in the absence (a) or presence (b) of Ni(II) ions in a 1 : 1 ratio.

hydrolytic cleavage and formation of a square-planar complex with the resulting product, similar to the specific Ni(II)-assisted hydrolysis of the -Glu–Ser- peptide bond of -TESHHK-.[16,17]

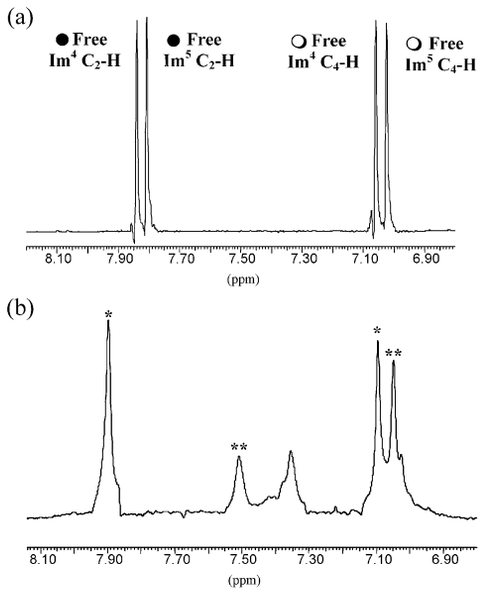


Fig. 6 High chemical shift region of ^{1}H-NMR spectra of -TEAHHK- at pH* 10.0 in 99.9% D_2O in the absence (a) or presence (b) of Ni(II) ions in a 1 : 1 ratio. ●: Free Im C_2-H, ○: free Im C_4-H, * uncoordinated His-4 and ** coordinated His-5.

This is supported by synthesizing the tetrapeptides -SAHK-am and -SHHK-am which both gave identical square-planar complexes to the ones produced by hydrolysis (as indicated by their ^{1}H-NMR spectra at pH* 10.8). These NMR results indicate that while substitution of His in position 4 or Glu residue by an Ala residue has no influence on the hydrolysis reaction, the substitution of His in position 5 or Ser residue seems to be critical for this reaction (no hydrolytic ability). Detailed results of other techniques studying the hydrolysis reaction of the hexapeptides will be presented elsewhere.

Conclusion

In this paper we have studied the interactions of Ni(II) ions with the hexapeptides -TESHHK-, -TASHHK-, -TEAHHK-, -TESAHK- and -TESHAK- models of the -ESHH- motif of histone H2A. The systematic changes in the peptide sequence allowed us to compare the effect of Glu, Ser and His residues on the stability and hydrolytic abilities of the complexes formed, as compared to the ones with -TESHHK-.[16]

In weakly acidic and neutral solutions, all peptides bind to Ni(II) ions initially through one or two imidazole nitrogens forming slightly distorted octahedral complexes. The d–d transitions near 440–450 nm and the CD parameters (Table 4) correspond to $NiH_{-2}L$ species and suggest the simultaneous coordination of an imidazole and three amide nitrogens in the equatorial plane, forming square-planar complexes. In $NiH_{-3}L$ species, Ni(II) ions seem to have similar coordination as in $NiH_{-2}L$ complexes. The only difference between these two square-planar complexes seems to be the deprotonated but uncoordinated ε-amino group of Lys in $NiH_{-3}L$ complexes. The square-planar configuration proposed implies coordination of only one imidazole nitrogen to Ni(II) ions, in the case of all peptides. The log$K$* of the $NiH_{-2}L$ complexes have similar values except the one with -TASHHK- which is at least one log unit higher than the values of the other peptides, indicating that the absence of a Glu residue is stabilizing this species.

It is noteworthy to report that the available NMR data on one- and two-dimensional ^{1}H-NMR spectra of the hexapeptides at pH* 10.0 in the absence or presence of Ni(II) ions are in good agreement with the proposed coordination modes in square-planar complexes supported also from potentiometric measurements and other spectroscopic techniques.

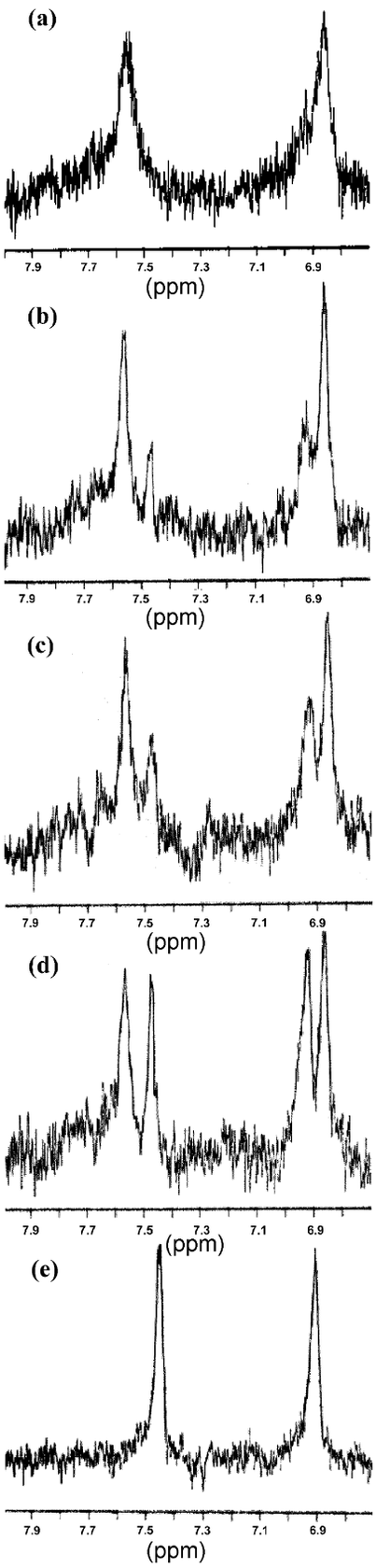


Fig. 7 ^{1}H-NMR spectra (Bruker AC 250 MHz) of samples containing -TESAHK-/Ni(II) in a 1 : 1 ratio (c = 3.4 mM) at different incubation times at 37 °C and pH* 10.8. (a) 0, (b) 2.8, (c) 3.5, (d) 5.3 and (e) ~8 h.

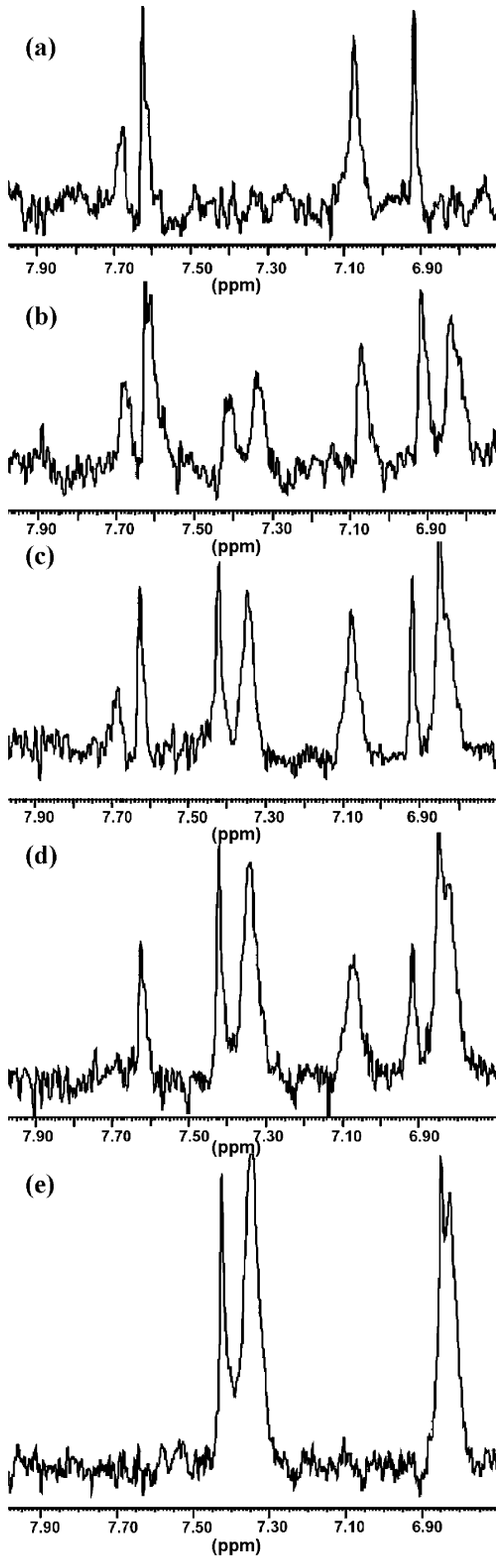
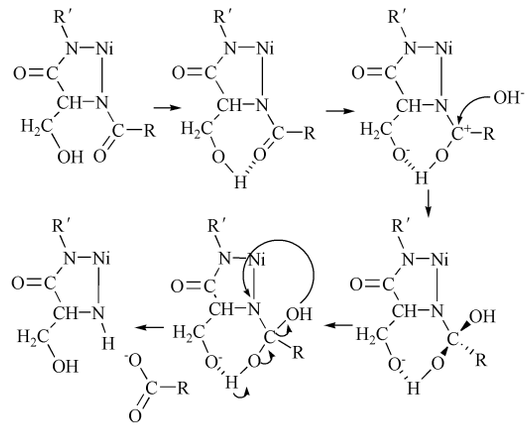


Fig. 8 ^{1}H-NMR spectra (Bruker AMX 400 MHz) of samples containing -TASHHK-/Ni(II) in a 1 : 1 ratio (c = 4 mM) at different incubation times at 37 °C and pH* 10.8. (a) 0, (b) 2, (c) 2.75, (d) 3.5 and (e) ~6.4 h.

In a separate experiment, the series of one dimensional spectra of freshly prepared samples containing each one of the hexapeptides with Ni(II) ions at pH* 10.8 after incubation at 37 °C indicated that metal complexation with the peptides -TASHHK- and -TESAHK- is accompanied by a hydrolytic cleavage, similar to the specific Ni(II)-assisted hydrolysis of the -Glu–Ser- peptide bond of -TESHHK-.[16,17] In contrast, no hydrolytic processes were found in the peptides -TEAHHK- and -TESHAK- where the Ser or His-5 residues are replaced with the Ala residue. Obviously, the substitution of His in position 4 or Glu residue by an Ala residue has no influence on the hydrolysis reaction, unlike with the substitution of His in position 5 or Ser residue which may be critical for this reaction.



Scheme 2 A possible hydrolysis mechanism including a hydrogen bond between the hydroxyl group of a Ser residue and the Glu or Ala carbonyl oxygen. *R* = Ac-Thr-Glu- or Ac-Thr-Ala- and *R′* = -His-His-Lys-am or -Ala-His-Lys-am. For clarity, only the moiety involved in hydrolysis is presented.

Thus, a possible hydrolysis mechanism may be proposed, as presented in Scheme 2, including a possible hydrogen bond between the hydroxyl group of the Ser residue and the glutamic or alanine carbonyl oxygen. This may be necessary for the cleavage of the -Glu–Ser- bond, increasing the positive charge on the carbon atom of the glutamic or alanine carbonyl, making it more sensitive to nucleophilic attacks by hydroxyl groups or water molecules. The next possible reaction may include the involvement of the hydroxyl group of the Ser residue in the formation of an intermediate O-acyl bond as had been reported for the hydrolytic abilities of Cu(II) ions against peptides containing the -Xaa-Ser-His- or -Xaa-Thr-His- sequence, cleaving the -Xaa–Ser(Thr)- bond.[35]

The efficient binding and hydrolytic abilities of Ni(II) ions towards these peptide models led us to conclude that possible interactions of Ni(II) ions with the whole histone octamer inside the cell nucleus, may cause possible damage to the nucleosome. This may provide a novel mechanism of genotoxicity, combining the damage to the nucleosome with the generation of further toxic Ni(II) species.

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