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Sequence-Specific Cu(II)-Dependent Peptide Bond Hydrolysis: Similarities and Differences with the Ni(II)-Dependent Reaction

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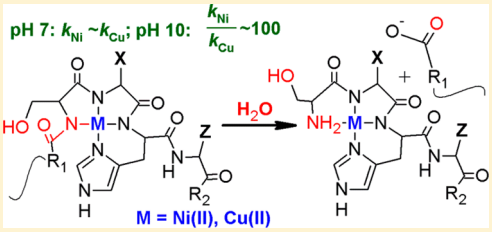
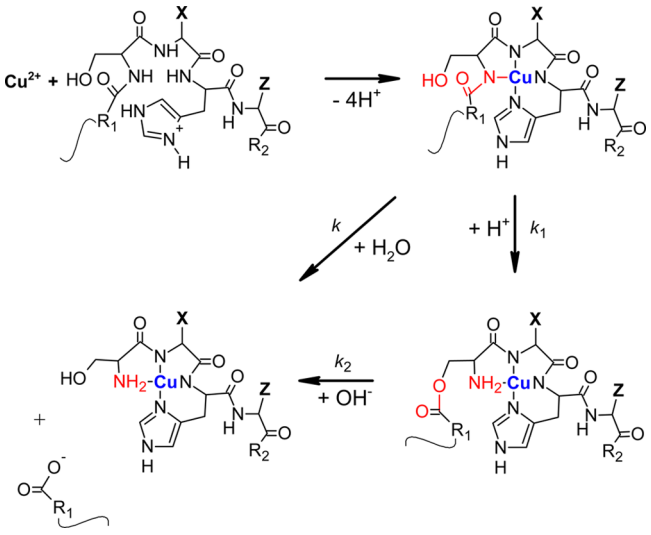
ABSTRACT: Potentiometry and UV−vis and circular dichroism spectroscopies were applied to characterize Cu(II) coordination to the Ac-GASRHWKFL-NH_2 peptide. Using HPLC and ESI-MS, we demonstrated that Cu(II) ions cause selective hydrolysis of the Ala-Ser peptide bond in this peptide and characterized the pH and temperature dependence of the reaction. We found that Cu(II)-dependent hydrolysis occurs solely in 4N complexes, in which the equatorial coordination positions of the Cu(II) ion are saturated by peptide donor atoms, namely, the pyridine-like nitrogen of the His imidazole ring and three preceding peptide bond nitrogens. Analysis of the reaction products led to the conclusion that Cu(II)-dependent hydrolysis proceeds according to the mechanism demonstrated previously for Ni(II) ions (Kopera, E.; Krężel, A.; Protas, A. M.; Belczyk, A.; Bonna, A.; Wysłouch-Cieszyńska, A.; Poznański, J.; Bal, W. *Inorg. Chem.* **2010**, *49*, 6636−6645). However, the pseudo-first-order reaction rate found for Cu(II) is, on average, 100 times lower than that for Ni(II) ions. The greater ability of Cu(II) ions to form 4N complexes at lower pH partially compensates for this difference in rates, resulting in similar hydrolytic activities for the two ions around pH 7.



INTRODUCTION

Over the last 15 years we have been studying the reaction of nickel-dependent peptide bond hydrolysis occurring before Ser/Thr in the R_N-(Ser/Thr)-Xaa-His-Zaa-R_C generic sequence. The reaction was discovered in the course of studies of Ni(II) interactions with histones. We found that the hexapeptide Ac-TESHHK-NH_2 derived from the C-terminal tail of human histone H2A is unstable in the presence of Ni(II) ions, yielding a square-planar Ni-SHHK-NH_2 complex.[1] The reaction proceeds with no external agents and no detectable side products. In subsequent work, we found that the reaction studied was the peptide bond hydrolysis occurring specifically before the serine residue, independent of the extension of the C- and N-terminal sequences (R_C and R_N, respectively).[2] We also found that the presence of Ser and His residues in the Ser-Xaa-His unit is crucial for the reaction to proceed and that Ser can be substituted with Thr.[3,4] In these studies we also observed a specific pH dependence of the hydrolysis reaction, which indicated that it requires the initial formation of a square-planar complex containing a Ni(II) ion coordinated to four nitrogen atoms of the peptide (the pyridine-like nitrogen of the His imidazole and three preceding main-chain peptide nitrogens). Subsequently we demonstrated that amino acid substitutions in positions Xaa and Zaa of the generic sequence significantly affect the reaction rate but do not alter its mechanism. Bulky and aromatic residues were found to be the optimal substituents for fast hydrolysis, and the Ser-Arg-His-Trp sequence was found to be optimal for fast hydrolysis.[5] The isolation of the intermediate product of the reaction and the demonstration of its ester character empowered us to propose a molecular mechanism for the hydrolysis consisting of an intramolecular acyl shift of the carbonyl group of the peptide bond preceding the Ser/Thr residue to its alcoholic function, followed by spontaneous hydrolysis of the ester.[6] This mechanism is presented in Scheme 1. Follow-up studies demonstrated that a Cys residue can replace His as the residue anchoring the Ni(II) ion to the hydrolysis site,[7] that the addition of another aromatic residue upstream of Trp is beneficial for the hydrolysis,[8] and that D-amino acid substitutions within the core Ni(II) binding sequence Ser-

Scheme 1. Main Steps of Cu(II)-Assisted Peptide Bond Hydrolysis (The Meanings of the Rate Constants k, k_1, and k_2 Are Shown)



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Arg-His significantly decrease the rate of hydrolysis or even prevent the reaction.[9] The latter result was of particular interest, demonstrating that the occurrence of hydrolysis depends solely on the spatial arrangements of amino acid side chains around the coordinated Ni(II) ion. In our latest study, we also demonstrated that the coordination of the metal ion to the resulting amino nitrogen (Scheme 1) prevents reversal of the acyl shift by engaging the nitrogen lone pair.[10] Parameters usually related to the reactivity of a metal ion, such as Lewis acidity, complex stability, and bond enthalpy, contribute to the hydrolysis process only via the square-planar organization of the equatorial coordination sphere about the metal ion.

In parallel, we demonstrated that the studied reaction can occur with similar specificity in proteins if the Ni(II) binding sequence is accessible to the Ni(II) ion and has sufficient conformational freedom to adopt the appropriate square-planar structure upon binding of Ni(II). The proteins studied in this respect included histone H2A in vitro and in the living cell,[2,11] zinc fingers,[12] and denatured ubiquitin.[13] These studies, especially the one dealing with zinc fingers, suggested that Ni(II)-dependent peptide bond hydrolysis may be one of the molecular mechanisms of nickel toxicity.[12] At the same time, we employed this reaction as a tool for affinity-tag cleavage in an improved method of purification of recombinant proteins.[13]

In our exploratory work, we observed that Cu(II) hydrolyzes the peptide derived from histone H2A in a fashion similar to that of Ni(II) but that the Cu(II) reaction is significantly slower.[2] Interestingly, specific hydrolysis in the presence of Cu(II) and Ni(II) ions was reported previously for peptide bonds preceding the Ser or Thr residue in peptides containing direct Ser-His and Thr-His dipeptide sequences.[14−17] In that reaction, Cu(II) ions were more reactive than Ni(II) ions, but the absence of the intervening amino acid residue Xaa between the key residues in those sequences suggests significant differences in its molecular mechanism compared with our reaction. In this study, we decided to investigate the Cu(II)-dependent reaction with R_N-(Ser/Thr)-Xaa-His-Zaa-R_C sequences in order to investigate its molecular mechanism, to find out whether the Cu(II)-dependent reaction (if detected) might be useful in biotechnology, and to check whether it might be of toxicological relevance.

EXPERIMENTAL SECTION

Materials. The Ac-GASRHWKFL-NH_2 peptide was synthesized according to the Fmoc strategy.[18] Details of the synthesis and purification were presented previously.[6] Acetonitrile (HPLC grade) was obtained from Rathburn Chemicals Ltd. Pure sodium hydroxide was obtained from Chempur. HEPES (≥99.5%) was purchased from Carl Roth GmbH. Other reagents were purchased from Sigma-Aldrich.

Potentiometry. Potentiometric titrations were performed on a Titrando 907 automatic titrator (Metrohm) using a combined glass−Ag/AgCl electrode (InLabMicro, Mettler Toledo, Switzerland), which was calibrated daily by nitric acid titrations.[19] CO_2-free NaOH (0.1 M) was used as the titrant. Sample volumes of 1.0−1.5 mL were used. The samples contained 0.5−1.0 mM peptide dissolved in 4 mM HNO_3/96 mM KNO_3. The Cu(II) complex formation was studied for the 1:1 stoichiometry using a 5−10% excess of peptide over Cu(II), which was added as $Cu(NO_3)_2$. All of the experiments were performed under argon at 25 °C over the pH range from 2.3 to 12.2. The collected data were analyzed using SUPERQUAD and HYPERQUAD.[20,21] Three titrations were included simultaneously into the calculations, which were performed separately for protonation and Cu(II) complexation.

UV−Vis and CD Spectroscopies. UV−vis spectra were recorded over the range of 300−850 nm on a PerkinElmer UV−vis spectrometer. The solutions contained 1.0 mM peptide and 0.9 mM Cu(II). The pH of the solution was adjusted manually over the range from 2 to 12 by addition of small amounts of concentrated NaOH. The experiment was performed in duplicate. The circular dichroism (CD) spectra were recorded over the range of 300−800 nm on a Jasco 815 spectropolarimeter. The samples contained 1 mM peptide and 0.9 mM Cu(II). The pH was set to 8.2 using NaOH. Concentrations of stock solutions of the peptide were determined by potentiometry.

HPLC Measurements of Ac-GASRHWKFL-NH_2 Hydrolysis Rates. The hydrolysis tests were performed in a fashion analogous to the previous Ni(II) studies.[1−10] Samples containing 1 mM peptide and 1 mM Cu(II) in 20 mM HEPES at pH 9.5 were incubated at temperatures of 37, 45, 60, and 70 °C (controlled within ±0.2 °C) in a thermoblock (J.W. Electronics). Additional experiments were done in a 100 mM borate buffer (pH 9.5). In all cases, the pH of the buffer was adjusted after the complex formation. The pH of the HEPES buffer was measured at 25 °C and not corrected for the temperature effect, as HEPES buffer has a relatively weak temperature dependence.[22] The pH stability of these buffers was also controlled over time. The downward drift did not exceed 0.5 log unit over the reaction time.

Another series of experiments was performed at various pH values between 6 and 10 at 70 °C. The aliquots were collected at various time points, up to 7 days for slow reactions. Previous studies demonstrated that the peptide alone is fully resistant to hydrolysis under such conditions.[6,9] Each 20 μL aliquot was added to 20 μL of 2% (v/v) trifluoroacetic acid (TFA) to stop the reaction, and the final acidified solutions were stored at 4 °C. For analysis, the reaction mixtures were diluted with water (20 μL of water to 20 μL of solution) in inserts and placed in the autosampler carousel. A 10 μL aliquot of each sample was injected into the HPLC system (Empower, Waters), which was equipped with an analytical C18 column. The eluting solvent A was 0.1% (v/v) TFA in water, and solvent B was 0.1% (v/v) TFA in 90% (v/v) acetonitrile. The chromatograms were obtained at 220 and 280 nm. The relative amounts of product (P), intermediate product (IP), and substrate (S) in each chromatogram were calculated by peak integration using Origin 8.1.

The calculations of reaction rates were done in several ways in order to obtain data comparable to those of previous approaches applied in the Ni(II)-dependent hydrolysis studies.[4−10] The equations used depended on the presence of the IP. For the reactions where the IP was detected, the set of three equations Kinet A, Kinet B, and Kinet C was used to obtain the rate constants k_1 and k_2 describing IP formation and decay, respectively. For reactions without detectable IP, Kinet A was used to find the substrate decay rate constant. As a double check, the product formation constant, which in principle is identical to k_1, was fitted using Kinet D. This approach was also used for simplified analysis when the IP was present. In this case, the IP was integrated together with the substrate or the product. The equations Kinet A−D are given below:

$$\text{Kinet A:}\quad y = A_0 e^{-k_1 t}$$

$$\text{Kinet B:}\quad y = \left(\frac{k_1 A_0}{k_2 - k_1}\right)(e^{-k_1 t} - e^{-k_2 t})$$

$$\text{Kinet C:}\quad y = A_0\left[1 + \left(\frac{1}{k_1 - k_2}\right)(k_2 e^{-k_1 t} - k_1 e^{-k_2 t})\right]$$

$$\text{Kinet D:}\quad y = A_0(1 - e^{-k_2 t})$$

In these equations, y is the mole fraction of a given species, t is the time, and A_0 denotes the initial concentration of the substrate.

RESULTS

Cu(II) Complexation of the Ac-GASRHWKFL-NH_2 Peptide. The formation of Cu(II) complexes of Ac-GASRHWKFL-NH_2 was followed using UV−vis spectroscopy over the wavelength range from 300 to 850 nm at pH values

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ranging from 3 to 11 (Figure 1). The spectra indicate the presence of the Cu(II) aqua ion at low pH (λ_{max} = 816 nm), a

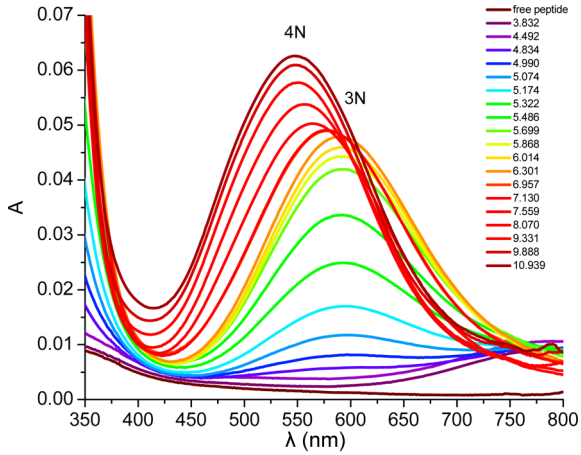

Figure 1. UV−vis spectra obtained by pH-metric titration of 1 mM Ac-GASRHWKFL-NH_2 peptide in the presence of 0.9 mM Cu(II) ions, recorded at 25 °C.

complex species under weakly acidic conditions (pH 5−7; λ_{max} = 593 nm), and another complex under neutral to alkaline conditions (λ_{max} = 550 nm). On the basis of a vast body of literature, we can assign coordination modes of these spectroscopic species as those with three and four nitrogen atoms coordinated equatorially to the Cu(II) ion (in brief, 3N and 4N complexes, respectively).[23,24]

The potentiometric results confirm the spectroscopic ones very well, as illustrated in Figure 2, which matches the

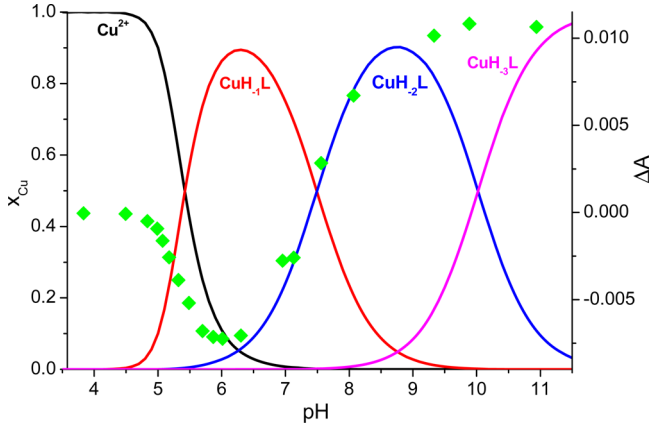

Figure 2. Species distribution for 1 mM Ac-GASRHWKFL-NH_2 peptide and 0.9 mM Cu(II) ions at 25 °C with I = 0.1 M as derived from potentiometry (lines, left scale), compared to the parameter $\Delta A = A_{550} - A_{593}$ derived from UV−vis spectra recorded under the same conditions (green diamonds, right scale).

potentiometric species distribution with the differential absorption at characteristic wavelengths: 593 nm for the 3N complex and 550 nm for the 4N complexes. Table 1 collects the protonation constants of the peptide, which are in good agreement with the data published previously,[6] and the stability constants of three Cu(II) complexes. The distribution of complex species is shown in Figure 2. The first of them corresponds to the spectroscopic 3N species and the latter two to the 4N species. Table 1 also presents the parameters of the

Table 1. Protonation and Cu(II) Stability Constants of the Ac-GASRHWKFL-NH_2 Peptide Obtained from Potentiometric Titrations, the Corresponding pK_a Values, and the Parameters of the UV−Vis Spectra of the Complexes[a]

species	log β	pK_a	λ_{max} (nm)	ε_{max} (M^{-1} cm^{-1})
HL	9.921(5)	9.92[b]		
H_2L	15.984(7)	6.06[c]		
$CuH_{-1}L$	3.112(6)	*5.332(8)*[d]	593	50
$CuH_{-2}L$	−4.380(9)	7.49[e]	550	60
$CuH_{-3}L$	−14.40(1)	10.02[b]	550	67

[a]Standard deviations in the last significant digits of constants are given in parentheses. The pK_a value in italics was obtained from the spectroscopic titration. [b]Lys NH_3^+. [c]His imidazole. [d]Formation of the 3N complex. [e]Formation of the 4N complex.

UV−vis spectra and the assignments of the pK_a values based on literature data and our previous work.[6,23,24] Figure 3 presents

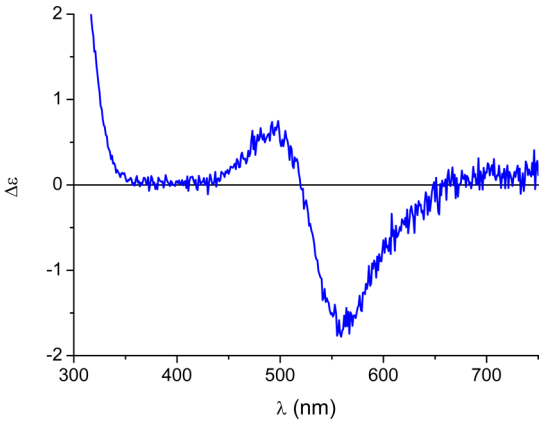

Figure 3. CD spectrum of 1 mM Ac-GASRHWKFL-NH_2 peptide and 0.9 mM Cu(II) ions recorded at pH 8.2 and 25 °C.

the CD spectrum of the $CuH_{-2}L$ complex, recorded at pH 8.5. Its d−d bands, localized at 561 nm ($\Delta\varepsilon$ = −1.6 M^{-1} cm^{-1}) and 492 nm ($\Delta\varepsilon$ = +0.6 M^{-1} cm^{-1}), are also typical for 4N complexes of peptides.[23,24]

Cu(II)-Dependent Peptide Bond Hydrolysis in the Ac-GASRHWKFL-NH_2 Peptide. The hydrolytic activity of Cu(II) ions toward the Ac-GASRHWKFL-NH_2 peptide was studied at various pH values between 6.0 and 10.0 to find out the species specificity of the hydrolysis. These experiments were performed at 70 °C. Another series of kinetic experiments was performed at 37, 45, 60, and 70 °C at pH 9.5 to establish the temperature dependence of the reaction rates. Typically, 1 mM peptide was incubated in the presence of 1 mM Cu(II) in 20 mM HEPES buffer. As the buffering capacity of HEPES is poor at high pH, control experiments were also performed at selected temperatures in a borate buffer at pH 9.5. The results were identical within the standard error limits. The stability of the pH of HEPES-buffered solutions at pH 9.5 was also verified over time. A slow downward drift of pH was observed, not exceeding 0.5 pH unit. The reaction products were separated and quantified using HPLC, and their identities were verified by electrospray ionization mass spectrometry (ESI-MS). Figure 4 shows examples of chromatograms recorded at pH 7.0 and 70 °C. Three major peaks were observed in the chromatograms. The one at the longest retention time (ca. 17 min) belonged to the

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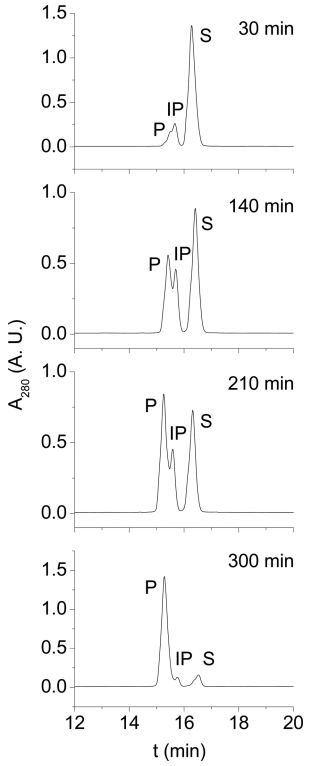

Figure 4. Examples of chromatograms of samples containing 1 mM Cu(II) and 1 mM Ac-GASRHWKFL-NH_2 peptide in 20 mM HEPES (pH 7) incubated at 70 °C. Incubation times are indicated on the respective plots. S denotes the reaction substrate, IP the intermediate reaction product (Ser ester), and P the C-terminal reaction product (SRHWKFL-NH_2 peptide).

reaction substrate, Ac-GASRHWKFL-NH_2 (S). The intermediate reaction product (IP), which was previously characterized as an ester resulting from the acyl shift of the N-terminal Ac-GA moiety to the Ser hydroxyl group, was found at the intermediate retention time.[5] The C-terminal product of hydrolysis, SRHWKFL-NH_2 (P), was detected at the shortest time. The cleaved-off N-terminal product Ac-GA was too hydrophilic to be retained by the C_{18} HPLC column. The IP was not observed above pH 8 because of the fast ester hydrolysis, as noted under these conditions in our previous Ni(II) studies.[5,6,9]

Figure 5 presents examples of calculations of a single first-order rate constant k at pH 9.5 for the purpose of determining the maximum reaction rates at various temperatures. The equations Kinet A and Kinet D were used in this case because of the absence of the IP in the chromatograms. In the presence of the IP, the reaction rates were calculated in two ways. The full treatment according to equations Kinet A, Kinet B, and Kinet C, illustrated in Figure 6, allowed us to calculate two rate

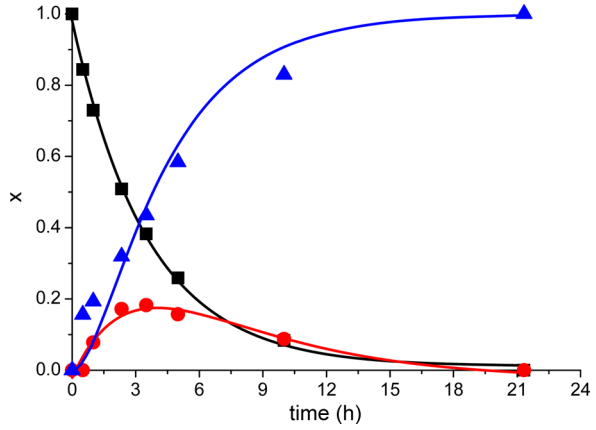

Figure 6. Kinetic plots of S decay (black), IP formation and decay (red), and P formation (blue) and corresponding fits to Kinet A, Kinet B, and Kinet C (lines) for incubation of 1 mM Ac-GASRHWKFL-NH_2 with 1 mM Cu(II) at pH 7.0 and 70 °C.

constants: k_1, which describes the formation of the IP, and k_2, which describes its hydrolysis into P. This approach was complemented by simplified calculations in which a single rate

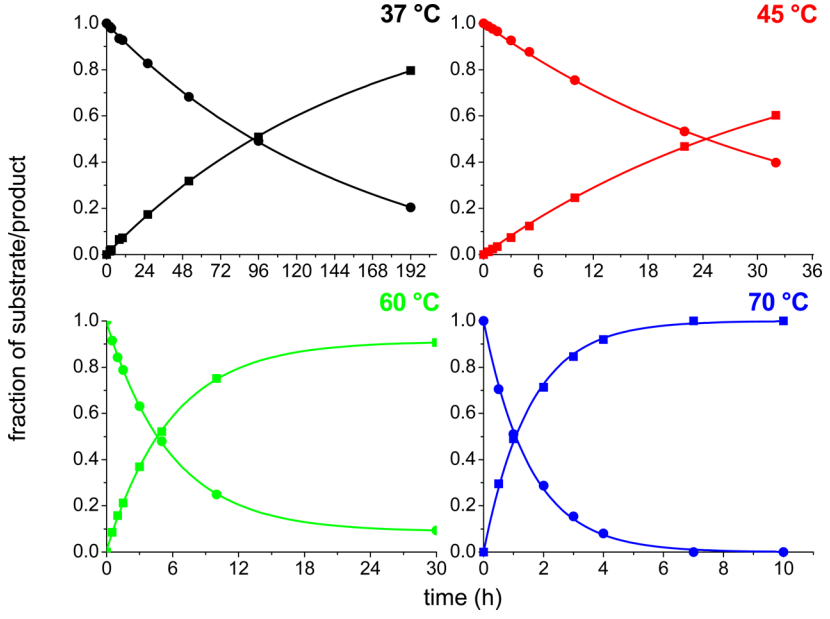

Figure 5. Kinetic plots of S decay and P formation (symbols) and corresponding fits to first-order rate expressions using Kinet A and Kinet D (lines) at various temperatures of incubation of 1 mM Ac-GASRHWKFL-NH_2 with 1 mM Cu(II) at pH 9.5.

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constant for the formation of P, denoted as k, was calculated using Kinet D. Such calculations were performed to maintain a uniform way of rate determination over the broad pH range. We found them also useful for the kinetic analysis at the lowest pH values, because prolonged incubations of the samples at 70 °C resulted in a slight nonspecific deterioration of the reaction substrate at long incubation times. HEPES is known to redox-activate Cu(II) ions under certain conditions.[25] This reactivity may be responsible for the peptide decay at low pH, where a large proportion of Cu(II) ions is not complexed to the peptide and is thus available to interact with HEPES. The kinetic results are summarized in Tables 2 and 3. We found that the simplified

Table 2. First-Order Rate Constants (k) and Corresponding Reaction Half-Times ($t_{1/2}$) for Cu(II)-Dependent Peptide Bond Hydrolysis in the Ac-GASRHWKFL-NH_2 Peptide Incubated at Various Temperatures in 20 mM HEPES Buffer (pH 9.5)

T (°C)	k (10^{-5} s^{-1})[a]	$t_{1/2}$ (h)
70	18.0 ± 0.3	1.1
60	4.74 ± 0.09	4.1
45	0.790 ± 0.008	24.2
37	0.213 ± 0.004	90.4

[a]Error bars are standard deviations.

Table 3. First-Order Rate Constants and Reaction Half-Times for Cu(II)-Dependent Peptide Bond Hydrolysis in the Ac-GASRHWKFL-NH_2 Peptide Incubated at Various pH Values at 70 °C[a]

pH	k (10^{-5} s^{-1})[b]	$t_{1/2}$ (h)	k_2 (10^{-5} s^{-1})[b]
6.0	0.8 ± 0.2	24.9	3.3 ± 0.3
6.3	1.3 ± 0.3	14.9	9.8 ± 0.6
6.6	2.1 ± 0.2	9.0	7.2 ± 1.3
7.0	4.3 ± 0.4	4.5	22.8 ± 6.2
7.3	7.8 ± 0.6	2.5	48.8 ± 8.8
7.5	10.6 ± 0.9	1.8	82.0 ± 12.4
7.7	13.1 ± 0.9	1.5	99.9 ± 11.6
8.0	15.7 ± 0.7	1.2	213 ± 32
9.0	18.5 ± 0.5	1.0	–
10.0	18.0 ± 0.3	1.1	–

[a]The values of k were calculated using Kinet D and represent the formation of the final product P; the IP was integrated together with S. The values of k_2 were calculated using Kinet B and Kinet C and describe the decay of the IP. The values of $t_{1/2}$ were calculated from the k values. [b]Error bars are standard deviations.

treatment provided more accurate results because the low extent of IP formation (below 20% of total peptide in all cases) led to significant uncertainties in the separation of the kinetic process into two steps with rate constants k_1 and k_2. Therefore, Figure 7 contains only k values to illustrate the pH dependence of the hydrolysis. Table 3 provides values of k and k_2. The logarithmic dependence of the latter on pH is illustrated in Figure 8. The comparison of the rate of formation of the hydrolysis product with the potentiometric species distribution (Figure 7) clearly indicates that the 4N species, $CuH_{-2}L$ and $CuH_{-3}L$, are the sole sources of hydrolytic reactivity.

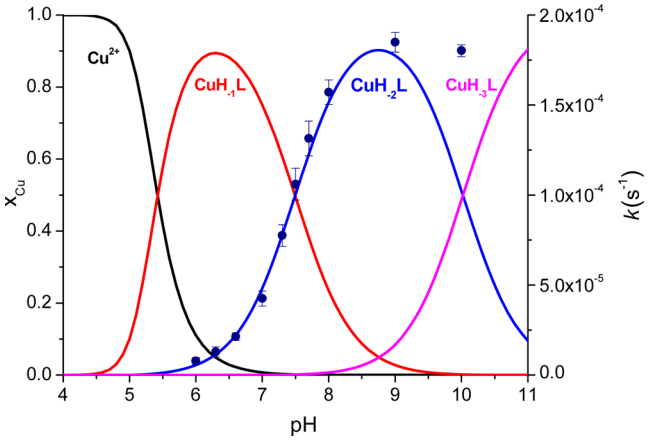

Figure 7. Comparison of the pH dependence of the hydrolysis rate (expressed as reaction product formation) calculated using Kinet D (circles) with the potentiometric species distribution (lines). The error bars indicate statistical errors of rate constant fitting.

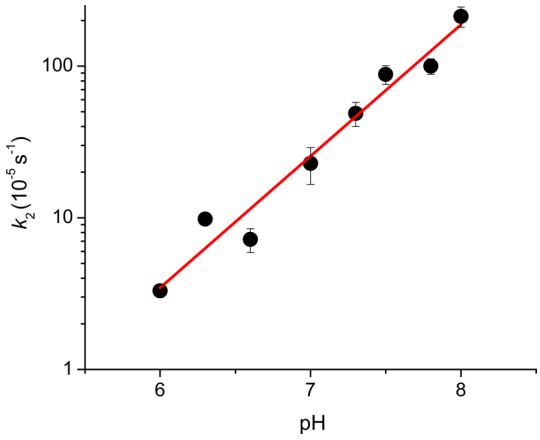

Figure 8. Logarithmic pH dependence of k_2, the first-order rate constant for IP decay.

DISCUSSION

Acid–Base and Cu(II) Complexation Equilibria of Ac-GASRHWKFL-NH_2. The Ac-GASRHWKFL-NH_2 peptide was studied previously in our laboratory in the context of Ni(II)-dependent peptide bond hydrolysis. The pK_a values for the Lys and His side chains determined in this work are 0.1 and 0.03 pH unit higher than those obtained previously, which constitutes a satisfactory agreement.[6]

The stoichiometry and UV–vis spectroscopic properties of the 3N and 4N complexes are fully consistent with coordination of the Cu(II) ion to the pyridine-like nitrogen in the His imidazole and two or three preceding amide bond nitrogens, respectively.[23,24] It is, however, interesting to note that complexes with lesser numbers of coordinated nitrogens (the 1N and 2N complexes) were not detected. The cooperativity of peptide nitrogen coordination to the Cu(II) ion due to the formation of fused chelate rings is a well-known phenomenon in complexes of histidine peptides. It is maximal in peptides containing His at position 3 with respect to the free N-terminus, where 4N complexes tend to form directly in the reaction of the Cu(II) aqua ion and the peptide.[23,24,26−28] In peptides containing a single His residue in the middle of the peptide chain, partial cooperativity has often been detected. The initially formed 1N complex, containing the Cu(II) ion coordinated to the imidazole nitrogen, releases two protons simultaneously, yielding a 3N complex with two preceding peptide nitrogens, typically at pH 6–7. Examples include short

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and long model peptides, prion and histone protein fragments, and angiotensin II.[23,29−43] The cooperativity was even seen in the simplest peptides containing only Gly residues upstream of His.[36] This demonstrates that the propensity to form 3N complexes over 2N complexes originates from conformational preferences of the main peptide chain. In rare cases, however, the cooperativity is partial or even absent.[44,45] This suggests that side chains can sometimes intervene to stabilize the intermediate 2N complex. However, in all previous studies known to us, the 1N complex was easily detectable by UV−vis spectroscopy. The UV−vis spectra and potentiometric data presented in Figures 1 and 2 clearly demonstrate the opposite for the studied peptide.

The absence of the 1N complex may be due either to its particularly low stability or to high stability of the 3N complex. When the fact that the 1N complex with the His imidazole is monodentate is taken into account, its stability must be related to the acidity of the ligand. The His imidazole in the studied peptide is indeed acidic. Its pK_a is 6.06, which is at the bottom of the range of values determined for analogous peptides (6.16−7.13),[29−45] although the difference is small if we take into account the observation that a peptide characterized with pK_a = 6.16 formed a relatively strong 1N complex and exhibited very significant cooperativity of formation of the 3N complex.[40] In contrast, the pK_a of the 3N complex formation measured in this work, 5.33, is extremely low. The complexes studied previously typically exhibited values in the range of 5.8 to 7.[29−44] The lowest values were found for a very strongly cooperative complex of the Ac-IKQHT-NH_2 peptide derived from the human prion protein (5.80)[34] and for the non-cooperative KLAHFG peptide (5.88).[44] Therefore, we can safely state that the 3N complex of the studied peptide forms with a particularly high cooperativity, which includes simultaneous deprotonation and coordination of all three of its nitrogen donors. The 4N complex is formed with pK_a = 7.49, which is also lower than those of most of its counterparts (typically above 8) but, for example, identical to that exhibited by a peptide derived from the Cap43 protein.[33]

The low values of ε_{max} for the $CuH_{-2}L$ and $CuH_{-3}L$ complexes (60 and 67 M^{-1} cm^{-1}, respectively) require consideration, because typical values for His-anchored 4N complexes are in the range of 100−150 M^{-1} cm^{-1}. However, lower values were also found on several occasions for peptides forming such complexes. Values of 79, 84, 86, and 105 M^{-1} cm^{-1} were published for a series of alloferon analogues.[46] An even lower value, 53 M^{-1} cm^{-1} was obtained for one of the model peptides derived from prion Cu(II) binding sequences (other peptides studied in that series had typical values of >100).[47] In our recent study on a hydrolytic peptide derived from the sequence of filaggrin, Ac-QAASSHEQA-NH_2, we found ε_{max} values of 81 and 88 M^{-1} cm^{-1} for the Cu(II) complexes of the hydrolysis substrate and product, respectively.[10] In another of our recent studies, regarding Cu(II) complexes of peptides containing β-alanine, we determined values as low as 65 and 74 M^{-1} cm^{-1}.[48] The low ε_{max} values are related to a relatively higher complex symmetry and may indicate that the geometry of the nitrogen donors around the Cu(II) deviates less from the tetragonal one than usual.

We can summarize this part of the discussion by stating that the Ac-GASRHWKFL-NH_2 peptide binds Cu(II) with a very high affinity in its class, 1−2 log units stronger than most of the peptides containing a single His residue in the middle of their peptide chains. This ability is analogous to that seen by us previously with respect to Ni(II) ions.[6,9]

Cu(II)-Dependent Peptide Bond Hydrolysis in Ac-GASRHWKFL-NH_2. The perfect correlation of the pH-dependent distribution of the 4N complexes with the rate of hydrolysis of the Ala-Ser bond in the Ac-GASRHWKFL-NH_2 peptide convincingly demonstrates that the 4N complex is the sole species enabling this reaction. Taking into account the fact that the course of hydrolysis, including the pH-dependent detection of the intermediate reaction product, was identical to that seen previously for Ni(II) reactions with this and other related peptides,[1−10] we can propose that the molecular mechanisms of the reactions enabled by Ni(II) and Cu(II) ions are also identical and include the (N−O) acyl shift leading to the formation of the intermediate Ser ester, which hydrolyses to yield the final reaction product. Figure 8 shows that the rate constant of ester hydrolysis depends exponentially on pH, which is a standard behavior in the hydrolysis of simple esters catalyzed by hydroxide ions.[49] Therefore, we can see that this ester hydrolysis is spontaneous. This result is in agreement with previous Ni(II) data, which showed that the k_2 values for different peptides recorded under the same conditions were very similar to each other.[9] Scheme 1, adapted from our previous papers, presents the main steps of this reaction, in which the metal ion plays a structural role.[6,10] With this fact established, we can compare the hydrolytic abilities of Cu(II) and Ni(II) ions. Figure 9 presents the Arrhenius plot showing

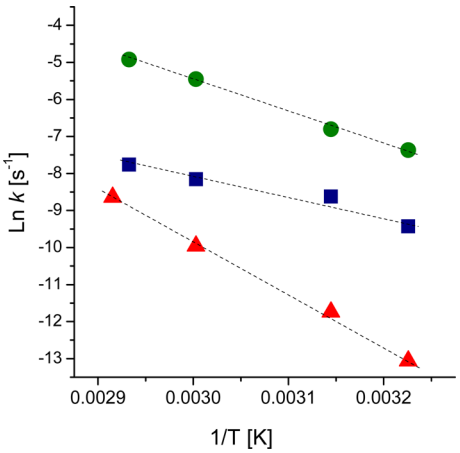

Figure 9. Arrhenius plots showing the temperature dependence of the first-order rate constant of Cu(II) hydrolysis of 1 mM Ac-GASRHWKFL-NH_2 peptide at pH 9.5 (triangles) compared with the results of analogous Ni(II) hydrolysis studies of this peptide at pH 8.2 (squares) and 11.6 (circles).

the temperature dependence of the Cu(II) reaction rate k at pH 9.5 and comparing it with the previously published Ni(II) data for pH 8.2 and 11.6.[6] Table 4 provides the corresponding numerical values.

The data in Figure 6 and Table 3 show that the maximum reaction rate for Cu(II) ions is obtained at pH 8.5 and higher because of the 100% abundance of the hydrolytically active complex. For Ni(II) ions, the analogous situation takes place at pH 11.6. Therefore, these two sets of data provide a comparison of the hydrolytic abilities of these two metal ions. This is expressed by the ratios of rate constants at pH 11.6 (last column of Table 4). Because of a slightly different temperature dependence, the ratio changes from 300 at 37 °C to 41 at 70

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Table 4. Comparison of First-Order Hydrolysis Rate Constants Obtained under Analogous Conditions for Cu(II) and Ni(II) Hydrolyses of 1 mM Ac-GASRHWKFL-NH_2 Peptide [Ni(II) Data Were Taken from Our Previous Study[6]]

	k (10^{-5} s^{-1})			fold higher by Ni(II)	
T (°C)	Cu(II), pH 9.5	Ni(II), pH 8.2	Ni(II), pH 11.6	pH 8.2	pH 11.6
70	17.7	42.7	727	2.4	41
60	4.74	28.7	428	6	90
45	0.79	18	111	23	140
37	0.21	8.05	63	38	300

°C. The ratio at slightly alkaline pH 8.2 is about 10 times lower, which is due to the small extent of formation of the hydrolytic complex for Ni(II) at this pH. On average, the Ni(II) ions hydrolyze the peptide bond preceding the Ser residue about 100 times faster than the Cu(II) ions, but the difference is smaller at higher temperatures. On the other hand, the Cu(II) ion is capable of forming 4N complexes at much lower pH than Ni(II). We found that the hydrolysis rate constant k is strictly proportional to the relative abundance of 4N complexes for both Cu(II) and Ni(II). Therefore, using the ratios of maximal reaction rates at high pH, given in the last column of Table 4, we can calculate the pH values at which Cu(II) might be a more efficient hydrolytic agent than Ni(II). These calculations are illustrated in Figure 10. Depending on the temperature, this

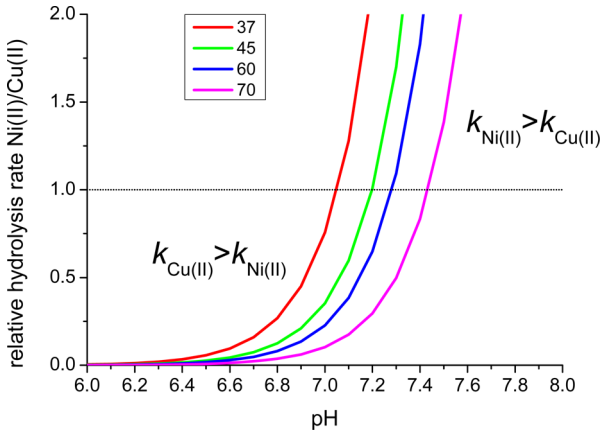

Figure 10. pH dependence of the relative reaction rate for Cu(II)- and Ni(II)-dependent hydrolysis of Ac-GASRHWKFL-NH_2 at various temperatures.

happens below pH 7.45 (at 70 °C) to 7.05 (at 37 °C). We previously demonstrated that Ni(II) ions can cleave proteins in the nucleui of living cells, which have pH around 7.[11] This striking coincidence suggests that Cu(II) ions should be taken into serious consideration as hydrolytic agents for cellular proteins.

We previously established that the hydrolysis reaction rate depends very strongly on the spatial orientation of side chains of the metal-ion-binding amino acid residues.[9] The bond lengths and overall metal binding geometries in the respective Cu(II) and Ni(II) complexes shown in Scheme 1 are similar.[50,51] Therefore, the difference between the reaction rates for the Cu(II) and Ni(II) complexes is due to details of their structures, most likely the conformations of chelate rings.[6,9] Further research will be required to explain this effect fully. Such research is warranted by the fact that Cu(II)-dependent hydrolysis may be relevant in the physiological pH range.

CONCLUSION

Our prior research established the sequence specificity and molecular mechanism of nickel-dependent peptide bond hydrolysis before Ser/Thr in the R_N-(Ser/Thr)-Xaa-His-Zaa-R_C sequences. The -SRHW- sequence embedded in the Ac-GASRHWKFL-NH_2 test peptide was found to be the most prone to hydrolysis within the library of -SXHZ- and -TXHZ- tetrapeptide sequences.[5,6] The experiments using this reference peptide that have been presented above allowed us to extend the reaction itself, as well as its molecular mechanism established for Ni(II), over to Cu(II) ions. The reaction occurs solely in 4N complexes and involves an acyl shift of the N-terminal peptide moiety to the alcoholic (Ser or Thr) side chain. However, despite the close similarity of the structures of the active complexes, the maximum rate of the Cu(II)-dependent reaction is on average ca. 100 times lower than that of the Ni(II)-dependent one. The greater ability of Cu(II) ions to form 4N complexes at lower pH partially compensates for this difference, resulting in the similar hydrolytic activities of the two ions around pH 7. These interesting facts will be the basis for further research into the molecular mechanism and biological relevance of the studied reaction. In regard to biotechnological applications, despite the requirement of harsh reaction conditions, copper hydrolysis may have practical applications in the field of thermostable proteins and in cases where Ni(II) ions may be unsuitable because of their potential toxicity.

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Notes

The authors declare no competing financial interest.

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