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# Sequence-specific Ni(II)-dependent peptide bond hydrolysis in a peptide containing threonine and histidine residues

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**Previously we demonstrated that Ni(II) complexes of Ac-Thr-Glu-Ser-His-His-Lys-$NH_2$ hexapeptide, representing residues 120–125 of human histone H2A, and some of its analogs undergo E-S peptide bond hydrolysis. In this work we demonstrate a similar coordination and reactivity pattern in Ni(II) complexes of Ac-Thr-Glu-Thr-His-His-Lys-$NH_2$, its threonine analogue, studied using potentiometry, electronic absorption spectroscopy and HPLC. For the first time we present the detailed temperature and pH dependence of such Ni(II)-dependent hydrolysis reactions. The temperature dependence of the rate of hydrolysis yielded activation energy $E_a$ = 92.0 kJ mol$^{-1}$ and activation entropy $\Delta S^{\neq}$ = 208 J mol$^{-1}$ K$^{-1}$. The pH profile of the reaction rate coincided with the formation of the four-nitrogen square-planar Ni(II) complex of Ac-Thr-Glu-Thr-His-His-Lys-$NH_2$. These results expand the range of protein sequences susceptible to Ni(II) dependent cleavage by those containing threonine residues and permit predictions of the course of this reaction at various temperatures and pH values.**

**Keywords**: nickel(II), peptide bond hydrolysis, complex formation, activation parameters

## INTRODUCTION

We previously discovered Ni(II)-dependent hydrolysis of the Glu-Ser peptide bond in the Ac-Thr-Glu-Ser-His-His-Lys-$NH_2$ hexapeptide, representing residues 120–125 of the major variant of human histone H2A (Bal *et al.*, 1998). Subsequently, we found that sequence specificity of this reaction was retained in the whole histone H2A *in vitro* (Bal *et al.*, 2000a) and in cell cultures exposed to nickel(II) salts (Karaczyn *et al.*, 2003). Using a series of alanine-substituted hexapeptides we demonstrated that the hydrolysis of the peptide bond preceding the Ser residue occurred in -Xaa-Ser-Yaa-His- sequences, where Xaa was Glu or Ala, and Yaa was His or Ala (Mylonas *et al.*, 2002a). The prior formation of a square-planar Ni(II) complex involving the coordination of amide nitrogens was required for the hydrolysis to occur. A substitution of Ser or His-5 in these hexapeptides with Ala residue abolished the peptide bond hydrolysis completely, although these peptides retained Ni(II) binding abilities. We also studied the coordination of Cu(II) ions (Mylonas *et al.*, 2002b) and Zn(II) ions (Mylonas *et al.*, 2004a) to these peptides and investigated the oxidative reactivity of their Cu(II) complexes (Kaczmarek *et al.*, 2005). These studies were summarized recently (Mylonas *et al.*, 2005a). Below we present novel results demonstrating that the substitution of the active serine residue with threonine yields peptides equally susceptible to Ni(II)-dependent hydrolysis. For the first time we provide the pH and temperature dependence of the reaction rate. These results greatly expand our knowledge on Ni(II) dependent peptide bond cleavage.

**Abbreviations**: $k^1$, 1st order rate constant; 3N, three-nitrogen complex; 4N, four-nitrogen complex.

## MATERIALS AND METHODS

**Peptide synthesis**. The peptides Ac-Thr-Glu-Thr-His-His-Lys-$NH_2$ and Ac-Phe-Thr-Glu-Thr-His-His-Lys-Tyr-$NH_2$ were synthesized in the solid state on an H-linker-chlorotrityl resin (1.5 g per each peptide with the loading capacity 0.5 mmol/g) using Fmoc strategy (Fields, 1997; Chan & White, 2000), with 4- and 8-fold excess of amino acids and coupling agents, respectively. The resin and protected amino acids, Fmoc-Lys(Boc)-OH, Fmoc-His(Mtt)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Glu(tBu)-OH, Fmoc-Phe(tBu)-OH, Fmoc-Tyr(tBu)-OH, and Fmoc-Ser(tBu)-OH were purchased from CBL Chemical, coupling agents, 1-hydroxybenzatriazole (HOBt) and dicyclohexylcarbodiimide (DCC) were from Merck AG, *N,N*-dimethylformamide (DMF), dichloromethane (DCM), acetic acid (AcOH) and acetonitrile (MeCN) were obtained from Riedel-de Haen, trifluoroacetic acid (TFA), 2,2,2-trifluoroethanol (TFE) and piperidine *N,N*-diisopropylethylamine (DIEA) were from Sigma-Aldrich. Fmoc protecting groups were removed with 20% piperidine in DMF. The coupling with DCC and HOBt, performed in the presence of 4-fold excess of DIEA over 4 h, was monitored by the ninhydrin (Keiser) test and TLC. Peptides were terminated with acetic anhydride in DMF containing DIEA. N-terminal acetylated resin-bound peptides were cleaved from the resin with 35 ml of 1:2:7 mixture of TFE, AcOH and DCM (by vol.), followed by evaporation under vacuum. The removal of protecting groups was performed in each case by adding 35 ml of 13:5:1:1 mixture of TFA, DCM, TFE and anisol (by vol.) and incubating for 24 h at room temperature. The resin was removed by filtration and washed with 50% TFA in DCM (2 × 2 ml), which was combined with the filtrate. The filtrate was then poured into 150 ml of cold ether, yielding a precipitate, which was subsequently dissolved in water and lyophilized to give about 300 mg of raw product as a powder. Peptides were purified using HP series 1100 HPLC system (Hewlett-Packard) on a preparative Alltech Apollo C18 column (22 mm × 250 mm, 5 μm) eluting with 0.1% TFA/water (A) and 0.1% TFA/90% MeCN/water (B), using a linear gradient from 0 to 100% of B over 60 min at a flow rate of 1ml/min, with detection at 220 nm. The identities and purities of peptides were confirmed using mass spectrometry, on a TSQ 700 ESI-MS spectrometer (Finnigan MAT). The *m/z* values found (calculated) of $(M+H)^+$ ions were: 793.2 (793.4) and 1103.5 (1103.4) for Ac-Thr-Glu-Thr-His-His-Lys-$NH_2$ and Ac-Phe-Thr-Glu-Thr-His-His-Lys-Tyr-$NH_2$, respectively. The purities of the final peptide samples were additionally controlled by $^1H$ NMR, on an Inova 400 MHz spectrometer (Varian). The synthesis of Ac-Thr-Glu-Ser-His-His-Lys-$NH_2$, Ac-Thr-Ala-Ser-His-His-Lys-$NH_2$, and Ac-Thr-Glu-Ser-Ala-His-Lys-$NH_2$ peptides was described previously (Mylonas *et al.*, 2002b).

**Potentiometry**. Potentiometric titrations of Ac-Thr-Glu-Thr-His-His-Lys-$NH_2$ and its Ni(II) complexes in the presence of 0.1 M $KNO_3$ were performed under a purified argon atmosphere at 25°C within the pH range of 2.8 to 11.5 on a Molspin automatic titrator, with 0.1 M NaOH as titrant. Changes in pH were monitored with a combined glass-Ag/AgCl electrode (InLab 422, Mettler-Toledo), calibrated daily in hydrogen ion concentrations by titrations of 4 mM $HNO_3$ solutions in 96 mM $KNO_3$ (Irving *et al.*, 1967). Sample volumes of 1.5–2.0 ml, peptide concentrations of 1 mM and peptide-to-Ni(II) molar ratios of 1.2–1.8 were used. The data were analyzed using SUPERQUAD program (Gans *et al.*, 1985). Standard deviations of constants computed by SUPERQUAD refer to random errors.

**Spectroscopic studies.** UV-Vis spectra of Ni(II) complexes of Ac-Thr-Glu-Thr-His-His-Lys-$NH_2$ were recorded on a Cary 50 Bio spectrophotometer, using peptide and Ni(II) concentrations of 1.5 mM. For pH values below 7.0 the samples were kept at room temperature and the spectra were recorded at 25°C. At higher pH values the samples were kept on ice between measurements in order to quench the hydrolysis reaction and allow for the reliable recording of spectra of the unhydrolyzed initial complexes.

**Determination of rate constants.** Samples containing 1 mM peptide and 1 mM Ni(II) in 50 mM phosphate buffer at pH values between 7.0 and 11.0 were incubated at 25°C for up to five days. In separate experiments, analogous samples at pH 9.5 were incubated at temperatures between 10 and 75°C. In either case, 20 μl aliquots of reaction mixtures were collected, mixed with 180 μl of 0.1 M $H_3PO_4$ and stored on ice until analyzed (within 3 h) on a series 1100 HPLC (Hewlett Packard) with a Hypersil BDC C18 analytical column, 4.6 × 250 mm (Merck). The substrate and the products were separated isocratically using 50 mM phosphate buffer in 10% MeOH, pH 2.6, at the flow rate of 1 ml/min and detection at 200 nm. The rate constants were determined by fitting the integrals of substrate and product peaks to the 1st order rate law.

## RESULTS AND DISCUSSION

### Protonation and complex formation

Table 1 presents the protonation and Ni(II) stability constants of the Ac-Thr-Glu-Thr-His-His-Lys-$NH_2$ peptide, Fig. 1 presents selected UV-Vis spectra, while Fig. 2 compares the potentiometry-

**Table 1. Cumulative and stepwise protonation and Ni(II) binding constants for Ac-Thr-Glu-Thr-His-His-Lys-$NH_2$, determined at 25°C and $I$ = 0.1 M ($KNO_3$).**

Standard deviations of the last digit are given in parentheses. The previously published data for the analogous Ac-Thr-Glu-Ser-His-His-Lys-$NH_2$ system (Mylonas *et al.*, 2002a) are included for comparison.

| Species | Ac-TETHHK-$NH_2$ | | Ac-TESHHK-$NH_2$ | |
|---|---|---|---|---|
| | log β[a] | p$K_a$[b] | log β[a] | p$K_a$[b] |
| HL | 10.465(6) | 10.47 | 10.28 | 10.28 |
| $H_2L^+$ | 17.178(7) | 6.71 | 17.06 | 6.78 |
| $H_3L^{2+}$ | 23.022(8) | 5.84 | 22.96 | 5.90 |
| $H_4L^{3+}$ | 26.983(9) | 3.96 | 26.81 | 3.85 |
| $NiHL^{2+}$ | 14.24(2) | | 14.04 | |
| $NiH_{-1}L$ | –1.90(2) | 8.07[c] | –2.16 | 8.10[c] |
| $NiH_{-2}L^-$ | –10.88(2) | 8.98 | –11.52 | 9.36 |
| $NiH_{-3}L^{2-}$ | –21.71(3) | 10.83 | –22.80 | 11.28 |

[a]β = $[Ni_iH_jL_k]/([Ni]^i[H]^j[L]^k)$; [b]p$K_a$ = log β$\{Ni_iH_{j+1}L_k\}$-log β$\{Ni_iH_jL_k\}$; [c]average value for two simultaneous deprotonations

based complex species distribution with UV-Vis spectral parameters characteristic for octahedral and square-planar complexes, $A_{638}$ and $A_{453}$, respectively, demonstrating the correctness of potentiometric data analysis. Table 2 provides the UV-Vis spectral parameters, calculated for individual complexes with the use of the potentiometric species distribution. The identical sets of potential Ni(II) binding sites, identical complex stoichiometries and very similar binding constant values (compared in Table 1) and UV-Vis spectroscopic profiles (compared in Table 2) indicate that analogous complexes were formed for the Ac-Thr-Glu-Thr-His-His-Lys-$NH_2$ peptide and for its serine analog, Ac-Thr-Glu-Ser-His-His-Lys-$NH_2$. Therefore, according to studies on the latter one (Bal *et al.*, 1998, Mylonas *et al.*, 2002a), the high-spin $NiHL^{2+}$ complex contains the Ni(II) ion coordinated to nitrogens of both imidazole rings and to the Glu side chain carboxylate. The release of two hydrogen ions from this species yields the $NiH_{-1}L$ complex, which involves the Ni(II) coordination to one imidazole and two deprotonated amide nitrogens (3N complex). The UV-Vis spectroscopic parameters of this complex (Table 2) indicate that this species is a mixture of high- and low-spin complexes sharing the same stoichiometry. Such phenomena are typical for Ni(II) complexes. Judging from the data obtained previously for similar complexes, the participation of both forms in the $NiH_{-1}L$ species is approximately equal (Mylonas *et al.*, 2002a). The next species, $NiH_{-2}L^-$ is formed upon the deprotonation and coordination of the third amide nitrogen (4N complex). The final $NiH_{-3}L^{2-}$ complex is formed upon the Lys amine deprotonation, which does not affect the binding mode, but influences spectroscopic properties of Ni(II). The parameters of spectra of $NiH_{-2}L^-$ and $NiH_{-3}L^{3-}$ complexes are consistent with typical square-planar structures of low-spin Ni(II). The molar absorption coefficient of the *d-d* band of the $NiH_{-3}L^{3-}$ complex is higher than that of the $NiH_{-2}L^{2-}$ complex by about 50%. The increases of ε values accompanying Lys deprotonations in complexes of related peptides were smaller (Mylonas *et al.*, 2002a). This difference might be due to the low temperature at which the samples were incubated here, which can be expected to augment intramolecular electrostatic interactions between side chains, and thereby sensitize the Ni(II) ion to Lys charge. Previously, we calculated the distribution of electrostatic potential in the structurally related N-terminal Ni(II) complex of protamine HP2 and demonstrated that it provides a template for such interactions with amino-acid side chains (Bal *et al.*, 2000b).

**Table 2. UV-Vis spectroscopic parameters of Ni(II) complexes of Ac-Thr-Glu-Thr-His-His-Lys-$NH_2$.**

Spectral shoulders are denoted by sh.

| Species | $\lambda_{max}$ (nm) | ε ($M^{-1}$ $cm^{-1}$) |
|---|---|---|
| $NiHL^{2+}$ | sh725 | 2.0 |
| | 638 | 3.9 |
| | 390 | 14.0 |
| $NiH_{-1}L$ | sh560 | 4.4 |
| | 443 | 28.5 |
| | sh382 | 16.7 |
| $NiH_{-2}L^-$ | 453 | 96 |
| $NiH_{-3}L^{2-}$ | 457 | 147 |

The presence of His residues in positions 4 and 5 may result in the formation of two different square-planar complexes, one at the -Asp-Thr-

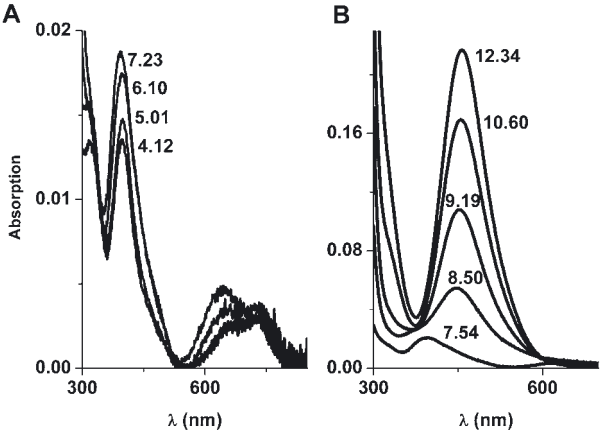


**Figure 1. Selected UV-Vis spectra for Ac-Thr-Glu-Thr-His-His-Lys-$NH_2$ peptide and Ni(II) ions, both at 1.5 mM, in the ranges of existence of high-spin (A) and low-spin (B) complexes.**
The pH values are indicated near the respective peaks.

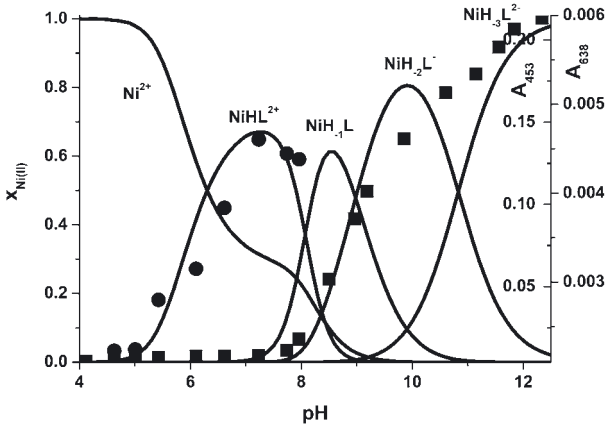


**Figure 2. The species distribution for the Ac-Thr-Glu-Thr-His-His-Lys-$NH_2$ peptide and Ni(II) ions at 25°C, both at 1.5 mM, with values of $A_{638}$ (●) and $A_{453}$ (■) overlaid.**

His- sequence (anchored at His-4), and another at the -Thr-His-His- sequence (anchored at His-5). As discussed previously (Mylonas *et al.*, 2002a), the simultaneous coordination of both His imidazoles is not compatible with the square-planar structure. The NMR data for the Ac-Thr-Ala-Ser-His-His-Lys-$NH_2$ peptide presented there suggest a preference for the latter option, which is most likely retained for all similar peptides, including the one studied here.

As shown in Table 1, the threonine analog binds Ni(II) a little more strongly than the serine analog. In particular, the p$K_a$ of formation of the four nitrogen (4N) complex of the threonine peptide is lower by 0.38 log units than that of the serine peptide. This difference can be explained by an additional shielding of amide nitrogens from water molecules, provided by the methyl group of the threonine side chain. The shielding by bulky substitutents was demonstrated previously to protect such

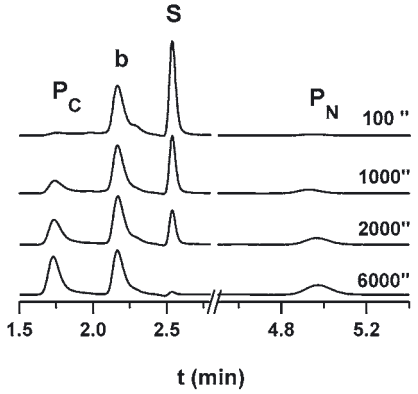


**Figure 3. Typical chromatograms of the sample containing 1 mM Ni(II) and 1 mM Thr-Glu-Thr-His-His-Lys-$NH_2$ peptide, incubated at 25°C and pH 9.5 for time periods indicated on the graph, in a 50 mM phosphate buffer.**

$P_C$, Thr-His-His-Lys-$NH_2$ peptide, $P_N$, Ac-Thr-Glu peptide; S, Thr-Glu-Thr-His-His-Lys-$NH_2$ peptide, b, unidentified buffer component.

complexes from reprotonation of amide nitrogens leading to Ni(II) dissociation (Raycheba & Margerum, 1980; Bal *et al.*, 1996).

**Peptide bond hydrolysis**. Figure 3 shows a selection of typical chromatograms of reaction mixtures containing equimolar Ni(II) and Ac-Thr-Glu-Thr-His-His-Lys-$NH_2$, at 1 mM, following incubation in a 50 mM phosphate buffer for various times. The identities of individual peaks were confirmed by ESI-MS. Chromatographic results confirmed the absolute sequence specificity of peptide bond hydrolysis in the system studied, at all pH values.

The pH dependence of the 1st order rate constant ($k^1$) for hydrolysis is shown in Fig. 4. The comparison of this profile with pH profiles of formation of 3N+4N and 4N complexes confirms the strict requirement of the latter complex, which contains the Ni(II) ion bonded to the imidazole nitrogen-1 of His-5 and three preceding amide nitrogens, for the Glu-Thr peptide bond hydrolysis to occur. The rate of this reaction is maximal at high pH values, where such a square planar complex predominates.

Figure 5 presents the temperature profile of $k^1$, determined at pH 9.5 for temperatures of incubation between 10 and 75°C. The strict sequence specificity of the reaction was maintained in this broad temperature range. As shown in this figure, $k^1$ depended strongly on the temperature. The linear Arrhenius plot of these data (R = –0.993, n = 6), allowed to estimate the activation energy $E_a$ = 92.0 kJ mol$^{-1}$ and activation entropy $\Delta S^{\neq}$ = 208 J mol$^{-1}$ K$^{-1}$. The non-catalytic character of the hydrolysis reaction studied here justifies the comparison of the activation energy obtained above with those determined previously for uncatalyzed peptide bond hydrolysis

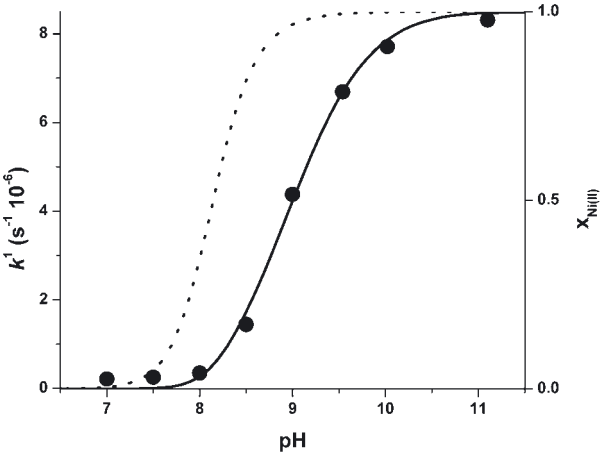


**Figure 4. The pH dependence of the 1st order rate constant of -Glu-Thr- peptide bond hydrolysis in 1 mM Thr-Glu-Thr-His-His-Lys-$NH_2$ incubated with 1 mM $Ni(NO_3)_2$ at 25°C.**

The pH was controlled by 50 mM phosphate buffer and monitored during hydrolysis. The dotted and solid lines represent potentiometry-based pH profiles of molar fractions of 3N+4N and 4N complexes, respectively.

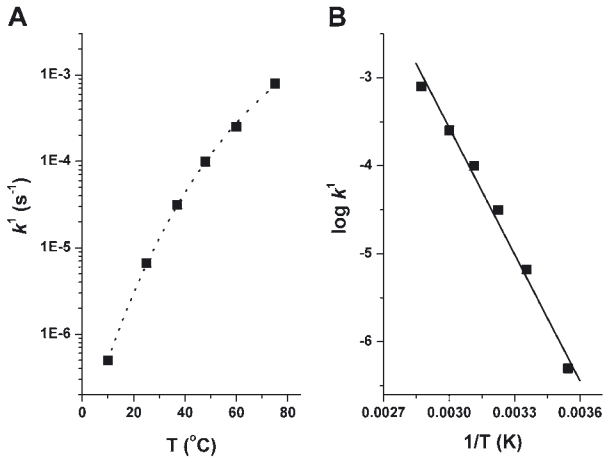


**Figure 5. The temperature dependence of the 1st order rate constant of Glu-Thr peptide bond hydrolysis in 1 mM Ac-Thr-Glu-Thr-His-His-Lys-$NH_2$ incubated in the presence of 1 mM Ni(II) at pH 9.5 in a 50 mM phosphate buffer (A), along with the Arrhenius plot of these data and their linear fit (solid line) (B).**

**Table 3. A review of 1st order rate constants and activation energies for non-catalytic peptide bond hydrolysis, determined under various conditions.**

| Peptide | $k_1$ ($s^{-1}$) | $E_a$ (kJ $mol^{-1}$) | Conditions |
|---|---|---|---|
| *Ala-Ala*[a] | $2.1 \times 10^{-6}$ | 70[a] | 75°C, 0.1 M NaOH |
| *Gly-Gly*[b] | $1.8 \times 10^{-5}$ | 80 | 75°C, 2 M HCl |
| *Gly-Gly*[b] | $9.7 \times 10^{-5}$ | 59 | 75°C, 2 M NaOH |
| *Gly-Gly*[c] | $1.3 \times 10^{-6}$ | 98 | 120°C, pH 5 |
| *PA-11 polymer*[d] | no data | 81–87 | 120°C, pH 7 |
| *Ac-TETHHK-$NH_2$*[e] | $7.5 \times 10^{-4}$ | 92 | 75°C, pH 9.5 |

[a]Hartmann *et al.* (1962); $E_a$ values in the 62–80 kJ $mol^{-1}$ range reported for several analogous simple dipeptides; [b]Radzicka & Wolfenden (1996); [c]Hammel & Glasstone (1954), $E_a$ value estimated from the published graph; [d]Meyer *et al.* (2002); [e]this work.

under a variety of conditions. A selection of relevant literature data is presented in Table 3. The $E_a$ values at neutral and acidic pH were similar to that obtained herein, while the alkaline hydrolysis proceeded with a lower activation barrier (Hammel & Glasstone, 1954; Hartmann *et al.*, 1962; Radzicka & Wolfenden, 1996; Meyer *et al.*, 2002). The $\Delta S^{\neq}$ values determined for simple dipeptides were in the range of about 130–170 J $mol^{-1}$ $K^{-1}$ (Hartmann *et al.*, 1962; Radzicka & Wolfenden, 1996). Despite these relatively small differences, the 1st order rate constants for spontaneous processes were substantially lower than that facilitated by Ni(II) ions. Therefore, the crucial effect introduced by the metal must be related to the effectivity of the bond cleavage once the energy barrier is overcome. It is probably due to a strong increase of Ni(II) binding constant for the C-terminal reaction product, so called His-3 peptide, compared to the starting complex. This phenomenon is due to a weaker competition of $H^+$ ions for the Ni(II)-bound amine nitrogen, present in the hydrolysis product ($pK_a$ ~ 7–9), compared to that for the corresponding amide nitrogen, present in the substrate ($pK_a$ ~ 14–15; Sigel & Martin, 1982). The broad literature on such complexes indicates that this gain of stability, roughly one million-fold, corresponds to a difference between the above $pK_a$ values, typically about six log units (Kozłowski *et al.*, 1999). This notion was confirmed for hydrolyzable serine analogs of the peptide studied (Mylonas *et al.*, 2004b; Mylonas *et al.*, 2005b). Figure 6 depicts the structures of substrate and product of the Thr peptide studied here, along with their spectroscopic parameters, which substantiate the difference in coordination modes. The conversion of the N-terminal amide in the starting Ni(II) complex into the N-terminal amine complex can thus be treated as a reaction coupled to hydrolysis, which provides additional free energy of about 34 kJ $mol^{-1}$ at 25°C and 40 kJ $mol^{-1}$ at 75°C.

Table 4 compares the rate constants and reaction half-times of several peptides containing the active Ser residue with that containing the active Thr residue. Among these peptides, the Ni(II) dependent hydrolysis of Ac-Thr-Glu-Ser-His-His-Lys-$NH_2$, Ac-Thr-Ala-Ser-His-His-Lys-$NH_2$ and Ac-Thr-Glu-Ser-Ala-His-Lys-$NH_2$ was studied previously only qualitatively (Mylonas *et al.*, 2002a), and that of the terminally extended peptide Ac-Phe-Thr-Glu-Ser-His-His-Lys-Tyr-$NH_2$ was not studied at all.

The rate for the Ac-Thr-Glu-Thr-His-His-Lys-$NH_2$ peptide was severalfold higher than that of the

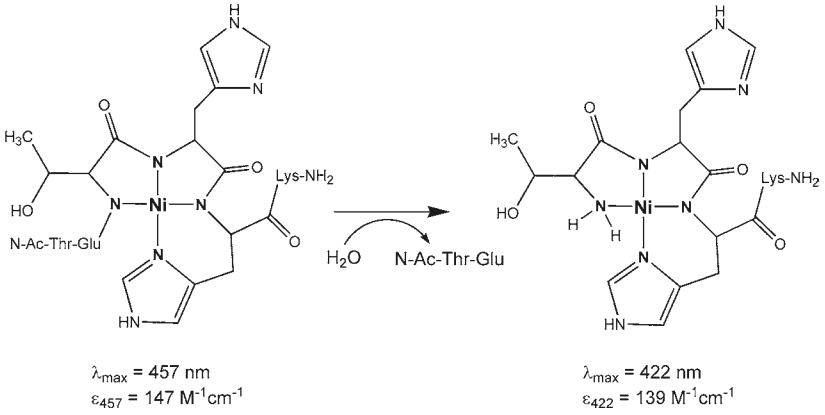


**Figure 6. Schematic representation of active square planar Ni(II) complex (fully deprotonated, $NiH_{-3}L^{2-}$) with Ac-Thr-Glu-Thr-His-His-Lys-$NH_2$ and its hydrolysis product.**
The conversion of the N-terminal amide donor into the amine results in a change of spectroscopic parameters of these planar complexes.

**Table 4. First order rate constants and corresponding reaction half-times for peptide bond hydrolysis, determined by HPLC at pH 9.5 (25 mM phosphate buffer) and 25°C, for peptide and Ni(II) concentrations of 1 mM.**

The N- and C-terminal hydrolysis products are marked in italic and plain characters, respectively, and the crucial Ser/Thr residue is marked in bold.

| Peptide | $k^1$ ($s^{-1} \times 10^{-6}$) | $t_{½}$ (h) |
|---|---|---|
| *Ac-Thr-Glu*-**Ser**-His-His-Lys-$NH_2$ | 2.0 ± 0.3 | 96 |
| *Ac-Phe-Thr-Glu*-**Ser**-His-His-Lys-Tyr-$NH_2$ | 8.0 ± 0.2 | 24 |
| *Ac-Thr-Ala*-**Ser**-His-His-Lys-$NH_2$ | 4.3 ± 0.8 | 44 |
| *Ac-Thr-Glu*-**Ser**-Ala-His-Lys-$NH_2$ | 4.3 ± 0.5 | 44 |
| *Ac-Thr-Glu*-**Thr**-His-His-Lys-$NH_2$ | 6.7 ± 0.7 | 29 |

sister Ac-Thr-Glu-Ser-His-His-Lys-$NH_2$ peptide, but slightly lower than that of the serine peptide elongated at both termini with aromatic residues. The serine peptides substituted with alanine residues before and after the Ser residue gave intermediate rate constants. Notably, the comparison of the rates for Ac-Thr-Glu-Ser-His-His-Lys-$NH_2$ and Ac-Thr-Ala-Ser-His-His-Lys-$NH_2$ indicates that the N-terminal part of the peptide, preceding the Ser residue, had no influence on the course of the reaction. Overall, Table 4 demonstrates that the threonine residue is at least equivalent to serine as a site of Ni(II)-dependent peptide bond hydrolysis in general sequences -Xaa-Thr/Ser-Yaa-His-Zaa-.

## CONCLUSIONS

In the present paper we demonstrated that the peptide containing a threonine residue in the -Thr-Xaa-His- sequence is susceptible to Ni(II) dependent hydrolysis at least as much as Ser-containing analogs reported previously. We also demonstrated that this reaction is strictly dependent on the formation of a 4N planar Ni(II) complex of the peptide and determined its activation parameters. The detailed knowledge of pH and temperature dependence of the rate of hydrolysis obtained in this work provides a strong basis for further studies leading to elucidation of the mechanism of Ni(II)-dependent peptide bond hydrolysis. With respect to Ni(II) toxicity, the results provided herein expand the range of protein sequences potentially susceptible to Ni(II) dependent cleavage by those containing threonine residues and provide the basis for correlating the extent of cleavage of histone H2A and other hypothetical Ni(II) hydrolytic targets with temperature and local pH values. These results will help direct further toxicological studies of Ni(II) dependent peptide bond hydrolysis.

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