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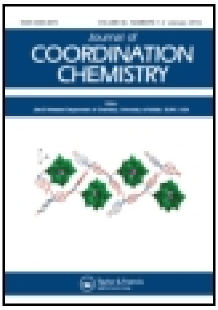

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Studies of new phosphothreonine complexes formed in binary and ternary systems including biogenic amines and copper(II)

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Binary and ternary systems of copper(II) with O-phospho-L-threonine (Thr-P) and biogenic amines (putrescine or spermidine or spermine) have been investigated. The studies were performed in aqueous solution. Overall stability constants of the complexes were determined by pH-metric study and the coordination sites were identified by ^{31}P NMR, visible and electron paramagnetic resonance spectroscopies. Results of the equilibrium and spectral studies have shown that in the phosphothreonine binary system Cu(HThr-P), Cu(Thr-P) and Cu(Thr-P)(OH) are formed. In the binary system at low pH, only the phosphate group is involved in coordination but at higher pH, the efficiency of $-PO_4^{2-}$ is low and the amine and carboxylate are the main sites of coordination. Formation of complexes was established in ternary systems with all bioamines studied. In these systems the reaction centers of phosphothreonine are the same as in the binary systems: phosphate, carboxylate and amine. Additionally, amine groups from the polyamines (PAs) are involved in the complex formation. Besides the heteroligand complexes with intermolecular interactions, also molecular complexes with the protonated bioamine in the outer coordination sphere appeared. In these molecular species, PA was involved in non-covalent interactions with the anchoring phospho-threonine complex.

Keywords: Phosphothreonine; Copper complexes; Polyamine; Stability constants; Coordination mode

1. Introduction

Almost every aspect of cell metabolism, including modification and functioning of proteins, is regulated by phosphorylation and dephosphorylation processes [1]. About 30% of the proteins encoded by the human genome contain covalently bound phosphate. Phosphothreonine and phosphoserine belong to naturally occurring phosphorylated amino acids. The mode of interaction of phosphorylated amino acids with other species (e.g. metal ions and small organic ligands like polyamine (PA)) could help to understand the role of phosphorylation in biological processes [2].

Putrescine (Put), spermidine (Spd) and spermine (Spm) show a wide range of biological activities in many metabolic processes [3–6], involved in replication, transcription and translation processes whose intensity depends rather significantly on the concentrations of these amines. Bioamines stabilize membranes, activate some enzymes, catalyze and control biosynthesis of proteins and are indicators of tumor progression and carcinogenesis [7–9].

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The cellular level of PAs drastically increases in tumors in comparison with that in normal tissue. Higher levels of PAs have been detected in a wide range of tumors [10–12]. PA analogs are used in anticancer therapy, but the mechanisms of their activities are still not well known.

Copper is a trace metal which plays an important role in the human body. Copper in trace amounts is essential to sustain life, for example, in lipoprotein metabolism and in cardiovascular problems such as atherosclerosis [13]. On the other hand, toxic accumulation of copper can be deleterious to human health (e.g. Wilson and Menkes' disease genes [14] and copper binding to β-amyloid protein is considered as inducing Alzheimer's disease [15]). The majority of Cu(II) ions in ceruloplasma are in the form of mixed complexes with amino acids, peptides, and other biomolecules [16].

This paper presents potentiometric and spectroscopic results of a study on the complexation of phosphoserine and biogenic amines to copper(II). Carboxylate, amine and phosphate groups of phosphorylated threonine are potential coordination sites. Identification of the coordination mode in the system Thr-P copper(II) ion (occurring in living organisms) is the first step for explanation of the function of phosphate in bioinorganic processes. The reactions between amino acids and metal ions are considered as models of the processes which take place at the molecular level in the metal–protein system. On the other hand, biogenic amines have amine groups capable of formation of complexes as well as in protonated form – capable of entering into non-covalent interactions with another bioligand (free or complexed). Understanding complexation processes of amino acids and their natural derivatives, biogenic amines and metal ions is important for understanding the role of these species in living organisms.

2. Experimental

2.1. *Materials*

O-phospho-L-threonine (Thr-P) as well as Put, Spd and Spm were purchased from Sigma and used without purification. PA nitrates were prepared by dissolving a proper amount of free amine in methanol and addition of an equimolar amount of HNO_3. The precipitate obtained was recrystallized, washed with methanol and dried in a desiccator over P_4O_{10}. The nitrate salts were checked by elemental analysis with % C, % N, % H in agreement with the theoretically calculated values (±0.5%). Elemental analyses were performed on an Elemental Analyzer CHN 2400, Perkin–Elmer. Copper(II) nitrate from Merck was used without purification. The concentration of copper(II) ions was determined by inductively coupled plasma optical emission spectrometry. All solutions and experiments were prepared using demineralized, carbonate-free water.

2.2. *Equilibrium study*

Potentiometric measurements were carried out using a Titrino 702 Metrohm equipped with an autoburette with a combined glass electrode – Metrohm 6.233.100. Prior to each series of measurements, the pH-meter was corrected with two standard buffer solutions of pH 4.002 and pH 9.225, and the electrode was calibrated in terms of H^+ concentration [17]. All potentiometric titrations were made under helium at constant ionic strength of $\mu = 0.1\,M$

(KNO_3), temperature 20 ± 1 °C (titration dish placed in thermostatic bath set at this temperature), in the pH range from 2.5 to 10.5, using CO_2-free NaOH at a concentration of 0.231 M as a titrant. The concentration of phosphothreonine in binary systems was 2×10^{-3} M and the metal to ligand ratios used were 2 : 1, 1 : 1 and 1 : 2. In ternary systems concentrations of PAs as well as phosphothreonine were 2×10^{-3} M and the metal to phosphothreonine and PA ratio was 1 : 1 : 1. Selection of the model and determination of the stability constants of the complexes were made using the HYPERQUAD program which uses nonlinear least squares [18]. The determined ionic product of water was $pK_w = 13.89$. Calculations were performed using 150–350 points for each job, taking into account only the part of titration curves that corresponded to conditions in which no precipitate appeared in the solutions. The hydrolysis constant for copper(II) was taken from our previous publication [19]. In all cases the testing began with the simplest hypothesis and then in the following steps the models were expanded to include progressively more species, and the results were scrutinized to eliminate those species that were rejected by the refinement processes. The model assumed was verified by analysis of the standard deviations; the convergence of the experimental data with those obtained for the model was evaluated by the Hamilton test (figure 1) and Chi-squared test [20]. Distributions of particular species were obtained by the HySS program [21].

2.3. *NMR measurements*

Samples for ^{31}P NMR studies were obtained by dissolving relevant species (*Thr-P*, *PA* and copper salt) in D_2O. The pH values of the samples were corrected according to $pD = pH_{meter\ readings} + 0.4$ [22] adjusted by NaOD and DCl. The concentration of ligands for NMR study was 0.05 M and molar ratios of Cu : Thr-P and Cu : Thr-P : PA were 1 : 75 and 1 : 75 : 75,

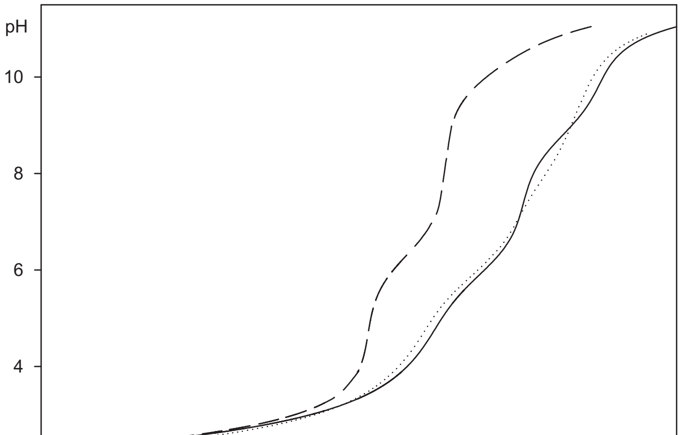

Figure 1. Experimental and simulated titration curves for the binary system: solid line – experimental curve, dotted line – simulated curve (complex formation was taken into account), dashed line – simulated curve (complex formation was not taken into account).

respectively. A considerable decrease in copper ion concentration has no significant effect on the pH ranges of a given species [23], relative to those in the 1 : 1 : 1 system. NMR spectra were recorded on an NMR Gemini 300VT VARIAN spectrometer using orthophosphoric acid as an external standard. The changes in the chemical shifts were calculated for the ligands in the investigated systems in relation to free ligands at the same pH (ppm scale). Significant changes in the chemical shifts occur only in the pH ranges of complex formation (the ranges had been determined from potentiometric studies).

2.4. *FT–IR measurements*

Samples for FT–IR studies were obtained by dissolving *Thr-P, PA* and copper salt in D_2O and the pH values of the samples were corrected as for the NMR samples. The concentration of ligands was 0.05 M and molar ratio was 1 : 1 in binary and 1 : 1 : 1 in ternary systems. The spectra were taken on an IFS 113v FT–IR Bruker spectrophotometer equipped with a DTGS detector; resolution $2\,\mathrm{cm}^{-1}$. A cell with Si windows (thickness 100 μm) was used.

2.5. *Visible (Vis) spectroscopy*

Samples for visible studies were prepared in H_2O at $M:L=1:1$ in binary systems and $M:L:L'$ ratio 1 : 1 : 1 in ternary systems (concentration of Cu(II) was 0.05 M). The spectra were recorded at 20 °C in a PLASTIBRAND PMMA cell with 1 cm path length on an Evolution 300 UV–Vis ThermoFisher Scientific spectrometer equipped with a xenon lamp (range 450–950 nm, accuracy 0.2 nm, sweep rate $120\,\mathrm{nm\,min}^{-1}$).

2.6. *Electron Paramagnetic Resonance (EPR) spectroscopy*

EPR studies were carried out at −196 °C using glass capillary tubes (volume $130\,\mu\mathrm{m}^3$). The concentration of Cu(II) was 0.005 M in water : glycol mixture (3 : 1) and the metal : ligand ratios were 1 : 1 in binary and 1 : 1 : 1 in ternary systems. The spectra were recorded on an SE/X 2547 Radiopan instrument.

2.7. *Density Functional Theory (DFT) calculations*

A full optimization of the isolated structures of Cu(Thr-P) and Cu(Thr-P)(PA) complexes were performed with GAUSSIAN 03 (Ground State DFT and B3LYP/Set 6–311G level). As starting points, structures pre-optimized semi-empirically with HYPERCHEM-7.52 (PM3 hamiltonian) were used [24, 25].

3. Results and discussion

Phosphothreonine is a phosphorylated amino acid which is a methyl derivative of phosphoserine (figure 2). The sequence of protonations of phosphothreonine functional groups is the same as for phosphoserine: $–NH_2$, $–PO_4^{2-}$, $–COO^-$ and $–PO_4H^-$ (the second site at the phosphate). The values of successive protonation constants: $\log K_1 = 10.03$,

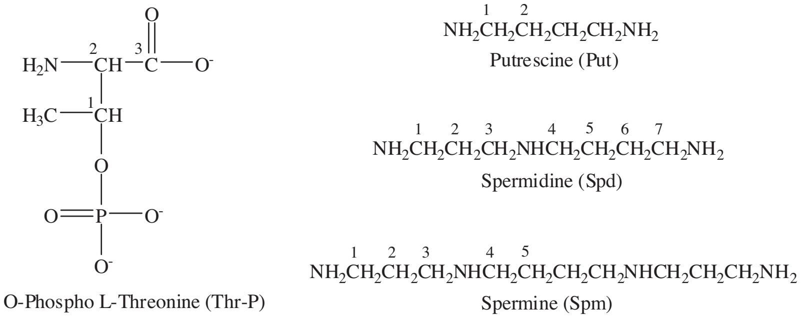

Figure 2. The ligand studied in the totally deprotonated forms.

$\log K_2 = 6.18$, $\log K_3 = 2.24$ were determined by computer analysis of the potentiometric data ($\log K_4$ was not determined as deprotonation of the first proton takes place at pH values lower than reached in this study). The value of $\log K_1$ is the same as that reported [26]; the other values of the successive protonation constants have not been determined yet. The overall and successive protonation constants of Thr-P are given in table 1.

Protonation constants of biogenic amines and stability constants of Cu(II) PA binary complexes are given in Supplementary material. Computer analysis of the potentiometric titration data was performed taking into consideration the protonation constants of ligands, stability constants of copper complexes formed in the binary systems, as well as the known constants for Cu(II) hydrolysis [19]. The coordination mode in the binary and ternary complexes formed in the systems was concluded on the basis of analysis of d–d energy transitions in the Vis spectra, $g_{||}$ as well as $A_{||}$ values in EPR investigation (taking into consideration the relation of these values to the number of coordinated donors [27–29]). Good correlation between the d–d transition energy and EPR spectral parameters permits determination of the composition of the inner coordination sphere of copper. In complexes with N or O donating ligands, with increasing number of donors coordinated to Cu^{2+}, the values of λ_{max} and $g_{||}$ decrease, while that of $A_{||}$ increase [27, 30, 31]. When oxygens (not originating from water molecules) are replaced by nitrogen in the tetragonal

Table 1. Overall and successive stability and equilibrium constants of Cu(II) complexes and protonation constants of Thr-P.

Species[a]	Overall protonation constants $\log\beta$	Reactions	Successive protonation constants $\log K_{1-3}$
H(Thr-P)	10.03(1)	Thr-P + $H^+ \leftrightarrows$ H(Thr-P)	10.03
H2(Thr-P)	16.21(1)	H(Thr-P) + $H^+ \leftrightarrows H_2$(Thr-P)	6.18
H3(Thr-P)	18.66(2)	H_2(Thr-P) + $H^+ \leftrightarrows H_3$(Thr-P)	2.45
Species	Overall stability constants $\log\beta$	Reactions	Equilibrium constants $\log K_e$
Cu(HThr-P)	15.70(3)	Cu^{2+} + H(Thr-P) $\leftrightarrows$ Cu(HThr-P)	5.67
Cu(Thr-P)	9.74(3)	Cu^{2+} + Thr-P $\leftrightarrows$ Cu(Thr-P)	9.74
Cu(Thr-P)(OH)	0.95(3)	Cu^{2+} + Thr-P + $H_2O \leftrightarrows$ Cu(Thr-P)(OH) + H^+	14.84

[a] For the sake of clarity the charges of individual species have been omitted.

geometry of copper, the maximum absorption wavelength shifts to smaller values, indicating a stronger ligand field, and the molar absorptivity usually increases (λ_{max} for (3 N) coordination it is 600 nm, for (4 N) 550 nm and for (3N1O) λ_{max} is 584 nm) [32–34]. The proposed mode of coordination was supported with analysis of IR spectra and chemical shifts in the ^{31}P NMR spectra of the ligand in the complexes with respect to those of the free ligand, interpreted according to our experience and critical analysis of the results for a number of systems with paramagnetic ions [33–36]. Good agreement of the results obtained by different spectroscopic methods permits description of the ligand arrangement in the complex with high probabilitySupplementary material.

3.1. *Complexes in binary systems*

Computer analysis of the potentiometric titration data for the binary system Cu(II)/Thr-P has revealed formation of Cu(HThr-P), Cu(Thr-P) and Cu(Thr-P)(OH); (where M=Cu and L=Thr-P) the overall stability constants of these complexes are given in table 1.

3.2. *Cu(HThr-P) complex*

The complexation reaction in the system Cu/Thr-P begins with formation of the protonated complex Cu(HThr-P) at pH 2 (for the sake of clarity, the charges of individual species have been omitted). This complex dominates at pH close to 5.0 and binds 75% of the copper ion in solution (figure 3).

The Vis and EPR spectral data for Cu(HThr-P): $\lambda_{max} = 719$ nm, $g_{||} = 2.304$ and $A_{||} = 170 \times 10^{-4}$ cm^{-1}, table 2, indicate that only oxygens are engaged in coordination, as can be concluded from literature data on coordination of Cu(II) with O-donor ligands [37]. Analysis of changes in the chemical shifts in the ^{31}P NMR spectra (taken in the pH range

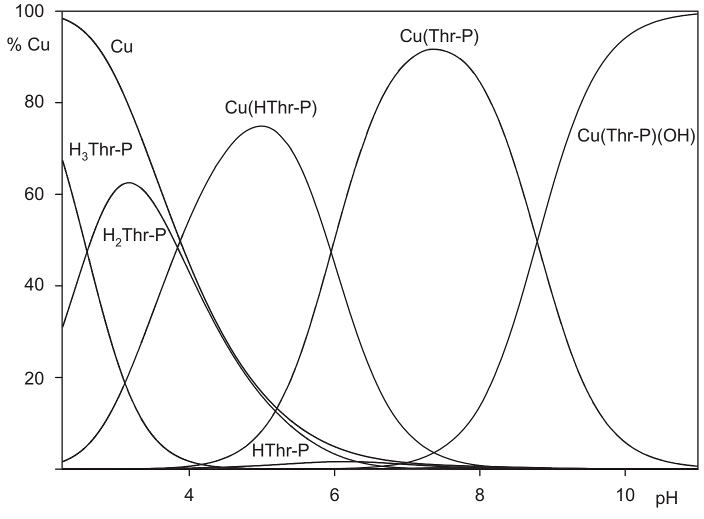

Figure 3. Distribution diagram for the Cu/Thr-P/system (1 : 1 ratio). The percentage of the species refers to the relative concentration of the metal; $c_{Cu} = 0.002$ M.

Table 2. Spectral data and modes of interactions in the complexes studied (differences between NMR chemical shifts for the ligands in the investigated systems in relation to the free ligands at the same pH (ppm)).

Species	pH	λ_{max} (nm)	ε ($M^{-1} cm^{-1}$)	$g_{\parallel}$	$A_{\parallel} \times 10^{-4}$ (cm^{-1})	Chromophore	P $\Delta\delta$ (ppm)
Cu(HThr-P)	5.0	719	26.1	2.304	170	{O*x*}	+0.489
Cu(Thr-P)	7.5	689	28.3	2.251	175	{N1,O*x*}	−0.005
Cu(Thr-P)(OH)	10.0	673	32.9	2.245	180	{N1,O*x*}	+0.022
Cu(Thr-P)H_4(Put)	3.5	752	10.7	2.390	136	{O*x*}	+4.759
Cu(Thr-P)H_3(Put)	5.0	716	23.8	2.293	159	{O*x*}	+3.650
Cu(Thr-P)H_2(Put)	7.5	691	26.1	2.288	163	{N1,O*x*}	−0.062
Cu(Thr-P)H(Put)	9.0	605	33.3	2.233	181	{N1,O*x*}	+0.006
Cu(Thr-P)(Put)	10.5	610	50.5	2.212	190	{N3,O*x*}	+0.085
Cu(Thr-P)H_4(Spd)	4.0	719	21.2	2.292	162	{O*x*}	+0.094
Cu(Thr-P)H_3(Spd)	6.5	698	24.7	2.285	157	{N1,O*x*}	−0.081
Cu(Thr-P)H(Spd)	8.5	628	61.3	2.221	190	{N2,O*x*}	+0.060
Cu(Thr-P)(Spd)	10.5	584	63.2	2.210	194	{N3,O*x*}	–
Cu(Thr-P)H_6(Spm)	5.0	710	23.3	2.321	165	{O*x*}	+0.277
Cu(Thr-P)H_4(Spm)	7.5	641	36.4	2.235	188	{N1O*x*}	−0.126
Cu(Thr-P)H_3(Spm)	8.25	592	68.5	2.218	197	{N2,O*x*}	+0.045
Cu(Thr-P)H_2(Spm)	9.75	586	74.8	2.216	199	{N2,O*x*}	–

of complex domination) of phosphorus from phosphothreonine +0.489 indicates that the phosphate from Thr-P is involved in coordination, table 2.

The IR spectrum of Cu(HThr-P) revealed that the asymmetric stretching band $\nu_{asC\text{–}O}$ assigned to –COO shifted only from 1626 to 1621 cm^{-1}, indicating that carboxylate was not involved in coordination. In spectra of the free ligand the asymmetric stretching vibration assigned to phosphate of Thr-P was at 1034 cm^{-1}. As a result of coordination this band disappeared, confirming the conclusions from NMR measurements, indicating that phosphate was involved in metallation (table 2).

The conditions of IR experiments prevented observation of changes in far infrared bands assigned to Cu–O and Cu–N. From analysis of spectroscopic data, phosphothreonine in Cu(HThr-P) is coordinated only by the phosphate, analogous to that of phosphoserine in the analogous protonated form [38].

3.3. *Cu(Thr-P) complex*

Cu(Thr-P) forms in solution from pH 4 and becomes dominant at pH close to 7.5 binding almost 90% of the copper ions (figure 3). The Vis and EPR spectral data for Cu(Thr-P) ($\lambda_{max} = 689$ nm, $g_{\parallel} = 2.251$ and $A_{\parallel} = 175 \times 10^{-4}\,cm^{-1}$, table 2) indicate that the chromophore for this complex is $\{1\,N, O_x\}$. Similar values of spectral parameters of Cu(Thr-P) and the earlier studied Cu(Ser-P) suggest that the mode of coordination in both complexes is the same [38]. A small but specific shift of $\nu_{asC\text{–}O}$ (from 1621 to 1617 cm^{-1}) points to the involvement of carboxylate in complex formation. The asymmetric bands assigned to uncoordinated –NH_2 group [39] (1580 cm^{-1}) undergo a significant shift in the spectra of the systems with copper ions, confirming coordination of copper(II) to –NH_2. The change in the signal coming from phosphorus in the ^{31}P NMR spectrum with respect to that in the spectrum of the free ligand by -0.005 ppm (table 2) and the lack of significant changes in the IR spectrum of Cu(Thr-P) in the range characteristic of the phosphate group exclude involvement of phosphate (asymmetric stretching band at 1023 cm^{-1} in the IR spectrum of the complex and at 1022 cm^{-1} in the spectrum of the free ligand). The results of the spectroscopic study prove that the amine and carboxylate groups are involved in coordination, which means that phosphothreonine and the earlier studied phosphoserine have uncoordinated phosphates starting from a pH of physiological values. The coordination sites are analogous to those in unphosphorylated amino acids [40]. The conclusions on the solution structure were supported by DFT calculations (GAUSSIAN). The energy was optimized

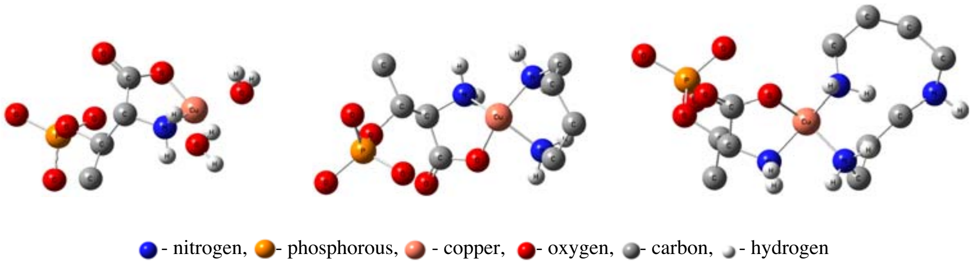

Figure 4. Optimized solution structure. (a) Cu(Thr-P). (b) Cu(Thr-P)(Put). (c) Cu(Thr-P)(Spd) (hydrogens bonded to carbons were omitted for clarity).

for the structure in which metal was coordinated via the amine and carboxylate groups from phosphothreonine and water, figure 4.

3.4. *Cu(Thr-P)(OH) complex*

Cu(Thr-P)(OH) starts forming in solution from pH of 7.0 and dominates above pH 10.5 where it binds all copper ions introduced into the solution (figure 3). Analysis of the Vis and EPR spectral data for this complex ($\lambda_{max} = 643\,nm$, $g_{\|} = 2.245$ and $A_{\|} = 180 \times 10^{-4}\,cm^{-1}$, table 2) indicates {N, O_x} coordination. Participation of the phosphate in coordination has been excluded on the basis of NMR study, table 2. Additionally, no differences in position of the band assigned to the asymmetric stretch of phosphate have been found in the IR spectra of free Thr-P and Cu(Thr-P)(OH). In IR spectra, a shift of the asymmetric stretch $\nu_{asC\text{–}O}$ from 1620 to 1616 cm^{-1} characteristic of COO$^-$ and a significant shift of the asymmetric bands assigned to uncoordinated –NH$_2$ point to involvement of –COO$^-$ and –NH$_2$ in coordination.

As follows from the spectral analysis, the main coordination site in Cu/Thr-P at low pH is phosphate, which becomes inactive with increasing pH and then the coordination sites become COO and –NH$_2$. In the binary system with Cu ions, phosphothreonine shows the same coordination properties as phosphoserine.

3.5. *Complexes in the ternary systems*

Stability constants of the complexes forming in the Cu/Thr-P/PA system, table 3, were calculated on the basis of protonation constants of the ligands and overall stability constants (logβ) of the complexes formed in the binary systems, Cu/Put, Cu/Spd and Cu/Spm (Supplementary material) [41] as well as logβ of the complexes formed in Cu/Thr-P system discussed above, table 1. In all systems studied the potential coordination centers are the same as in the binary systems, i.e. phosphate, carboxylate and amine from phosphothreonine and also amine from PAs. Groups that are potential sites of coordination should also be considered as possible sites of weak non-covalent interactions between bioligands. The possibility of such non-covalent interactions between the anchoring phosphothreonine complex (phosphothreonine metallation takes place at low pH values, close to 2.0, lower than the pH values of the PA metallation) and the protonated biogenic amine should be taken into consideration.

Table 3. The overall stability constants of the ternary complexes as well as statistical parameters (Σ, χ^2).

	Put $\Sigma = 0.83$, $\chi^2 = 13.75$	Spd $\Sigma = 0.89$, $\chi^2 = 16.28$	Spm $\Sigma = 3.82$, $\chi^2 = 28.81$
Overall stability constants logβ of the ternary complexes			
Cu(Thr-P)H$_6$L	–	–	68.76 (10)
Cu(Thr-P)H$_5$L	–	–	–
Cu(Thr-P)H$_4$L	46.50 (6)	48.58 (4)	55.47 (10)
Cu(Thr-P)H$_3$L	42.33 (6)	43.16 (3)	47.73 (10)
Cu(Thr-P)H$_2$L	36.23 (6)	35.82 (4)	38.85 (9)
Cu(Thr-P)HL	27.79 (6)	28.54 (2)	–
Cu(Thr-P)L	18.10 (6)	18.74 (3)	
Cu(Thr-P)L(OH)	6.98 (7)	7.50 (4)	

3.6. *Cu/Thr-P/Put system*

In the Cu/Thr-P/Put system, formation of the following complexes was found Cu(Thr-P) H_x(Put), where x varies from 4 to -1, figure 5. The values of the overall stability constants of these species are given in table 3. For Cu(Thr-P)H_4(Put), forming as the first in this system, figure 5, the results of the Vis and EPR studies ($\lambda_{max} = 782$ nm, $g_{\parallel} = 2.395$ and $A_{\parallel} = 134 \times 10^{-4}$ cm^{-1}, table 2) indicate formation of a species with the oxygen chromophore $\{O_x\}$. Changes in the chemical shift in the ^{31}P NMR spectra of the phosphorus from phosphothreonine, table 2, point to the phosphate group from Thr-P being the main site of copper coordination in this complex.

The formation of oxygen chromophore concluded from the Vis and EPR spectral study excludes coordination from the amine groups from the PA and phosphothreonine. It means that Put is involved in non-covalent interactions with coordinated phosphothreonine. The presence of Put outside the inner coordination sphere was confirmed by the additional Vis spectral study. Addition of Put in equimolar amount to Cu/Thr-P at the pH of Cu(Thr-P) H_4(Put) domination, pH = 3.5, has no significant change in the position of the absorption band ($\lambda_{max} = 775$ nm and $\lambda_{max} = 782$ nm for binary Cu/Thr-P and ternary Cu/Thr-P/Put systems, respectively). Moreover, insignificant changes in the IR spectrum of bands characteristic of the carboxylate indicate that this group takes part in interaction with amine, making the complex Cu(H_2Thr-P)|||||(H_2Put). A similar mode of interaction was found in Cu/Ser-P/Put, in which the fully protonated amine was outside the inner coordination sphere and interacted with the anchoring phosphoserine complex [37].

At pH 5.0 the dominant species is Cu(Thr-P)H_3(Put), figure 5. The spectral parameters of this complex and the changes in the chemical shifts of the phosphate, table 3, suggest that in the inner coordination sphere there are only oxygens. The decrease in λ_{max} means that more than one oxygen is involved in coordination. Similarly as for Cu(Thr-P)H_4(Put), the amine is outside the inner coordination sphere and involved in non-covalent interac-

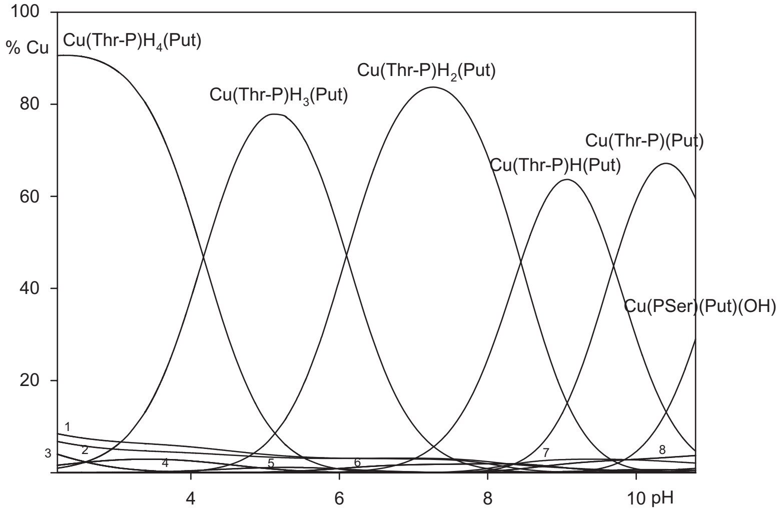

Figure 5. Distribution of the species formed in the system Cu/Thr-P/Put (1 : 1 : 1 ratio); 1 – Cu, 2 – H_2Put, 3 – H_3Thr-P, 4 – H_2Thr-P, 5 – Cu(HThr-P), 6 – Cu(Thr-P), 7 – Put, 8 – Thr-P; the percentage of the species refers to the relative concentration of the metal $c_{Cu} = 0.002$ M.

tions with the anchoring Cu(HThr-P). The value of the equilibrium constant for formation of Cu(HThr-P) + H_2(Put) $\leftrightarrows$ Cu(Thr-P)H_3(Put) is logK_e = 6.12, which is higher than that for the analogous complex with phosphoserine logK_e = 5.08 [37].

With increasing pH, the dominant species becomes Cu(Thr-P)H_2(Put), figure 5. The spectral parameters of this species are λ_{max} = 691 nm, $g_{\|}$ = 2.288 and $A_{\|}$ = 163 × 10^{-4} cm^{-1}, table 2, and indicate formation of the $\{1N,O_x\}$ chromophore. The lack of changes in λ_{max} after addition of PA to the binary system confirms that it is outside the inner coordination sphere. LogK_e of 5.98 for this species is similar to logK_e for Cu(Thr-P)H_3(Put) also with Put outside the coordination sphere. Changes in the IR spectrum of signals characteristic of carboxylate imply that this group together with the amine from Thr-P take part in coordination. Relatively small changes in the ^{31}P NMR spectrum point to involvement of the phosphate only in non-covalent interaction with Put.

The spectral parameters obtained for Cu(Thr-P)H(Put), dominant at pH 9.0, table 2, indicate formation of $\{2N,O_x\}$ chromophore. At pH 9.0, the addition of Put to Cu/Thr-P causes a shift in the absorption by about 35 nm (681 nm for the binary system and 647 nm for the ternary one), which means that the partly diprotonated Put is involved in coordination through its amine. The presence of PA in the inner coordination sphere is confirmed by the logK_e obtained for Cu(Thr-P)H(Put) of 7.22 which is higher than logK_e = 6.12 and logK_e = 5.98 obtained for Cu(Thr-P)H_3(Put) and Cu(Thr-P)H_2(Put), respectively, where in the latter two complexes amine was outside the inner coordination sphere.

The spectral parameters of Cu(Thr-P)(Put), dominant at pH 10.5, table 2, indicate formation of the $\{3N,O_x\}$ chromophore, which means that in this complex Put is bonded to copper via two amines. Participation of the two amine groups from Put was confirmed by Vis study of Cu/Thr-P. The addition of Put to this system resulted in a shift of λ_{max} by about 68 nm. Moreover, the equilibrium constant of Cu(Thr-P)(Put) formation logK_e = 8.36 is higher than that of Cu(Thr-P)H(Put) logK_e = 7.22, confirming the involvement of another nitrogen from Put in coordination. Participation of carboxylate from phosphothreonine in the metallation was confirmed by analysis of IR spectra revealing a shift of the bands assigned to carboxylate. As for Cu/Ser-P/Put, for Cu/Thr-P/Put, deprotonation of the amine leads to coordination. The coordination in Cu(Ser-P)(Put) was confirmed by calculations using GAUSSIAN and the solution structure obtained is presented in figure 4.

The hydroxo complex forming from a pH of 10.5, figure 5, reaches a maximum complexation at the pH value of Cu(Thr-P)(Put) domination, which makes it impossible to perform spectral studies to get information on the mode of coordination.

3.7. *Cu/Thr-P/Spd system*

Computer analysis of the potentiometric data shows the formation of Cu(Thr-P)H_x(Spd) complexes with x from 4 to -1 in the Cu/Thr-P/Spd system; the relevant overall stability constants are given in table 1. At the lowest pH Cu(Thr-P)H_4(Spd) is formed, figure 6. The Vis and EPR spectral parameters characteristic of this species, table 2, indicate coordination with the chromophore $\{O_x\}$. No changes in λ_{max} for the binary system Cu/Thr-P and ternary one at the same pH with λ_{max} = 724 and 719 nm, respectively, mean that Spd is outside the inner coordination sphere. Changes in the chemical shifts of the ^{31}P signals of phosphate of Thr-P indicate that copper is coordinated to phosphate. Changes in the position of the IR band characteristic of carboxylate confirm that Cu(HThr-P)|||||(H_3Spd) is

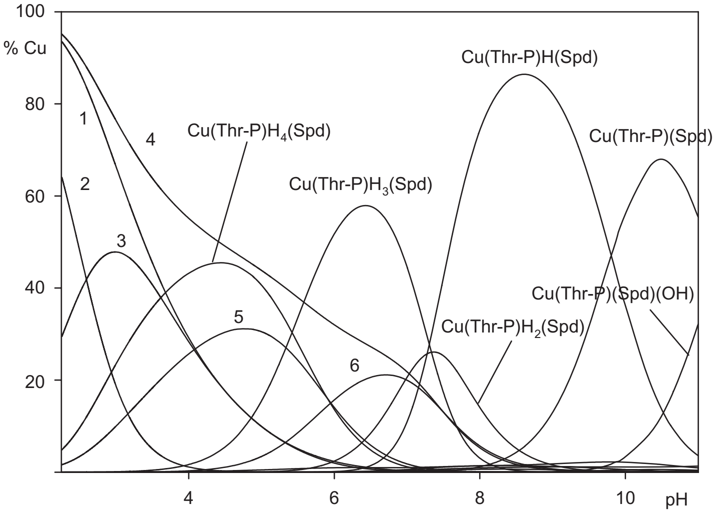

Figure 6. Distribution of the species formed in Cu/Thr-P/Spd (1 : 1 : 1 ratio): 1 – Cu, 2 – H_3Thr-P, 3 – H_2Thr-P, 4 – H_3Spd, 5 – Cu(HThr-P), 6 – Cu(Thr-P); the percentage of the species refers to the relative concentration of the metal $c_{Cu} = 0.002$ M.

formed in which the fully protonated amine is engaged in non-covalent interactions with the carboxylate of the anchoring Cu(HThr-P) complex.

This type of interaction in Cu(HThr-P)|||||(H_3Spd) is also confirmed by the equilibrium constant of formation of Cu(HThr-P) + H_3Spd $\leftrightarrows$ Cu(Thr-P)H_4(Spd) $\log K_e = 3.27$. This value is lower than those of Put complexes in which a non-covalent interaction was also observed ($\log K_e = 6.12$ and $\log K_e = 5.98$ for Cu(Thr-P)H_3(Put) and Cu(Thr-P)H_2(Put), respectively, and Cu(Ser-P)H_4(Spd) $\log K_e = 4.87$). This lowest $\log K_e$ value suggests that not all protonated Spd groups take part in non-covalent bonding.

With deprotonation of phosphothreonine, Cu(Thr-P)H_3(Spd) is formed, figure 6. The spectral parameters of this species ($\lambda_{max} = 698$ nm, $g_{\|} = 2.285$ and $A_{\|} = 157 \times 10^{-4}$ cm^{-1}, table 2) indicate $\{N,O_x\}$ chromophore. Addition of Spd to the binary system Cu/Thr-P at pH = 6.5 ($\lambda_{max} = 702$ nm) does not change the position of the d–d band, which means that Spd is not involved in coordination. Higher $\log K_e = 6.43$ for this complex formation than that of formation of Cu(Thr-P)H_4(Spd), $\log K_e = 3.27$, implies a different mode of coordination of phosphothreonine. Relatively small changes in the chemical shift of the signal assigned to phosphate in the spectrum of Cu(Thr-P)H_3(Spd), table 2, smaller than those in the spectrum of Cu(Thr-P)H_4(Spd), mean that this group in the former complex does not take part in coordination but acts as a center of weak negative interaction with the protonated amine from Spd. From IR spectra analysis, the copper is coordinated with carboxylate (–COO$^-$ bands in the IR spectrum). In comparison with Cu(Thr-P)H_4(Spd), in Cu(Thr-P) H_3(Spd) the coordination sites change from phosphate to carboxylate and amine groups from Thr-P and the center of weak interaction changes from carboxylate to phosphate.

Above pH 6.0, formation of Cu(Thr-P)H_2(Spd) and Cu(Thr-P)H(Spd) takes place, figure 6. The presence of a diprotonic complex at the pH value for which the dominant species are Cu(Thr-P)H_3(Spd) and Cu(Thr-P)H(Spd) does not permit determination of the mode of coordination.

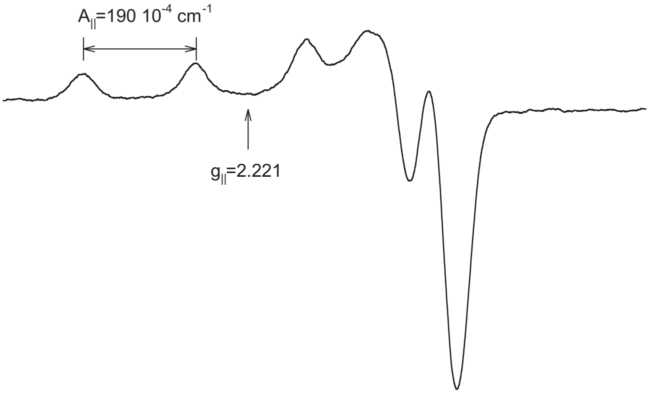

Figure 7. EPR spectra of Cu(Thr-P)H(Spd), pH = 8.5.

The spectral parameters of Cu(Thr-P)H(Spd), table 2, indicate the {2N,O$_x$} chromophore, figure 7, which means that Spd is involved in coordination. Addition of this PA to the binary system Cu/Ser-P at pH 8.5 (λ_{max} = 681 nm) resulted in a shift of λ_{max} by 53 nm. The value of the shift toward higher energies indicates one nitrogen from Spd has been included in the inner coordination sphere. Inclusion of Spd in the inner coordination sphere is also manifested by logK$_e$ = 7.83, higher than logK$_e$ = 6.43 for Cu(Thr-P)H$_3$(Spd), in which the amine was involved in non-covalent interactions with the anchoring complex. Involvement of the second nitrogen from Spd in coordination leads to much greater increase in the equilibrium constant (logK$_e$ = 9.68 for Cu(tn) [42]). Addition of Spd to the binary system Cu/Thr-P causes a shift of the signal by about 100 nm, which means that the second nitrogen from phosphothreonine has been included in the inner coordination sphere. Analysis of the changes in the chemical shift in the ^{31}P NMR spectrum and changes in the position of the carboxylate band in the IR spectrum characteristic of –COO indicates that copper is coordinated to carboxylate and amine, forming an energetically beneficial five-membered ring, and to one amine from Spd, while the protonated fragments of Spd are engaged in weak interaction with the phosphate of phosphothreonine.

Above pH 8.0, formation of Cu(Thr-P)(Spd) and Cu(Thr-P)(Spd)(OH) takes place, figure 6. As the hydroxo complex is present in the system at the pH of domination by Cu (Thr-P)(Put), it was impossible to draw conclusions on the mode of its coordination. The spectral parameters of Cu(Thr-P)(Spd), table 2, point to the {3N,O$_x$} chromophore. The addition of amine to the binary system Cu/Ser-P results in shift of the absorption toward lower energies by 89 nm, which means that two nitrogens of Spd are involved in coordination. The logK$_e$ = 9.00 obtained for Cu(Thr-P)(Spd), close to that of the Cu(tn) complex formation, logK$_e$ = 9.68 and higher than logK$_e$ of the Cu(Thr-P)H(Spd) formation, logK$_e$ = 7.83, means that in Cu(Thr-P)(Spd) two amines from the PA are involved in coordination, forming a five-membered ring. Relatively small changes in the chemical shifts of NMR spectra, table 2, mean that the phosphate of Thr-P is engaged neither in coordination nor in non-covalent bond formation. The coordination mode in Cu(Ser-P)(Spd) was confirmed by calculations using the GAUSSIAN program and the solution structure obtained is presented in figure 4.

3.8. *Cu/Ser-P/Spm system*

In the ternary system Cu/Thr-P/Spm only protonated complexes of the Cu(Thr-P)H$_x$(Spm) type are formed, with $x = 6$, 4–2; the values of the overall stability constants of the complexes are given in table 3.

Cu(Thr-P)H$_6$(Spm) is present in the system at the start of the potentiometric measurements, figure 8, and binds all copper ions present in the solution up to pH 5.5. The Vis and EPR results obtained for this complex ($\lambda_{max} = 710\,\text{nm}$, $g_{||} = 2.321$ and $A_{||} = 165 \times 10^{-4}\,\text{cm}^{-1}$, table 2) indicate formation of a species with the oxygen chromophore $\{O_x\}$. Similar to Put and Spd at low pH, addition of Spd to Cu/Thr-P does not change the position of λ_{max}, so the amine is outside the inner coordination sphere. Changes in the chemical shift of the ^{31}P NMR spectra show that the main coordination site for copper is the phosphate from phosphothreonine, table 2. The lack of refinement for Cu(Thr-P)H$_5$(Spm) can be a result of its presence in low concentrations, preventing detection. An analogous species in the system with phosphoserine has been refined and its relative concentration is close to 35%.

Cu(Thr-P)H$_4$(Spm) dominates at pH 7.5, figure 8, and spectral parameters for this species, table 2, show that besides oxygens there is a nitrogen in the inner coordination sphere ($\{1N,O_x\}$ chromophore), table 2. Addition of Spm to Cu/Thr-P at pH 7.5, $\lambda_{max} = 689\,\text{nm}$, shifts the maximum absorption wavelength by 48 nm, which suggests that Spm is coordinated by one nitrogen. Relatively high $\log K_e = 9.38$ of formation of Cu(Thr-P)H$_4$(Spm): Cu(HThr-P) + H$_3$Spm $\leftrightarrows$ Cu(Thr-P)H$_4$(Spm) indicates that Spm is involved in the inner coordination sphere and that there are weak interactions between the protonated amine groups of Spd and the complexed phosphothreonine. Changes in the chemical shift in ^{31}P NMR spectra confirm the involvement of phosphate from Thr-P in coordination, whereas changes in the IR spectra confirm the participation of carboxylate from Thr-P in non-covalent interactions.

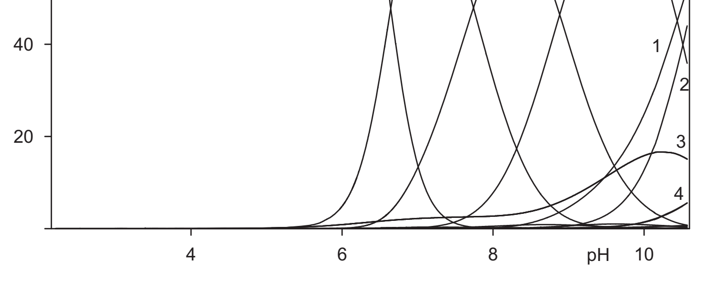

Figure 8. Distribution of the species formed in Cu/Thr-P/Spm (1 : 1 : 1 ratio): 1 – Cu(Spm), 2 – Thr-P, 3 – HThr-P, 4 – CuH(Spm)2; the percentage of the species refers to the relative concentration of the metal $c_{Cu} = 0.002\,\text{M}$.

From a pH of about 6.0, formation of Cu(Thr-P)H_3(Spm) was detected. The spectral parameters, table 2, obtained for this complex imply the presence of the {2N,O} chromophore. Addition of Spd to Cu/Thr-P causes a shift of the maximum absorption by 67 nm to lower energies, indicating that one nitrogen of Spm is involved in coordination. The $\log K_e = 7.60$ obtained for Cu(Thr-P)H_3(Spm) being lower than that for Cu(Thr-P)H_4(Spm) $\log K_e = 9.38$, confirms that although the mode of Spm coordination is the same (one $-NH_2$ involved in coordination), the coordination of phosphothreonine to Cu is different, and indicates that there are no non-covalent interactions between the bioligands. Changes in the chemical shifts observed in the ^{31}P NMR and IR spectra confirm the conclusions drawn on the basis of the equilibrium and spectral studies. In Cu(Thr-P)H_3(Spm), amine and carboxylate of phosphothreonine and one amine of Spm are involved in coordination (the phosphate of Thr-P is inactive).

Cu(Thr-P)H_2(Spm) dominates in the system at pH 9.75, figure 8. The spectral parameters, table 2, obtained for this complex imply the presence of {2N,O} chromophore. Addition of Spd to Cu/Thr-P causes a shift of the maximum absorption by 73 nm to lower energies, indicating that one nitrogen of Spm is involved in coordination, as in Cu(Thr-P) H_3(Spm). The $\log K_e = 7.83$ obtained for Cu(Thr-P)H_2(Spm), similar to Cu(Thr-P)H_3(Spm), means that only one nitrogen of Spm and amine and carboxylate of Thr-P participate in coordination. Changes in the chemical shifts of the ^{31}P NMR spectra confirm the conclusions that amine and carboxylate of phosphoserine and one amine of Spm are involved in coordination, and there are no non-covalent interactions in this complex.

4. Conclusions

Formation of complexes in binary systems of copper(II) with phosphothreonine has been established. In the complexes present below the physiological pH, Cu(HThr-P) and Cu (Thr-P), $-PO_4^{2-}$ is involved in coordination. Thus, phosphorylation of the endogenic threonine (which at low pH coordinates via the carboxylate) results in changes in the mode of metal binding. At physiological pH, a different type of coordination is observed. This process is a result of changes in the effectiveness of the competing donors and the carboxylate and amine of the ligands are involved in coordination, while the activity of the phosphate decreases. An analogous change in coordination preference was observed in the earlier system of Cu(II)/phosphoserine.

Similarly, in the ternary systems Cu/Thr-P/PA, the mode of phosphothreonine complexation depends on pH. Below physiological pH, *Thr-P* coordinates only via the phosphate and with increasing basicity the main reaction centers become the amine and carboxylate. In all ternary systems at low pH, formation of molecular complexes ML… L’ was established (where L = Thr-P, L’ = PA). Totally protonated PA with the active centers blocked for metallation was concluded to be in the outer coordination sphere and interact non-covalently with the binary phosphothreonine complexes via a negatively charged carboxylate. The phosphate from the phosphorylated amino acid is blocked as a center of non-covalent interactions via copper(II) ions.

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