Peer-reviewed version available at *Int. J. Mol. Sci.* **2018**, *19*, 2271; doi:10.3390/ijms19082271

Research Article

Investigations of the copper peptide hepcidin-25 by LC-MS/MS and NMR[1]

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Abstract:

Hepcidin-25 was identified as the main iron regulator in the human body by binding to the sole iron-exporter ferroportin. Studies showed that the N-terminus of hepcidin is responsible for this interaction, the same N-terminus that encompasses a small copper(II)-binding site known as ATCUN (amino terminal Cu(II)- and Ni(II)- binding) motif. Interestingly, this copper-binding property is largely ignored in most papers dealing with hepcidin-25. In this context, detailed investigations of the formed complex of hepcidin-25 with copper could reveal insights into its biological role. The present work is mainly focused on the study of the metal-bound form of hepcidin-25, which could be considered the biologically active form. The first part is devoted to the reversed-phase chromatographic separation of copper-bound and copper-free hepcidin-25, which was achieved by applying basic mobile phases containing 0.1% ammonia. Further, mass spectrometry (tandem mass spectrometry MS/MS, high resolution mass spectrometry HRMS) and nuclear magnetic resonance (NMR) spectroscopy were employed to characterize the copper-peptide. Lastly, a 3D model of hepcidin-25 with bound copper(II) is presented. The identification of metal complexes and potential isoforms and isomers, from which the latter usually are left undetected by mass spectrometry, led to the conclusion that complementary analytical methods are needed to characterize a peptide calibrant or reference material comprehensively. Quantitative nuclear magnetic resonance (qNMR), inductively-coupled plasma mass spectrometry (ICP-MS), ion-mobility spectrometry (IMS) and chiral amino acid analysis (AAA) should be considered among others.

Keywords: Hepcidin-25; copper; nickel; ATCUN motif; metal complex; MS; NMR structure; metal peptide, metalloprotein; metallopeptide, isomerization, racemization, purity, reference material

[1] Dedicated to Dr. Norbert Jakubowski on the occasion of his 65th birthday.

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Peer-reviewed version available at Int. J. Mol. Sci. 2018, 19, 2271; doi:10.3390/ijms19082271

1. Introduction

Hepcidin as main iron regulator

Hepcidin has attracted much attention ever since its discovery in 2001 [1] as the main player in iron homeostasis. The production of this peptide hormone is activated in the liver by highly iron-saturated transferrin, the carrier protein of iron, and by inflammation stimuli. The release of interleukin-6 (IL-6) induces the synthesis and secretion of hepcidin-25 (Hep-25) by hepatocytes [2, 3]. Circulating hepcidin binds to ferroportin-1, also known as the iron-regulated transporter-1, expressed mainly on the membrane surface of duodenum, spleen and liver cells (Fig. 1). The interaction of plasmatic hepcidin with ferroportin - the sole protein responsible for cellular iron efflux into the blood stream - induces its internalization and proteolysis, resulting in inhibition of intestinal iron absorption [4].

Hepcidin isoforms and their bioactivity

N-terminal truncated isoforms (mainly Hep-20, -22, -24) have been described as breakdown products of Hep-25 [5, 6]. In cellular studies, the bioactivity of these isoforms was assessed by monitoring the internalization of ferroportin. Experiments using alanine mutants, in which each residue of the N-terminal region of hepcidin-25 was individually replaced with alanine, established the importance of the structure of the N-terminus for the interaction of Hep-25 with ferroportin and thus for the biological activity of this 25 amino acid peptide in iron homeostasis [7]. Recently, similar studies reported that Hep-24 presents a 10-fold lower activity compared to Hep-25, and the activities of both Hep-20 and Hep-22 were at least 10-fold lower than that of Hep-24 [6]. In addition, it was shown that the antimicrobial activity of human Hep-25 was enhanced in presence of copper [8]. A recent study on trout hepcidin-25 showed similar results [9]. Melino et al. have demonstrated that the same N-terminal region is responsible for the metal binding capacity of hepcidin-25. In that study, the authors addressed the affinity of hepcidin-25 for Ni^{2+} and Cu^{2+} based on the presence of a small amino-terminal metal binding site known as ATCUN motif in the structure of the peptide [10].

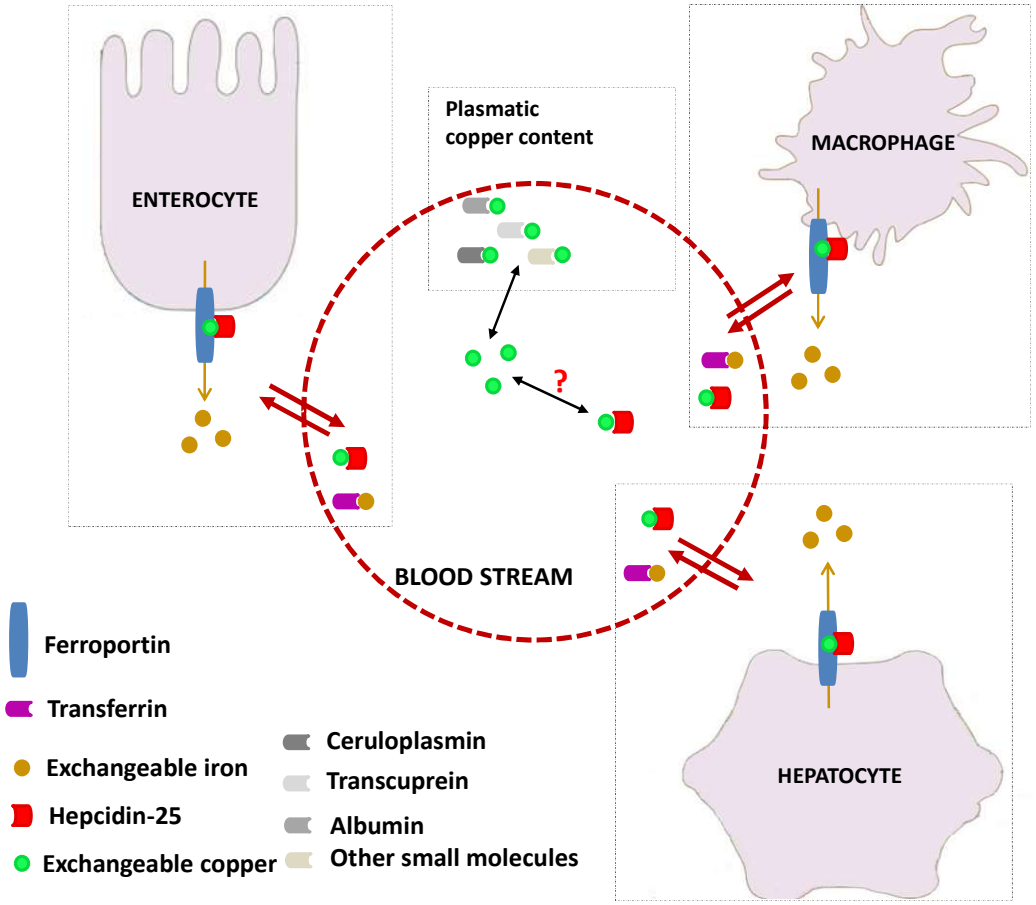

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Fig 1 – Iron homeostasis. Suggested iron and copper traffic in the blood stream. Ferroportin (blue) is expressed on the membrane surface of iron releasing cells (enterocytes, hepatocytes and macrophages), allowing the iron ions (yellow) to exit the cell (yellow arrow) and bind transferrin (pink) for transport through the blood stream. Circulating hepcidin-25 (red) blocks the iron export by binding to ferroportin. Copper ions (green) released from intestinal cells are bound by various molecules in blood. Iron and copper ions are exchanged between various complexing agents based on chemical affinities and biological needs.

Disulfide connectivity

Intensively discussed work was performed over almost a decade to reveal the solution structure of this peptide hormone. The latest findings show the major form of hepcidin (Hep-25) as a cationic peptide consisting of 25 amino acid residues that forms a distorted beta sheet stabilized by four intramolecular disulfide bonds [11]. Earlier structural studies of Hep-25 reported the connectivity of the disulfide bonds to be Cys7 to Cys23, Cys10 to Cys22, Cys11 to Cys19, in addition to a rather unusual vicinal bond between Cys13 and Cys14 [12]. Seven years later, Jordan et al proved this connectivity to be incorrect and reported the currently accepted disulfide pairing for human hepcidin-25 as follows: Cys7 and Cys23, Cys11 and Cys19, Cys10 and Cys13, and Cys14 and Cys22 [11], forming a rather compact structure. It is important to note that, recent publications refer (only) to the older 3D structure illustrating that the community is still not fully aware of the current state of structural knowledge and its relevance. Curiously, the paper from Jordan et al. does not refer to the interaction of Hep-25 to metals, despite of the ATCUN motif established several years before within the structure of the peptide [10]. The practical reason why synthetic and natural hepcidin-25 have not been found to contain metals is the low pH needed for extraction and purification, which leads to a quantitative loss of any metals bound to the peptide. Although metal ions are lost under acidic pH, their presence should not be ignored at physiological or higher pH.

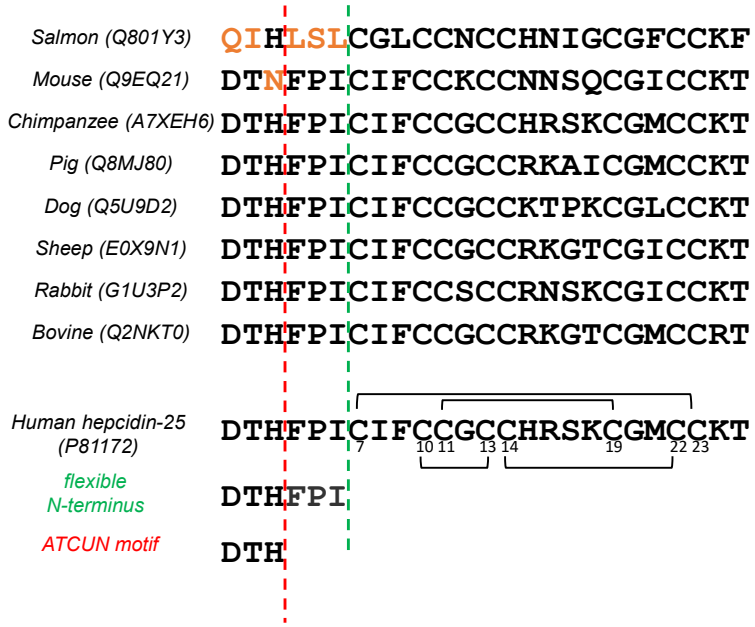


Fig. 2 - *Amino acid sequences of human hepcidin-25 [11], its N-terminus and the ATCUN motif. Hepcidin sequences of various species are shown [13].*

ATCUN motif

The ATCUN motif has been studied for more than 50 years [14-17]. It is a metal binding site specific for the coordination of Cu^{2+} and Ni^{2+}, present at the amino terminus of several naturally occurring proteins [18-20]. It consists of four nitrogen atoms in the first three N-terminal amino acids, involving the free α-NH_2 nitrogen, two following amide nitrogen atoms and the imidazole nitrogen of a histidine residue in the third position. The nitrogen atoms act as metal ligands, forming a distorted square planar geometry [14, 21]. Several biological functions were reported in peptides and proteins

due to the presence of the ATCUN motif, such as anticancer and antimicrobial activity or Cu chelation and transportation [22].

The most well-known and highly abundant protein containing this metal-binding site is human serum albumin (HSA), with the ATCUN motif responsible for its role in the transport of metal ions, including copper [16]. HSA accommodates Cu^{2+} in its binding site, and this represents only 15% of the total plasmatic copper. The rest is bound to ceruloplasmin (65%), transcuprein (12%), and other serum components of low molecular weight [23] (Fig.1). McMillin et al. report a "free" extracellular copper concentration of 1-2 μM ("unbound" fraction), that was determined by separating the bound fractions of copper through ultrafiltration, using a molecular weight cutoff of 30 kDa, which eliminates albumin, ceruloplasmin, and other proteins expected to bind copper [24], and would lead the access of other small molecules such as Hep-25 to copper ions. However, due to above described limitations to isolate the Hep-25-Cu(II) complex, it is still a matter of discussion whether hepcidin-25 (2.8 kDa) does exist in the human body in the copper-bound form or not (Fig.1). In humans, the physiological level of this peptide is varying from <0.5 nM to around 15 nM [25].

Analysis of hepcidin-metal complexes

The interaction of human Hep-25 with metals was explored by some previous studies. Initially, Farnaud et al. suggested that linear hepcidin-25 binds iron, using MALDI-MS (matrix-assisted laser desorption/ionization mass spectrometry) analysis [26, 27]. Tselepis et al. tested the ability of several transition ions (Cu^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+} and Ni^{2+}) to form complexes with the folded hepcidin-25. Their study employing FTICR-MS (Fourier-transform ion cyclotron resonance mass spectrometry) showed no evidence of complex formation in the case of ferrous and ferric ions, while copper, nickel and zinc ions all bound Hep-25 with a sequence of highest-to-lowest affinity as follows: $Cu^{2+} > Ni^{2+} > Zn^{2+}$ [28]. The dissociation constant of the hepcidin-25-copper(II) complex was reported to be $<< 10^{-6}$ M. Kulprachakarn et al. further characterized the affinity of Hep-25 for copper using MALDI-MS, with a reported dissociation constant of $10^{-7.7}$ M. However, the measurements lacking an internal standard provide a poor peptide quantitation which lead to a higher uncertainty in affinity determination. Recently, Plonka et al. employed potentiometric titration and UV-Vis spectroscopy, which are widely recognized as the standard procedure for binding constant calculations [22, 29], and characterized the flexible N-terminal hexapeptide of hepcidin-25 (DTHFPI) as the strongest ATCUN motif ever reported with a dissociation constant of $10^{-14.66}$ M, which is even higher than the affinity of albumin for copper ($K_D=10^{-12}$ M) [30]. Moreover, this study showed a rapid transfer of Cu^{2+} from HSA to the 6-residue N-terminus [31]. Such extraordinary affinity of the N-terminus of hepcidin-25 for copper is supported by reports which showed that the conserved presence of aspartic acid (D) as residue 1 in the ATCUN motif increases the basicity of the nitrogen atoms involved in the metal complex and thus the copper binding is enhanced [32]. Considering this high affinity and the concentration of Cu^{2+} in blood, it is reasonable to assume that a significant fraction of hepcidin-25 is present in the copper-bound form under physiological conditions. In this regard, ESI tandem mass spectrometry (MS/MS) was also employed to characterize the Hep-25-copper complex. Collision induced dissociation (CID) applied to the copper complex resulted in the fragment DTH-copper, representing the last three residues of the N-terminus of Hep-25 bound to a copper ion. This indicates the stability of the copper-binding motif that is preserved even during MS fragmentation. Furthermore, the affinity of hepcidin-25 for copper allowed the quantification of the peptide in serum samples using inductively coupled plasma mass spectrometry (ICP-MS) by employing direct biomolecule labeling [33].

Publications regarding N-terminus of Hep-25 reported this motif to be unstructured in contrast to the rest of the molecule which is highly rigid due to four intramolecular disulfide bridges [11, 12]. This suggests that the N-terminal fragment could serve as a model for metal binding and structural studies, the results of which should then be transferrable to full-length hepcidin-25. Interestingly, the alignment of hepcidin-25 sequences of various species revealed a highly conserved N-terminus. Not only the ATCUN motif (Asp-Thr-His), but even the 6-residue peptide DTHFPI seems to be preserved among most of the mammals (Fig 2), which suggests a specific receptor interaction.

Immobilized metal ion affinity chromatography (IMAC) or His-Tag, based on the affinity of the imidazole group of histidine for transition metal ions such as nickel and copper, was also exploited for the purification of hepcidin-25 taking advantage of the Cu^{2+}-affinity of the ATCUN motif and the presence of histidine at its NH_2-terminus. IMAC protein chips loaded with copper led to the enrichment of hepcidin from mouse or human serum [34, 35]. As mentioned before, bioactivity evaluation of structurally altered hepcidin-25 variants revealed the functional importance of the histidine residue at this sequence position [7, 36].
Despite several studies stating the high affinity of Hep-25 for copper ions, the structure of the human hepcidin-copper complex was so far not elucidated in detail. In studies for the structural analysis of Hep-25 using NMR spectroscopy and molecular dynamics simulations [11, 24], the presence of the copper ions was largely ignored. Interestingly, a 3D model of the copper complex of trout hepcidin-25 was proposed [9], yet based on the outdated structural template [12].

Scope of the present work

Increasing evidence indicates that the high affinity of hepcidin-25 for copper(II) contributes to its active form and function. Thus, the need remains to further examine the interaction of human hepcidin with metals and supports the importance of the correct structure when working with a biochemical compound. This could have a meaningful impact on hepcidin-25 assays and contribute to solving the problems associated to hepcidin-25 quantification [37, 38]. Therefore, the present study investigated the complex formation of hepcidin-25 with metals based on the hypothesis that the natural, bioactive form of human hepcidin-25 contains a copper(II) ion. LC-MS and NMR analysis are employed to investigate the composition and the 3D structure of hepcidin-25 in the metal-bound form with copper(II) and nickel(II). In addition to the structural characterization of Cu^{2+} binding to Hep-25 or its N-terminal ATCUN peptide fragment, we employed Ni^{2+} that is also bound by Hep-25 with high affinity and serves an appropriate substitute for Cu^{2+} forming a diamagnetic ATCUN complex amenable to high-resolution structure determination by NMR. The copper(II) and nickel(II) complexes of Hep-25 were investigated by NMR spectroscopy under physiological conditions (pH of 7.4). However, a LC separation of the transition metal complexes of hepcidin performed at neutral pH was not possible. Therefore, we have chosen to perform LC-MS at basic pH (≈11) in order to avoid abrogation of the metal binding by standard, acidic conditions. These conditions lead to a very high stability of the metal complexes. Additionally, high resolution (HR) MS measurements using FTICR-MS were conducted at a pH of 7.4 by employing direct infusion.

2.1. Chemicals

Human hepcidin-25 was purchased from Bachem (Bubendorf, Switzerland, H5926.0500, lot number 1060499, >95%) and dissolved in 1% acetic acid (AppliChem, Darmstadt, Germany, 361008.1611, HPLC grade) according the manufacturer's instructions. Linear hepcidin-25 was purchased from peptides&elephants (Hennigsdorf, Germany, lot number 1006P06, >95%). Hexapeptide (DTHFPI) was also purchased from peptides&elephants (Hennigsdorf, Germany, >99%). Copper sulfate solution 0.1 M (35185, Titripur®) and copper sulfate pentahydrate were obtained from Sigma-Aldrich, Taufkirchen, Germany. Nickel sulfate hexahydrate and ammonia solution 30% (hN66.2, LC-MS grade) were purchased from Carl Roth, Karlsruhe, Germany. Purified laboratory water was obtained from a Milli-Q water-purification system (Millipore, Bedford, USA). Reduced glutathione (Thermo Fischer, Darmstadt, Germany, BP2521-10, >98%) and oxidized glutathione (Sigma-Aldrich, Taufkirchen, Germany, G4376 >98%) were used. Acetonitrile (ACN, 2697, LC-MS grade) was obtained from Chemsolute, Germany and trifluoroacetic acid (TFA, 14264, LC-MS grade) was purchased from Honeywell, USA. Sodium dihydrogen phosphate (Carl Roth, Karlsruhe, Germany) and sodium phosphate tribasic 12-hydrate (J. T. Baker®) were used. Trimethylsilylpropanoic acid (TSP) was obtained from Sigma-Aldrich, Taufkirchen, Germany. Deuterium oxide (D_2O) was obtained from Euriso-top, France.

2.2. Synthesis of hepcidin-metal complexes (LC-MS)

Hepcidin-25 was dissolved in 1% acetic acid solution to obtain a stock solution of 1 mg/mL. Aliquots of 50 µL were stored at -20°C. The thawed aliquots were used immediately. The synthesis of the Cu^{2+}:hepcidin-25 complex was carried out by adding a 1.8-180 µM $CuSO_4$ solution in a mixture of the LC mobile phases (defined below) 5% B/ 95% A (v/v) to a hepcidin-25 solution of 18 µM (0.1 to 10-fold molar excess). For experiments at different pH values, hepcidin folded in house was used (see Supplementary Material Fig. 1). A solution of 18 µM $CuSO_4$ in different buffer solution was added in a molar ratio of 1:1. 100 mM phosphate buffer at pH of 2.2, 100 mM citrate buffer at pH of 4.2 and 10 mM ammonium acetate at pH of 7.4 were used respectively.

2.3. LC-MS

LC-MS/MS experiments were carried out on an Agilent 1260 Infinity LC system coupled to a Triple Quad™ 6500 MS (AB Sciex, Darmstadt, Germany). Electrospray ionization was performed in the positive mode (ESI+) with a source temperature of 400°C. Further parameters used for ionization were 4500 V ion spray voltage, an entrance potential (EP) of 10 V, a curtain gas (CUR) with 35 psi, a nebulizer gas (GS1) with 62 psi, a turbo gas (GS2) with 62 psi and a collision gas (CAD) with 10 psi. Parameters used to produce fragment ions were a declustering potential (DP) of 40 V, a collision cell exit potential (CXP) of 6 V and a collision energy (CE) of 45 V. Data acquisition and analysis were carried out using the software Analyst ® 1.6.2 (AB Sciex, Darmstadt, Germany). Chromatographic separation was achieved by an UltraCore 5 µm SuperC18 column (50 mm x 2,1 mm) with an ACE Excel UHPLC pre-column. The mobile phases used for chromatography were composed of 0.1% ammonia in water (pH 11) (A) and in ACN/water 90/10 (v/v) (B) respectively. The peptide-copper complexes were eluted applying a flow rate of 600 µL/min as follows: 5% B, isocratic for 2 min, linear increase to 95% B within 5 min, kept at 95% B for 2 min, return to the initial conditions within 0.5 min, and kept for 5.5 min. The column oven temperature was set to 50°C and the injection volume employed was 10 µL.

2.4. FTICR-MS

A Thermo LTQ FTICR Ultra MS was operated in positive mode. Mass calibration was achieved by using a Pierce LTQ ESI Positive Ion Calibration solution. The system was tuned using a hepcidin-25 solution 5 mg/L dissolved in water/ACN/TFA 60/38/2 v/v/v. Typically, a scanning range of m/z 400-2000 was detected. Direct infusion was employed using the integrated syringe pump set at a flow rate of 30 µL/min. Hepcidin-25-copper complex solutions at pH of 11 and 7.4 respectively, prepared as described previously, were analyzed.

2.5. NMR Spectroscopy

All NMR experiments were performed on a Bruker AVANCE III 600 spectrometer equipped with a 5 mm BBI probe. Chemical shifts were referenced to internal trimethylsilylpropanoic acid (TSP) at 0.0 ppm. NMR samples were prepared as aqueous solutions containing 5 % D_2O for experiments detecting exchangeable protons. Metal titrations were performed by adding small amounts of concentrated solutions of metal salts to the peptide sample. Ni^{2+} or Cu^{2+} titrations were performed by adding 10 mM $NiSO_4$ 6 H_2O or $CuSO_4$ stock solution, respectively, to the 2.5 mM phosphate-buffered solution of the peptide, at pH of 7.4, until a 1:1 complex was formed. Chemical shift assignments were achieved by analyzing 1D-^{1}H, ^{1}H-^{13}C HSQC, ^{1}H, ^{1}H-TOCSY and ^{1}H, ^{1}H-ROESY spectra. The ROESY mixing time was 400 ms. In case of metal-free hepcidin-25, NMR spectra were recorded at 273 K and 325 K. The spectra of hexapeptide, hexapeptide-metal complex and hepcidin-25-metal complex were acquired at 273 K. In case of the metal-free peptides, the majority of chemical shift assignments could be transferred from published data [11]. Acquired data were processed and analyzed using Bruker Topspin (v3.5) and the CARA software [39].

2.5. Molecular modelling

Structures were calculated with Xplor-NIH v2.43. The model of metal-bound hepcidin-25 was generated by joining the 3D structure of the Ni^{2+}-bound hexapeptide that was calculated based on

experimental restraints with the C-terminal part of hepcidin-25 with its coordinates fixed to the conformation reported by Jordan et al. [11]. The 3D structure of the Ni^{2+}-bound N-terminal hexapeptide (DTHFPI) was obtained by simulated annealing with r^{-6} averaging of distances within Xplor-NIH. Distance restraints were derived from ROESY cross-peak volumes, which were multiplicity corrected and calibrated with reference to cross peak volumes corresponding to known distances in the peptide [40]. For all internuclear distances we defined upper and lower distance bounds with a large margin (d±1 Å) to take into account offset-dependent and other distance uncertainties specific to the ROESY experiment [41, 42]. Modifications of the Xplor-NIH parameter and topology files were made to incorporate the nickel or copper ion in a square planar complex, using distance and torsion angle restraints, involving the four nitrogen atoms. The correct square planar geometry of nickel or copper coordination was maintained by imposing fourteen restraints including bond distances, bond angles and dihedral angles. The final ensemble of ten hexapeptide structures had a backbone r.m.s.d. of 1.17 Å, 75 % of back bone dihedral angles were in the most favored region of the Ramachandran plot. Complete structural statistics are provided in the online Supplementary Material (Table 1.). Chemical shifts were translated into backbone dihedral angle restraints by using the software TALOS [43]. Calculations were performed according to the standard protocol of Xplor-NIH. The PDB structure of hepcidin-25 (code 2KEF) [11] was used as a starting structure for simulated annealing. Simulated annealing was done with 24000, 40-psec, steps at 1000 K during heating and 3000, 0.8-psec, steps during cooling to 100 K.

3. Results and discussions

3.1. Chromatographic separation of hepcidin-25-copper(II) complexes

MS studies of the hepcidin-25-copper complex using FTICR or MALDI-MS were initially performed without separating the copper-bound from the copper-free peptide. This hinders an accurate and sensitive analysis of a mixture of the peptide species, especially in more complex matrices such as biological samples. The LC separation improves detection and reduces ion suppression effect in LC-MS analysis.

Anion exchange chromatography was previously employed for the separation of the copper-labeled peptide from the excess of copper in an LC-ICP-MS study for the quantification of Hep-25 by measuring the amount of copper bound to the peptide [33]. Unfortunately, this method provides no molecular information on the peptide species present in solution.

We introduced a RP-HPLC (reversed-phase high-performance liquid chromatography) separation step complementary to MS detection for a sensitive and robust analysis of the species resulting from the complexation of Hep-25 with copper. This is based on an LC-MS/MS quantification method developed in our laboratory for the quantification of hepcidin-25 using mobile phases containing 0.1% ammonia (pH of 11) [44].

For chromatographic separation of the obtained metal species, a neutral or basic pH of the mobile phases is required for complex stability. Previous publications have shown that hepcidin-25-copper(II) is not stable at pH <5.2 [10]. Unfortunately, a separation of the peptide species at pH 7.4 could not be achieved. It is known that a good chromatographic resolution is achieved, when fully ionized or fully non-ionized species are used. Hepcidin-25 is a basic peptide with an isoelectric point of 8.5. Hepcidin will be >99% ionized at a pH value approximately 2 pH units above its isoelectric point (10.5). Such high pH values are usually not suitable for commonly used reversed phase chromatography columns, stable up to a pH of 8. However, the authors employed new generation HPLC columns for performance at high pH. An ACE Super C18 HPLC column with extended pH stability allowed the HPLC analysis at a pH of 11.

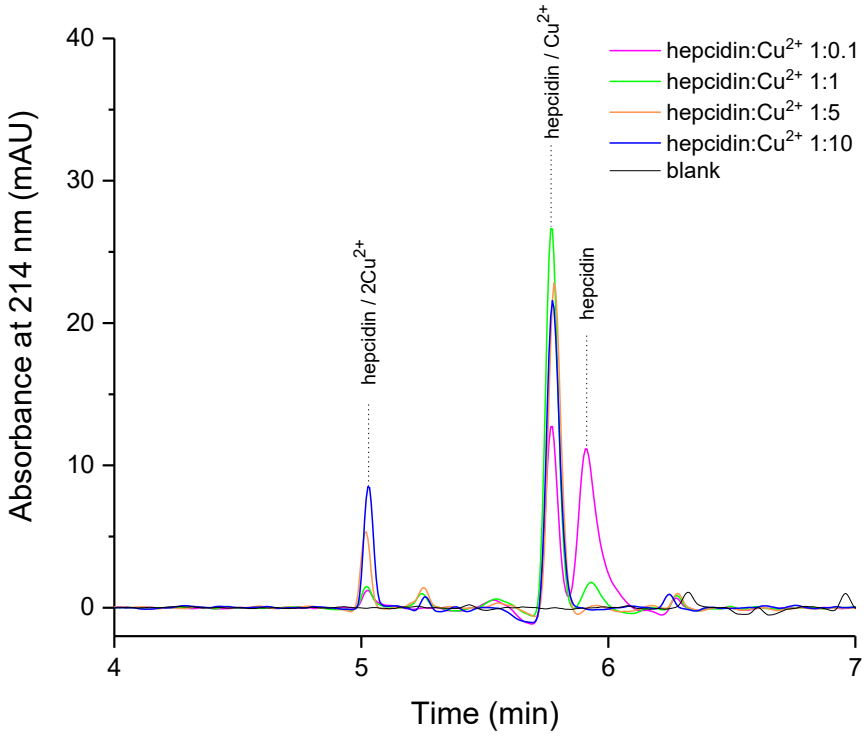


Fig. 3 – The influence of molar ratio on hepcidin-copper complex behavior (HPLC separation, mobile phase A: H_2O/NH_3 100/0.1 v/v, pH=11, B: ACN/H_2O/NH_3 90/10/0.1, concentration of hepcidin-25 of 50 mg/L (18 µM)).

Synthetic hepcidin-25 was titrated with copper ions to monitor the formation of the metal complex(es) by addition of a 1.8-180 µM Cu^{2+} solution to a hepcidin-25 solution of 18 µM (50 mg/L) at a pH of about 11 (0.1% ammonia). Three peaks were identified (Fig. 3) that were subsequently attributed via LC-MS analysis to free hepcidin-25, a 1:1 hepcidin-25:copper complex and the complex of hepcidin-25 with two copper ions.

As expected, the DAD signal at 214 nm shows a decrease in the apo-hepcidin peak upon the addition of Cu^{2+} due to metal complex formation (Fig. 3). Also, the decrease in hepcidin signal at molar ratio 1:5 and 1:10 coincides with significantly increased amounts of a new species identified further by MS/MS as the complex of hepcidin with two copper ions. This species was briefly mentioned previously in the literature [30], but no clear significance was attributed to the complex. To our best knowledge, this is the first reported chromatographic separation and MS/MS analysis of the peptide complex with two copper ions.

As mentioned before, the interaction of hepcidin-25 with copper is pH-dependent. Varying results were reported in the literature for quantitative hepcidin-25-copper complex formation. Some authors applied FTICR-MS measurements at pH of 6.8 and found total complex formation at molar ratio hepcidin-25:copper of 1:1 [28], while other authors reported the presence of unreacted peptide at equimolar concentrations, in a q-ToF-MS (Quadrupol-Time-of-flight mass spectrometry) experiment at pH of 8.2. [33]. Fig. 3 shows that at pH of 11 there is unreacted peptide present, while the excess of copper (1:5) leads to quantitative complex formation.

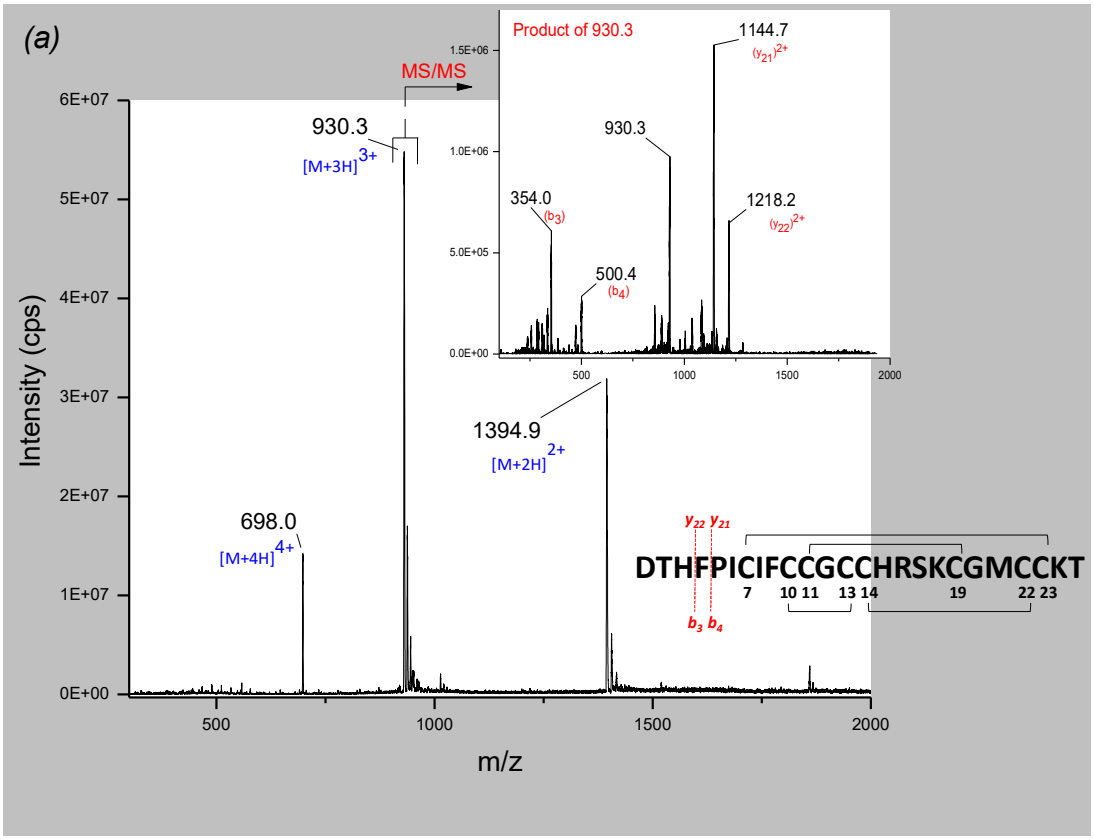
We investigated further the behavior of the metal complex solution prepared at different pH values. At physiological pH (7.4), the species present is mainly the mono-copper complex in accordance with the previously described results at pH of 6.8 [28] (see Supplementary Material Fig. 2). We report the formation of the peptide complex with two copper ions preferably at high pH (11), but not at physiological pH. Further, we characterized the three peaks obtained by tandem mass spectrometry (MS/MS).

3.2. MS/MS characterization of hepcidin-25 and hepcidin-25-copper(II) species

Hepcidin, hepcidin-Cu^{2+} (1:1) and hepcidin-Cu^{2+} (1:10) were analyzed on a triple quadrupole in positive mode using a 50 mg/L solution (18 µM) in 14% acetonitrile and 0.1% ammonia (starting conditions of the HPLC gradient).

The spectra of hepcidin-25 revealed the doubly, triply and quadruply charged quasi-molecular ions with m/z 1394.9 ($[M+2H]^{2+}$), 930.3 ($[M+3H]^{3+}$) and 698.0 ($[M+4H]^{4+}$) respectively. $[M+3H]^{3+}$ was the most abundant quasi-molecular ion and was selected as precursor ion for fragmentation (Fig. 4a). Product ion spectra resulted in the loss of b_3 (354 Da) to yield y_{22}^{2+} (1218.2 Da).

Further, the analysis of the hepcidin-Cu^{2+} solution (molar ratio 1:1) showed similar series of ions in full scan mode. The quasi-molecular ions with m/z 1425.0 ($[M+C^{2+}$), 950.3 ($[M+H+Cu]^{3+}$) and 713.1 ($[M+2H+Cu]^{4+}$) were identified (Fig. 4b). The fragmentation of the triply charged quasi-molecular ion as the most intense precursor ion resulted in the same fragment y_{22}^{2+} (1218.2 Da) compared to the fragmentation pattern of hepcidin-25, and a fragment of 415 Da which corresponds to a copper-adduct of the N-terminal fragment containing the three amino acids of the ATCUN motif (Asp-Thr-His). The difference of 61 Da corresponds indeed to the addition of one copper (62.9 Da) and the loss of 2 Da and 2 positive charges (2 H^+), in accordance with previous findings [28, 33].



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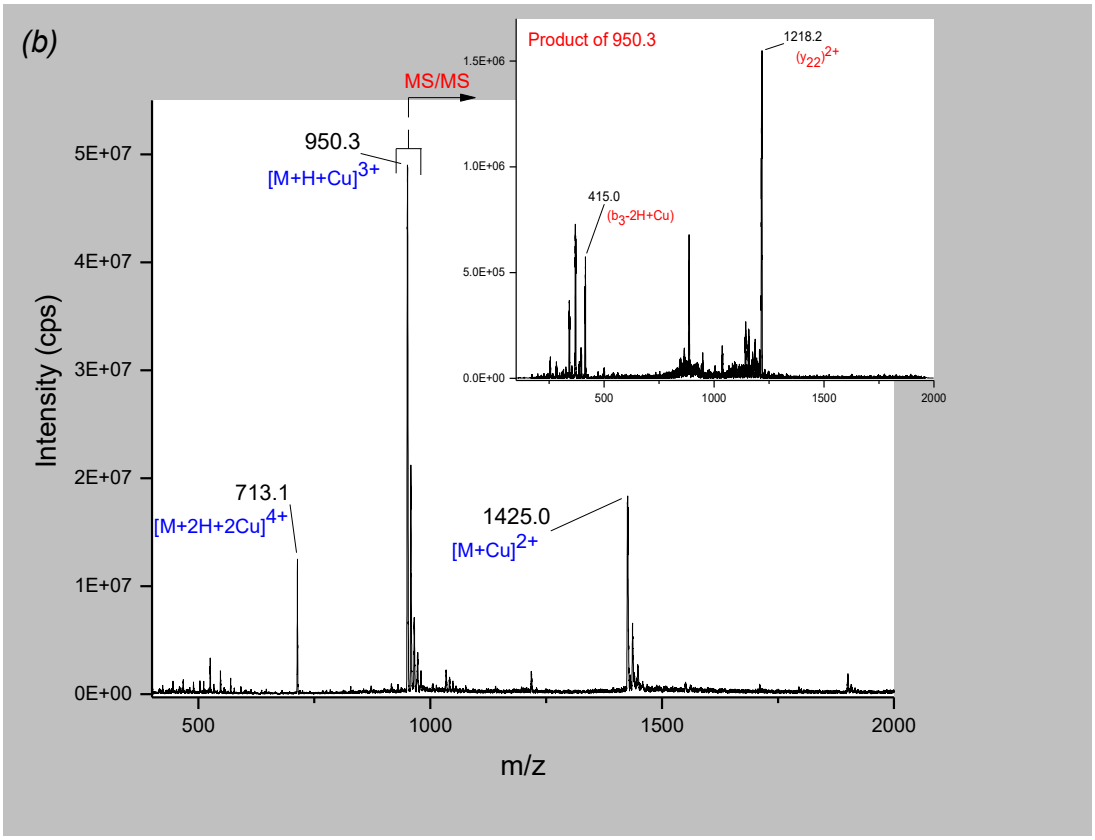

Fig 4 – Full scan (MS) and product ion spectra (MS/MS) of (a) hepcidin-25 and (b) hepcidin-25-Cu^{2+}. The fragmentation pattern presented in (a) is valid for both species.

Analyzing the solution of hepcidin-Cu^{2+} (molar ratio 1:10) revealed a hepcidin-25 species complexed with two Cu^{2+}-ions. This compound was previously reported in literature using MALDI-TOF analysis [30], but never separated and characterized using LC-MS/MS.

The doubly, triply and quadruply charged quasi-molecular ions were observed at m/z 1456.1, 971.0 and 728.5, respectively, but the signal intensity was lower compared to the other two species (see Supplementary Material Fig. 3). The product ion spectra of the triply charged quasi-molecular ion allowed identification of the 415 Da fragment corresponding to the copper-adduct of the DTH motif. Another fragment of m/z 1248.7 was identified that could be attributed to a copper-adduct of the fragment $(y_{22})^{2+}$, taking into consideration the mass difference of 30.5 Da for the doubly charged fragment. This indicates a two Cu^{2+}/hepcidin-25 species with one copper ion complexed at the ATCUN motif and a second Cu^{2+} binding at a different site of the peptide. This fact has never been reported before and it can be speculated that the second copper ion binds the histidine residue in position 15, due to the high affinity of the imidazole side chain for copper.

Remarkably, even at pH of 11, the ESI ionization process in positive mode works efficiently leading to a sensitive detection of protonated hepcidin species. The negative ion mode was also examined (data not shown) with the triply charged quasi-molecular ion as the most abundant ion, but the sensitivity was poor and no specific fragmentation of the precursor ion could be achieved.

However, the uncertainty of the unit mass resolution provided by the triple quadrupole does not allow accurate determinations of the exact (monoisotopic) mass and the isotope composition. Thus, in order to validate the results obtained with the triple quadrupole and to confirm the isotope pattern of the complexes of hepcidin-25 with copper(II) ions, high resolution MS (HR-MS) was employed. FTICR-MS investigations showed an isotope pattern for hepcidin-25-copper(II) complexes corresponding to the following chemical formulas: $C_{113}H_{168}N_{34}O_{31}S_9Cu$ and $C_{113}H_{168}N_{34}O_{31}S_9Cu_2$ (see Supplementary Material Fig. 4). The mass difference between the complex of the peptide with one and two copper ions and hepcidin-25 (chemical formula $C_{113}H_{170}N_{34}O_{31}S_9$) was 60.9 Da and 123.8 Da

respectively (mass accuracy < 4 ppm). This indicates that the interaction with the first copper ion occurs with the loss of two H^+ as confirmed previously by LC-MS/MS, and the second copper ion (62.9 Da) binds without proton expulsion at pH 11.

Additionally, FTICR-MS experiments were performed using direct infusion for comparison of the behavior of hepcidin-25 complexed with one Cu^{2+} at basic and physiological pH in the absence of the LC mobile phases (pH 11). Very similar isotope patterns of hepcidin-Cu^{2+} at pH values of 7.4 (see Supplementary Material Fig. 5) compared to 11 were obtained, indicating no interference of the chromatographic solvents used in the LC-MS/MS analysis on the copper-bound form of the peptide.

FTICR offers superior resolution compared to the triple quadrupole used previously in the LC-MS/MS experiments. However, HR-MS is less attractive for quantification purposes, mainly due to lower sensitivity. In future, the chromatographic separation of the hepcidin-25 species developed employing basic mobile phases, coupled to tandem mass spectrometry MS/MS using the triple quadrupole operated in MRM mode (multiple reaction monitoring) and complemented by the use of an internal standard, could represent a valuable tool for the quantification of the copper-bound form of hepcidin-25 in biological samples. This would require, however, the development of a sample preparation protocol employing a suitable pH to maintain the copper-bound form.

3.3. Metal coordination of hepcidin-25 and its N-terminal hexapeptide fragment monitored by NMR spectroscopy

With the objective to complement our (LC)-MS studies, the copper(II) and nickel(II) complexes of Hep-25 were investigated by NMR spectroscopy under physiological conditions (pH of 7.4). In order to find out how hepcidin's ATCUN motif accommodates metal ions at neutral pH, we investigated Cu^{2+} and Ni^{2+} binding of the peptide fragment comprising the six N-terminal amino acids (DTHFPI) and full-length hepcidin-25 by NMR spectroscopy.

First, sequence specific assignments were achieved by analyzing 1D-^{1}H, ^{1}H-^{13}C-HSQC, 2D-TOCSY and 2D-ROESY spectra. In case of the hexapeptide, these were acquired at room temperature, and a virtually complete assignment was obtained (Supplementary Material Fig. 10). In case of Hep-25, spectra were acquired at room temperature as well as at 325 K in order to minimize line broadening caused by intermediate dynamics of the loop region [11]. We were able to transfer 84% of the published resonance assignments, however, because of limited sensitivity/peptide concentration in our experiments, the resonance assignment of Hep-25 is still incomplete.

It was possible to detect the proline [Pro-5] *cis-trans* isomerization in the hexapeptide and hepcidin-25 by NMR spectroscopy. With respect to the NMR chemical shift time scale, the proline [Pro-5] *cis*/*trans* isomerization is slow, and therefore two sets of resonances are observed in NMR spectra at 298K as well as at 325 K. The *cis* and *trans* isomers in hexapeptide were distinguished from each other by comparison of ^{13}C chemical shifts of proline in ^{1}H-^{13}C HSQC spectra [45] and the *cis*/*trans* ratio is about 30%/70%. It was possible to distinguish the *cis* and *trans* proline isomers in hepcidin-25 by comparison of the TOCSY spectra of hepcidin-25 and hexapeptide (see Supplementary Material Fig. 14). The ratio of the *cis*/*trans* isomers in hepcidin-25 is about 10%/90%, due to the lower flexibility of hepcidin-25 in comparison to the hexapeptide, as determined from peak intensities. Neither the temperature nor the pH value affect the *cis*/*trans* isomer ratio to significant extent. Proline *cis-trans* isomerization is a well-known phenomenon that is frequently observed especially for proline residues in flexible parts of polypeptides. It is of high biological relevance in folding, denaturation and renaturation of peptides and proteins [46, 47].

To characterize metal binding of Cu^{2+} and Ni^{2+}, the metals were titrated into solutions of the two peptides and spectral changes were followed by acquiring 1D-^{1}H- and 2D-TOCSY spectra. As expected, both peptides show affinity for Cu^{2+} as well as Ni^{2+}. In case of Cu^{2+}, a paramagnetic complex is formed with the ATCUN motif leading to severe line broadening of residues in close proximity to the coordination site. When Cu^{2+} is added in sub-stoichiometric amounts, two sets of signals arise with one species corresponding to the metal-free peptide showing sharp NMR resonances and on species

showing broadened lines of amino acid residues remote from the coordination site and completely vanishing signals of most other residues (see Supplementary Material Fig. 7 – 8).

From the TOCSY spectrum taken at equimolar concentration of Cu^{2+} and the hexapeptide (see Supplementary Material Fig. 9) it becomes obvious that only resonances of Ile-6, the residue located furthest away from the Cu^{2+} center, and the signals of Pro-5-Hβ and –Hγ retain detectable intensity. All others are completely broadened out. The fact that two sets of signals exist under sub-stoichiometric conditions indicates that the exchange of Cu^{2+} between individual ATCUN motifs is slow on the NMR time scale.

As a high-resolution structure determination of Cu^{2+}-bound Hep-25 and its N-terminal hexapeptide is impossible because of paramagnetic line broadening, we used Ni^{2+} as a substitute of Cu^{2+}. It is known from literature that both metal ions are bound by ATCUN motifs and show very similar coordination geometries [18-20]. Figure 5 illustrates that Ni^{2+} forms a stable complex with hepcidin's ATCUN motif, both with the DTHFPI hexapeptide (Fig. 5A) as well as with full-length Hep-25 (Fig. 5B) (See also Supplementary Material Fig. 6, 11 and 12) confirming the results by LC-MS shown above. As with Cu^{2+}, also the Ni^{2+} complex shows a slow exchange on the NMR time scale.

Coordinating Ni^{2+} causes chemical shift perturbations as a consequence of changes in the electronic structure and by stabilizing a specific conformation. The most strongly affected amino acids are those of the ATCUN motif, specifically aspartic acid [Asp-1], threonine [Thr-2] and histidine [His-3] (Fig. 5A).

It is worth noting that upon complexation, the two amino protons of Asp-1 become visible showing correlations to Asp-1's α- and β-protons in the TOCSY spectrum.

Chemical shift changes due to the metal complexation decrease further away from the metal binding site with the smallest effect observed at phenylalanine [Phe-4], proline [Pro-5], and isoleucine [Ile-6]. Similar results were obtained for the hepcidin-25-nickel(II) complex by a series of nickel titrations. One-dimensional ^{1}H NMR spectra (see Supplementary Material Fig. 12) and 2D-TOCSY again revealed the largest chemical shift perturbation at the N-terminal amino acids aspartic acid [Asp-1], threonine [Thr-2] and histidine [His-3] (Fig. 5B and Supplementary Material Fig. 13). For reasons of sensitivity, we can currently not exclude small effects on the structure of the C-terminal part of hepcidin-25.

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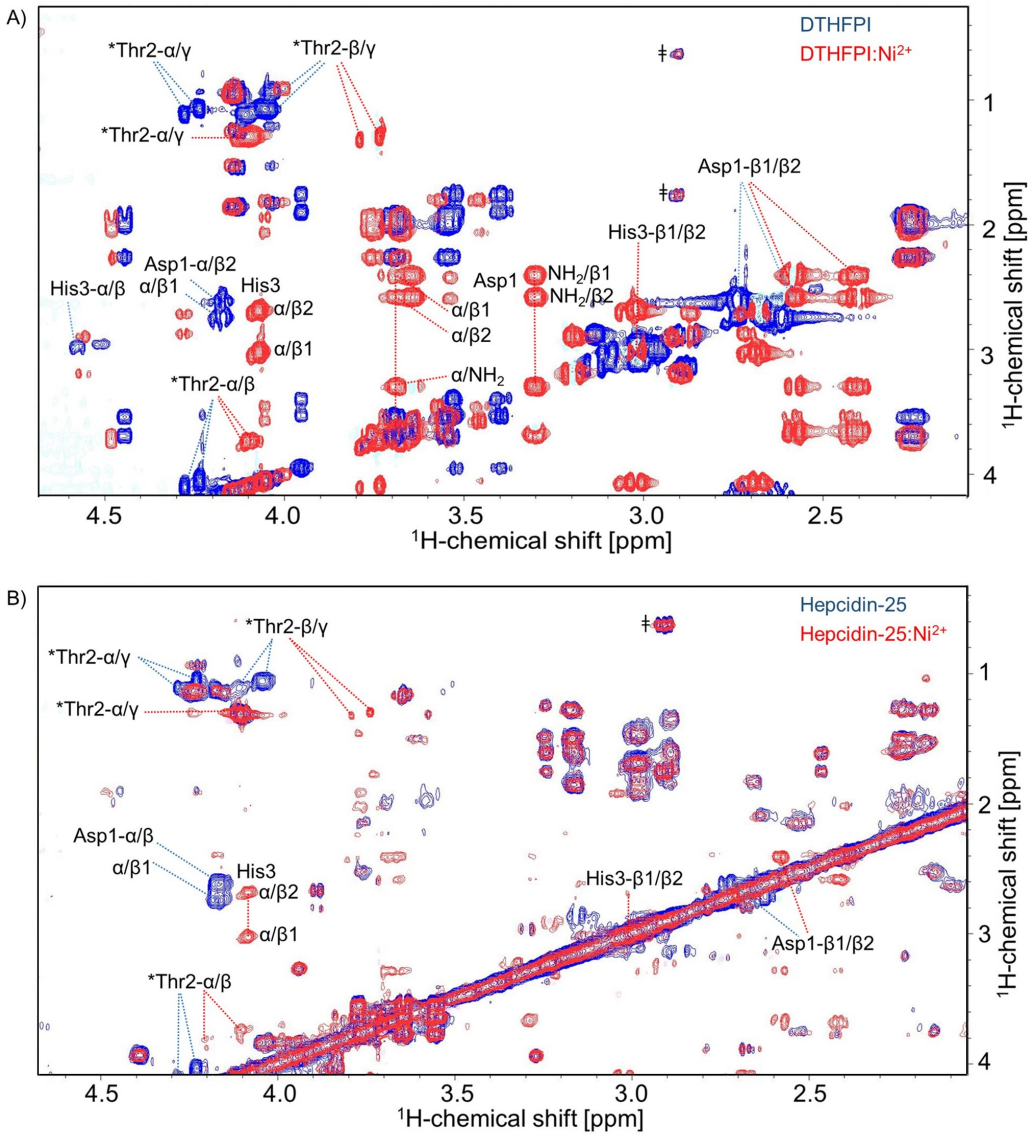

*Fig. 5 – ^{1}H-^{1}H TOCSY spectra of A) hexapeptide DTHFPI and B) hepcidin-25 in the presence of Ni^{2+} (red) and in the absence of Ni^{2+} (blue). The most strongly affected amino acids in both cases are those of the ATCUN motif aspartic acid [Asp-1] (sidechain protons (Hb)), threonine [Thr2] (alpha proton (Ha) and sidechain protons (Hb and Hg) shift) and histidine [His3]. * Contains a double set of peaks for each amino acid and suggest a presence of cis-trans proline isomerization [11]. ‡ Indicates the presence of impurities.*

3.4. Model-structure of hepcidin-25-copper complex

Based on the combination of several NMR spectra, structural models of the Cu^{2+}-bound hepcidin-25 were constructed. First, the solution structure of the N-terminal hexapeptide complexing Ni^{2+} was determined. Internuclear distance restraints were calculated from ROESY cross peak volumes, and dihedral angle restraints of the peptide backbone were generated from backbone chemical shifts using the program TALOS. In addition, the square-planar coordination known to be present in metal-bound ATCUN motifs was imposed by additional distance and dihedral angle restraints. The resulting 3D structure is shown in the Supplementary Material Fig. 15 and the structure determination statistics is summarized in the Supplementary Material Table 1.

Subsequently, models of full-length hepcidin complexing Cu^{2+} were generated by combining the 3D structure of the N-terminal hexapeptide, described above, with the 3D structure of the 19 C-terminal amino acids as determined by *Jordan* et al. (PDB code: 2KEF) [11].

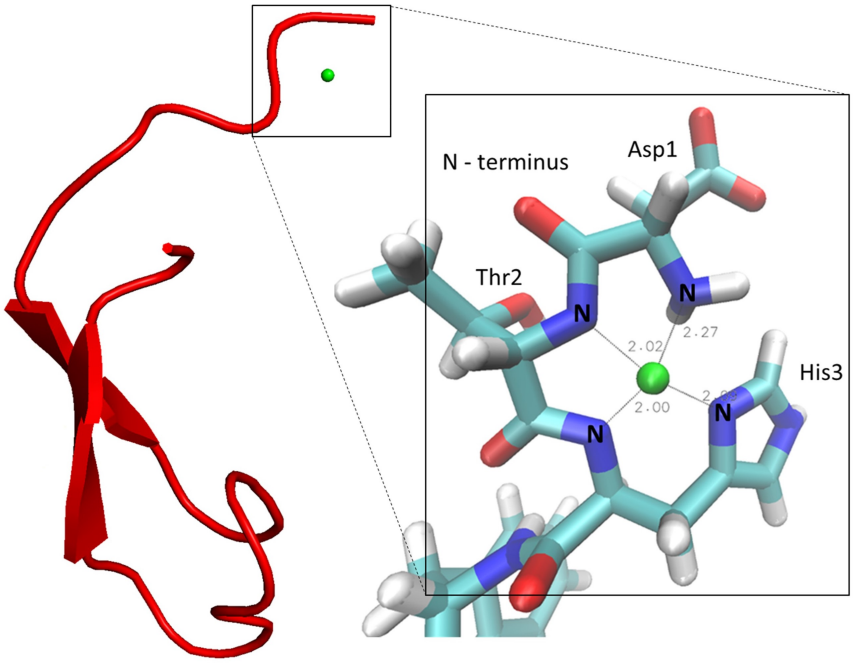

Fig. 6 – The lowest energy structure of the Cu^{+2}-hepcidin 25 model.

Specifically, we performed simulated annealing calculations with the C-terminus kept fixed by appropriate coordinate restraints. The model has been generated with proline [Pro-5] in the *trans* configuration, because this configuration is thermodynamically strongly preferred (~90%). In the N-terminal part, we applied the same distance and dihedral angle restraints that we had previously determined for the Ni^{2+} complex of the hexapeptide. In addition, Ni^{2+} was replaced by Cu^{2+}, and the square planar coordination was imposed by restraining distances and dihedral angles as described [17-21, 48]. Cu^{2+}-N-distances were adjusted to standard values found in X-ray structures of ATCUN motifs. [15]. This strategy is justified as it is known that both Ni^{2+} and Cu^{2+} form high affinity square planar complexes with very similar coordination geometries and metal-ligand bond lengths [15]. The resulting model with lowest restraint violation energy of copper-bound hepcidin-25 is shown in Fig. 6. As a consequence of the coordinate restraints, the C-terminal part corresponds almost perfectly to the 3D structure determined by *Jordan* et al. [11]. The N-terminus extends away from the disulfide-stabilized C-terminus with some degree of conformational variation as shown in the Supplementary Material Fig. 16. This suggests that the linker region connecting the ATCUN motif to the C-terminus is flexible. *Laussac* et al. suggested that the carboxylate group of the aspartate residue may have some interactions with the metal atoms in the DAH motif, forming a penta-coordinated structure [16]. Currently, we have no evidence in favor of such an additional interaction. However, it cannot be excluded that other residues of hepcidin-25 are involved in weak coordinative interactions to the metal atom in the motif.

3.5. Discussions

The structure and behavior of hepcidin-25 and its metal complexes, particularly of copper(II), were investigated to support the hypothesis that the native form of hepcidin-25 contains a Cu^{2+} ion. In this vein, we present the first LC separation of copper-bound and copper-free hepcidin-25 employing mobile phases containing 0.1% ammonia (pH 11). In future, this could lead to the LC-MS/MS quantification of copper-bound hepcidin-25 in biological samples, which might be a superior biomarker in relation to hepcidin-25. At high pH, we identified a new species corresponding to hepcidin-25 complexed with two copper ions. HR-MS experiments showed that two protons are lost during the complex formation of hepcidin-25 with one Cu^{2+}, while the second metal ion binds the peptide without H^+ losses. The first NMR analysis of hepcidin-25 complexed with metal ions was

performed. The NMR data recorded for the N-terminus (DTHFPI) complexed with nickel (II) allowed the construction of a 3D model of human hepcidin-25 containing a copper(II) ion coordinated by the ATCUN motif.

By combining LC-MS/MS and NMR spectroscopy, copper (and nickel) complexation by hepcidin-25 could be confirmed and examined in more detail taking advantage of the complementarity of these analytical techniques.

In accordance with previous reports, it could be shown by NMR spectroscopy that Pro-5 of hepcidin undergoes *cis*/*trans* isomerization (about 10%/90%). This is documented by the doubling of NMR resonances observed in the region centered around [Pro-5] [11, 12]. It is known that the imide bond of the proline residue can in many instances exist in both the *trans* and the *cis* configuration, in contrast to the peptide bonds involving other amino acid residues, which are rarely found in the *cis* configuration [49]. This *cis*/*trans* isomerization of [Pro-5] in the case of Hep-25 could be of interest for its biological activity and its chromatographic retention. *Lüders* et al. showed that, in the case of salmon antimicrobial peptide [SAMP H1], the isomerization of the proline residue was necessary for the activation of the synthetic peptide. The antimicrobial activity of the synthetic standard was enhanced by treatment with peptidylproline *cis*/*trans*-isomerase [50].

Also, the disulfide connectivity represents another possible cause for hepcidin-25 variation. Ion mobility spectrometry (IMS) studies of two Hep-25 standards from two synthesis pathways showed different mobility profiles. Although the different mobilograms obtained were not further identified, the authors suggested that multiple distinct conformers were present in these peptide standards [51].

In addition, the isomerization of the aspartate residue (Asp) to iso-aspartate (IsoAsp) is known to occur often, leading to problems such as protein aging or loss of activity [52]. Whether this is relevant in case of the storage of hepcidin should be clarified in future studies. During synthesis and storage, some racemization of amino acids can occur. To our knowledge, none of the materials used for hepcidin analysis has been characterized in this respect, yet. Even simple (non-chiral) amino acid analysis can be very useful for the traceable quantitation or calibration of proteins and particularly peptides [53].

In addition to the formation of metal complexes, several non-enzymatic post-translational modifications could contribute to the heterogeneity of Hep-25. Not only that these structural modifications could affect the biological activity, but also the quantification of the native peptide in biological samples needs to be evaluated accordingly. The current discrepancies between Hep-25 measurements [37] might be caused by the presence of different hepcidin isoforms. Recently, Smith *et al.* [54] proposed the term “proteoform” to designate all the related molecular forms of a protein product arising from a specific gene. While MS analysis often does not detect possible isomers, the added value of NMR spectroscopy to the chemical identity allows for a high-level purity to be achieved (uncertainties <0.1% [55]). In this context, quantitative NMR (qNMR) is recommended by the metrological community as universal and powerful method to be used for purity determination of organic compounds [56, 57]. The validity and broader acceptance of qNMR is supported by the use of this methodology by the pharmaceutical and chemical industries in their GMP/GLP settings [58]. Furthermore, the use of ICP-MS could be beneficial for compound-independent calibration based on sulfur or the copper complex [59], and the determination of metal traces [60], particularly copper, potentially introduced from process chemicals and nickel from stainless steel equipment. For the isotopically labelled internal standard, a determination of the isotopic purity would be helpful. Thus, the efforts towards hepcidin-25 standardization [38] could be complemented by the development of a reference material additionally characterized by qNMR, ICP-MS [61] and amino acid analysis [53]. We would like to raise the awareness that synthetic and natural peptides can contain a large number of different proteoforms, from which isomers and metal complexes are particularly difficult to identify. Under different conditions, such as temperature, solvents, pH or salt content, their ratios can be highly variable and might lead to a wrong quantification, even with high-resolution MS. In addition, the use of an insufficiently characterized peptide standard as a calibrator could introduce a hidden bias and might lead to inconsistent results between different laboratories.

Acknowledgments: I.M.A. and M.V. were supported by the SALSA (School of Analytical Sciences Adlershof) program funded by DFG (German Research Foundation).

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