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A comprehensive study of the role of phosphorylation in the coordination and hydrolytic behaviour of the human tau(28-34) protein fragment

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Abstract

Phosphorylation of tau protein by kinases is an abnormal biological process that can significantly alter the chemical properties and conformation of the protein. However, in the context of Alzheimer's disease (AD), phosphorylation may play an important role in protein aggregation and the development of neurodegenerative diseases. Literature data have demonstrated that potential phosphorylation of the alcoholic side chains of threonyl and seryl residues in the tau protein sequence has a significant impact on the accumulation of the protein. The present article investigates the role of phosphorylation of the threonyl residue in the -TMH- motif in the human tau(28-34) protein fragment, in the chemical interaction of the tau peptide with metal ions. The interaction of the native tau(28-34) protein fragment (Ac-GYTMHQD-NH₂), as well as the phosphorylated tau(28-30T(P)-34) peptide (Ac-GYT(P)MHQD-NH₂), with essential Cu(II), Zn(II), and toxic Ni(II) ions has been thoroughly investigated. Thermodynamic equilibrium studies have shown that phosphorylation of threonine considerably improves metal ion binding affinity and increases the stability constants of M(II)-N_{im} complexes. Furthermore, there were unambiguous differences in the CD and UV-Vis spectra of Cu(II) and Ni(II) complexes compared to those of the native tau peptide complexes. The hydrolytic stability of the amide bond between tyrosyl and threonyl residues was investigated for the native peptide in the presence of Ni(II) at 25°C and different pH values. However, the irreversible hydrolytic reaction was inhibited in the case of the phosphorylated tau peptide.

1. Introduction

Metal ion-protein interactions are very common in the human body and are involved in many biological processes, such as enzymes activation, redox reactions, hydrolytic processes, and protein aggregation [1,2]. Amyloid-beta, prion, and tau are significant proteins present in the

human brain and are environmentally more exposed to metal ion interactions. Many studies have demonstrated that the interaction of metal ions with A β and prion proteins has an unambiguous role in the protein aggregations and development of neurodegenerative diseases; however, the potential role of the microtubular tau protein remain significantly unclear [3,4,5].

Microtubules are essential components of the neuronal cellular structure in the human brain and perform many crucial functions in developing neuronal system. Inside the neurons, microtubules mediate pivotal cellular processes such as neuronal migration, differentiation, and synapse formation [6]. Tau protein maintains and stabilizes neuronal microtubules in the human brain under physiological conditions. It directly associates tubulin protein, the main component of microtubules, and prevents microtubules from damage or dissociation [7]. However, through poorly understood pathological mechanisms, tau undergoes structural alteration, essentially by hyperphosphorylation process induced by kinase enzymes [8]. This process ultimately leads to protein destruction, accumulation of toxic protein aggregates, and subsequent generation of neurofibrillary tangles, resulting in neurodegenerative diseases commonly known as tauopathies [9]. Despite not being a huge protein, tau comprises of six soluble isoforms, with sequences ranging from 352 to 441 amino acids. The protein structure contains four major regions: an acidic N-terminal region, a proline-rich region, a microtubule-binding domain (MTBD), and a C-terminal region [10]. The MTBD consists of four repeat domains (R1-R4), which are essential for the interaction of tau with microtubules [11].

Phosphorylation of tau protein is a common post-translational modification that significantly regulates the normal function of the protein and enhances its binding to microtubules [12]. However, abnormal phosphorylation by kinases alters the protein's conformation due to the large, negatively charged phosphate groups in the protein side chains [8]. As phosphorylation of tau changes its conformation and leads to abnormal functions, the chemical structure of the protein sequence contains many potential sites for phosphorylation, such as seryl, threonyl, and tyrosyl residues [13]. There are eighty-five potential phosphorylation sites in the tau sequence, fifty-five of which have been identified under pathological conditions. Many of these are located in the proline-rich region, the C-terminal region of the protein, and the microtubule-binding domain [14]. Phosphorylation at certain sites, such as Ser214 by PKA kinase or Ser262 and Ser356 by MARK kinase, was described to decrease the microtubule-binding affinity of tau protein [15,16].

The role of metal ions in promoting neurodegenerative diseases has been demonstrated by many clinical and analytical studies. High concentrations of metal ions such as Cu(II), Zn(II), Fe(II), and Ni(II) have been found in plaques, which are hallmarks of Alzheimer's disease [17,18]. The extent of tau protein aggregation is influenced by several factors, including the concentration and oxidation state of metal ions, hyperphosphorylation of the protein, biological redox reactions of specific metal ions, and hydrolytic processes [19,20]. Furthermore, phosphorylation of several threonyl and seryl residues in the protein can significantly modify its metal-binding ability, thereby increasing the affinity of the protein for metal ions. However, there is insufficient information on the interaction between phosphorylated tau protein and metal ions. Tau contains

twelve histidyl residues, which serve as primary binding sites-anchors-for metal ions, in addition to numerous hydroxyl and carboxyl groups, which enhance this chemical interaction [21].

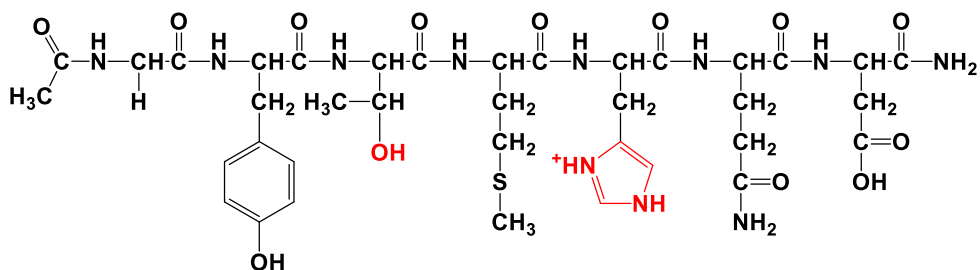
Copper(II) and zinc(II) are essential metal ions in the human body and are involved in many biological functions [22,23], whereas nickel(II) ions are not essential for the human body, despite this, the body naturally excretes nickel(II) ions under physiological conditions. The *Helicobacter pylori* bacterium is the sole microorganism containing Ni(II) ion in its natural environment and present in the human stomach during infections [24]. These three metal ions are involved in our study, since a comprehensive analysis showed high concentrations from these metal ions in biological samples of patients diagnosed with neurodegenerative diseases [23,25]. Nickel(II) ions can significantly promote hydrolysis of the amide bond in the -T(S)XH- region in protein fragments. Literature data have affirmed that the Ni(II)-induced hydrolytic process is significant in the protein sequence -Z1aaThr(Ser)XaaHisZ2aa- in which Z1aa and Z2aa are amino acid residues and Xaa refers to any amino acid residue excluding proline. The hydrolyzed amide bond is between the Z1aa and Thr or Ser residue in the protein fragment [26, 27].

Initiation of the Ni(II)-induced hydrolytic process in the protein fragment requires the formation of a Ni(II) complex with (3N⁻, N_{im}) binding sites by the -T(S)XH- moiety of the protein fragment. This complex starts to form under slightly basic conditions. According to the stereochemistry of this complex, the threonyl or seryl hydroxyl group is positioned close to the preceding amide bond in the protein fragment and acts as a strong nucleophile. Structural alteration and ester formation then occur, enabling the development of albumin-type binding sites around Ni(II) ion (NH₂, 2N⁻, N_{im}), which is a highly active form for hydrolysis in the presence of a base [28]. The rate of the hydrolysis induced by Ni(II) ions depends on the reaction temperature, pH, and nature of the amino acid residue close to the hydrolytic site [29,30].

Both our preliminary studies and data from literature have shown that, under the conditions of solution equilibrium studies, hydrolysis of the ester group does not occur [31]. The -TMH- motif in the human tau(28-34) protein fragment (Ac-GYTMHQD-NH₂) contains not only Thr(30) – a potential phosphorylation site – but also His(32), which serves as an anchor for metal ions. Moreover, this protein fragment may undergo hydrolytic processes induced by M(II) ions. This present paper reports the effect of phosphorylation of threonine in the human tau(28-34) protein fragment on the metal ion binding affinity and hydrolytic processes. The coordination chemistry behaviour of the protein fragment and its threonyl-phosphorylated derivative with essential Cu(II), Zn(II), and toxic Ni(II) ions has been thoroughly investigated. In addition, the hydrolytic processes of the native and phosphorylated protein fragments were examined in the presence of Ni(II) ion at room temperature and at different pH values. pH-potentiometry, spectroscopic techniques (UV-Vis, CD), RP-HPLC, ¹HNMR, and ESI-MS were used for a comprehensive analysis.

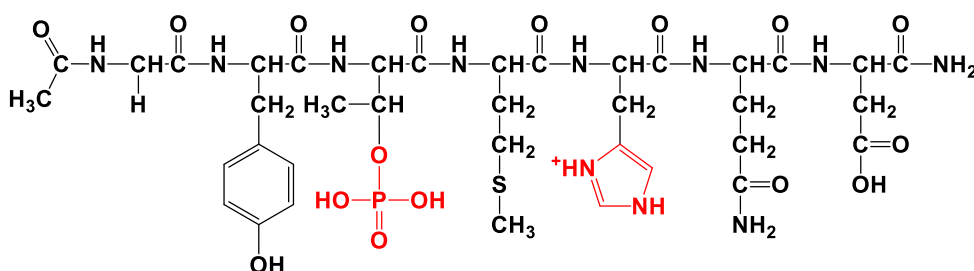
The two peptides examined in this paper were tau(28-34) (Ac-GYTMHQD-NH₂) = L1) and tau(28-30T(P)-34) (Ac-GYT(P)MHQD-NH₂) = L1P) (see Figure 1). For comparison, we also use data from two peptides we previously studied (tau(Y29A-34), Ac-ATMHQD-NH₂ = L2 and tau(371-376), Ac-IETHKL-NH₂) = L3) [32], as well as data from two peptides reported in the literature (tau(261-

268), Ac-GSTENLKH-NH₂ = R1 and tau(261-262S(P)-268), Ac-GS(P)TENLKH-NH₂) = R1P) [31]. Table S1 lists the abbreviations used for these ligands.



Tau(28-34), Ac-GYTMHQD-NH₂ (L1)

(a)



Tau(28-30T(P)-34), Ac-GYT(P)MHQD-NH₂ (L1P)

(b)

Figure 1: Chemical structures of the native tau(28-34) peptide (L1) (a) and phosphorylated tau(28-30T(P)-34) peptide (L1P) (b).

2. Experimental Part

2.1. Materials

All solvents and chemicals used for synthetic purposes were obtained from commercial sources in the highest available purity and used without further purification. All of the N-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids, including (Fmoc-Gly-OH, Fmoc-Tyr(Trt)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Meth-OH, Fmoc-His(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Asp(OtBu)-OH), the Rink Amide resin and 2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) were purchased from Novabiochem (Switzerland). N-methylpyrrolidone (NMP), 2,2'-(ethylenedioxy)diethanethiol (DODT), 2-methyl-2-butanol, N-hydroxybenzotriazole (HOBt) and triisopropyl-silane (TIS) were purchased from Merck, while N,N-diisopropylethylamine (DIPEA) and trifluoroacetic acid (TFA) were Merck Millipore Co. products. Peptide-synthesis grade N,N-dimethylformamide (DMF) and acetic anhydride (Ac₂O) were bought from VWR International, while piperidine, dichloromethane (DCM), diethyl ether (Et₂O), acetic acid (AcOH) and acetonitrile (ACN) from Molar Chemicals Ltd.

The two peptides were in the N-acetylated and C-amidated form to remove any end-charge effects. The native tau(28-34) peptide Ac-GYTMHQD-NH₂ was synthesized by the

technique of solid phase synthesis, while the phosphorylated tau(28-30T(P)-34) peptide Ac-GYT(P)MHQD-NH₂ was purchased from SynPeptide Co. Ltd. with purity level above 95%. Stock solutions (CuCl₂, NiCl₂, ZnCl₂) were prepared from analytical-grade reagents (Reanal Ltd.), and their concentrations were verified gravimetrically via the precipitation of oxinate or complexometric titrations.

2.2. Peptide Synthesis (SPPS)

The synthesis of the human tau (28-34) protein fragment (Ac-GYTPMHQD-NH₂) was conducted manually via solid-phase peptide synthesis (SPPS), following the (Fmoc)/tert-butyl (tBu) strategy. The Rink amide AM resin (800 mg, 0.70 mmole/g, 100-200 Mesh Number) was placed in a plastic polypropylene reaction vessel equipped with a filtration system capable of withstanding the organic solvents employed. After resin swelling in DMF for 40 min, Fmoc-protected amino acids were introduced through the following activation protocol: Fmoc-Xaa-OH (4 equiv.), TBTU (4 equiv.) and DIPEA (8 equiv.) in DMF for 30 min.

The L-amino acids were coupled in the following order: Fmoc-Asp(OtBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-His(Trt)-OH, Fmoc-Meth-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(Trt)-OH, Fmoc-Gly-OH. After each coupling, the resin was washed with DMF (3 × 8 ml). Fmoc group removal was performed by treating the resin with 20% piperidine in DMF (1 × 5 min + 1 × 15 min) and washing with DMF (5 × 2 min).

The Fmoc group at the N-terminus of the peptide sequence was also removed. The final acetylation was performed directly on the solid phase by adding a solution containing Ac₂O 1.4 mL in 5.6 mL DMF (20%) (2 × 10 min). The resin was then filtered and washed with DMF, diethylether, and 2-methyl-2-butanol.

The peptide was cleaved from the resin using a mixture of (94% TFA, 2.5% TIS, 2.5% H₂O, and 1% EDT V/V) for a period of two and half hours at room temperature. This process also resulted in the removal of the side chain protective groups. The crude product from the peptide was precipitated by cold diethylether from the filtered TFA solution. The solid product was also washed with cold diethylether and dried. It was then dissolved in water, separated by preparative RP-HPLC and finally frozen for lyophilization.

Analytical reverse-phase high-performance liquid chromatography (RP-HPLC, detailed in the section below) analysis was conducted to confirm the purity of the synthesized protein fragment. The active substance content of the product was greater than 95%, as confirmed by pH-potentiometric measurements, which also identified the protonation sites.

The ESI-MS measurements (detailed in the section below), performed in positive mode, yielded exact mass (m/z) value for the synthesized protein fragment.

2.3. Potentiometric Measurements

pH-potentiometric titrations were carried out in 3.00 mL samples. The ligands concentration was in the range from 1 mM to 2 mM by using carbonate free stock solution of potassium hydroxide of known concentration approximately 0.2 M. The selected ratio for M:L

was 1:1 for the titration, Argon gas was being bubbled above the surface of the solution to ensure the absence of CO₂ gases. The samples were being stirred by a VELP scientific magnetic stirrer. All pH-potentiometric measurements were conducted at 298 K and at constant ionic strength of 0.2 M KCl. The pH measurements were made using a MOLSPIN pH-meter equipped with a 6.0234.100 and 6.0234.110 combination glass electrode (Metrohm) and the titrant dispensed by means of a computer controlled MOL-ACS burette.

During the titrations, the following equilibrium conditions were used: the system was in equilibrium if the maximum change of potential is 0.03 mV for 9 seconds. The recorded pH readings were converted into hydrogen ion concentration as described by Irving et al. [33]. Protonation constants of the ligands and overall stability ($\log \beta_{pqr}$) constants of the metal complexes were calculated by means of general computational programs (PSEQUAD [34] and SUPERQUAD [35]) based on the Equations (1) and (2).



$$\beta_{pqr} = [M_p H_q L_r] / [M]^p [H]^q [L]^r \quad (2)$$

Some derived constants were calculated according to Equations (3) and (4).

$$\log K(M-N_{im}) = \log \beta(MH_x L) - \log \beta(H_x L), (x = 0 \text{ or } 1) \quad (3)$$

$$pK_{amide} = (\log \beta(MH_x L) - \log \beta(MH_{x-n} L)) / n (n = 1, 2 \text{ or } 3) \quad (4)$$

The concentration distribution curves were generated by the MEDUSA program [36] using the protonation constants of the ligands and the stability constants of the metal ion complexes.

2.4. Spectroscopic Measurements

2.4.1. UV-Vis Spectroscopy

The same concentration range used for the pH-potentiometry was selected to register the UV-Vis spectra of the copper(II) and nickel(II) complexes. The measurements were conducted using a VWR UV1600 PC scanning spectrophotometer at a wavelength range of 200-900 nm and at room temperature. The wavelength and absorption data were recorded using software provided by the manufacturer, and the absorption spectrum data were plotted using MS Excel software.

2.4.2. CD Spectroscopy

A JASCO J-810 spectropolarimeter was used to perform circular dichroism (CD) measurements. The CD spectra of the Cu(II) and Ni(II) complexes were acquired in the range from 280 to 800 nm using 1 cm cell and from 220 to 300 nm using 1 mm cell. The same concentration used as applied for pH-potentiometric measurements. The CD measurements were performed in all cases at room temperature. The CD spectra were recorded at different pH values, however in the hydrolytic studies the CD spectra were recorded as a function of time. The data of the experiments were evaluated using the Spectral Manager software provided by the manufacturer, and the CD spectra were plotted using MS Excel software.

2.4.3. Electrospray Ionization-Mass Spectrometry (ESI-MS)

The Bruker maXis II QTOF ESI-TOF instrument was used to perform the ESI-TOF-MS analyses in the positive mode for the synthesized ligand with the concentration of 0.1 μM , and also for the products of the hydrolytic studies. The temperature of the drying gas (N_2) was 100 $^\circ\text{C}$. The pressure of the nebulizing gas (N_2) was 30 psi. The voltages that were applied at the capillary entrance, capillary exit, and the first and the second skimmers were -4500, 120, 40, and 30 V, respectively. The spectra were accumulated and recorded with a digitalizer at a sampling rate of 2 GHz. Sodium formate was used as an external calibrator cluster ion ($[(\text{HCOONa})_n + \text{Na}]^+$) generated from solution under ESI conditions served as a reference. The MS signals were identified based on their m/z values using the DataAnalysis 4.4 (Bruker) computer software.

2.4.4. ^1H -NMR spectroscopy

^1H -NMR experiments were performed with a Bruker Avance II 500 spectrometer operating at 500 MHz ^1H frequency and equipped with a 5 mm z-gradient TXI probe. The ligands were dissolved in D_2O with 99.8% isotopic purity. Solutions with a concentration of 0.003 mol/dm ^{3} from the ligands were prepared for recording the ^1H -NMR spectra. The pH^* of the solutions was adjusted to the desired value using different molar concentrations from DCl and NaOD solutions in D_2O . The pH^* values (direct pH meter readings in a D_2O solution of a pH meter calibrated in H_2O according to Irving et al. [37]) were converted into pH values using the following equation: $\text{pH} = 0.936\text{pH}^* + 0.412$ [38]. TSP- d_4 (Sodium 3-(trimethylsilyl)propionate-2,2,3,3- d_4) was used as a standard ($\delta_{\text{TSP}} = 0$). The NMR spectra were analyzed using the MestreNova program.

2.5. High Performance Liquid Chromatography Measurements (RP-HPLC)

The application of the RP-HPLC technique was important to prove the purity of the synthesized peptide. For the purification of the protein fragment (L), a semi-preparative and an analytical Jasco instrument were used, monitoring the wavelength at 222 nm. A gradient elution with 0-20-0% of solvent B was conducted using solvent A (0.1 V/V% TFA in Milli-Q $^\circ$ water) and solvent B (0.1 V/V% TFA in acetonitrile) at a flow rate of 1.00 mL/min. A Teknokroma EUROPA PROTEIN C18 chromatographic column (250 x 4.6 mm, 300 $\text{\AA}$ pore size, 5 μm particle size) was used for analytical measurements. A flow rate of 3 mL/min and two semi-preparative columns (EUROPA PROTEIN C18 chromatographic column (250 x 10 mm, 300 $\text{\AA}$ pore size, 5 μm particle size), EUROPA PROTEIN C8 chromatographic column (250 x 10 mm, 300 $\text{\AA}$ pore size, 5 μm particle size) in linear succession are used to separate and collect the synthesized product with the instrument.

For hydrolysis studies, the measurements were performed on a Waters Alliance 2995 separations module equipped with a Waters Alliance Series Column Heater/Cooler, a Waters 2996 PDA detector, and a Waters 2487 Dual λ Absorbance Detector. Chromatographic data acquisition and handling were performed using Empower $^\text{TM}$ 3 CDS (Waters, Milford, MA, USA). Separation of the components was achieved on an XBridge BEH Shield RP18 (130 $\text{\AA}$, 2.5 μm , 4.6

mm × 75 mm) column equipped with an ACQUITY Column In-Line Filter (Waters, Milford, MA, USA). A gradient method (0-20-0% of solvent B) was used for the separation with 0.1% TFA in water as solvent A and 0.1% TFA in acetonitrile as solvent B. The flow rate of the mobile phase was 1.00 mL/min, and the column temperature was set to 25°C. Injection volume was 20.0 µL, and the detection wavelength on the UV detector was 222 nm.

2.6. Samples Preparation for Hydrolysis Studies

In all cases, the ratio was equimolar (1:1), with a slight excess of the peptide to eliminate the formation of nickel(II) hydroxide precipitate. The peptides were dissolved in 20 mM HEPES buffer to achieve a concentration of 1 mM, which was then adjusted to pH 8.0 with 0.2 M KOH and HCl, but in the case pH 11.0, there was not any need to use HEPES buffer. After pH adjustments, the samples were incubated at 25°C. Finally, the metal ion was added (in the case of the control samples, the same volume of three time-distilled water was added instead), marking the $t = 0$ point. The reaction vessels were hermetically sealed to prevent the evaporation of water and were only opened for the purpose of taking samples of 50 µL. The reaction was halted by the addition of 200 µL of 1% solution of HPLC-grade trifluoroacetic acid, after which the samples were stored in a refrigerator at 7°C until the analytical RP-HPLC and ESI-MS measurements were conducted. The final concentration of the protein fragment (L) at the $t = 0$ point, following the halting of the reaction, was approximately 0.21 mM.

3. Results and Discussion

3.1. Acid-base behaviour of native and phosphorylated protein fragments

3.1.1. Potentiometry

The values of the formation and deprotonation constants of the ligands were determined using the pH-potentiometric technique, which are listed in Table 1. Regarding the chemical structures of the peptides (Figure 1), they are protected from the N-terminal part by an acetyl group, while the C-terminal part is protected by an amide group. In a strongly acidic environment, where all donor groups in the side chains of the ligands are in their fully protonated forms ($[H_3L]^+$ for L1 and $[H_5L]^+$ for L1P), 3 deprotonation steps are possible for the native enzyme and 5 for the phosphorylated derivative. These peptides have one histidyl residue, which is the effective binding site for metal ions, in addition to other polar side chains that enhance the coordination process, like the negatively charged aspartate and phosphate groups, together with the tyrosyl residue, participate in the equilibrium processes for these ligands. The L1 fragment contains three ionizable groups belonging to aspartyl, histidyl, and tyrosyl residues, while the threonyl-phosphorylated L1P peptide possesses the same above-mentioned ionizable groups with an extra two deprotonation site corresponding to the phosphate group ($-OPO(OH)_2$) in acidic medium.

The data show that the pK value of the imidazole-N donor group in the side chain of the L1 is in good agreement with the pK value of terminally protected peptides containing one histidine and glutamic or aspartic acid residues (e.g. L2) [32]. The carboxylic groups of the side chains are considered to form weak hydrogen bonds with the imidazole-N atom and hinder its deprotonation process [39]. However, the pK values of imidazole-N donors in the absence of any other side chains are usually between 6.0 and 6.3 [40]. The imidazole pK value of L1P ligand was significantly higher, than that of L1, and this is due to the presence of phosphate group in the vicinal position to histidyl residue. The difficulty of the proton removal process probably could be because of the formation of hydrogen bonds between the negative charged phosphate group and imidazole protons, which is also consistent with literature (pK(His): 6.46 for R1 and 6.74 for R1P) [31]. The deprotonation of the carboxylic group of aspartyl residue occurs in the slightly acidic solution, where pH value is below 4.0. The first deprotonation of threonyl-phosphorylated residue takes place in strong acidic solution, which falls outside the pH range measurable by pH-potentiometry, so we were unable to determine the corresponding pK value. The second deprotonation of the same group occurs at pH value 5.70, which also depends on the position of (-PO₃H⁻) group from histidyl residue [31]. The pK value of the tyrosyl residue is high, due to the weak acidic character of hydroxyl group of tyrosyl residue in oligopeptides.

Table 1. Formation (log β) and deprotonation constants (pK) of native L1, phosphorylated L1P and some tau peptides for comparison (T=298 K, I = 0.2 M KCl).

Species	L1	L1P	R1 [31]	R1P [31]	L2 [32]
HL	9.70 (1)	9.79 (7)	10.23	10.25	6.51
H ₂ L	16.13 (2)	16.83 (1)	16.69	16.99	10.27
H ₃ L	19.75 (3)	22.53 (1)	20.74	22.63	—
H ₄ L	—	26.31 (2)	—	26.39	—
pK(Asp)	3.62	3.78	—	—	3.76
pK(Glu)	—	—	4.05	3.76	—
pK(HOPO ₂ ⁻)	—	5.70	—	5.64	—
pK(His)	6.43	7.04	6.46	6.74	6.51
pK(Tyr-OH)	9.70	9.79	—	—	—
pK(Lys-NH ₃ ⁺)	—	—	10.23	10.25	—

3.1.2. ¹H-NMR spectroscopy

Besides pH-potentiometry, ¹H-NMR measurements as a function of pH were conducted in order to follow the deprotonation processes of the native and phosphorylated protein fragments.

The NMR titration data for the two ligands are consistent with the solution equilibrium results. Deprotonation of the carboxylic group of aspartic acid residues in both native and phosphorylated protein fragments started in strongly acidic solution above pH 3, as indicated by the upfield shift of the methylene group (β -CH₂) of the aspartic acid residues (marked by x signals in the NMR spectrum and in Figure S2) from δ = 2.88 to 2.69 ppm, which is consistent with pK

value = 3.62-3.78. These signals do not show any change in other ^1H -NMR spectra until basic pH Figure 2 and Figure S1.

The resonating frequency of the doublet β -methylene signal of the histidyl residue (**Q**) in the native protein fragment exhibits an upfield shift in a very slightly acidic medium at pH 6.55. This upfield shift was more pronounced at higher pH values. Furthermore, the two aromatic singlet imidazolyl proton signals (* and Δ) show a significant shift consistent with the β -CH₂ of the histidyl residue. These NMR results corroborate the pK value for the deprotonation of the imidazole proton (pK 6.45) as shown in Figure S1 and Table S2.

On the other hand, deprotonation of the imidazole proton in the phosphorylated protein fragment is accompanied by a significant upfield shift of the doublet His-CH₂ at pH $\sim$ 7.94, along with an obvious change in the chemical shifts of the singlet aromatic signals. This gives indication that the pK value for the imidazole proton is higher in the phosphorylated ligand. Between pH 4.89 and 6.36, there is a distinct upfield shift of the doublet Thr-CH₃ signal ($\odot$), indicating the second deprotonation of the phosphate group. It is therefore plausible to assume that this process occurs at a lower pH than that of imidazole-nitrogen deprotonation. These findings confirm that the pH-potentiometric and ^1H NMR results are in good agreement Figure 2 and Table S3.

On increasing the pH, the final deprotonation process in both native and phosphorylated ligands is related to the hydroxyl group of the tyrosyl residue, which occurs at high pH. This is evident in the ^1H -NMR spectra by changes of the resonating frequency of the aromatic protons and CH₂ group of the tyrosyl residue, as shown in Figure 2 and Figure S1. The chemical shift of the doublet Tyr-CH₂ signal (**h**) was changed from 3.00 to 2.94 ppm in the native ligand, and from 2.97 to 2.91 ppm in the phosphorylated peptide in last in NMR spectra at pH $\sim$ 11.50. Additionally, the doublet signals of the aromatic protons (**•** and **S**) of the tyrosyl residue exhibited a big shift at this pH, with a considerable overlap with one of the singlet protons of the imidazole part of the molecule.

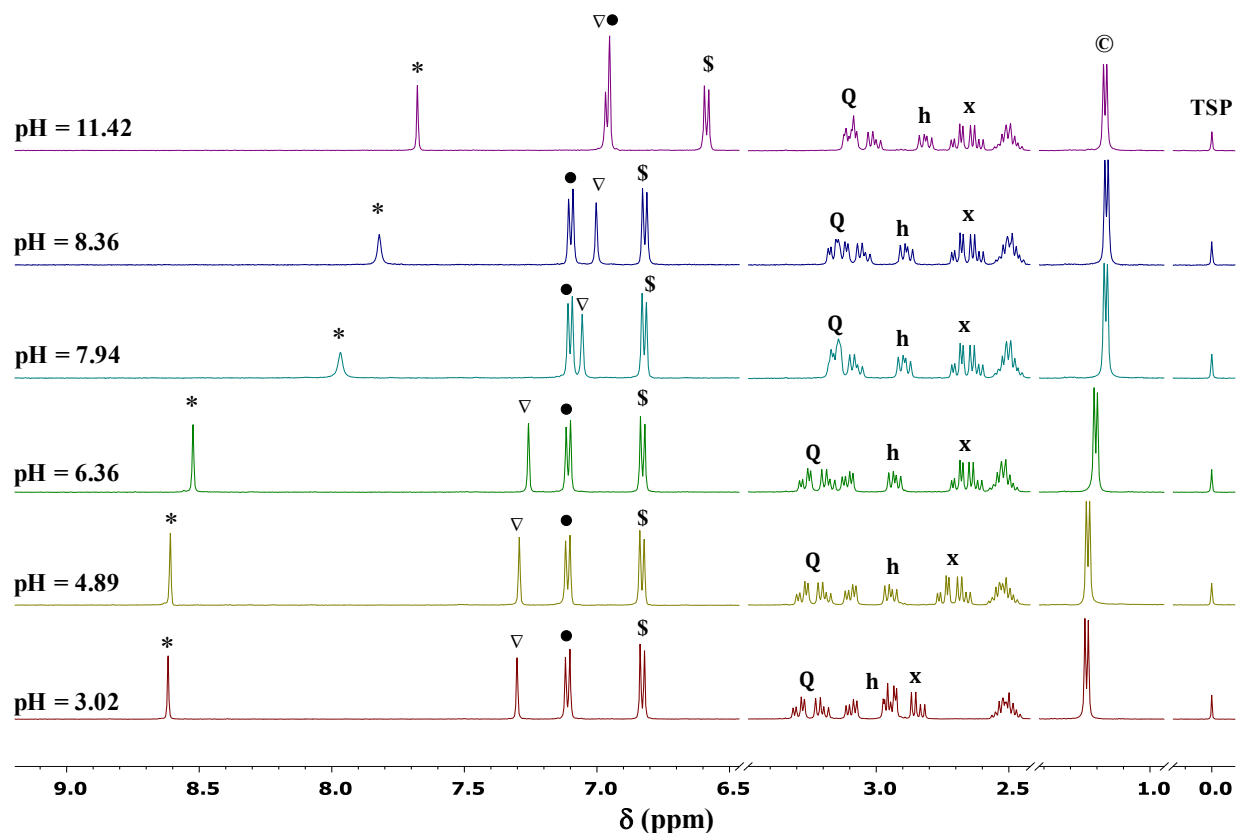


Figure 2: pH-dependence of the ^1H -NMR spectra registered for L1P ligand (* and Δ are His-aromatic protons signals (s), $\bullet$ and S are Tyr-aromatic proton signals (d), Q is His- CH_2 signal (d), h is Tyr- CH_2 signal (d), x is Asp- CH_2 signal (d), © is Thr- CH_3 signal (d)).

3.2. Coordination chemistry properties of Cu(II)-peptide complexes

Equilibrium investigations (pH-potentiometry) were conducted to determine the stability constants of the Cu(II) complexes of the ligands, and the derived constants are presented in Table 2. It is also worth mentioning that the complex formation processes in both Cu(II): tau(28-34) and Cu(II): tau(28-30T(P)-34) systems are reversible. This is greatly obvious in Figure S3 and Figure S4, where the titration curves of the two systems are presented. The first titration curve for both systems was obtained through normal potentiometric titration, while the second titration was performed after reacidification of the titrated sample. The figures confirm that no irreversible reaction occurred in the pH range studied.

Table 2. Stability constants ($\log\beta$) of the Cu(II) complexes of L1, L1P, and some tau peptides for comparison (T=298 K, I = 0.2 M KCl).

Species	L1	L1P	R1 [31]	R1P [31]	L2 [32]	L3 [32]
[CuH ₂ L]	—	20.74(7)	—	20.52(2)	—	—
[CuHL]	14.57(3)	15.37(5)	14.57(3)	15.29(1)	—	14.56(2)
[CuL]	—	—	7.99(2)	8.45(1)	5.09(2)	—

[CuH ₋₁ L]	3.37(2)	2.08(5)	1.99(3)	2.01(1)	—	2.15(2)
[CuH ₋₂ L]	-4.42(3)	-7.50(6)	-6.54(5)	-6.67(2)	-6.02(2)	-6.57(2)
[CuH ₋₃ L]	-14.01(4)	-17.61(6)	-16.92(7)	-17.00(2)	-14.47(3)	-16.47(2)
pK(HOPO ₂ ⁻)	—	5.37	—	5.23	—	—
pK ₁₂ (amide)/av.	5.60	6.65	6.29	6.64	5.56	6.21
pK ₃ (amide)	7.79	9.58	8.53	8.68	8.45	8.72
pK(Tyr-OH)*	9.59	10.11	—	—	—	—
pK(Lys-NH ₃ ⁺)**	—	—	10.38	10.33	—	9.90
logK(Cu(II)-N _{im})	4.87	5.58	4.34	5.04	5.09	4.23

* $\log\beta([\text{CuH}_{-1}\text{L}]) - \log\beta([\text{CuH}_{-2}\text{L}])$

** $\log\beta([\text{CuH}_{-2}\text{L}]) - \log\beta([\text{CuH}_{-3}\text{L}])$

The ligands coordinate to copper(II) ions through histidyl residue (His32), which is the anchor to metal ions. The Cu(II) complex formation process of L1 molecule is in a good agreement with those observed in other terminally protected peptides, containing -TXH- moiety [32]. The derived constants calculated for the imidazole-coordinated [CuLH] complex ($\log K(\text{M-N}_{\text{im}})$) (see Equation 3) were slightly higher for L1 due to the polar side chain of the aspartic acid residue.

The phosphorylation of threonine residue affects the complex formation processes. The phosphorylated threonine in the L1P ligand significantly increased the stability of the Cu(II)-N_{im} complexes, with a $\log K(\text{Cu(II)-N}_{\text{im}})$ (Equation 3) value of 5.58. A similar increase in $\log K(\text{Cu(II)-N}_{\text{im}})$ is also observed for the R1 and R1P ligands (See Figure 12).

The distribution curves of Cu(II): L1 and L1P systems are plotted in Figure 3a and Figure 3b. The percentage of the Cu(II)-N_{im} coordinated complex increased for phosphorylated derivative, which indicates the involvement of the phosphate group in the coordination process. Deprotonation and metal ion coordination of the first two amide functions takes place in a cooperative manner for both L1 and L1P peptide, forming [CuH₋₁L] = [CuH₋₂(HL)] species, where the tyrosyl residue remains protonated. Cooperative deprotonation of the two amide groups is generally observed in copper(II) complexes of other terminally protected peptides containing a single histidine as well [41,42]. However, the average pK₁₂(amide) values determined for the Cu(II) complexes of the L1 and L1P peptides (5.60 and 6.65, see in Table 2), and the distribution curves of copper(II) complexes of the peptides (Figure 3) clearly show that the deprotonation of the two amide nitrogens preceding histidine in the peptide backbone significantly shifted to higher pH values in case of phosphorylated L1P peptide. On the other hand, the copper(II)-induced deprotonation of the amide nitrogen in the L1 peptide, which contains the -TMH- sequence, occurs at a lower pH than in peptides that do not contain this sequence (e.g., L3). However, this is consistent with the results of our previous studies (e.g., the L2 peptide).

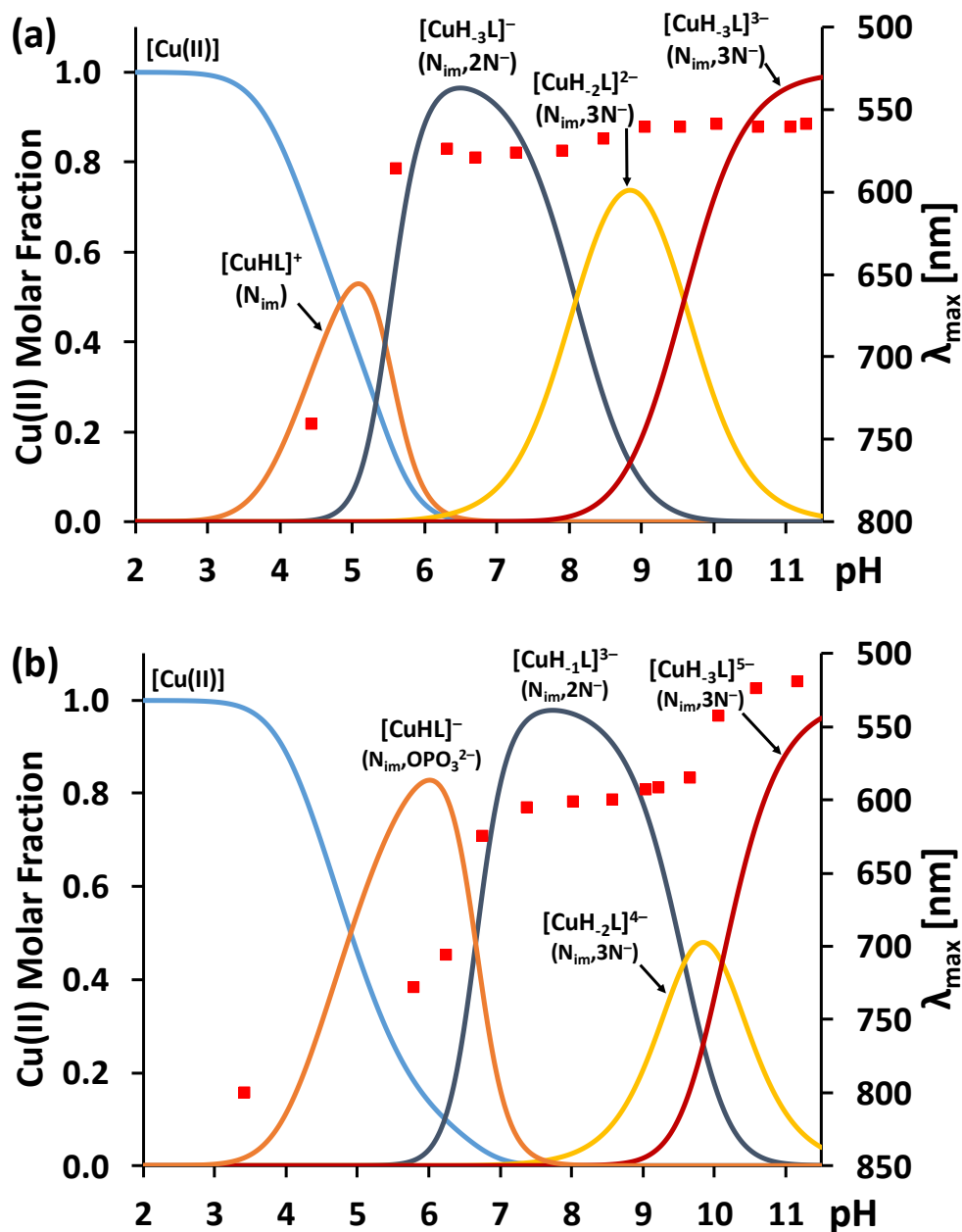


Figure 3: Metal ion distribution curves in the Cu(II): L1 (c(L) = c(M) = 1.09 mM) (a) and Cu(II): L1P (c(L) = c(M) = 1.33 mM) (b) systems, plotted in parallel with the change of wavelengths belonging to absorption maxima (marked with red squares).

The deprotonation of the third amide nitrogen occurred at pH 7.79 for L1, forming the $[\text{CuH}_{-2}\text{L}] = [\text{CuH}_{-3}(\text{HL})]$ species; however, this value rose significantly (9.58) for L1P peptide (Table 2). These results indicate that the phosphorylation of threonyl residue improved the stability of Cu(II)– N_{im} complex species and hindered the amide deprotonation processes, which are compatible with the other phosphorylated R1P peptide [31]. The deprotonation of the hydroxyl group of the tyrosyl residue in the native L1 peptide occurred in strongly basic medium at pH

9.59, giving $[\text{CuH}_3\text{L}]$ species; however, in the L1P peptide, this process was shifted to pH 10.11, which might be due to interaction between the hydroxyl group and the adjacent, negatively charged phosphate group.

The UV-Vis absorption maxima detected in the solution of copper(II) complexes of L1 and L1P as a function of pH are plotted in Figure S5a and Figure S5b respectively. The change of the binding sites from $(\text{N}^-, \text{N}^-, \text{N}_{\text{im}})$ -coordinated species to form the $(\text{N}^-, \text{N}^-, \text{N}^-, \text{N}_{\text{im}})$ -coordinated species is accompanied by a significant blue shift of the absorption maxima (Table 3, Figure S5). For the native L1 peptide, the maximum absorption of the $(\text{N}^-, \text{N}^-, \text{N}^-, \text{N}_{\text{im}})$ -coordinated complex appears at higher wavelength than that of the phosphorylated L1P peptide. A slight redshift in the maximum absorption usually indicates an axial interaction in the copper(II) complexes [43]. This weak axial interaction is caused by coordination of the $-\text{OH}$ group in the threonyl residue to $\text{Cu}(\text{II})$ ion, that's why, it causes change in the spectral parameters. However, this interaction was inhibited by the phosphate group in the threonyl residue (See Figure 4).

Table 3. UV-Vis parameters (λ_{max} [nm] and ϵ [$\text{M}^{-1} \cdot \text{cm}^{-1}$]) of $\text{Cu}(\text{II})$ complexes L1, L1P, and some tau peptides for comparison ($T=298 \text{ K}$, $I = 0.2 \text{ M KCl}$).

Binding Sites	L1	L1P	L2 [32]	L3 [32]
$\text{N}_{\text{im}} (+ \text{OPO}_3^{2-})$	730(38)	728(49)	713(20)	742(36)
$(\text{N}^-, \text{N}^-, \text{N}_{\text{im}})$	586(77)	605(106)	592(44)	603(94)
$(\text{N}^-, \text{N}^-, \text{N}^-, \text{N}_{\text{im}})$	560(91)	519(149)	561(52)	519(149)

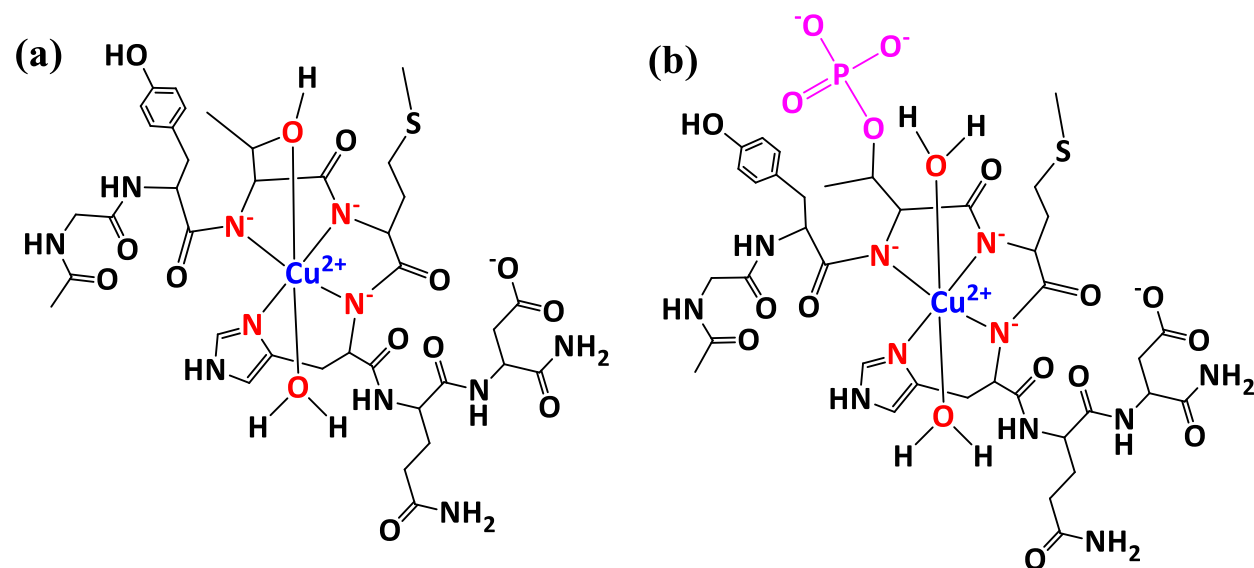


Figure 4: Chemical structures of $(\text{N}^-, \text{N}^-, \text{N}^-, \text{N}_{\text{im}})$ coordinated $\text{Cu}(\text{II})$ complexes $[\text{CuLH}_2]$ of L1 (a) and L1P (b) peptides.

The spectral data of circular dichroism (CD) measurements for the studied ligands proved the same aforementioned coordination modes (Table S4). Observed measured CD spectra were

detected in slightly acidic pH in parallel with the formation of amide coordinated complexes. These spectra are plotted in Figure S6a for the native tau(28-34) protein fragment and Figure S6b for the phosphorylated derivative. There was a big change in the CD spectra due to the change of the coordination modes from (N^-, N^-, N_{im}) -coordinated species to (N^-, N^-, N^-, N_{im}) -coordinated species by increasing the pH of the reaction solution.

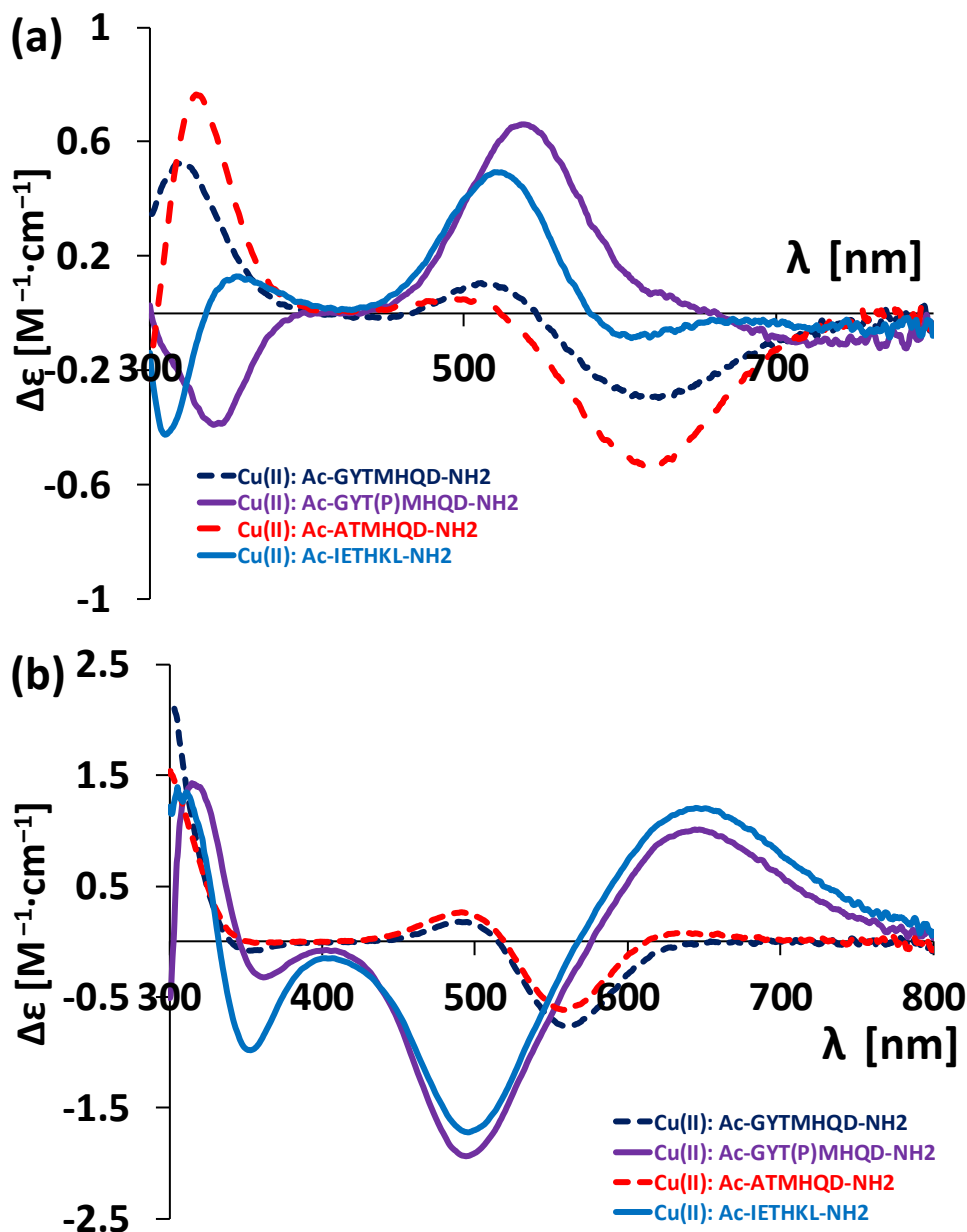


Figure 5: CD spectra of (N^-, N^-, N_{im}) -coordinated Cu(II) complexes (a) and (N^-, N^-, N^-, N_{im}) -coordinated Cu(II) complexes (b) of various peptides.

These CD spectra proved that 3N- and 4N-coordinated Cu(II) complexes have different spectral characteristics, suggesting that the coordination of the first two amide-N donor atoms takes place in a single step, which are the major complex of all peptides studied. The deprotonation process of third amide takes place under alkaline conditions, resulting in a significant change in the spectra.

The CD spectra of (2N[−],N_{im})-coordinated and (3N[−],N_{im})-coordinated Cu(II) complexes of the native L1 peptide show a significant difference from those of the phosphorylated L1P peptide (Figure 5). The spectra of L1 are like peptides containing the –T(S)XH– motif [32,44]. However, the CD spectra of L1P have a contradictory effect similar to other tau peptides, which proves that phosphorylation of threonine blocked the effect of hydroxyl group and prevented the axial interaction with copper(II) ion.

3.3. Coordination chemistry properties of Zn(II)–peptide complexes

The stability constants of complexes formed in zinc(II)-L1 and L1P systems are collected in Table 4. These data show that zinc(II) has a low binding affinity. The histidine side chain is the anchor group and imidazole coordinated complexes are formed. The stability of Zn(II)-N_{im} complex of L1 with formula [ZnLH] is moderately high, where the ligand binds monodentately to the Zn(II) ion through the N-donor of the imidazole ring; the $\log K(\text{Zn(II)-N}_{\text{im}}) = 3.47$, which could be due to the weak axial interaction of aspartyl carboxylate and threonyl –OH group [32]. This value is typically less than 3.0 for terminally protected peptides containing a single histidine [18]. In the event of the L1P ligand, the stability constant of Zn(II)-N_{im} binding was significantly increased to $\log K(\text{Zn(II)-N}_{\text{im}}) = 4.26$. This strong interaction could be explained by the contribution of phosphate group in the coordination process in slightly acidic and neutral solution.

The formation of a zinc(II)-hydroxide precipitate in the slightly alkaline medium (pH around 7.8) suppressed the formation of any other species for native L1 peptide; however, the formation of a mixed hydroxido complex for the phosphorylated L1P peptide was detected ([ZnL]), and the pK of hydroxido complex agrees with –TXH– motif containing peptide [32]. According to the coordination chemistry behavior of the zinc(II) ion with peptides, it cannot promote the deprotonation of peptide amide bonds even in the presence of phosphate groups.

Table 4. Stability constants ($\log \beta$) of the Zn(II) complexes of L1, L1P, and some tau peptides for comparison (T=298 K, I = 0.2 M KCl).

Species	L1	L1P	L2 [32]	L3 [32]
[ZnHL]	13.17(4)	14.05(2)	—	13.27
[ZnL]	—	6.01(4)	3.06	—
[ZnH _{−1} L]	—	—	−4.96	—
pK(hydroxido)*	—	8.04	8.02	—
$\log K(\text{Zn(II)-N}_{\text{im}})$	3.47	4.26	3.06	2.94

* $\log \beta([\text{ZnHL}]) - \log \beta([\text{ZnL}]$ for L1P and $\log \beta([\text{ZnL}]) - \log \beta([\text{ZnH}_{-1}\text{L}])$ for L2)

3.4. Coordination chemistry properties of Ni(II)-peptide complexes

The stability constants of the nickel(II) complexes of L1 and L1P ligands are presented in Table 5. The complex formation processes between Ni(II) and tau peptides illustrated that the Ni(II)-N_{im} coordinated complexes are the present in the physiological pH range for both native and phosphorylated peptides (Figure 6).

Table 5. Stability constants ($\log\beta$) of the Ni(II) complexes of native L1, phosphorylated L1P, and some tau peptides for comparison (T=298 K, I = 0.2 M KCl).

Species	L1	L1P	L2 [32]	L3 [32]
[NiLH]	12.56 (8)	13.33 (3)	—	12.99
[NiL]	—	—	3.03	—
[NiLH ₋₁]	—	-4.59 (3)	—	-4.53
[NiLH ₋₂]	-11.55 (3)	-13.69 (2)	-13.20	-13.32
[NiLH ₋₃]	-21.40 (3)	-24.19 (2)	-21.40	-23.64
pK ₁₂ (amide)/av.	—	8.96	8.115	8.76
pK ₃ (amide)	—	9.1	8.20	8.79
pK ₁₂₃ (amide)/av.	8.04	9.01	—	8.77
pK(Tyr-OH)*	9.85	10.50	—	—
pK(Lys-NH ₃ ⁺)**	—	—	—	10.32
logK(Ni(II)-N _{im})	2.86	3.54	3.03	2.66

* $\log\beta([NiH_{-1}L]) - \log\beta([NiH_{-2}L])$

** $\log\beta([NiH_{-2}L]) - \log\beta([NiH_{-3}L])$

In the case of L1, the $\log K(Ni(II)-N_{im})$ value was low ($\log K(Ni(II)-N_{im}) = 2.86$) compared to those of Cu(II) complexes, which indicates that the imidazole-coordinated Ni(II) complex of L1 has low stability. This data is consistent with those for other monodentate Ni(II)-imidazole peptide complexes [45], however, it is more stable than those of terminally protected peptides containing only histidine without polar side chains. Moreover, the value of $\log K(Ni(II)-N_{im})$ was significantly increased in case of phosphorylated peptide ($\log K(Ni(II)-N_{im}) = 3.54$). These results corroborate the involvement of the phosphate group in the coordination process with Ni(II) ions around physiological pH (See Table 5).

The nickel(II) ion induced deprotonation of the three amide nitrogens in the peptide backbone that precede histidine from the N-terminus direction, forming a yellow-colored square planar Ni(II)-4N complex ($[NiH_{-2}L] = [NiH_{-3}(HL)]$) at basic medium. The tyrosyl side chain remained protonated during the Ni(II) ion complexation process. Deprotonation of the side chain results in the $[NiH_{-3}L]$ species, in which the coordination mode remains unchanged.

The pK_{amide} values of deprotonation processes indicate that the deprotonation steps occur cooperatively in the native peptide. For the phosphorylated peptide, the stability constant of the

($N_{im,2N^-}$) coordinated $[NiH_{-1}L]$ complex could be determined, but as the distribution curve shows (Figure 6b), this species exists only parallel with other complexes. The ($N_{im,3N^-}$) coordinated nickel(II) complexes of L1 appear around pH 8.0; however, in the case of L1P, this process shifted to around pH 8.5 (Figure 6b). This means that deprotonation of the amide nitrogens of L1P ligand was slightly inhibited by the presence of phosphate groups.

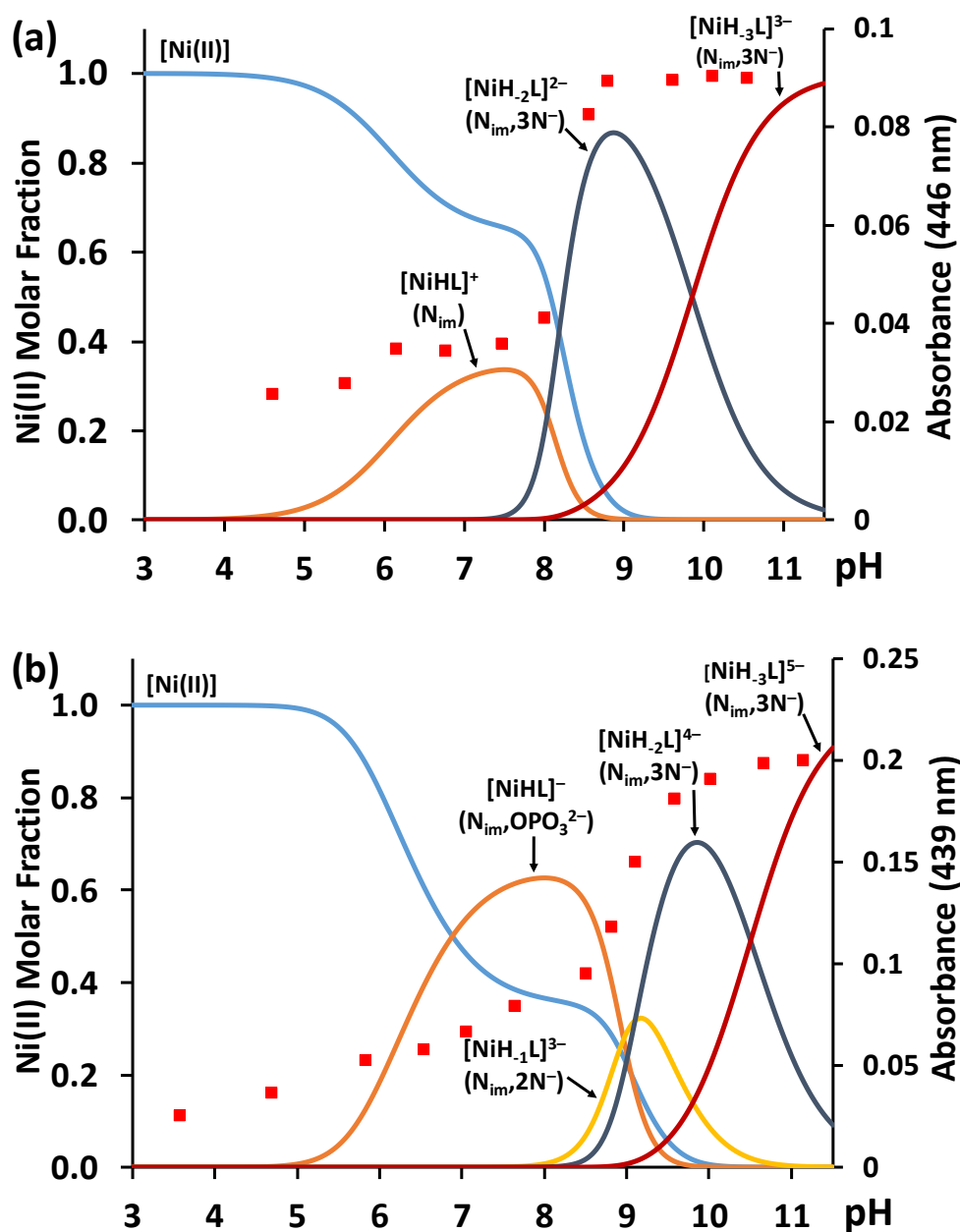


Figure 6: Concentration distribution curves in the Ni(II): L1 ($c(L) = c(M) = 1.07 \text{ mM}$) (a) and Ni(II): L1P ($c(L) = c(M) = 1.28 \text{ mM}$) (b) systems with the change in absorbance at λ_{max} wavelength (marked with red squares).

This is well reflected by the average $pK_{123}(\text{amide})$ (equation 4) values of deprotonation of the three amide nitrogen atoms preceding histidine in the peptide sequence, which significantly increased in the case of phosphorylated peptide.

The UV-Vis spectra support that the square planar Ni(II)-4N complexes exist above pH 8. In the UV-Vis spectra, absorption maximum appears at approximately 450 nm for the native peptide and around 439 nm in the phosphorylated peptide, which can be attributed to the 4N square planar Ni(II) complexes (Figures S7). The intensity of these peaks increased in parallel with the increase in the concentration of these complexes.

On the other hand, the disparate CD spectra of the chiral active Ni(II) complex are also observed above pH 8, corresponding to the formation of square planar nickel(II) complexes coordinated by the $(3N^-, N_{im})$ donors. The spectra of Ni(II) complexes of L1 are significantly different from those of the phosphorylated L1P peptide, as shown in Figure S8. The pH-dependent changes in the CD spectra of the L1 peptide are similar to those of the nickel(II) complexes of peptides containing the $-TXH-$ moiety. However, phosphorylation of threonine completely reverses the CD spectra, making them similar to those of peptides lacking the $-TXH-$ moiety, such as L3 ligand. This is illustrated by comparison of the CD spectra of the Ni(II)-peptide systems, as shown in Figure 7. The CD spectra of the L1 and L2 peptides display intense positive and negative peaks around 430 and 490 nm, respectively. This phenomenon has been observed previously in similar peptides and can be explained by the presence of the threonine $-OH$ group [18,46]. However, the CD spectra of the Ni(II) complexes of the L1P and L3 tau fragments show a virtually mirror image, which corroborates that the phosphorylated threonine has no effect on the 4N Ni(II)-complexes.

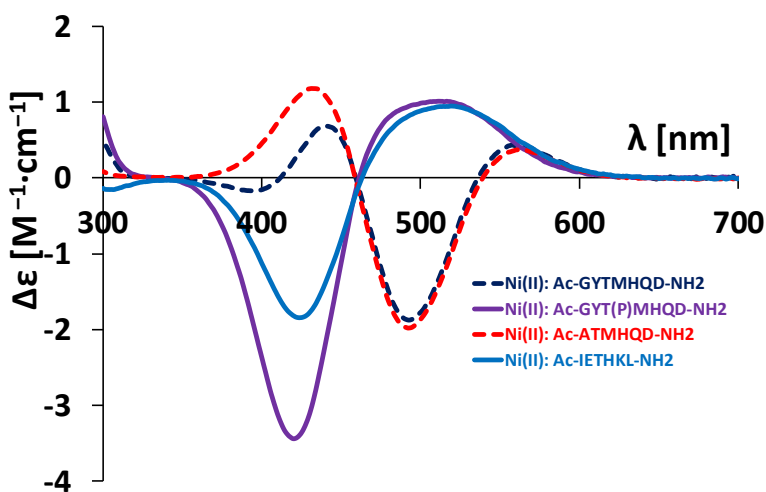


Figure 7: CD spectra of (N^-, N^-, N^-, N_{im}) coordinated Ni(II) complexes of various terminally protected peptides containing single histidine at around pH = 10.5.

In contrast to Cu(II) complexes, the formation of Ni(II) complexes of native peptide in a strongly basic solution is accompanied by an irreversible process. This is clearly demonstrated by the difference between the titration curve of a freshly prepared sample and that of a sample that has been reacidified (Figure 8). This suggests that the square planar Ni(II)–4N complex of L1 ligand formed in the alkaline medium induces hydrolysis of the peptide amide bond. However, phosphorylation of threonine inhibited this process, preventing both the irreversible reaction and hydrolysis of the amide bond in the case of L1P peptide (Figure S9). These findings were corroborated by CD studies, time-dependent RP-HPLC, and ESI-MS studies at 25 °C in the basic medium.

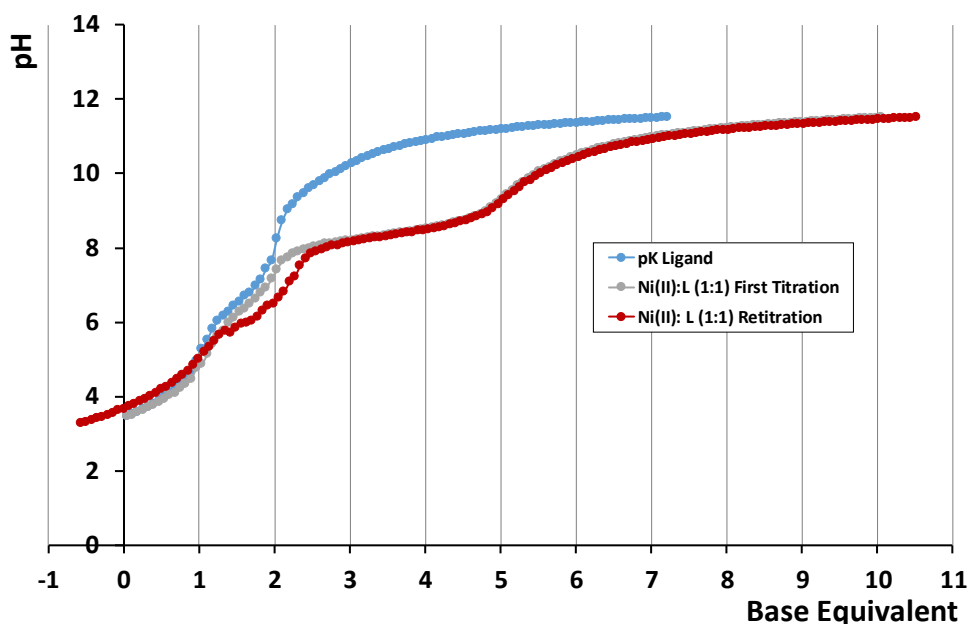


Figure 8: Titration curves of the equimolar Ni(II): Ac-GYTMHQD-NH₂ (L1) system (1:1) and its repeated series after reacidification indicated with a red color.

3.5. Role of phosphorylation in the Ni(II)-induced hydrolytic processes

Because of phosphorylation of the threonyl residue is a crucial factor affecting the metal ion binding affinity to histidyl residue in the –TMH– moiety of the protein fragment, as mentioned previously, it was deemed essential to determine the effect of threonine phosphorylation on hydrolytic process in the protein fragment (L), which undergoes hydrolysis by the effect of Ni(II) ions (see later). Accordingly, the effect of threonine phosphorylation in the –TMH– motif on the Ni(II)-induced hydrolytic reaction was investigated for L1P tau protein fragment. The studies were performed by reverse-phase high performance liquid chromatography (RP-HPLC) at room temperature (25°C) and at pH 11.0. The hydrolytic reaction was running for 7 days, and the HPLC chromatograms of phosphorylated L1P peptide recorded at pH 11.0 are shown in Figure 9.

The HPLC measurements show that from the start of the reaction (time = 0 min) until the end of the HPLC studies (after 7 days), there was not any change in the HPLC chromatograms,

and the HPLC measurements showed that only a large peak observed at a retention time of 14.02 min that characterize the L1P ligand (Figure 9). These results corroborate that phosphorylation of the threonyl residue in the active part of the protein molecule (–TMH–) halted the hydrolytic reaction induced by nickel(II) ions, however the reaction was significantly clear for the non-phosphorylated L1 ligand (see next). Control samples (in which nickel(II) ions were replaced by three-time distilled water) of the phosphorylated tau fragment, prepared as described in the experimental section, also showed similar results and there was not any change in the HPLC chromatograms during the hydrolytic study period (Figure S10). It's worth to mention that the peak of the phosphorylated L1P ligand elutes at lower retention time ($t = 14.02$ min) than that of the native L1 ligand ($t = 15.08$ min) in the RP-HPLC due to the high polarity caused by phosphate group.

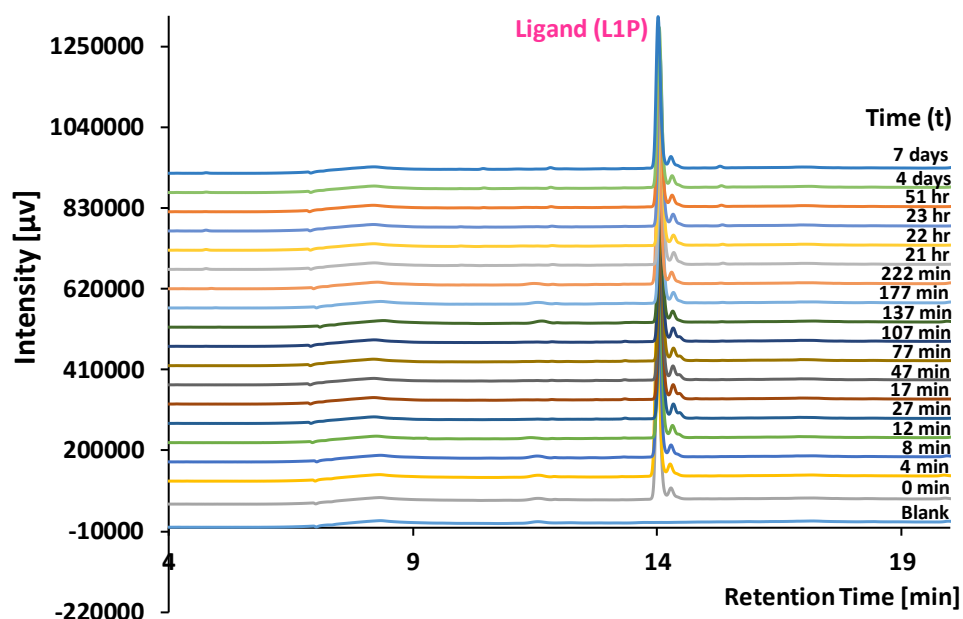


Figure 9: Time-dependent HPLC chromatograms for the Ni(II): L1P (1:1) ratio system at pH = 11.0 at T = 25°C..

3.6. Ni(II) ion induced hydrolytic processes of the native protein fragment

Regarding the native tau(28-34) protein fragment (L1) containing the –TMH– moiety, which is susceptible to hydrolysis of the amide bond between tyrosyl and threonyl residues, the hydrolytic process in the presence of Ni(II) ions was investigated in more details. The studies were performed at room temperature (25°C) and at two different pH values: pH 8.0, as at this point the hydrolytic reaction initiates clearly and close to physiological environment, and the other value is under strongly basic conditions, around pH 11.0 in order to observe the catalytic effect of the base on the rate of the hydrolytic reaction. In this case, RP-HPLC and CD spectroscopy were the core techniques to clearly follow the hydrolytic reaction.

The initial HPLC measurements of L1 containing system at pH 8 illustrated that the ligand peak (L) appeared at a retention time of 15.08 minutes. As the reaction progressed, a new peak was detected at 47 minutes, with a retention time of 13.58 minutes, corresponding to the formation of the intermediate ester (IP), while the intensity of the ligand peak (L) decreases (Figure 10a). After 21 hours, a new low-intensity peak appeared in the chromatogram at a retention time of 13.19 minutes, which corresponds to the first product (P1) of the hydrolytic reaction. However, the formation of the second product (P2) in significant concentration was not observed until 51 hours had elapsed, at a retention time of 9.43 minutes.

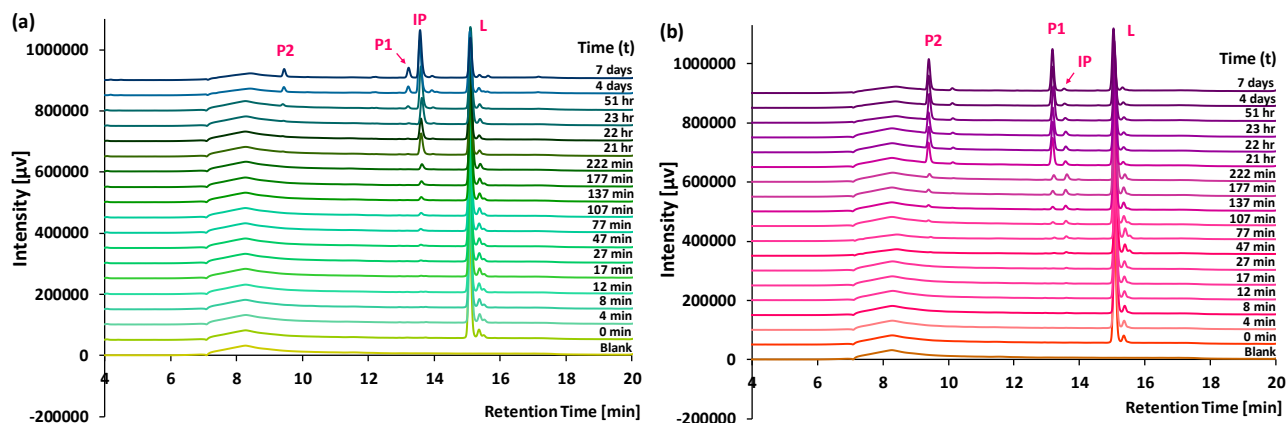


Figure 10: Time-dependent HPLC chromatograms for the Ni(II): L1 = 1:1 system at pH = 8.0 (a) and pH = 11.0 (b) at T = 25°C.

At pH 11, the hydrolytic reaction proceeded much more rapidly (Figure 10b), confirming the irreversibility of the reaction, as evidenced by the repeated titration curves. Compared to the chromatograms recorded at pH 8, the corresponding peaks appeared on the chromatograms after a much shorter time. The signal for the intermediate ester (IP) appeared as early as 27 minutes, with a retention time of 13.58 minutes. Shortly thereafter, at 47 minutes, the signal for product P1 (retention time 13.19 minutes) became visible, followed by the signal for product P2 (retention time 9.43 minutes) in the chromatogram recorded at 137 minutes. In the chromatograms recorded at pH 8, these last two peaks are detectable only after one and two days, respectively. As the hydrolytic reaction continues, the peak intensity of both first and second products (P1 and P2) significantly increases, while the amount of ligand (L) decreases as it is consumed during the Ni(II)-induced hydrolytic reaction. The intensity of the intermediate ester (IP) does not increase significantly during the experiments, as it is catalytically hydrolyzed into the final products within a short time, indicating the very rapid hydrolysis of the intermediate ester (IP) under strongly alkaline conditions.

As the conclusion of seven days experiment it was found that under strongly alkaline conditions, nickel(II) ions induce the hydrolysis and cleavage of the amide bond between tyrosyl and threonyl residues of the original L1 protein fragment (L) very rapidly, whereas at pH 8 this process takes longer. Based on previous results, it can be concluded here as well that during

Ni(II)-induced hydrolysis, an extremely stable ATCUN complex forms, creating an intermediate ester in the first step, followed by hydrolysis of the ester bond within the peptide and its cleavage into two fragments (Figure 11) [26].

The formation of an ester functional group inside the protein fragment (L) increases its polarity; therefore, the intermediate ester form (IP) elutes with a lower retention time than the initial ligand (L) in the HPLC chromatogram. The second product (P2) of the hydrolytic reaction elutes faster than the first product (P1) as it is more polar and has a lower molecular weight. Detection of small amounts of the intermediate ester under strongly alkaline conditions indicates the catalytic role of the base in cleaving the ester bond of the peptide molecule.

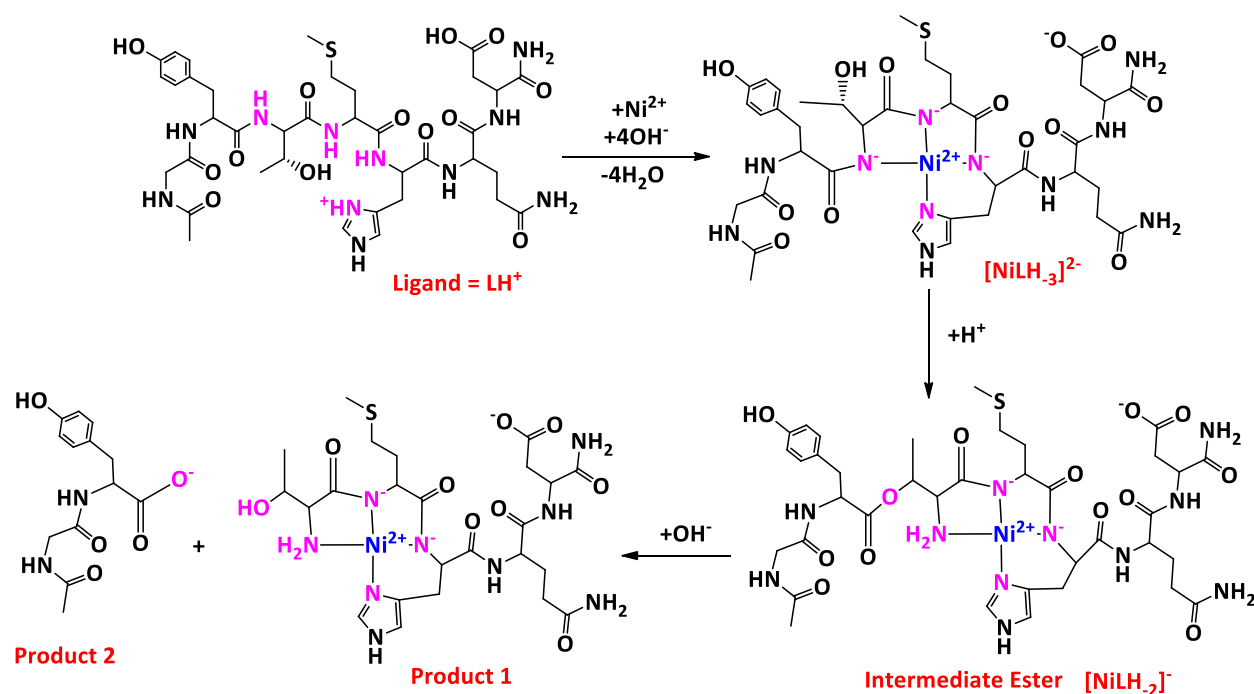


Figure 11: The mechanism of Ni(II) ion induced hydrolysis [26] of L1 peptide.

Table 6. Retention times and the measured MS data for the products of the hydrolytic reaction, ester and the original peptide.

Compound	Molecular Formula	Retention Time (min)	Measured MS (M/Z)	Calculated MS (M/Z)
Peptide Ac-GYTMHQD-NH ₂	[C ₃₇ H ₅₃ N ₁₁ O ₁₃ S] ⁺	15.07	892.3606	892.3617
Ester Ac-GYTMHQD-NH ₂	[C ₃₇ H ₅₃ N ₁₁ O ₁₃ S] ⁺	13.58	892.3585	892.3617
Product (1) TMHQD-NH ₂	[C ₂₄ H ₃₉ N ₉ O ₉ S] ⁺	13.19	630.2664	630.2664
Product (2) Ac-GY-COOH	[C ₁₃ H ₁₆ N ₂ O ₅] ⁺	9.43		281.1131

In order to obviously demonstrate that Ni(II) ions are necessary to initiate and induce the hydrolytic process, identical HPLC measurements were conducted on control samples in which nickel(II) ions were replaced by three-time distilled water. The HPLC measurements showed only a large peak at a retention time of 15.08 min, corresponding to the starting reactant (L), while the HPLC peaks for the intermediate ester (IP) in addition to the final products (P1 and P2) were completely absent (Figure S11). The products of the irreversible hydrolytic reaction of the protein fragment (L) were collected from HPLC and identified by HR-ESI-MS (LC-MS) (Table 6). During the hydrolytic reaction, molecular rearrangement of the ligand (L) forms an ester intermediate (IP) in the presence of Ni(II) ions. The molecular formulae of the new ester form (IP) stay identical to that of the ligand (L), as confirmed by m/z values. Similarly, the molecular weights of the final products corroborate the hydrolysis process and were identified by m/z values according to the reaction mechanism (Figure 11).

During the reaction of the hydrolytic process, the intermediate ester (IP) and the first product (P1) have the same binding sites (NH_2 , 2N^- , N_{im}), which provide an albumin-like binding sites for nickel(II) ions (Figure 11). Typically, complexes formed with this type of binding exhibit exceptionally high stability [42,47]. Moreover, the CD spectra of Ni(II) complexes formed with albumin-like coordination modes (NH_2 , 2N^- , N_{im}) significantly differ from those of (3N^- , N_{im})-coordinated complexes, giving the accessibility to CD spectroscopy to monitor the hydrolytic reaction. Accordingly, CD measurements tracked the hydrolytic process induced by Ni(II) ions. Temporal changes in the CD spectra were recorded for the Ni(II) complexes of native tau(28-34) fragment at four pH values and at different times. The CD measurements indicate the formation of the square planar Ni(II) complex with (3N^- , N_{im}) coordination modes at the start of the reaction under basic conditions (Figure 12 and Figure S12). As time progresses, the observed changes in the shape of the CD spectra, as well as the blue shift corroborate that the hydrolytic process induced by Ni(II) is ongoing. After 24 hours, the recorded CD spectra were completely different and were similar to those of Ni(II):MKHM- NH_2 complexes, indicating only albumin-type coordination, and also that Ni(II)-induced hydrolysis is a time-dependent reaction (Figure 12). However, CD spectroscopy can only detect the change in coordination modes as a result of hydrolysis, it cannot distinguish between the intermediate ester (IP) and the first product (P1), as both structures have the same coordination modes around the Ni(II) (Table S5).

Despite its ability to promote hydrolytic reactions, the conditions of the high stability of copper(II) complexes with (3N^- , N_{im}) coordination modes under alkaline conditions make hydrolytic investigations of copper(II) systems much slower and more complicated, even at high temperatures. According to pH-potentiometric studies—as mentioned previously—the complex formation processes occurring in the systems are reversible for both native and phosphorylated protein fragments. The hydrolytic behaviour of Zn(II) systems cannot be studied, as the zinc(II) ions are incapable of forming Zn(II) complexes coordinated by (3N^- , N_{im}) donor set, which are the fundamental prerequisite for initiating the hydrolytic reaction.

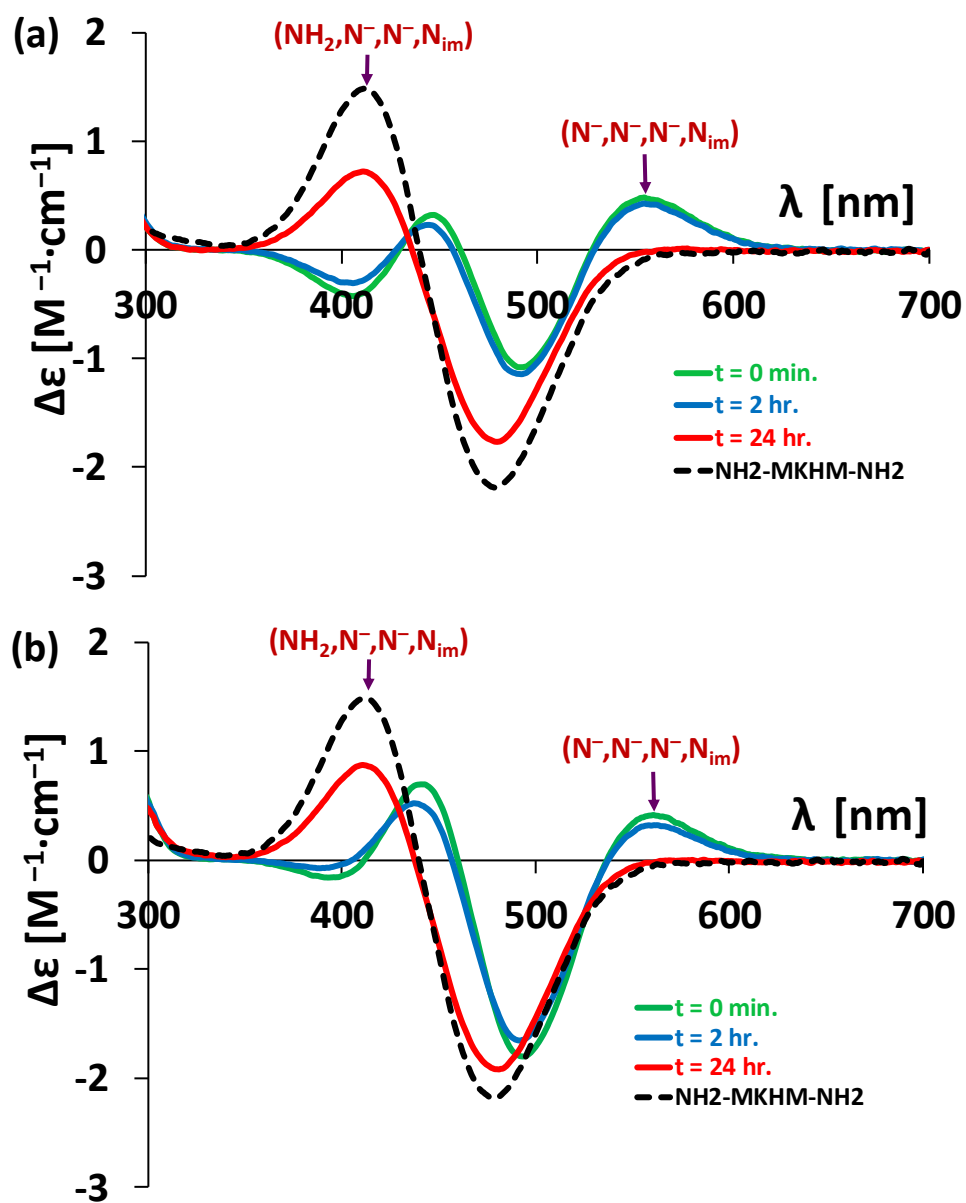


Figure 12: Time-dependent CD spectra for the Ni(II): L1 system at 1:1 ratio at two pH values: pH 8.9 (a), pH 11.0 (b) in function of time at $T = 25^\circ\text{C}$. The CD spectrum of the $(\text{NH}_2, \text{N}^-, \text{N}^-, \text{N}_{\text{im}})$ coordinated Ni(II) complex of $\text{NH}_2\text{-MKHM-NH}_2$ peptide is also shown (dash black curve).

4. Conclusion

The present paper investigates the effect of phosphorylation of threonine on the behaviour of the native human tau(28-34) protein fragment in the presence of essential copper(II) and zinc(II) ions, in addition to the toxic nickel(II) ions. The research focused on two main directions: The first involved an examination of the complex formation processes in solution and determining the stoichiometry and stability constants of the complexes formed with both native tau(28-34) (L1) and phosphorylated tau(28-30T(P)-34) (L1P). The second direction focused on examining the hydrolysis of the two ligands in the presence of Ni(II) ions at pH 8.0 and pH 11.0.

Based on the deprotonation constants determined for the L1 and L1P ligands, we established that the presence of the phosphate group significantly increased the pK value of imidazolium nitrogen (N_{im}). ^1H -NMR studies proved that the deprotonation of the phosphorylated threonyl residue undergoes in the second step, while the third deprotonation step corresponds to the loss of proton from the imidazolium group. The equilibrium studies of metal complexes demonstrated that phosphorylation of threonine in the protein fragment enhanced the metal ion binding affinity and increased the thermodynamic stability of $M(II)-N_{im}$ complexes for all three metal ions. This is well illustrated by Figure 13, which shows the $\log K(M(II)-N_{im})$ values for L1 and L1P ligands. This figure also clearly reflects that the stability of imidazole-coordinated complexes follows the $Ni(II) < Zn(II) < Cu(II)$ order.

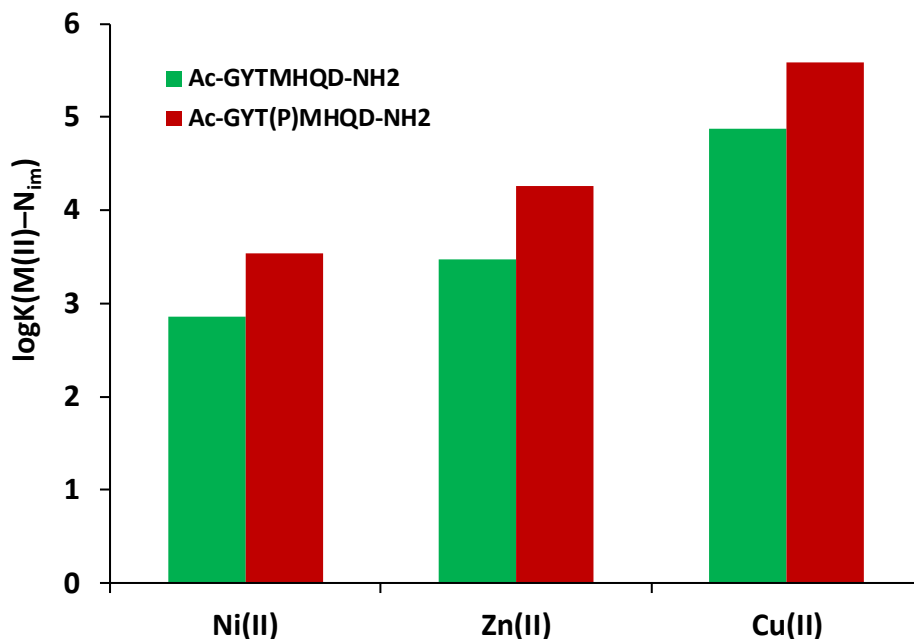


Figure 13: Stability constants of $M(II): N_{im}$ complexes of native tau(28-34) (L1) and phosphorylated tau(28-30T(P)-34) (L1P) protein fragments.

At the same time, the presence of phosphorylated threonine shifts the copper(II)- and nickel(II)-induced deprotonation processes of amide nitrogens toward a higher pH range. These facts

suggest that the phosphate group, located one amino acid away from the histidine side chain, participates in metal ion binding through weak axial coordination. Furthermore, phosphorylation of the peptide completely altered the CD spectra of the Cu(II) and Ni(II) complexes compared to that of the native peptide.

The hydrolytic processes of both peptides were also investigated in the presence of Ni(II), as the native L1 peptide contains the –TXH– sequence. It is already known that Ni(II)–induced hydrolysis of peptides containing the –TXH– sequence occurs in alkaline medium when a Ni(II) complex with coordination of (3N[−], N_{im}) donor set is formed. The findings of hydrolysis studies on the human tau protein containing the –TMH– motif showed that the protein fragment underwent hydrolytic reaction in nickel(II) systems. The hydrolytic reaction began at pH 8.0 and accelerated significantly at pH 11.0, indicating the exclusively catalytic role of the base in hydrolyzing the ester form of the ligand to produce the final reaction products. Structural elucidation and ester formation then occur within a few hours, enabling the development of albumin–type binding sites around Ni(II) ions. However, the phosphorylation of threonine residue located in the –TMH– region of the protein – in addition to enhancing the interaction between the M(II) ion and the protein – halted the hydrolytic reaction and prevented the peptide from cleaving into small fragments.

Our results show that the presence of even a single phosphorylated threonine within a heptapeptide influences complex formation but has an even greater impact on hydrolytic processes. These findings may thus contribute to clarifying the role of metal ions and abnormal phosphorylation of specific amino acids in the human tau protein.

Author Contributions

Conceptualization, G.E.A. and K.V.; methodology, G.E.A. and K.V.; validation, G.E.A. and K.V.; formal analysis, G.E.A. and K.V.; investigation, G.E.A. and V.K.; resources, K.V.; data curation, K.V.; writing—original draft preparation, G.E.A. and K.V.; writing—review and editing, G.E.A. and K.V.; visualization, G.E.A. and K.V.; supervision, K.V.; project administration, K.V.; funding acquisition, G.E.A. and K.V. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The original contributions presented in this study are included in the article/supplementary material. The raw data supporting the conclusions of this article will be made available by the authors on request.

Conflicts of Interest

The authors declare no conflicts of interest.

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