Thermodynamic Study of Cu$^{2+}$-Binding to the DAHK and GHK Peptides by Isothermal Titration Calorimetry (ITC) with the Weaker Competitor Glycine

Ana Trapaidze$^{a,b,c}$, Christelle Hureau$^{a,b,*}$, Wojciech Bal$^{d}$, Mathias Winterhalter$^{c}$, Peter Faller$^{a,b,*}$

$^{a}$ CNRS ; LCC (Laboratoire de Chimie de Coordination) ; 205, route de Narbonne, F-31077 Toulouse, France
$^{b}$ Université de Toulouse ; UPS, INPT ; LCC ; F-31077 Toulouse, France
$^{c}$ Jacobs University Bremen, Campusring1, D-28759 Bremen, Germany
$^{d}$ Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawinskiego 5a, 02-106 Warsaw, Poland

*corresponding authors:
P. Faller e-mail: peter.faller@lcc-toulouse.fr C. Hureau e-mail: christelle.hureau@lcc-toulouse.fr Fax: 0033/561553003
Abstract

The peptides Asp-Ala-His-Lys (DAHK) and Gly-His-Lys (GHK) are naturally occurring copper(II)-chelating motives in human serum and cerebrospinal fluid. Here the sensitive thermodynamic technique isothermal titration calorimetry (ITC) has been used to study the energetics of copper(II) binding to DAHK and GHK peptides in the presence of the weaker ligand glycine as a competitor. DAHK and GHK bind Cu(II) predominantly in a 1:1 stoichiometry with conditional dissociation constants (i.e. at pH 7.4, in the absence of the competing chelators glycine and HEPES buffer) of \( 2.6 \pm 0.4 \times 10^{-14} \) M and of \( 7.0 \pm 1.0 \times 10^{-14} \) M, respectively. Furthermore, the apparent \( \Delta H \) values were measured and the number of protons released upon Cu(II) binding was determined by performing experiments in different buffers. This allowed us to determine the conditional \( \Delta G, \Delta H, \) and \( \Delta S \), i.e. corrected for the contributions of the weaker ligand glycine and the buffer at pH 7.4. We found that the entropic and enthalpic contributions to the Cu(II)-binding to GHK and DAHK are distinct, with a higher enthalpic contribution for GHK. The obtained thermodynamic parameters correspond well to those in the literature obtained by other techniques, suggesting that the use of the weaker ligand glycine as a competitor in ITC provides accurate data for Cu(II)-binding to high affinity peptides, which cannot be accurately determined without the use of a competitor ligand.
**Introduction**

Copper is an important trace element in all aerobic organisms, involved in several vital processes [1]. On the one hand, copper is important as a catalytic center of many enzymes and its deficiency leads to a decline of metabolic activity of the cell or of the organism due to decreased production of active enzymes [2]. On the other hand, copper as cofactor has redox capabilities and its excess promotes uncontrolled production of potentially damaging ROS (reactive oxygen species) through Fenton and Haber–Weiss reactions. ROS induce oxidative stress and deleterious modifications in macromolecules [3].

A healthy human body contains up to 110 mg of copper, with about 9 mg present in the brain. The recommended intake of copper for adults is 0.9 mg/day with an upper limit of 10 mg/day [1]. Alterations in the regulation of copper homeostasis result in severe diseases, like Menkes and Wilson diseases, and may be involved in several neurodegenerative disorder, e.g. prion diseases, Alzheimer’s disease, etc. [4].

In Alzheimer’s disease, copper is shown to play an important role as it forms a complex with the peptide amyloid-β (Aβ) and modulates its aggregation [5]. In addition, Aβ-bound copper can catalyze the production of ROS via redox cycling of the Cu in the presence of natural reducing agents such as ascorbate [6]. And as a consequence, Cu²⁺ chelation leading to its redox silencing is investigated as a therapeutic approach for Alzheimer’s disease [7, 8].

![Scheme 1](image)

**Scheme 1**: Cu²⁺ coordination of peptides with the N-terminal sequence Xxx-Xxx-His (ATCUN motive like DAHK) (left) and Xxx-His (like GHK) (right). GHK has an equatorial labile coordination position (X) and it can form ternary complexes.

DAHK (Asp-Ala-His-Lys) is the N-terminal metal binding sequence of human serum albumin (HSA) [9, 10] which is the major protein in the blood and in the cerebrospinal fluid and is involved in Cu transport [11, 12]. Cu²⁺ is bound to the so called ATCUN motif [9], which is also found in many
other peptides/proteins and is characterized by a free NH$_2$-terminus, an amino acid different from proline in the second position and a histidine residue in the third position. Cu$^{2+}$ is bound in a slightly distorted square planar geometry by 4 nitrogen atoms: the N-terminal amine, the two amide functions from Asp-Ala and Ala-His peptide bonds and the N$_5$ from the imidazole ring of His (Scheme 1, left) [9].

In recent studies it was demonstrated that a chemically synthesized DAHK can inhibit copper-induced oxidative DNA double-strand breakage and telomere shortening in cell cultures [13]. It was also determined that DAHK prevents lipid oxidation in a copper-catalyzed oxidant system [14]. The DAHK peptide as well as HSA was shown to be able to remove copper rapidly and stoichiometrically from Aβ, to restrict Aβ aggregation, to suppress the catalytic ROS production and to reduce neurotoxicity [15].

The tripeptide GHK is a copper binding growth factor of plasma. In contrast to DAHK, GHK doesn’t possess the ATCUN motif. In solution it coordinates Cu$^{2+}$ acting as a tridentate chelator involving the N-terminal amino group of glycine, the amidyl nitrogen atom from the Gly-His peptide bond and the N$_5$ of the His imidazole ring (Scheme 1, right). There is evidence that the fourth equatorial position is preferably occupied by an oxygen donor provided by an external ligand [16, 17].

GHK has been shown to stimulate the growth and improve viability of several types of cultured cells and organisms, including neuronal cells. This tripeptide also actively participates in the processes of wound healing and tissue repair [16]. It was also reported to compete successfully with Aβ for Cu$^{2+}$ coordination, thus being able to act as a potential protector from Cu-Aβ toxicity [12, 15, 18].

Despite well characterized and widely studied functions of DAHK and GHK and of their copper complexes, the energetic parameters of DAHK and GHK coordination are less investigated. Remelli and coworkers [19] reported thermodynamics of Cu$^{2+}$ coordination to GHK by using potentiometry and isoperibolic calorimetry. For DAHK no data could be found, but the related Lys-Gly-His-Lys (KGHK) peptide was analyzed by the same methods [20]. Wilcox and coworkers studied the Cu$^{2+}$ binding to the related Gly-Gly-His and bovine serum albumin (having a DTHK motive) by ITC with the weaker chelator Tris (2-Amino-2-(hydroxyméthyl)propane-1,3-diol) at pH 9.1 [21, 22].

The aim of our study was to determine the thermodynamic parameters of Cu$^{2+}$ binding to DAHK and GHK peptides using isothermal titration calorimetry in the presence of the weaker ligand glycine at pH 7.4. A weaker ligand is necessary in order to measure the otherwise too strong Cu$^{2+}$ binding [23]. We were able to show that these ITC measurements were in agreement with the structural and thermodynamic values from the literature, suggesting that the here applied methodology might be useful for other less well-characterized copper-binding peptides, like amyloid-β [23, 24], prion, α-synculein etc.
Materials and Methods

Materials: GHK peptide (sequence Gly-His-Lys) was bought from Bachem Company (Bubendorf, Switzerland). DAHK peptide (sequence Asp-Ala-His-Lys) was bought from Bachem (Switzerland) or GeneCust (Dudelange, Luxembourg). N-terminal β-amyloid peptide Aβ16 (sequence Asp-Ala-Glu-Phe-Arg-His-Ser-Gly-Tyr-Glu-Val-His-Glu-Lys) was obtained from GeneCust (Dudelange, Luxembourg). Chemicals CuSO₄, glycine, buffers N-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS), 2-(4-(2-hydroxyethyl)-1-piperazinyl)ethanesulfonic acid (HEPES), 1,4-Piperazinediethanesulfonic acid (PIPES), and N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic Acid (TES) were obtained from Sigma-Aldrich.

Sample preparation: The stock solutions of peptides were prepared by dissolving appropriate amounts of solid peptides in deionized milli-Q water and stored at 253K in a freezer. Under these conditions aggregation and/or degradation of the peptides was avoided. Concentrations of GHK and DAHK were determined by titration followed by UV-Vis absorption spectroscopy. In a typical experiment, the stock solution was ten-fold diluted and aliquots of a Cu²⁺ solution of known concentration were added until no increase in the d-d band of the Cu²⁺-peptide complex and turbidimetry due to unbound Cu²⁺ precipitation in the buffer were observed. Measurements were performed in 0.1 M phosphate buffer (pH 7.4). This experimental determination led to concentration values that are 10-20% off compared to those obtained using the molecular mass of the peptide and its counterions, suggesting that counterion salts co-precipitate during the peptide synthesis. The concentration of Aβ16 was estimated by absorption spectroscopy at 276 nm using the molar extinction coefficient ε₂₇₆= 1410 L mol⁻¹ cm⁻¹ based on the Tyr residue absorption. In all experiments a concentrated (100 mM) Cu²⁺ solution was used, obtained by dissolving CuSO₄ in ultrapure water. Metal ions and buffer solutions were kept at 281 K (refrigerator).

Absorption Spectroscopy (UV-Visible) measurements: UV-Visible spectra were recorded at room temperature with single beam Agilent 8453 UV-Vis spectrophotometer. For absorption measurements a 500 µL volume quartz cuvette was used (1 cm path length). The blank measurements were conducted for the 80 mM 2-(4-(2-hydroxyethyl)-1-piperazinyl)ethanesulfonic acid (HEPES) buffer.

ITC measurements: Isothermal titration calorimetry measurements were performed at a constant atmospheric pressure and a constant temperature of 303±0.1 K, using an ultrasensitive VP-ITC micro calorimeter (Microcal, Northampton, USA). Copper(II) and peptides solutions were prepared in the same buffer. The pH was measured twice - before and after each experiment. All solutions were degassed for at least 5 minutes by stirring under vacuum to eliminate air bubbles right before each experiment. The reference power was set at 25 µcal/sec, which provided an accurate baseline and a sufficient power compensation for the exothermic binding events investigating. To achieve
homogeneous mixing in the cell, the stirrer speed was kept constant at 300 rpm. A 100 seconds pre-
tritation delay was set to allow for the creation of a pre-titration baseline. During each experiment up
to 30 injections of 10 µL titrant were performed with a spacing of 300 seconds. The heat of dilution
was determined under identical conditions by injecting the metal ion solution into the cell containing
only the sample buffer. The titration data were analyzed using the software provided by the
manufacturer (Origin for ITC). The corrected binding isotherms were fitted using least squares
regression for one set of the binding site model to obtain the apparent association constant (K app), the
number of peptide molecules bound to per Cu²⁺ complex (stoichiometry, n), and the enthalpy change
associated with the interaction (ΔH).

Study of Cu²⁺ binding to Aβ16 peptide: The binding of Cu²⁺ to Aβ16 was studied previously using
ITC [23-25]. Since to the scope of our work included a comparison of thermodynamic profiles of
Cu(II) binding to Aβ16 and DAHK/GHK peptides, we reproduced some experiments reported in the
literature[23]. For this reason a 0.7 mM Cu²⁺ solution stabilized by glycine at a four-fold molar excess
were titrated into 0.08 mM Aβ16 solution. Both solutions were prepared in 80 mM HEPES buffer at
pH 7.4.

Study of Cu²⁺ binding to DAHK and GHK peptides: The binding of Cu(II) to DAHK and GHK was
studied analogously to the Aβ16 experiments. 0.7 mM Cu(II) stabilized with 2.8 mM Gly was injected
into 0.08 mM peptide solution. 80 mM HEPES buffer at pH7.4 was used as a medium.

Influence of weaker ligand (Gly) concentration on Cu²⁺ binding affinity: For studying an effect of Gly
concentration on binding affinity of copper, a set of experiments were carried out in 80 mM HEPES
buffer with 2.8, 10, 20, 30, 40 or 50 mM Gly at pH 7.40±0.02. The same HEPES-Gly medium was
used for the Cu²⁺ (0.7 mM) and peptide (DAHK or GHK, 0.08 mM) solutions.

Calculations of affinity constant of Cu²⁺ binding to peptides in presence of competitor ligand

1-Definition of the absolute K_d for metal M and ligand L:

\[ M + L \rightleftharpoons ML \]

\[ K_d^L = \frac{[M][L]}{[ML]} \]

2-Definition of the conditional K_d for metal M and ligand L, in different protonation states:

\[ L + H^+ \rightleftharpoons LH^+ \]

\[ K_{d1}^L = \frac{[H^+][L]}{[LH^+]} \]
$\text{LH}^+ + \text{H}^+ \rightleftharpoons \text{LH}_2^{2+}$

$$K_{d_2}^{L} = \frac{[H^+]^n[LH]}{[LH_2^{2+}]}$$

$$[\text{LH}^+] = \frac{[H^+]^n[L]}{K_{d_1}^{L}}$$

$$[LH_2^{2+}] = \frac{[H^+]^n[\text{LH}^+]}{K_{d_2}^{L}} = \frac{[H^+]^2[L]}{K_{d_1}^{L} K_{d_2}^{L}}$$

$$cK_{d_1}^{L} = \frac{[M][L]}{[ML]} \left( 1 + \frac{[H^+]}{K_{d_1}^{L}} + \frac{[H^+]^2}{K_{d_1}^{L} K_{d_2}^{L}} \right)$$

$$cK_{d_2}^{L} = K_{d_1}^{L} \left( 1 + 10^{-\alpha H + pK_{d_1}^{L}} + 10^{-2\alpha H + pK_{d_1}^{L} + pK_{d_2}^{L}} \right)$$

More generally, for a ligand with $n$ protonation states: $cK_{d}^{L} = K_{d}^{L} \left( \sum_{i=0}^{n} 10^{-\alpha_i H + i \Sigma_{j=1}^{i} pK_{d_1}^{L}} \right)$

3-Definition of the apparent $\text{app} K_d$ for metal $M$, ligand $L$, competitor $C$ and buffer $B$:

$M + L \rightleftharpoons ML$

$$K_{d_1}^{L} = \frac{[M][L]}{[ML]}$$

$M + C \rightleftharpoons MC$

$$K_{d_1}^{C} = \frac{[M][C]}{[MC]}$$

$MC + C \rightleftharpoons MC_2$

$$K_{d_2}^{C} = \frac{[MC][C]}{[MC_2]}$$

$M + B \rightleftharpoons MB$

$$K_{d}^{B} = \frac{[M][B]}{[MB]}$$

$$\text{app} K_{d_1}^{L} = \frac{[L][M] + [MC] + [MC_2] + [MB]}{[ML]}$$

$$\text{app} K_{d}^{L} = \frac{[L][M]}{[ML]} \left( 1 + \frac{[C]}{K_{d_1}^{C}} + \frac{[C]^2}{K_{d_1}^{C} K_{d_2}^{C}} + \frac{[B]}{K_{d}^{B}} \right)$$
More generally, for a competitor and a buffer with complexes formation varying from MC to MCn and MB to MBn:

\[ appK^L_d = K^L_d \left( 1 + \sum_{i=1}^{n} \prod_{j=1}^{i} K^C_{d_i} + \sum_{p=1}^{m} \prod_{q=1}^{p} K^B_{d_i} \right) \]

In the particular case of the buffer, \([B] \approx [B]^0\) because B is in a high excess compared to M or L.

For competitor C, the equation is solvable when \([C] \gg [M] \Rightarrow [C] \approx [C]^0\). Then,

\[ appK^L_d = \frac{[L][M]}{[ML]} \left( 1 + \frac{[C]^0}{K^L_d} + \frac{[C]^0}{K^C_{d_1} K^C_{d_2} + K^B_{d_1}} \right) \]

Note that in the case of ligand L, competitor C and buffer B with several protonation states, \(K^L_d, K^C_d\) and \(K^B_d\) should be replaced by \(\epsilon K^L_d, \epsilon K^C_d\) and \(\epsilon K^B_d\), respectively.

**4. Definition of the experimental apparent \(appK_d\) for metal M, ligand L, competitor C which form a ternary MLC complex:**

\(M + L \rightleftharpoons ML\)

\[ K^L_d = \frac{[M][L]}{[ML]} \]

\(M + C \rightleftharpoons MC\)

\[ K^C_d = \frac{[M][C]}{[MC]} \]

\(ML + C \rightleftharpoons MLC\)

\[ K^{LC}_d = \frac{[ML][C]}{[MLC]} \]

\[ appK^L_d = \frac{[L][M] + [MC] + [MLC]}{[ML]} \]

\[ appK^L_d = K^L_d \left( 1 + \frac{[C]}{K^L_d} + \frac{[C][L]}{K^C_d K^{LC}_d} \right) \]

This equation is solvable if \([C] \gg [M] \Rightarrow [C] \approx [C]^0\) and \([L] \gg [M] \Rightarrow [L] \approx [L]^0\), then:

\[ expK^L_d = K^L_d \left( 1 + \frac{[C]^0}{K^C_d} + \frac{[C]^0[L]^0}{K^C_d K^{LC}_d} \right) \]
Note that in the case of ligand L and competitor C with several protonation states, $K_d^L$ and $K_d^C$ should be replaced by $^cK_d^L$ and $^cK_d^C$, respectively.

The apparent $^appK_d$ is the data obtained directly from measurement.

5-The case of the Cu-DAHK complex.

DAHK denotes the -COO$^-$ (C-term and Asp side chain), His, NH$_2$ (N-term) and NH$_3^+$ (Lys) protonation state of the peptide;

DAHK-H$^+$ denotes the -COO$^-$ (C-term and Asp side chain), HisH$^+$, NH$_2$ (N-term) and NH$_3^+$ (Lys) protonation state of the peptide;

DAHK-H$_2^+$ denotes the -COO$^-$ (C-term and Asp side chain), HisH$^+$, NH$_3^+$ (N-term) and NH$_3^+$ (Lys) protonation state of the peptide.

$$appK_d^{DAHK} = condK_d^{DAHK}$$

$$\times \left(1 + \frac{[Gly]_0}{K_{d_1}^{Gly}} \times \frac{1}{1 + 10^{-pH+pk_{a}^{Gly}}} + \frac{[Gly]_0^2}{K_{d_1}^{Gly} K_{d_2}^{Gly}} \times \frac{1}{1 + 10^{-pH+pk_{a}^{Gly}}^2} \right)$$

$$+ \frac{[Hepes]_0}{K_d^{Hepes} \left(1 + 10^{-pH+pk_{a}^{Hepes}}\right)}$$

Using values obtained for $[Gly]_0 \geq 10mM$; $[Hepes]_0 = 80mM$, $pk_{a}^{Hepes} = 7.41$, $K_d^{Hepes} = 10^{-3.22}$ [26]; $pk_{a}^{Gly} = 9.53$, $K_{d_1}^{Gly} = 10^{-8.2}$, $K_{d_2}^{Gly} = 10^{-6.9}$ (from the NIST data base [27]), we found an average conditional $condK_d^{DAHK}$ value of $2.6 \times 10^{-14}$ M.

6-The case of the Cu-GHK complex.

GHK denotes the -COO$^-$ (C-term), His, NH$_2$ (N-term) and NH$_3^+$ (Lys) protonation state of the peptide;

GHK-H$^+$ denotes the -COO$^-$ (C-term), HisH$^+$, NH$_2$ (N-term) and NH$_3^+$ (Lys) protonation state of the peptide;

GHK-H$_2^+$ denotes the -COO$^-$ (C-term), HisH$^+$, NH$_3^+$ (N-term) and NH$_3^+$ (Lys) protonation state of the peptide.
In the present case, \( K_{d}^{GHK} \) and \( K_{d}^{Gly, GHK} \) are not known. The value of the ratio \( \frac{[GHK]}{K_{d}^{Gly, GHK}} \) has been chosen in the way that the \( K_{d}^{GHK} \) value obtained for various \([Gly]_0\) have a minimal standard deviation over the mean value.

Using values obtained for \([Gly]_0 \geq 10mM; [Hepes]_0 = 80 mM, pK_{d}^{Hepes} = 7.41, K_{d}^{Hepes} = 10^{-3.22} \) (ref : [26]); \( pK_{d}^{Gly} = 9.53, K_{d1}^{Gly} = 10^{-8.2}, K_{d2}^{Gly} = 10^{-6.9} \) [27]; we found an average conditional \( K_{d}^{GHK} \) value of 7.0 \( \times 10^{-14} \).

It is worth noting that we haven’t taken into account the possibility of having the ternary GHK-Cu-GHK species which only predominates for high GHK to Cu ratio, and such do not disturb significantly determination of the \( K_d \). This holds also for the determination of the number of protons displaced via the measurement of \( \Delta H \) ion different buffers (see also below).

Calculation of conditional \( \Delta H \) from the literature: The reported absolute \( \Delta H \) for the species present at pH 7.4 of Cu\(^{2+}\) binding to GHK and KGHK (the closest analogue found for DAHK), i.e. - 94 kJ /mol and - 132 kJ/mol were corrected for the \( \Delta H \) of the protonation of the N-terminal amine, His and lysine(s) in GHK and KGHK by taking into account the protonation states at pH 7.4 ( via Henderson-Hasselbalch equation and the pKa’s reported in the same references) [19, 20].

Study of proton exchanged during Cu\(^{2+}\) binding to peptides: For studying proton exchanged during the binding of Cu\(^{2+}\) to DAHK and GHK peptides ITC experiments were performed in buffers with different ionization enthalpies (Table 1) [28]. For both peptides, the Cu\(^{2+}\) solution in the syringe and DAHK or GHK in the sample cell were dissolved in 80 mM TAPS, TES, HEPES or PIPES buffers at
pH 7.40±0.02. During each experiment 0.7 mM Cu$^{2+}$ solution stabilized with 2.8 mM Gly was injected into the cell containing 0.08 mM peptide solution.

**TABLE 1**: Ionization enthalpy change and ionization constants of buffers

<table>
<thead>
<tr>
<th>Buffers</th>
<th>PIPES</th>
<th>HEPES</th>
<th>TES</th>
<th>TAPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta H_{\text{ion}}$ kcal/mol</td>
<td>2.77</td>
<td>5.14</td>
<td>7.72</td>
<td>9.9</td>
</tr>
<tr>
<td>pK</td>
<td>6.71</td>
<td>7.45</td>
<td>7.42</td>
<td>8.38</td>
</tr>
</tbody>
</table>

Calculation of conditional binding affinity as function of formation constants $\beta$:

$$L + iH \rightleftharpoons LH_i$$

$$\beta_i^H = \frac{[LH_i]}{[L][H]^i}$$

$$M + L + jH \rightleftharpoons MLH_j$$

$$\beta_j^M = \frac{[MLH_j]}{[M][L][H]^j}$$

$$cK_d' = \frac{[M] \sum_i [LH_i]}{\sum_j [MLH_j]} = \frac{\sum_i \beta_i^H [H]^i}{\sum_j \beta_j^M [H]^j} = \frac{\sum_i 10^{-i\cdot pH + \log(\beta_i^H)}}{\sum_j 10^{-j\cdot pH + \log(\beta_j^M)}}$$
Results and Discussion

Isothermal titration calorimetry (ITC) is a very-well suited method to measure directly the enthalpy of a reaction and to deliver a model based binding stoichiometry as well as dissociation constants [29]. With these parameters the reaction entropy can be calculated. ITC can also be applied to study the interaction of metal ions with biomolecules (for a recent review see [30]). In our study, we used this approach to examine the interaction of Cu\(^{2+}\) with DAHK and GHK peptides.

The reported conditional dissociation constant of GHK and analogues of DAHK are about 1 x 10\(^{-14}\) M [19, 20, 31-36]. Although in a 100mM HEPES buffer the affinity will decrease to a \(^{\text{app}}\)K\(_d\) of about 10\(^{-12}\) M at pH 7.4 [26], this affinity is still too high to be measured directly by ITC. Thus we applied the competitive titration method using glycine as a competitor, following the approach published to study the Cu\(^{2+}\) binding to the A\(\beta\) peptide [23, 24]. In the presence of the weaker competitor glycine, the apparent \(^{\text{app}}\)K\(_d\) will be higher and hence accessible by ITC measurements.

First, the Cu\(^{2+}\)-binding to A\(\beta\)16 was measured under the same conditions applied by Hatcher et al. [23]. This enabled the comparison of our measurement with the literature data. We also could compare the Cu-binding to DAHK/GHK directly with that to A\(\beta\)16, which is of a physiological interest (see Introduction). Figure 1 (right) shows the integrated data of Cu\(^{2+}\)-A\(\beta\)16 (for raw data see Suppl. Mat.). The curve agrees nicely to the one in the literature [23].

ITC measurement of Cu\(^{2+}\)-binding to DAHK and GHK (Figure 1, left) under the same conditions as for A\(\beta\)16, showed an exothermic reaction with a steep titration curve. Obviously the enhanced steepness of the titrations suggests that both DAHK and GHK bind Cu\(^{2+}\) stronger than A\(\beta\)16. The fitting yielded the apparent \(^{\text{app}}\)K\(_d\) values of 21.2 x 10\(^{-9}\) M and 4.4 x 10\(^{-9}\) M for GHK and DAHK, respectively. This is about 400 (DAHK) and 80 times (GHK) stronger than the \(^{\text{app}}\)K\(_d\) of A\(\beta\)16 of 1.7 x 10\(^{6}\) M. In addition, the fitting also confirmed the copper-peptide stoichiometry of one binding site in each peptide.
These apparent Kd values were determined with Cu$^{2+}$ ion stabilized by four equivalents of Gly, acting as a weaker competitor. Its presence increased apparent Kd, but a higher excess was required for a better defined steepness and hence the apparent Kd of the titration curves. Regarding Cu$^{2+}$ binding to DAHK and GHK peptides, in order to obtain more accurate data, glycine concentration was further increased (Figure 2).

**Figure 1**: Binding isotherm curves of calorimetric titrations. Integrated data of 30 injections (10 µL per injection) of 0.7 mM copper with 2.8 mM glycine into 0.08 mM peptides: GHK and DAHK (left), and Aβ16 (right). Experiments were performed in 80 mM HEPES buffer at pH 7.4 and at 303K. Solid line represents least-square fits of the data to a one set of binding site model.
Experiments were carried out in presence of 2.8, 10, 20, 30, 40 and 50 mM glycine concentrations both in the syringe and reaction cell. Measurements were conducted in 80 mM HEPES at pH 7.4. As expected, the increase of glycine concentration decreased the steepness of the binding isotherms, suggesting that experimental binding affinities of peptides decreased as well (Figure 2 and Tables 1 and 2).

The dependence of the apparent dissociation constant (appK_a) of GHK and DAHK on the Gly concentration is listed in Tables 1 and 2. The higher the glycine concentration the higher are the appK_a for both DAHK and GHK peptides, in line with the competition of glycine for Cu^{2+}-binding. appK_a is lower for DAHK compared to GHK at all glycine concentrations, indicating that DAHK is a stronger Cu^{2+} ligand.

Figure 2: Integrated data of 30 injections (10 µL per injection) of 0.7 mM copper into 0.08 mM peptide GHK (A) and DAHK (B) in presence of 2.8, 10, 20, 30 and 40 mM glycine both in the syringe and the sample cell. Experiments were performed in 80 mM HEPES buffer at pH 7.4 and at 303K. Solid line represents least-square fits of the data to a one set of binding site model.
TABLE 2: Enthalpy, entropy, free energy changes and dissociation constants of copper binding to DAHK peptide in presence of different concentrations of the weaker ligand glycine. \( \text{app} K_d \): apparent dissociation constant; \( \text{cond} K_d \): conditional dissociation constant (see material and methods section for definitions).

<table>
<thead>
<tr>
<th>Gly (mM)</th>
<th>( \text{app} \Delta H ) (cal/mol)</th>
<th>( \text{app} \Delta S ) (cal/K/mol)</th>
<th>( \text{app} \Delta G ) (cal/mol)</th>
<th>( \text{app} K_d ) (M)</th>
<th>( \text{cond} K_d ) (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.8</td>
<td>-7.5 x 10^3</td>
<td></td>
<td></td>
<td>Not well defined</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>-8.3 x 10^3</td>
<td>3.1</td>
<td>-9.3 x 10^3</td>
<td>2.0 x 10^{-7}</td>
<td>2.9 x 10^{-14}</td>
</tr>
<tr>
<td>20</td>
<td>-8.7 x 10^3</td>
<td>-0.37</td>
<td>-8.6 x 10^3</td>
<td>5.9 x 10^{-7}</td>
<td>2.2 x 10^{-14}</td>
</tr>
<tr>
<td>30</td>
<td>-7.4 x 10^3</td>
<td>1.8</td>
<td>-7.9 x 10^3</td>
<td>1.8 x 10^{-6}</td>
<td>3.0 x 10^{-14}</td>
</tr>
<tr>
<td>40</td>
<td>-6.9 x 10^3</td>
<td>2.9</td>
<td>-7.8 x 10^3</td>
<td>2.5 x 10^{-6}</td>
<td>2.3 x 10^{-14}</td>
</tr>
<tr>
<td>50</td>
<td>-6.9 x 10^3</td>
<td>1.5</td>
<td>-7.4 x 10^3</td>
<td>4.9 x 10^{-6}</td>
<td>2.9 x 10^{-14}</td>
</tr>
</tbody>
</table>

* the curve was too steep to accurately determine \( \text{app} K_d \) and hence \( \Delta S \), \( \Delta G \), and \( \text{cond} K_d \).

TABLE 3: Enthalpy, entropy, free energy changes and dissociation constants of copper binding to GHK peptide in presence of different concentrations of the weaker ligand glycine. \( \text{app} K_d \): experimental dissociation constant; \( \text{cond} K_d \): conditional dissociation constant; (see material and methods section for definitions).

<table>
<thead>
<tr>
<th>Gly (mM)</th>
<th>( \text{app} \Delta H ) (cal/mol)</th>
<th>( \text{app} \Delta S ) (cal/K/mol)</th>
<th>( \text{app} \Delta G ) (cal/mol)</th>
<th>( \text{app} K_d ) (M)</th>
<th>( \text{cond} K_d ) (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.8</td>
<td>-9.1 x 10^3</td>
<td>-2.0*</td>
<td>-8.5 x 10^3*</td>
<td>7.5 x 10^{-7}*</td>
<td>6.0 x 10^{-14}</td>
</tr>
<tr>
<td>10</td>
<td>-9.3 x 10^3</td>
<td>-5.4</td>
<td>-7.6 x 10^3</td>
<td>3.1 x 10^{-6}</td>
<td>6.4 x 10^{-14}</td>
</tr>
<tr>
<td>20</td>
<td>-9.4 x 10^3</td>
<td>-7.8</td>
<td>-7.1 x 10^3</td>
<td>8.1 x 10^{-6}</td>
<td>7.6 x 10^{-14}</td>
</tr>
<tr>
<td>30</td>
<td>-9.7 x 10^3</td>
<td>-9.8</td>
<td>-6.7 x 10^3</td>
<td>1.4 x 10^{-5}</td>
<td>8.0 x 10^{-14}</td>
</tr>
<tr>
<td>40</td>
<td>-9.4 x 10^3</td>
<td>-9.6</td>
<td>-6.5 x 10^3</td>
<td>1.9 x 10^{-5}</td>
<td>7.3 x 10^{-14}</td>
</tr>
<tr>
<td>50</td>
<td>-8.5 x 10^3</td>
<td>-6.9</td>
<td>-6.4 x 10^3</td>
<td>2.3 x 10^{-5}</td>
<td>6.6 x 10^{-14}</td>
</tr>
</tbody>
</table>

* the curve was too steep to accurately determine \( \text{app} K_d \) and hence \( \Delta S \), \( \Delta G \), and \( \text{cond} K_d \).
It is important to note that the heat measured by ITC, and as a consequence the thermodynamic parameters $^{app}K_d$ and $^{app}\Delta H$, stem from the entire reaction occurring in the sample cell and therefore it is apparent. This means, that in the present case the measured heat is the sum of several events, including not only the release of the Cu$^{2+}$ from the glycine and Cu$^{2+}$ binding to the peptides, but also the changes in the protonation state of peptide, glycine and buffer. To determine the presence of protonation/deprotonation coupling, ITC titrations in buffers with different ionization enthalpy were performed. The difference observed in $^{app}\Delta H$ value intitrations, which differ only in their type of buffer, can be ascribed to proton exchange with the buffer [37].

Thus, to examine whether proton exchange is occurring when the copper binds to DAHK and GHK peptides a set of ITC experiments based on 0.7 mM Cu$^{2+}$ titrations into 0.08 mM peptide were conducted in presence of PIPES, TES and TAPS buffers at pH 7.4.

Buffer selection was made in the same family (Good’s buffer) reported to have weak interaction with Cu$^{2+}$ ions [38]. Despite their distinct ionization enthalpies, they have pKa’s in range of 6.7-8.4 and could be used as buffer at pH 7.4. In addition, their Cu$^{2+}$ coordination capability is low and therefore they don’t compete too much for metal binding with peptides. The analysis is based on the assumption, that by changing the buffer only the ionization enthalpies change. This means that buffers do not interact substantially with Cu$^{2+}$, with the peptide, or with Cu$^{2+}$-peptide.

Experimental data suggest that in the case of GHK, changing the buffer induces no significant difference among enthalpy (Fig. 3, left). However, for DAHK obvious dependence on buffer was shown (Fig. 3, right).
To analyze the data and calculate the number of protons exchanged, enthalpy changes of \( \text{Cu}^{2+} \) binding to GHK and DAHK peptides in different buffers were plotted as a function of the ionization enthalpy of the used buffer (Fig 4) (see Exp. Sect.). Since the character of the dependence should be linear, corresponding trend line was selected. The equations of these lines are given in the upper right corner of the graphs. The slope corresponds to the number of exchanged protons when one \( \text{Cu}^{2+} \) binds to the peptide and the intercept gives the value of the enthalpy change corrected for the contribution of the buffer protonation.

**TABLE 4**: Apparent Enthalpy of \( \text{Cu}^{2+} \) binding to DAHK and GHK peptides in presence of different buffer mediums.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>DAHK ( \Delta H ) (cal( \times )mol(^{-1} ))</th>
<th>GHK ( \Delta H ) (cal( \times )mol(^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIPES</td>
<td>(-6.3 \times 10^3)</td>
<td>(-9.4 \times 10^3)</td>
</tr>
<tr>
<td>HEPES</td>
<td>(-8.7 \times 10^3)</td>
<td>(-9.4 \times 10^3)</td>
</tr>
<tr>
<td>TES</td>
<td>(-9.4 \times 10^3)</td>
<td>(-8.8 \times 10^3)</td>
</tr>
<tr>
<td>TAPS</td>
<td>(-1.1 \times 10^4)</td>
<td>(-9.6 \times 10^3)</td>
</tr>
</tbody>
</table>
The analysis revealed a slope of 0.008 for GHK peptide, indicating that during the binding there is no proton exchange occurring with buffer, while in the case of DAHK peptide the slope was 0.56, which demonstrated that proton exchange occurred during the Cu$^{2+}$ binding.

To determine how many protons are released during the reaction, the pKa values of DAHK and GHK peptides need to be taken into consideration. For GHK the pKa values of amine and histidine are equal to 7.85 and 6.45 [19], while for DAHK these values are 7.53 and 6.28 [34]. Substituting these pKa into the Henderson-Hasselbalch equation, it is possible to estimate how many protons can be released by certain groups on average. Thus, at pH 7.4 GHK releases 1.87 protons (1 from the deprotonation of the Gly-His peptide bond and 0.89 from deprotonation of the -NH$_3^+$ and His side chain that are partially protonated) and DAHK releases 2.65 protons (2 from the deprotonation of the Asp-Ala and Ala-His peptide bonds and 0.64 from deprotonation of the -NH$_3^+$ and His side chain that are partially protonated) (See Appendix in the Supporting Information).

This seems not to fit with the experimental data. However, one has also to consider that Cu$^{2+}$ has been injected as a Cu$^{2+}$-Gly$_2$ complex, in which the amine is deprotonated. When Cu$^{2+}$ binds to DAHK and GHK peptides, two Gly ligands will be released and their amine functions will get protonated (pKa of 9.6) (Scheme 2).
As a consequence, the two protons released from GHK are taken up by two liberated glycine molecules and hence no proton exchange with buffer should be observed. Indeed, experimental data and their analysis proved absence of proton release in buffer.

In the DAHK case, two out of the 2.64 protons released are captured by the Gly ligands and thus 0.64 remaining protons are exchanged with buffer. The number of released protons in buffer obtained through analysis of experimental data is 0.56, which is in well agreement with theoretical calculations.

So far we discussed only the measured experimental thermodynamic parameters, which include the entire reaction. In order to obtain the thermodynamic parameter (here called conditional) of $\text{Cu}^{2+}$-binding to DAHK and GHK at pH 7.4, the measured parameter $\text{app} K_d$ and $\text{app} \Delta H$ have to be corrected by the reactions with glycine and buffer, which leads to $\text{cond} K_d$ and $\text{cond} \Delta H$. The contribution to $\text{app} \Delta H$ of the protonation of the buffer can be corrected by extrapolating the measurements with different buffer (see Fig. 4) to a buffer of $\Delta H_{\text{ionization}} = 0$, i.e. -9.3 kcal/mol for GHK and -5.2 kcal/mol for DAHK. The $\Delta H$ protonation and $\text{Cu}^{2+}$-binding to glycine are reported, and hence their contribution can be calculated [27]. These results summarized in Table 5 suggest that in the case of DAHK the $\text{Cu}^{2+}$ binding reaction is driven by entropy only, since enthalpy is positive. In contrast, for GHK entropic and enthalpic contributions are favorable. The purely entropic driven binding of $\text{Cu}^{2+}$ to DAHK might be explained by the release of water (from the aqua complex of $\text{Cu}^{2+}$ and the peptide) and release of protons from the peptide. Note that we corrected for the contribution of glycine and buffer. The entropic contribution is thus responsible for the stronger affinity of DAHK compared to GHK. This might be explained by the fact that in the case of GHK but not in DAHK a fourth

**Scheme 2**: Scheme of copper coordination by (A) GHK and (B) DAHK. At first copper is stabilized by two glycine molecules, when copper binds to peptide it liberates bound glycine molecules. Free glycine binds hydrogen displaced by copper during binding to peptide.
equatorial ligand is bound (see Scheme 1, termed X and also below) which might increase the order of the system.

The \( \Delta H \) for GHK has been reported in the literature, based on measurements with an isoperibolic calorimeter [19]. Calculating the \( \Delta H \) at pH 7.4 by including the \( \Delta H \) and protonation state of the Cu\(^{2+}\) ligands, yields a value of \( \Delta H = -1.3 \) kcal / mol [19]. No \( \Delta H \) for DAHK could be found, but for it has been determined for the analogue peptide KGHK [20], and based on the absolute values a \( \Delta H \) of -0.5 kcal was calculated (see Materials and Methods). These values are relatively similar to the one determined here, and the larger difference in the case of DAHK versus KGHK can be explained by the different pKa values of the ligands [20, 34].

**TABLE 5:** Conditional enthalpy, entropy, free energy changes and dissociation (and association) constants of Cu\(^{2+}\) binding to GHK and DAHK peptides deduced from ITC. Conditional means that values are at pH 7.4 but they are corrected for the influence of the weaker ligand glycine and HEPES buffer.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>( K_d ) (mol)</th>
<th>( K_a ) (mol(^{-1}))</th>
<th>( \Delta H ) (cal(\times)mol(^{-1}))</th>
<th>( \Delta S ) (cal(\times)K(^{-1})(\times)mol(^{-1}))</th>
<th>( \Delta G ) (cal(\times)mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAHK</td>
<td>2.6x10(^{-14})</td>
<td>3.9x10(^{-13})</td>
<td>2.7 x10(^{3})</td>
<td>72</td>
<td>-1.9x10(^{4})</td>
</tr>
<tr>
<td>GHK</td>
<td>7.0x10(^{-14})</td>
<td>1.4 x10(^{13})</td>
<td>-1.4 x10(^{3})</td>
<td>55</td>
<td>-1.8x10(^{4})</td>
</tr>
</tbody>
</table>

Similarly, the conditional dissociation constant (\( K_d \)) can be calculated from the present data because the dissociation constants of glycine and HEPES have been determined in the literature (see also material and methods section for details). For DAHK this calculation was more straightforward because ternary complexes of Cu-DAHK with either glycine or HEPES have not been reported (in line with a complete equatorial plane). The calculations showed that the \( K_d \) is identical (in the limits of the experimental error) under all conditions as expected with a value of 2.6 +/- 0.4 x 10\(^{-14}\) M. This is in excellent agreement with the \( K_d \) of 1.6 x 10\(^{-14}\) M reported for the C-terminal amidated DAHK by potentiometry [32] as well as other similar peptides (Table 6).

For GHK, the analysis is a little more complicated as it has been shown that Cu-GHK can make ternary complexes with other ligands like glycine, histidine [39], DAHK [31] or a second GHK, although with a lower affinity than the first one [19]. The \( K_d \) was calculated by correcting for the competition with glycine and HEPES, as well as for the formation of a ternary complex with glycine (the formation of [Cu(GHK)\(_2\)] was neglected as the slope of the isotherm responsible for the Kd is obtained for stoichiometry close to 1:1). The calculations showed that the \( K_d \) equals 7.0 +/- 1.0 x 10\(^{-14}\) M., in good agreement with the \( K_d \) calculated from the literature (based on potentiometry) (Table 6).
TABLE 6: Comparison of the conditional dissociation constants ($K_d^{\text{cond}}$) at pH 7.4 for Cu$^{2+}$ binding to GHK and DAHK peptides obtained by ITC with the constants calculated from the literature for GHK and analogues of DAHK peptides (See Experimental Sections for details).

<table>
<thead>
<tr>
<th>peptide</th>
<th>$K_d^{\text{cond}}$(7.4)</th>
<th>method</th>
<th>peptide</th>
<th>$K_d^{\text{cond}}$(7.4)</th>
<th>method</th>
</tr>
</thead>
<tbody>
<tr>
<td>GHK</td>
<td>0.7 x 10$^{-13}$ M</td>
<td>ITC</td>
<td>DAHK</td>
<td>2.6 x 10$^{-14}$ M</td>
<td>ITC</td>
</tr>
<tr>
<td>GHK</td>
<td>0.6/0.9 x 10$^{-13}$ M</td>
<td>[31] EPR/potentiometry</td>
<td>DAHK-$NH_2$</td>
<td>1.7 x 10$^{-14}$ M</td>
<td>[34] potentiometry</td>
</tr>
<tr>
<td>GHK</td>
<td>1.8 x 10$^{-13}$ M</td>
<td>[19] potentiometry</td>
<td>DAH-$NH_2$</td>
<td>2.0 x 10$^{-14}$ M</td>
<td>[35] potentiometry</td>
</tr>
<tr>
<td>GHK</td>
<td>1.9 x 10$^{-13}$ M</td>
<td>[40] potentiometry</td>
<td>DAH-$NMe$</td>
<td>1.9 x 10$^{-14}$ M</td>
<td>[36] potentiometry</td>
</tr>
<tr>
<td>GHK</td>
<td>2.4/3.0 x 10$^{-13}$ M</td>
<td>[33] EPR/potentiometry</td>
<td>KGHK</td>
<td>12 x 10$^{-14}$ M</td>
<td>[20] potentiometry</td>
</tr>
</tbody>
</table>

Conclusions

The binding of Cu$^{2+}$ to the two biological relevant peptides GHK and DAHK was analyzed by ITC at pH 7.4. As DAHK and GHK bind Cu$^{2+}$ too strongly for a direct measurement of $K_d$ by Cu$^{2+}$ titration, the weaker Cu$^{2+}$-ligand glycine was added, like it has been done for the peptide Aβ16 [24]. In contrast to most of the other, often longer, biologically relevant peptides, Cu$^{2+}$-binding to GHK and DAHK are structurally and potentiometrically well characterized. For GHK also enthalpy measurements by isoperibolic calorimetry are available [19]. The present ITC study provides thermodynamic parameters in line with most reports in the literature, validating the method of using the weaker Cu$^{2+}$-ligand glycine in ITC measurements. The present ITC measurements were performed at a concentration of 0.08 mM peptide, in contrast to 4-9 mM used for the isoperibolic calorimetry measurements [19]. Thus ITC with the weaker ligand glycine can be a useful method for determination of thermodynamic parameters for Cu$^{2+}$-binding to more complex systems, like the less well characterized peptides/proteins, Aβ, α-synuclein, prion etc., and can also give the information about the presence and affinities of an eventual ternary complex peptide-Cu-glycine.

In principle, the ITC measurements to determine the thermodynamic parameters the can be expanded to other metal ions and/or weaker competing ligands, under the conditions that the thermodynamic data of the interaction of the metal ion with the weaker ligands are well characterized and that the contribution of the weaker ligand and the one of the buffer are corrected for.

The here obtained conditional dissociation constants of copper(II) for the DAHK and GHK peptides by ITC were $2.6 +/- 0.4$ x 10$^{-14}$ M and of $7.0 +/- 1.0$ x 10$^{-14}$ M, respectively. In other words DAHK binds Cu(II) 2-3 times stronger than GHK. This indicates that the binding of an additional amidyl in DAHK compared to GHK does not increase dramatically the affinity, because the gain via
the chelate effect of an additional metal-cycle is counteracted by the energy needed to displace the proton from the amide, which has a very high pKa. By analogy, one would expect that the amidyl coordination in GHK does not contribute much to the affinity, which explains that the bidentate histidine ligand, which forms stable metallacycle with Cu²⁺, can compete effectively with GHK and DAHK for Cu²⁺-binding [41].

The stronger Cu²⁺-binding of DAHK compared to GHK at pH 7.4 might be modulated in biological conditions as GHK can make ternary complexes with other ligands (like glycine, histidine etc), and hence GHK is likely to be able to compete with DAHK type sites (ATCUN) in biological systems [40]. Moreover, human serum albumin (HSA) containing a DAHK motive has a lower affinity than DAHK, [32, 34] and hence GHK is perhaps a stronger Cu²⁺ chelator than HSA [40]. Moreover, the present measurements confirm clearly that either DAHK or GHK are much stronger Cu²⁺ chelators by at least an order of magnitude than the peptide Aβ at pH 7.4.

**Acknowledgements:** We would like to thank Prof. Dr. Laurent Paquerau (IPBS, Toulouse) for access to the ITC.
References

Supplementary Materials:

*Raw data of Isothermal titration calorimetry experiments*

Figure S1: Raw data of 0.7mM copper titration into 0.08 mM Aβ16 peptide. Copper was stabilized by 2.8mM Gly. Measurements were performed in 80 mM HEPES buffer at pH 7.4 and 303K.
Figure S2: Raw date of 0.7mM copper titration into 0.08 mM GHK (A, C, E) or DAHK (B, D, F) peptide in presence of 2.8 mM (A, B), 20 mM (C, D) and 50 mM (E, F) of Gly. Measurements were performed in 80 mM HEPES buffer at pH 7.4 and 303K.
Figure S3: Raw date of 0.7mM copper titration into 0.08 mM GHK (A, C, E) or DAHK (B, D, F) in 80 mM PIPES (A, B), TES (C, D) or TAPS (E, F) buffer at pH 7.4 and 303K.
**Calculation of protonation states of GHK and DAHK peptides**

In order to calculate protonation state of peptides was used Henderson-Hasselbalch equation.

\[
pH = pK_a + \log \frac{[A^-]}{[AH]} \quad (A1)
\]

Where \([A^-]\) is concentration of deprotonated species and \([AH]\) is concentration of protonated species at particular pH. Assuming, that in one mole of substance \([AH]\) is molar concentration of protonated species, then

\[\text{[A']} = 1 - [AH] \quad (A2)\]

Substituting equation A2 into A1 and solving it for \([AH]\), we get following:

\[\frac{[AH]}{1 + 10^{pH - pK_a}} = \frac{1}{1 + 10^{pH - pK_a}} \quad (A3)\]

\([AH]\) shows concentration of species in one mole substance, which can release proton and get deprotonated.

In both peptides (GHK and DAHK) there are two groups which might be protonated: NH\textsubscript{2}-terminus and imine nitrogen of imidazole ring.

In the case of GHK peptide, pKa values of NH\textsubscript{2} and N from imidazole are 7.85 and 6.45 respectively. However, in DAHK peptide pKa for NH\textsubscript{2} is 7.53 and for imidazole imine 6.27. Substituting these values in A3 equation, we get that at pH 7.4 in one mole GHK peptide amount of protonated NH\textsubscript{2} and imidazole imine is 0.76 and 0.13 mole correspondingly. This means that one mole GHK peptide is able to release in total of 0.89 mole protons from N terminus and imidazole ring. Similar calculations for DAHK show that from one mole peptide in total of 0.65 mole hydrogen atoms can be displaced from NH\textsubscript{2} and histidine imine (0.58 and 0.065 moles respectively).