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An overlooked hepcidin-cadmium connection

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Abstract

Hepcidin (DTHFPICIFCCGCCHRSKCGMCCKT), an iron regulatory hormone is a 25 amino acid peptide with 4 intramolecular disulfide bonds, circulating in blood. Its hormonal activity is indirect and consists of marking ferroportin-1 (an iron exporter) for degradation. Hepcidin biosynthesis involves N-terminally extended precursors prepro-hepcidin and pro-hepcidin, processed by peptidases to the final 25-peptide form. A sequence-specific formation of disulfide bonds and export of the oxidized peptide to the bloodstream follows. In this study we considered the fact that prior to export, reduced hepcidin may function as an octathiol ligand bearing some resemblance to the N-terminal part of α-domain of metallothioneins. Consequently, we studied its ability to bind Zn(II) and Cd(II) ions using the original peptide and a model for prohepcidin, extended N-terminally with a stretch of five arginine residues (5R-hepcidin). We found that both form equivalent mononuclear complexes with two Zn(II) or Cd(II) ions saturating all eight Cys residues. The average affinity at pH 7.4, determined from pH-metric spectroscopic titrations, is $10^{10.1}$ M^{-1} for Zn(II) ions, the Cd(II) ions bind with affinities of $10^{15.2}$ M^{-1} and $10^{14.1}$ M^{-1}. Using mass spectrometry and 5R-hepcidin we demonstrated that hepcidin can compete for Cd(II) ions with metallothionein-2, a cellular cadmium target. These studied empowered us to conclude that hepcidin binds Zn(II) and Cd(II) sufficiently strongly to participate in zinc physiology and cadmium toxicity under intracellular conditions.

Keywords

Hepcidin; prohepcidin; cadmium; affinity constant; metallothionein; MT2A

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Introduction

Metals in organisms (both essential and non-essential) share similarities that make their regulating pathways overlap to some extent [1]. Thus, all elements of the metallome puzzle ought to be extensively investigated. One of them, hepcidin, is an iron regulatory hormone. In its mature form it is a 25 amino acid peptide with 4 intramolecular disulfide bonds that circulates in blood (DTHFPICIFCCGCCHRSKCGMCCKT). Its hormonal activity is indirect and consists of marking ferroportin-1 (an iron exporter) for degradation. This stops the iron efflux from cells [2].

Hepcidin itself does not bind iron but has two distinct metal binding sites for other metal ions with potential high affinity. One of these sites is its N-terminal tripeptide sequence DTH, which belongs to the ATCUN (Cu and Ni binding site) family [3,4]. The mature hepcidin-25 has indeed high affinity for Cu(II), and can also weakly bind Ni(II) and Zn(II), the latter two unlikely to have a biological meaning [3,5].

The other putative metal binding site can be proposed based on the layout of thiol groups in the molecule. The eight cysteines in hepcidin are arranged in a similar manner to those in the N-terminal part of α-domain of metallothioneins, proteins involved in metal homeostasis and detoxification [6,7] (see Scheme S1). However, for the hepcidin to properly function as an iron hormone, only one of these cysteines is essential. The rest do not have an apparent function except to potentially increase the peptide stability in blood [8]. Nevertheless, all eight cysteines are evolutionarily conserved.

When hepcidin is circulating in blood, all the cysteines are arranged in disulfides within one molecule and with a specific pattern of these bonds [9]. However, before the release to the bloodstream, hepcidin and its immature forms: prepro- and pro-hepcidin are biosynthesized, processed, and trafficked in the reducing, thiol-rich environment of the cell. Thus, with the cysteines exposed it may be a potential molecular target of thiophilic metals like cadmium.

Cadmium is a toxic metal targeting a number of organs. Its toxicity is pleiotropic and manifests throughout the body, but mainly in kidneys, liver or lungs, dependent on the type of exposure [10]. Acute dietary cadmium poisoning damages liver while chronic exposure eventually reaches and damages kidneys [11]. The assumption is that it is excreted with metallothioneins and then filtered in nephrons but it can be also transported with other low and high molecular mass proteins [12].

It was reported that hepcidin is upregulated by cadmium exposure [13,14], although that observation was contested [15]. What is interesting, the hepcidin gene (*Hamp1*) silencing decreases the Cd^{2+} toxicity in $mIMCD_3$ cells. Similarly, overexpression of Hamp1 increases the Cd^{2+} toxicity [16]. A full mechanism for that is yet unknown.

The present paper explores a possibility of hepcidin binding Cd^{2+} ions on a molecular level, to provide a background for continued studies of the hepcidin-cadmium link. The Zn^{2+} binding was also explored, due to the intrinsic physiological zinc/cadmium link [7].

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Results

Hepcidin-25 was synthesized using standard Fmoc solid phase synthesis, and its identity, as well as the reduced state was confirmed using mass spectrometry (monoisotopic peak $+3H^+$ of the peptide with free thiols was 932.707). In the first spectroscopic experiment reduced hepcidin-25 (12.57 µM dissolved in the presence of 0.5 mM TCEP at pH 5.0 to maintain the reduced character of its all eight Cys residues) was titrated with a $CdCl_2$ solution. A characteristic charge-transfer (CT) Cd(II)-thiolate band emerged at 250 nm band (Figure 1A).

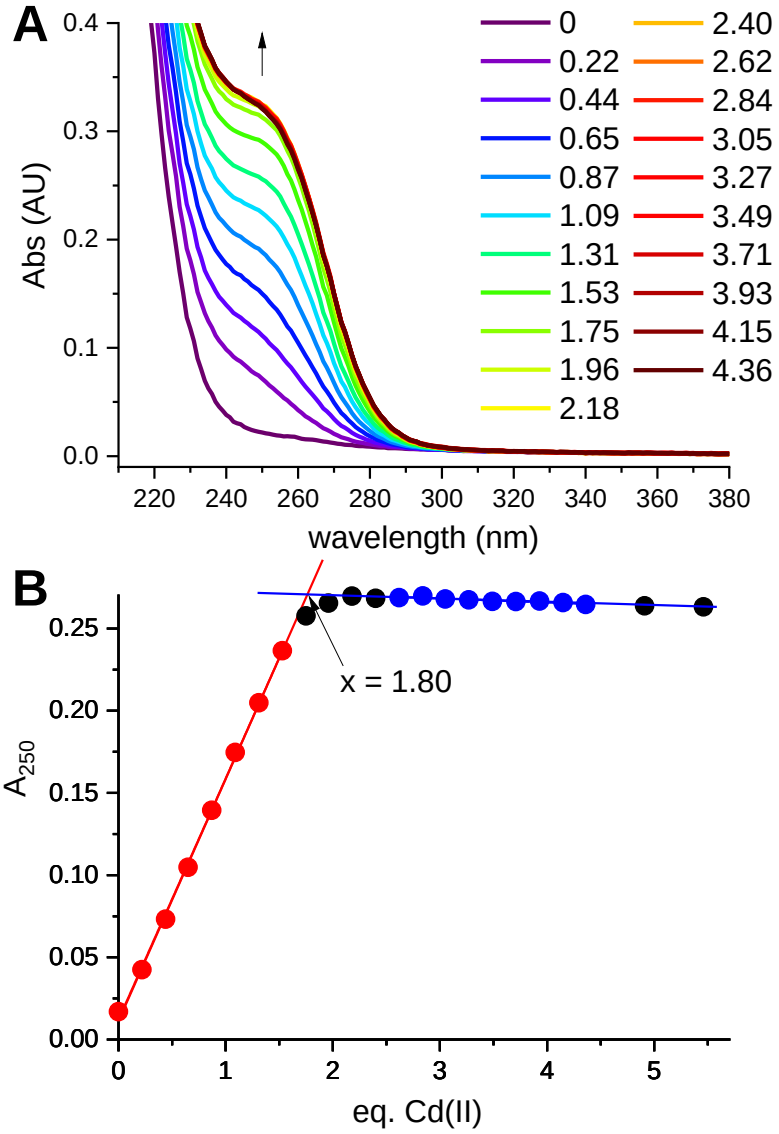

Figure 1. Titration of 12.57 µM hepcidin-25 with $CdCl_2$ in the presence of 0.5 mM TCEP at pH 5.0. A. the spectra featuring the CT band at 250 nm formed upon the increasing Cd(II)/hepcidin-25 ratios (color coded in the plot), the arrow marks the direction of changes; B. the titration curve at 250 nm. The red and blue segments of the curve were used to define the straight lines of the binding site saturation. Their crossing at 1.80 Cd(II) equivalents determines the saturation concentration.

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The full saturation of this band with two Cd(II) equivalents is visible in Figure 1B. The value 1.8 in the plot indicated a 10% deficit of hepcidin-25 and was used to correct its stock solution concentration in further experiments.

This experiment established the 2:1 Cd(II)-to-hepcidin-25 stoichiometry. The linear increase of A_{250} indicated that the Cd(II) sites are spectroscopically equivalent. In the next experiment hepcidin-25 was thus mixed with two Cd(II) equivalents at pH 2.0 and titrated pH-metrically using a concentrated NaOH solution. This titration was monitored with UV-vis and CD spectroscopies (Figure 2 A and B). Again, a distinct CT band appeared at 250 nm in UV-vis and CD spectra above pH 3. Figure 2C presents the titration curves generated from this titration, and Figure 2D provides the fitting of the two-sites binding model for the UV-vis data (less noisy than the qualitatively equivalent CD data). These titrations could not be extended beyond pH 6.0, due to the peptide precipitation.

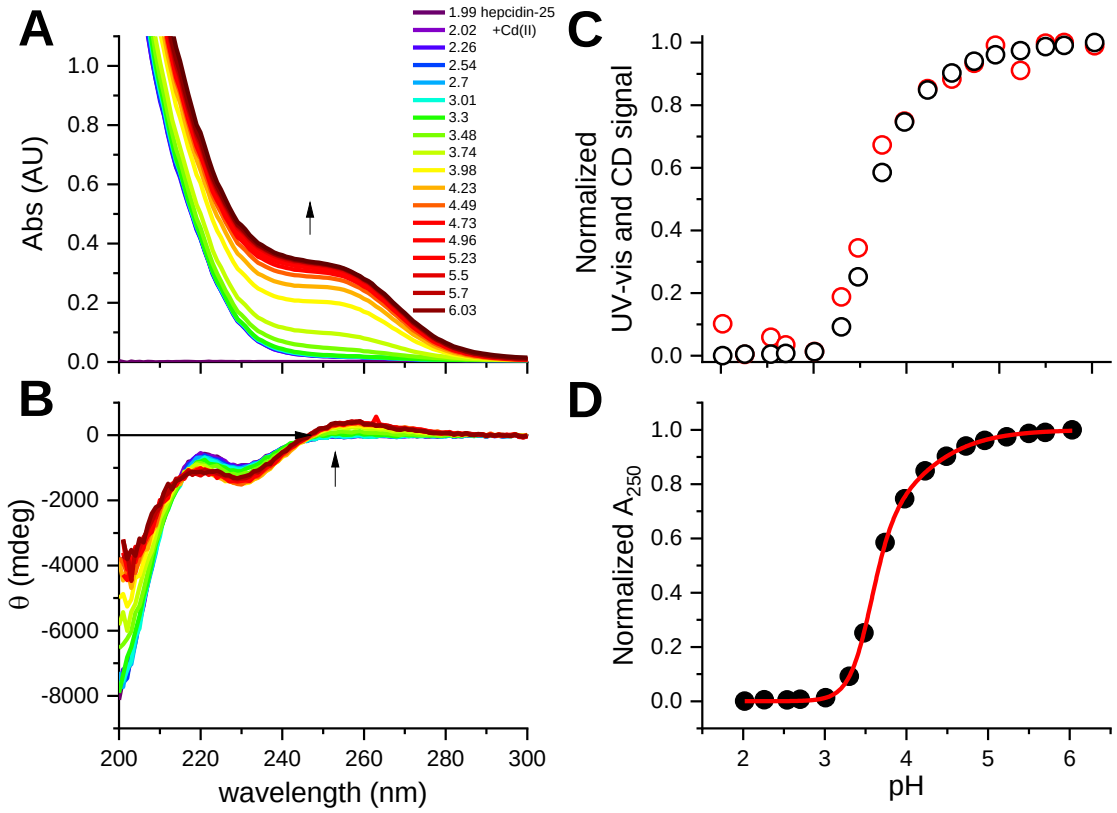

Figure 2. *The pH-metric titration of 12.2 μM hepcidin-25 with 2 eq. of $CdCl_2$ in the presence of 0.5 mM TCEP. A. UV-vis spectra; B. CD spectra, with the common color codes for pH in plot A; C. the pH dependence of CT bands at 250 nm derived from UV-vis and CD spectra, presented in the common relative scale; D. Fitting of the two sites binding model to the normalized UV-vis data at 250 nm.*

The fitting of the titration curve derived from the pH dependence of the Cd(II)-S CT band, presented in Figure 2D, yielded p*K* values for the binding of two Cd(II) ions as 3.60(1) and 3.88(2). Assuming the average p*K* of the thiol group in Hepc-25 as 8.9, by analogy with metallothinein [17], and the structurally equivalent binding of both Cd(II) ions to four Cys residues each, one can tentatively extrapolate these p*K* values into log *K* values for absolute Cd(II) affinity constants as high as 21.2

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and 20.1, and conditional constants at pH 7.4 as 15.2 and 14.1.

A similar pH-dependent experiment was carried out for hepcidin-25 with two equivalents of Zn(II) added (Figure 3). A CT band around 220 nm (Figure 3A) was hardly visible in the spectra due to its weakness, in accord with the previous data for tetrathiolate zinc fingers and metallothioneins [7,18] compounded by peptide precipitation above pH 5, clearly seen in Figure 3C. However, the change of ellipticity around 230 nm (Figure 3B) could be assigned to a combination of conformational effects in the peptide main chain and Zn(II) binding. The region of the titration curve tentatively assigned to be Zn(II)-dependent was used to fit the p*K* for Zn(II) binding to hepcidin-25, as presented in Figure 3D. The average log *K* value obtained from this titration, 4.88(3), can be analogously extrapolated into the log *K* value for absolute Zn(II) affinity constant of 16.1, and conditional constant at pH 7.4 of 10.1.

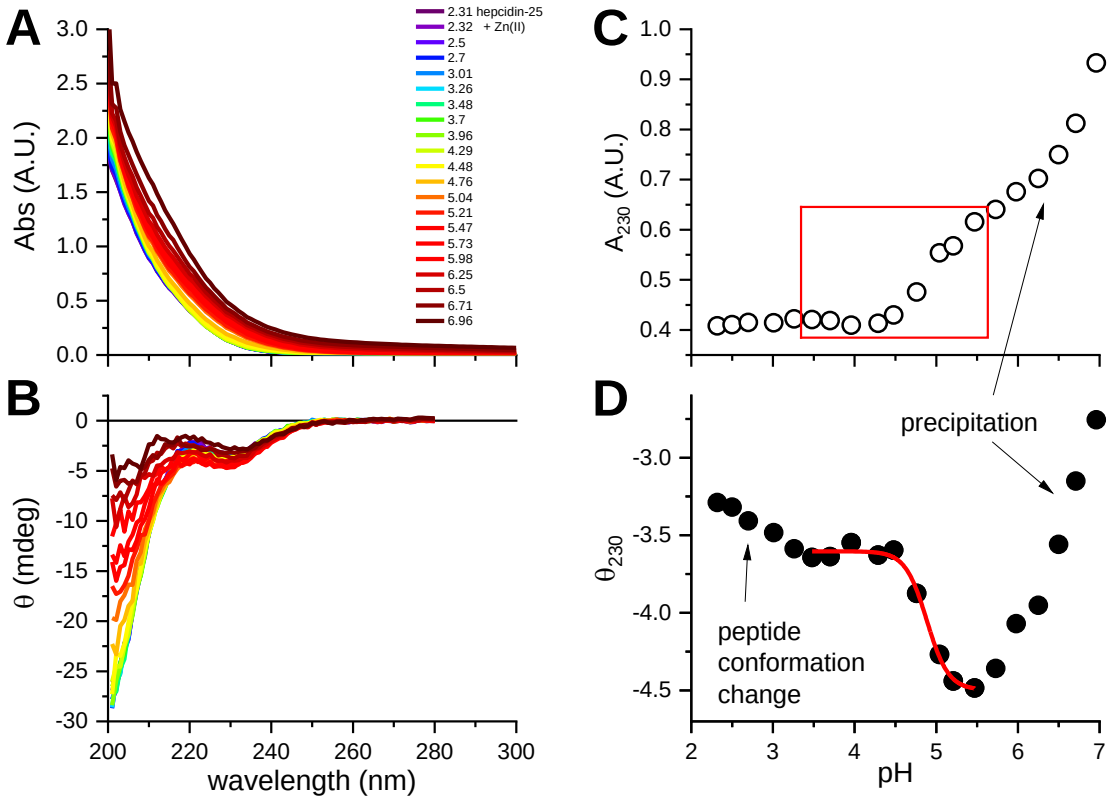

Figure 2. *The pH-metric titration of 15.5 μM hepcidin-25 with 2 eq. of $ZnCl_2$ in the presence of 0.5 mM TCEP. A. UV-vis spectra; B. CD spectra, with the common color codes for pH in plot A; C. the pH dependence of A_{230} in the UV-vis spectra, showing the Zn(II) binding range (red box) and the loss of solution transparency due to peptide precipitation; D. D. Fitting of the one site binding model to the CD data at 230 nm.*

A more direct estimation of affinity constants for Cd(II) and Zn(II) complexes with hepcidin-25 could not be done due to a very strong binding. Very poor solubility of the peptide near the neutral pH posed additional experimental problems. We therefore attempted several competition approaches. We first tried a stepwise competition experiment using Co(II) and Ni(II) ions, which that bind to thiol

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peptides with weaker affinities. We successfully applied this strategy before for XPA and PARP zinc finger peptides [18–20]. Unfortunately, this method failed for hepcidin-25, because of a different, 3:1 stoichiometry (hence non-equivalent substitutions) for Co(II) and peptide precipitation for Ni(II). We also tried to use small competitors with varying Cd(II) affinities, EDTA, HEDTA and EGTA, as described [21]. Unfortunately, all three anionic chelators caused a partial precipitation of hepcicin-25, and no meaningful conclusions could be drawn (data not shown).

In order to alleviate the problem of solubility of hepcidin-25, its sequence of was extended N-terminally by 5 arginines, which are the last five residues of prohepcidin. The new peptide (RRRRRDTHFPICIFCCGCCHRSKCGMCCKT) was labelled 5R-hepcidin. The arginine residues in prohepcidin are recognized by furin, the protease involved in hepcidin maturation [22]. What is worth noting, prohepcidin has no additional binding sites for metal ions, and exists intracellularly, without fully formed disulfides. Therefore, any interaction of metals with hepcidin thiols should be also represented by 5R-hepcidin. The 5R addition indeed increased the peptide solubility at least fivefold and improved its detection by electrospray mass spectrometry (MS) in the positive ions mode.

As expected, 5R-hepcidin retained the ability to bind Cd(II) and Zn(II) ions demonstrated for hepcidin-25 (Figure S1). This empowered us to perform a direct competition experiment between hepcidin-25 and R-hepcidin. Since both have essentially the same binding site, distinguishing it by spectroscopic methods would be impossible. Native mass spectrometry was thus employed to detect any differences. Cd_2hepcidin-25 in ammonium acetate at pH=7.4 was titrated with rising concentrations of 5R-hepcidin. The samples were injected on Q-exactive UHMR mass spectrometer designed to preserve non-covalent bonds. One should always be wary of potential errors and differences in ionization, bonds not being carried over to gas phase or general lack of solvent during measurement [23]. However, in this case Cd(II) is not a fast exchanging metal ion and the peptides were similar enough to consider this technique. This titration, illustrated in Figure 4 (see Figure S2 for the spectra), confirmed our assumption on equivalency of both peptides with respect to Cd(II) binding, with the relative binding affinity hepcidin-25/5R-hepcidin of 1.1 ± 0.3.

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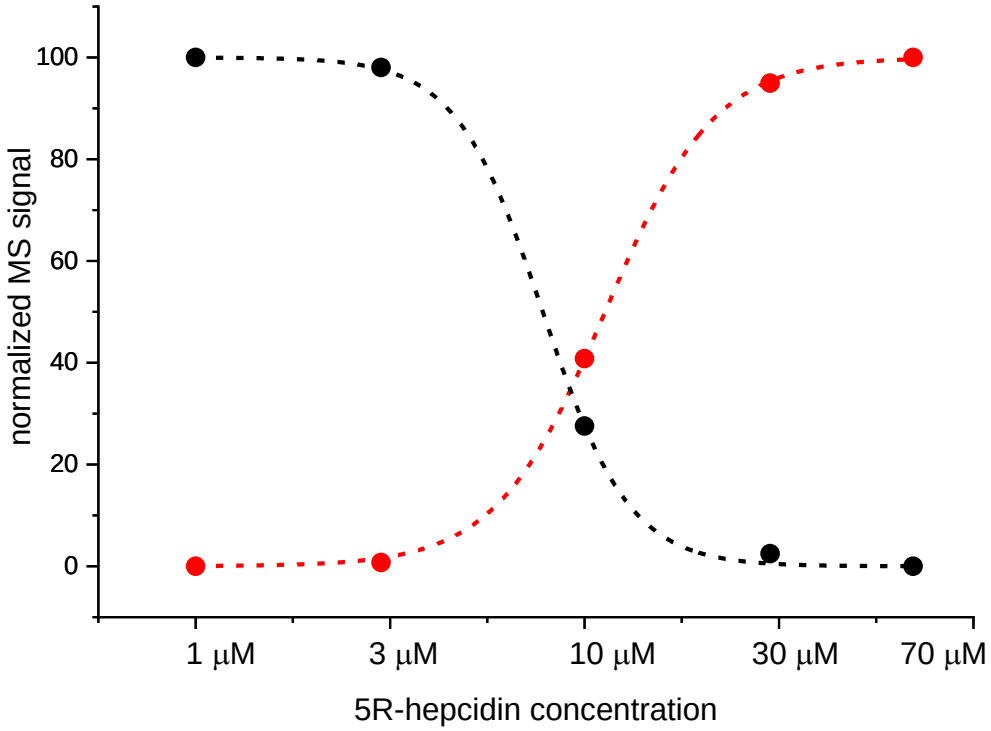

Figure 4. *Integrated native MS signals of Cd_2hepcidin-25 (black dots) and $Cd_2$5R-hepcidin (red dots) obtained from the titration of 10 μM Cd_2hepcidin-25 with 5R-hepcidin. The lines represent fits for the competition constant for the reaction Cd_2hepcidin-25 + 5R-hepcidin = hepcidin-25 + $Cd_2$5R-hepcidin. The MS spectra indicated only low amounts of other metallated species (see Figure S2)*

Taking advantage of a much better solution stability of 5R-hepcidin, we then attempted to establish its relative affinity to Zn(II) and Cd(II), by titrating $Cd_2$5R-hepcidin with Zn(II) ions, and vice versa. When Cd(II) ions were titrated to $Zn_2$5R-hepcidin, the CT band rose as if there were no competing Zn(II) ions present (Figure 5). The apparent elevation of the band in the competition titration is due of the release of Zn(II) ions by Cd(II) competition (Figure 5B). In the reverse experiment, a high excess of Zn(II) ions was necessary to achieve partial displacement of one Cd(II) ion (Figure 6). The unitless competition constant calculated from this experiment for the reaction $Cd_2$5R-hepcidin + Zn(II) = CdZn5R-hepcidin was 99±14, meaning that ca. hundredfold Zn(II) excess was necessary to displace one of the two Cd(II) ions bound to 5R-hepcidin. The displacement of only one Cd(II) ion was confirmed by the fact that the loss of the CT band asymptotically approached 50% of the initial value, corroborated by equal contribution of both Cd(II) ions to the CT band absorption (Figure 1).

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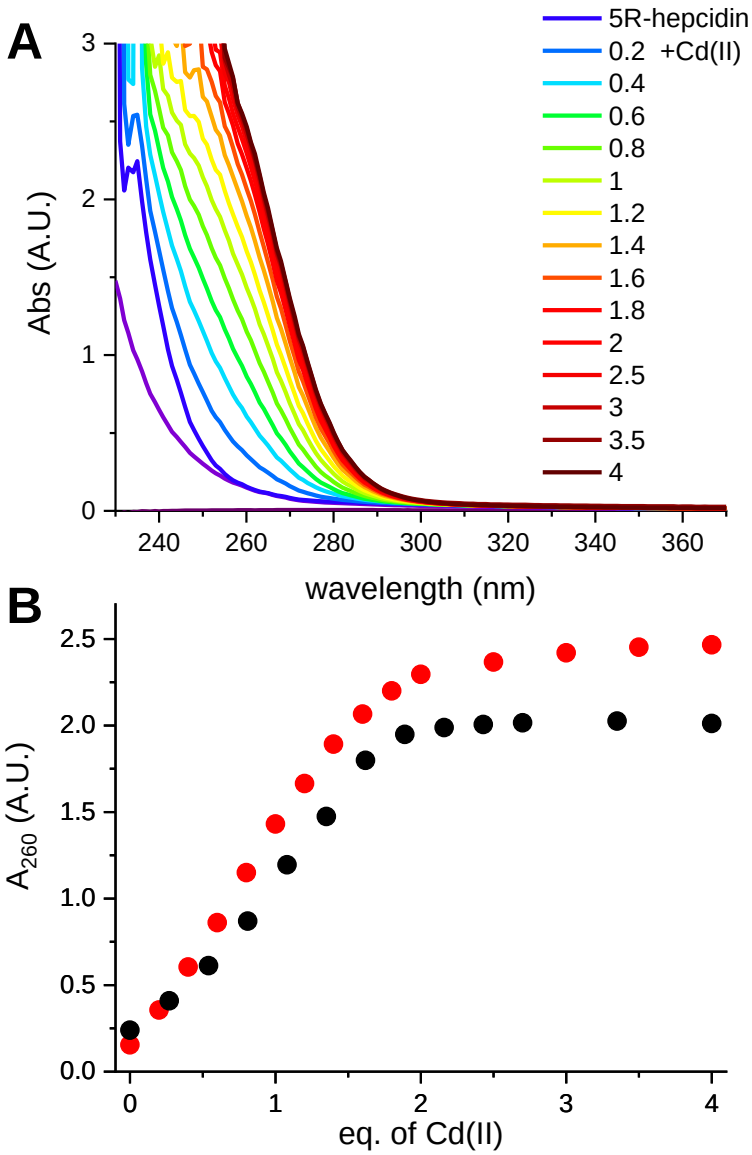

Figure 5. *A. 100 μM $Zn_2$5R-hepcidin titrated with Cd(II) acetate in 100mM HEPES pH 7.4. B. The comparison of A_{260} values for Cd(II) titrations of 100 μM 5R-hepcidin (black dots, data from Figure S1) and $Zn_2$5R-hepcidin (red dots).*

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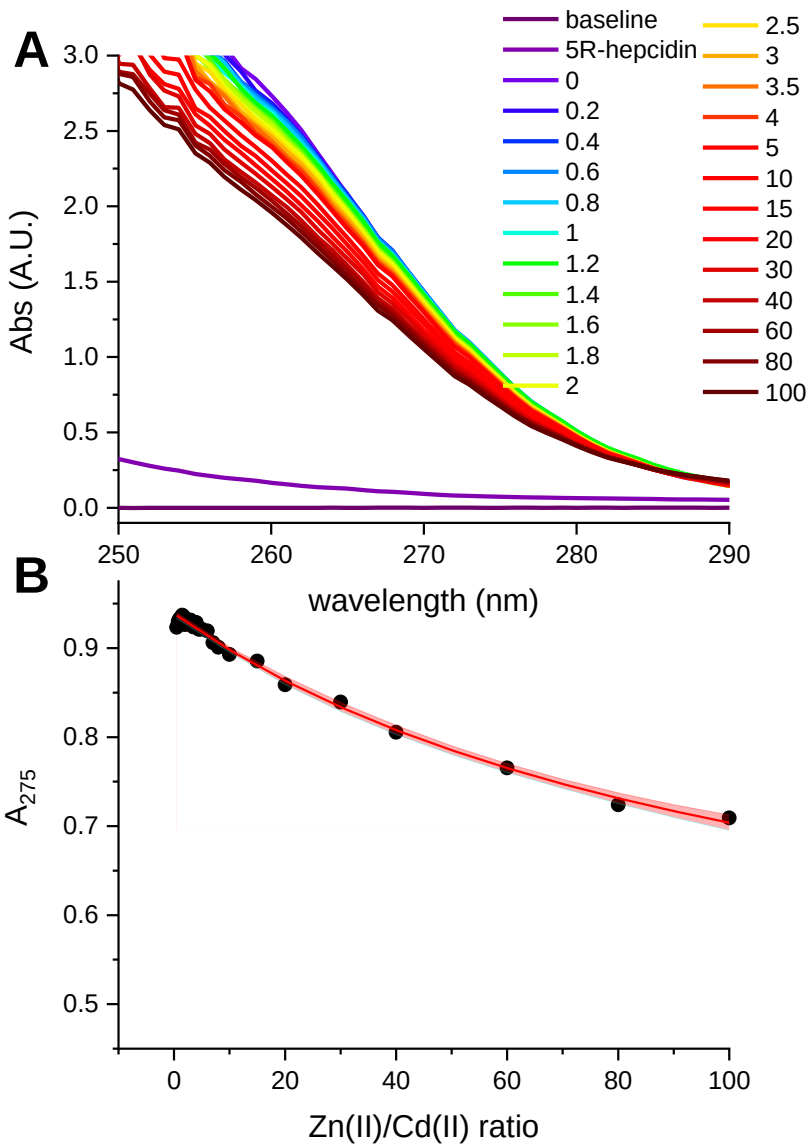

Figure 6. *A. 100 μM $Cd_2$5R-hepcidin titrated with $Zn(NO_3)_2$ in 100 mM HEPES pH 7.4. Numbers in the legend denote molar excess of Zn(II) ions. B. Titration curve at 275 nm and the fit for the competition reaction $Cd_2$5R-hepcidin + Zn(II) = CdZn5R-hepcidin. The 95% confidence bands are marked red.*

The resistance to atmospheric oxygen provided by Cd(II) binding was another test of the Cd(II) affinity to 5R-hepcidin. In theory, no disulfide bonds should be formed upon the Cd(II) protection, but the intrinsic dynamics of the coordination sites can lead to slow oxidation of the thiols. In case of 5R-hepcidin, two Cd(II) equivalents prevented the disulfide formation for more than 48 hours (Figure 7). This experiment additionally confirmed the initial assumption that two Cd(II) ions were bound to all eight Cys residues of hepcidin.

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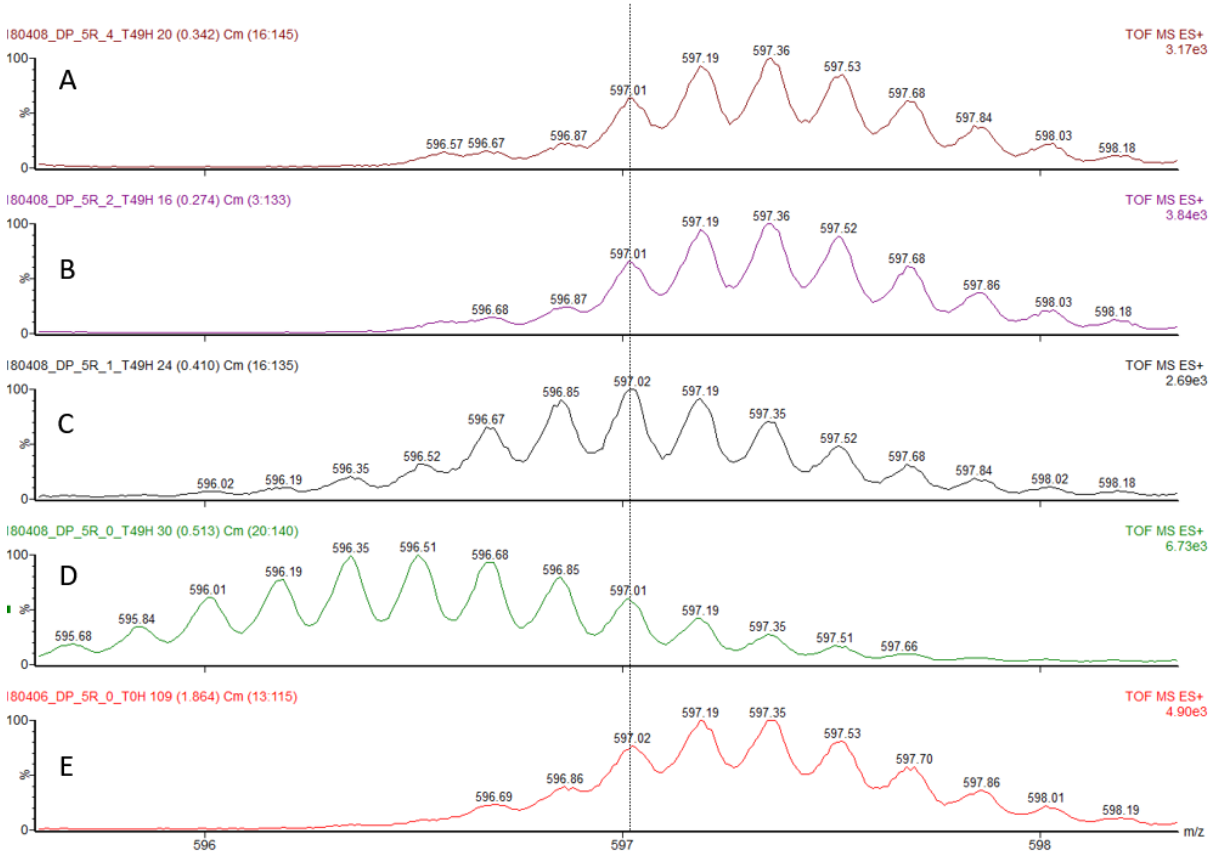

Figure 7. 5R-hepcidin (100 μM) incubated with 4, 2, 1 and 0 Cd(II) equivalents (A, B, C and D respectively) for 49 hours in pH 6, with the initial sample presented in E. The disulfide formation was monitored at ESI-QToF MS at 6+ charged peptide ions $[M+6H]^{6+}$. The isotopic distribution shift to left, indicating the loss of two H atoms for each disulfide formed. The monoisotopic peak of reduced peptide is marked with the dotted line. Small peaks left from the dotted line (one S-S bond) in all samples occurred during sample preparation process.

In the final experiment native mass spectrometry was applied to a series of measurements with constant 5R-hepcidin and Cd(II) acetate and increasing amounts of Zn_7MT2A in the presence of Zn(II) ion excess. MT2A is a natural Cd(II) ligand in cells exposed to cadmium. Despite the complicated character of this experiment which was aimed at reproducing physiological conditions to some extent, the Cd(II) complex of 5R-hepcidin could be monitored and quantified. The deconvoluted spectra are shown in Figure 8 (raw spectra in Figures S3 and S4) and the integrated signals of Cd_2R5-hepcidin in Figure 9. Very clearly, 5R-hepcidin was able for compete for Cd(II) ions with MT2A.

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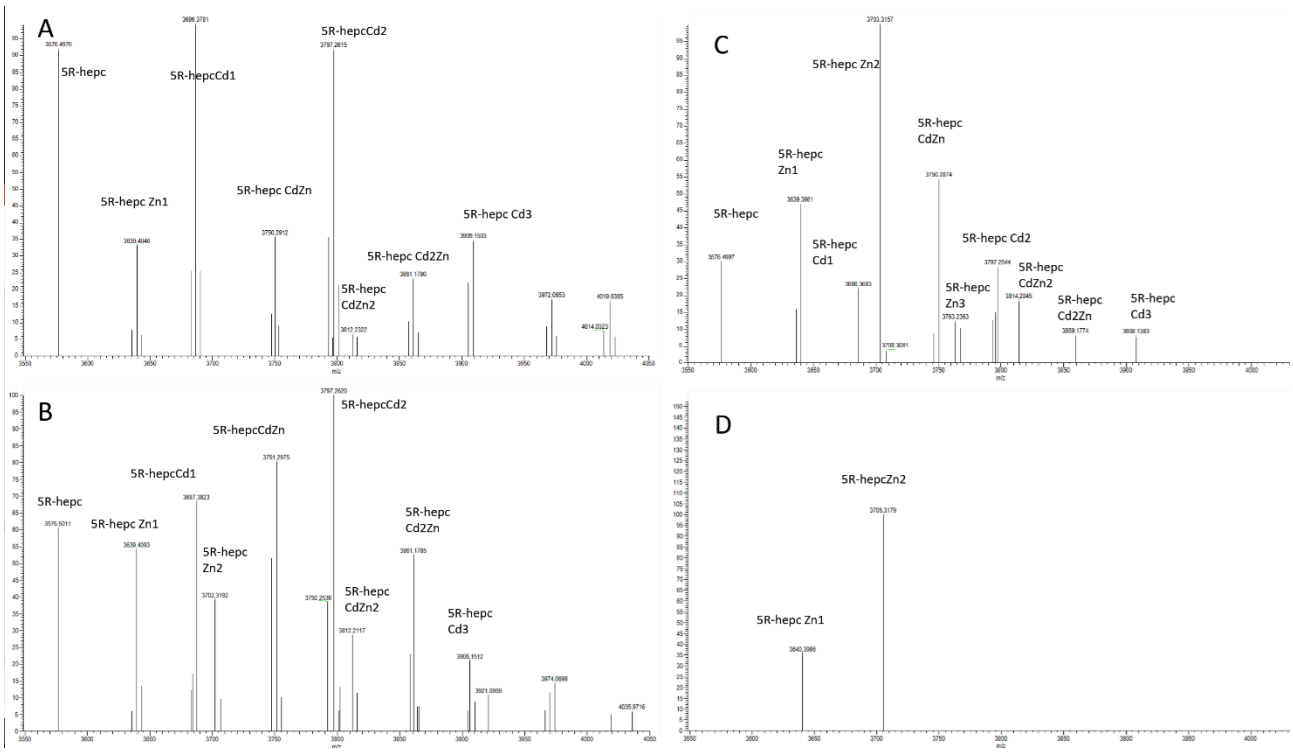

Figure 8. Deconvoluted Native MS spectra of 10 μM 5R-hepcidin and 20 μM Cd(II) acetate with following concentrations of Zn_7MT2A. A – 1μM, B – 3μM, C – 10μM, D – 30μM in ammonium acetate pH 7.4.

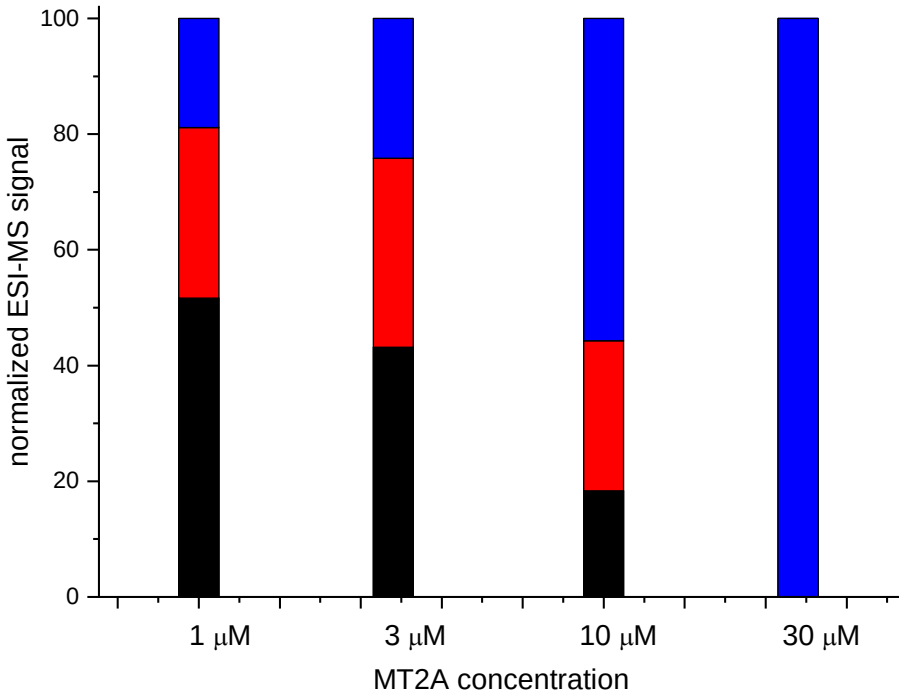

Figure 9. Quantitation obtained by careful integration of relevant signals from native MS experiment of 10 μM 5R-hepcidin and 20 μM Cd(II) acetate titrated with Zn_7MT2A in ammonium acetate, pH 7.4. 5R-hepcidin (blue), Cd5R-hepcidin (red), $Cd_2$5R-hepcidin (black).

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Discussion

So far, the discussion of the direct role of hepcidin in metallobiochemistry has been limited to its mature, extracellular form in which thiol groups of all eight Cys residues are locked in specifically arranged disulfide bonds [9]. The ATCUN motif is the only metal binding site in mature hepcidin-25 and its shorter model peptides, but its biological role remains to be seen [3,24]. In contrast, the set of experiments presented here demonstrates that the intracellular reduced form of hepcidin and/or its prepro- and pro- precursors is a strong ligand for biological Zn(II) and toxic Cd(II) ions. The combined study of spectroscopically and structurally equivalent hepcidin-25 and model 5R-hepcidin indicated that at physiological pH reduced hepcidin binds the Zn(II) ions with conditional log K of 10.1. This affinity is similar to the average affinity of Zn(II) ions to proteome at large, 10.3 [25], indicating that hepcidin-25 may be at least partially metallated by physiological Zn(II) ions during its intracellular maturation. This notion has interesting implications for the mechanism of disulfide bond formation in the course of hepcidin maturation, and for the possible relationship between hepcidin/iron physiology and liver zinc status.

Furthermore, our studies indicated that reduced hepcidin is a potent Cd(II) ligand. The affinities of its both sites for Cd(II) ions are similar to those of metallothionein [7]. The direct MS experiment demonstrated that hepcidin can actually compete with MT2 for Cd(II) ions. One should always be wary of mass spectrometry results as a substitute to solution studies, and take them with a grain of salt [23,26,27]. The abundance of Zn(II) ions, residual from MT2A purification process, which could not be fully removed from the MS system could also distort the quantitative results, but the consistency of the competition experiment with in-solution titration experiments confirms that Cd(II) affinity of reduced hepcidin is high enough to compete with MT2.

The primary function of metallothionein is to control cytoplasmatic availability of Zn(II) and Cu(I) ions [7]. Detoxifying cadmium was likely not an evolutionary pressure before industrial times, which is true for all cadmium related proteins in humans. Moreover, MT2A has multiple binding constants for Cd(II) [28], which means that although it is the reservoir for Cd(II) inside the cell, it is not an ultimate scavenger [27]. And our experiments confirm that Cd(II) can be retained by reduced hepcidin in the presence of MT2.

Despite some similarities of hepcidin and MTs regarding the Zn(II) and Cd(II) binding [28,29], they differ greatly. Biological experiments indicate that unlike the MTs, hepcidin apparently does not provide a cytoprotective effect. As opposed to MTs, hepcidin silencing decreases Cd(II) toxicity while overexpression of hepcidin decreases cell viability in the presence of cadmium [16]. Binding cadmium by hepcidin could result in a formation of a toxic compound suggested by reactive oxygen species formation in cell experiments. However, an opposite effect of hepcidin presence on

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iron-induced toxicity was reported. This cannot be due to the binding of iron, as hepcidin binds only Fe^{3+} in a reducing environment, a combination unlikely to happen *in vivo* [30].

The Cd(II)/Zn(II) competition experiment yielded an additional clue for the toxicity of Cd(II)-hepcidin. The pH titration indicated that the Zn(II) affinity to hepcidin-25 is ca. four orders of magnitude weaker than that of the weaker Cd(II) site (log K 10.1 vs. 14.1 at pH 7.4). In contrast, the titration of Zn(II) into $Cd_2$5R-hepcidin demonstrated the ratio of only two orders of magnitude. One can speculate that the binding of the Cd(II) ion could pre-organize the peptide for stronger Zn(II) binding, on the metallothionein level (log K ~12). Thus, hepcidin could somehow contribute to cadmium toxicity by additionally interfering with the zinc metabolism.

One could also hypothesize that cadmium can interfere with hepcidin maturation. Prohepcidin in blood correlates neither with iron loading, nor with inflammation, as normally hepcidin does [31,32]. It is correlated with renal failure [33], but has no clear function. Except for Cd-bound MTs, other sulfhydryl-rich proteins (this might include hepcidin) can transport cadmium from liver to kidneys [34]. A Cd(II):hepcidin complex can withstand oxidizing conditions (Fig. 7), so it would be theoretically possible for improperly maturated hepcidin to retain Cd(II) in blood.

We can safely conclude that at least partial exposure of hepcidin and, by extension, of prohepcidin to cadmium can lead to metalation of the peptide. Whether it results in improper folding and exporting of Cd-prohepcidin remains to be further investigated.

Materials and methods

Materials. Dimethylformamid (DMF) was purchased from Carl Roth. Acetonitrile from Avantor POCH. Peptide synthesis building blocks, Trifluoroacetic acid (TFA), Piperidine, HBTU, N,N-Diisopropylethylamine (DIEA), triisopropylsilane (TIS), 1,2-Ethanedithiol (EDT) from Merck. 4-(2-Pyridylazo)resorcinol (PAR), HEPES buffer, 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and ammonium acetate from Sigma-Aldrich. Fmoc Thr(t-Bu) TentaGel S PHB resin from Rapp polymere.

Methods. Peptide synthesis was carried out with standard Fmoc-SPPS protocol using Liberty1 synthesizer (CEM) [35]. Peptides were purified with acetonitrile:H_2O gradient with 0.1% TFA and the purity was confirmed using ESI-QToF MS on Premier (Waters).

Expression and purification of metallothionein MT2A from Addgene plasmid ID 105693 was carried out as before [36]. Concentration of metals was determined using PAR [37] and peptides using DTNB assay [38]. Metal titrations were carried out on Cary 50 Bio spectrophotometer (Varian) and J-815 spectropolarimeter (Jasco). Whenever no TCEP was used, all titrations were performed in anaerobic

chamber flushed constantly with N_2. In air oxidation experiment, reagents were mixed in anaerobic chamber, pH was adjusted with ammonia and/or formic acid, then taken to normal atmosphere and gently mixed. ESI-MS spectra were recorded on Premier ESI-QToF MS (Waters) All measurements were performed in the positive ion mode. The source temperature 80 °C was used for a complete desolvation of the peptide ions. The cone voltage was 30 V. The transmission of the ions was optimized on the quadrupole for the mass range m/z 300 to 2000. Mass spectra were accumulated over 2 min to improve the signal-to-noise ratio. The sample flow was 20 µL/min.

Native MS was performed on Q Exactive UHMR Hybrid Quadrupole-Orbitrap mass spectrometer (ThermoFisher Scientific). Target peptides were prepared by dissolving peptides in 20 mM ammonium acetate pH 7.4 in 10 µM concentration. To each sample a cadmium acetate was added to reach 2:1 ratio, and then a differing concentration of competing ligand. Measurements started with an 1 h delay to allow for reaching equilibrium. Samples were introduced into the mass spectrometer with a syringe pump using 10 µL/min flow rate, by electrospray ionization using positive mode in HESI source. MS measurements were conducted under the following settings: desolvation voltage: -20 V, capillary temperature 320 °C, detector m/z optimization: low m/z; ion transfer optimization to low m/z. The RF applied throughout the instrument were set to 150 Vp-p for injection flatapole, 300 Vp-p for bent flatapole, 250 for transfer multipole and HCD cell, and 2300 for C-trap. The ions transfer optics was to 5 V for injection flatapole, 4 V for intel flatapole, 2 V for bent flatapole and 0 V for transfer multipole. Integration of the resulting peaks was achieved with built-in software FreeStyle 1.4 (Thermo Scientific).

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Author contributions

Conceptualization, D.P., A.M.B and W.B.; methodology D.P. and W.B.; investigation D.P and M.D.W; formal analysis W.B. and D.P; resources A.M.B., M.D.P-D. and A.K.; data curation D.P; writing – original draft preparation D.P. and W.B.; writing – review and editing M.D.W., A.M.B., M.D.P-D. and A.K.; visualization D.P and W.B. project administration W.B.; supervision W.B.; funding acquisition D.P. All authors have read and agreed to the published version of the manuscript.

Data availability statement

The data presented in this study are available on request from the corresponding author. The data are stored offline due to online security reasons.

Conflicts of interests

Authors declare no conflicts of interests.

References

1. Maret, W. Metallomics: The Science of Biometals and Biometalloids. In *Advances in Experimental Medicine and Biology*; Springer New York LLC, 2018; Vol. 1055, pp. 1–20.
2. Nemeth, E.; Tuttle, M.S.; Powelson, J.; Vaughn, M.D.; Donovan, A.; Ward, D.M.V.; Ganz, T.; Kaplan, J. Hepcidin Regulates Cellular Iron Efflux by Binding to Ferroportin and Inducing Its Internalization. *Science (80-.).* **2004**, *306*, 2090–2093, doi:10.1126/SCIENCE.1104742.
3. Płonka, D.; Bal, W. The N-Terminus of Hepcidin Is a Strong and Potentially Biologically Relevant Cu(II) Chelator. *Inorganica Chim. Acta* **2018**, *472*, 76–81, doi:10.1016/j.ica.2017.06.051.
4. Gonzalez, P.; Bossak, K.; Stefaniak, E.; Hureau, C.; Raibaut, L.; Bal, W.; Faller, P. N-Terminal Cu-Binding Motifs (Xxx-Zzz-His, Xxx-His) and Their Derivatives: Chemistry, Biology and Medicinal Applications. *Chem. - A Eur. J.* **2018**, *24*, 8029–8041, doi:10.1002/CHEM.201705398.
5. Tselepis, C.; Ford, S.J.; McKie, A.T.; Vogel, W.; Zoller, H.; Simpson, R.J.; Diaz Castro, J.; Iqbal, T.H.; Ward, D.G. Characterization of the Transition-Metal-Binding Properties of Hepcidin. *Biochem. J.* **2010**, *427*, 289–296, doi:10.1042/BJ20091521.
6. Babula, P.; Masarik, M.; Adam, V.; Eckschlager, T.; Stiborova, M.; Trnkova, L.; Skutkova, H.; Provaznik, I.; Hubalek, J.; Kizek, R. Mammalian Metallothioneins: Properties and Functions. *Metallomics* **2012**, *4*, 739–750, doi:10.1039/c2mt20081c.
7. Krężel, A.; Maret, W. The Bioinorganic Chemistry of Mammalian Metallothioneins. *Chem. Rev.* **2021**, *121*, 14594–14648, doi:10.1021/acs.chemrev.1c00371.
8. Preza, G.C.; Ruchala, P.; Pinon, R.; Ramos, E.; Qiao, B.; Peralta, M.A.; Sharma, S.; Waring, A.; Ganz, T.; Nemeth, E. Minihepcidins Are Rationally Designed Small Peptides That Mimic Hepcidin Activity in Mice and May Be Useful for the Treatment of Iron Overload. *J. Clin. Invest.* **2011**, *121*, 4880–4888, doi:10.1172/JCI57693.
9. Jordan, J.B.; Poppe, L.; Haniu, M.; Arvedson, T.; Syed, R.; Li, V.; Kohno, H.; Kim, H.; Schnier,

P.D.; Harvey, T.S.; et al. Hepcidin Revisited, Disulfide Connectivity, Dynamics, and Structure. *J. Biol. Chem.* **2009**, *284*, 24155–24167, doi:10.1074/jbc.M109.017764.

10. Maret, W.; Moulis, J.M. The Bioinorganic Chemistry of Cadmium in the Context of Its Toxicity. *Met. Ions Life Sci.* **2013**, *11*, 1–29, doi:10.1007/978-94-007-5179-8_1.
11. Thévenod, F.; Lee, W.K. Toxicology of Cadmium and Its Damage to Mammalian Organs. *Met. Ions Life Sci.* **2013**, *11*, 415–490, doi:10.1007/978-94-007-5179-8_14.
12. Thévenod, F.; Fels, J.; Lee, W.K.; Zarbock, R. Channels, Transporters and Receptors for Cadmium and Cadmium Complexes in Eukaryotic Cells: Myths and Facts. *BioMetals* **2019**, *32*, 469–489, doi:10.1007/s10534-019-00176-6.
13. Balesaria, S.; Ramesh, B.; McArdle, H.; Bayele, H.K.; Srai, S.K.S. Divalent Metal-Dependent Regulation of Hepcidin Expression by MTF-1. *FEBS Lett.* **2010**, *584*, 719–725, doi:10.1016/j.febslet.2009.12.023.
14. Chen, J.; Shi, Y.H.; Li, M.Y. Changes in Transferrin and Hepcidin Genes Expression in the Liver of the Fish Pseudosciaena Crocea Following Exposure to Cadmium. *Arch. Toxicol.* **2008**, *82*, 525–530, doi:10.1007/s00204-008-0297-7.
15. Tokumoto, M.; Ohtsu, T.; Imai, S.; Honda, A.; Nagase, H.; Satoh, M. DNA Microarray Analysis of Hepatic Gene Expression in Mice Exposed to Cadmium for 30 Days. *J. Toxicol. Sci.* **2013**, *38*, 155–157, doi:10.2131/jts.38.155.
16. Probst, S.; Fels, J.; Scharner, B.; Wolff, N.A.; Roussa, E.; van Swelm, R.P.L.; Lee, W.K.; Thévenod, F. Role of Hepcidin in Oxidative Stress and Cell Death of Cultured Mouse Renal Collecting Duct Cells: Protection against Iron and Sensitization to Cadmium. *Arch. Toxicol.* **2021**, *95*, 2719–2735, doi:10.1007/s00204-021-03106-z.
17. Vašák, M.; Kägi, J.H.R. *Metallothioneins. In Encyclopedia of Inorganic Chemistry*; King, R.B., Ed.; John Wiley & Sons, Ltd: New York, NY, 1994;
18. Bal, W.; Schwerdtle, T.; Hartwig, A. Mechanism of Nickel Assault on the Zinc Finger of DNA Repair Protein XPA. *Chem. Res. Toxicol.* **2003**, *16*, 242–248, doi:10.1021/TX025639Q.
19. Kopera, E.; Schwerdtle, T.; Hartwig, A.; Bal, W. Co(II) and Cd(II) Substitute for Zn(II) in the Zinc Finger Derived from the DNA Repair Protein XPA, Demonstrating a Variety of Potential Mechanisms of Toxicity. *Chem. Res. Toxicol.* **2004**, *17*, 1452–1458, doi:10.1021/tx049842s.
20. Bossak, K.; Goch, W.; Piątek, K.; Frączyk, T.; Poznański, J.; Bonna, A.; Keil, C.; Hartwig, A.; Bal, W. Unusual Zn(II) Affinities of Zinc Fingers of Poly(ADP-Ribose) Polymerase 1 (PARP-1) Nuclear Protein. *Chem. Res. Toxicol.* **2015**, *28*, 191–201, doi:10.1021/tx500320f.
21. Wątły, J.; Łuczkowski, M.; Padjasek, M.; Krężel, A. Phytochelatins as a Dynamic System for Cd(II) Buffering from the Micro- to Femtomolar Range. *Inorg. Chem.* **2021**, *60*, 4657–4675, doi:10.1021/acs.inorgchem.0c03639.
22. Valore, E. V.; Ganz, T. Posttranslational Processing of Hepcidin in Human Hepatocytes Is Mediated by the Prohormone Convertase Furin. *Blood Cells, Mol. Dis.* **2008**, *40*, 132–138, doi:10.1016/j.bcmd.2007.07.009.
23. Płonka, D.; Kotuniak, R.; Dąbrowska, K.; Bal, W. Electrospray-Induced Mass Spectrometry Is Not Suitable for Determination of Peptidic Cu(II) Complexes. *J. Am. Soc. Mass Spectrom.* **2021**, *32*, 2766–2776, doi:10.1021/jasms.1c00206.
24. Melino, S.; Garlando, L.; Patamia, M.; Paci, M.; Petruzzelli, R. A Metal-Binding Site Is Present in the Amino Terminal Region of the Bioactive Iron Regulator Hepcidin-25. *J. Pept. Res.* **2005**, *66*, 65–71, doi:10.1111/j.1747-0285.2006.00328.x.
25. Mahim, A.; Karim, M.; Petering, D.H. Zinc Trafficking 1. Probing the Roles of Proteome,

Metallothionein, and Glutathione. *Metallomics* **2021**, *13*, 55, doi:10.1093/MTOMCS/MFAB055.

26. Jecklin, M.C.; Touboul, D.; Bovet, C.; Wortmann, A.; Zenobi, R. Which Electrospray-Based Ionization Method Best Reflects Protein-Ligand Interactions Found in Solution? A Comparison of ESI, NanoESI, and ESSI for the Determination of Dissociation Constants with Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **2008**, *19*, 332–343, doi:10.1016/J.JASMS.2007.11.007.
27. Konermann, L. Addressing a Common Misconception: Ammonium Acetate as Neutral PH "Buffer" for Native Electrospray Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **2017**, *28*, 1827–1835, doi:10.1007/S13361-017-1739-3.
28. Wang, Y.; Mackay, E.A.; Kurasaki, M.; Kägi, J.H.R. Purification and Characterisation of Recombinant Sea Urchin Metallothionein Expressed in Escherichia Coli. *Eur. J. Biochem.* **1994**, *225*, 449–457, doi:10.1111/J.1432-1033.1994.00449.X.
29. Krężel, A.; Maret, W. Dual Nanomolar and Picomolar Zn(II) Binding Properties of Metallothionein. *J. Am. Chem. Soc.* **2007**, *129*, 10911–10921, doi:10.1021/ja071979s.
30. Farnaud, S.; Rapisarda, C.; Bui, T.; Drake, A.; Cammack, R.; Evans, R.W. Identification of an Iron-Hepcidin Complex. *Biochem. J.* **2008**, *413*, 553–557, doi:10.1042/BJ20080406.
31. Kemna, E.; Pickkers, P.; Nemeth, E.; Van Der Hoeven, H.; Swinkels, D. Time-Course Analysis of Hepcidin, Serum Iron, and Plasma Cytokine Levels in Humans Injected with LPS. *Blood* **2005**, *106*, 1864–1866, doi:10.1182/blood-2005-03-1159.
32. Young, M.F.; Glahn, R.P.; Ariza-Nieto, M.; Inglis, J.; Olbina, G.; Westerman, M.; O'Brien, K.O. Serum Hepcidin Is Significantly Associated with Iron Absorption from Food and Supplemental Sources in Healthy Young Women. *Am. J. Clin. Nutr.* **2009**, *89*, 533–538, doi:10.3945/ajcn.2008.26589.
33. Taes, Y.E.C.; Wuyts, B.; Boelaert, J.R.; De Vriese, A.S.; Delanghe, J.R. Prohepcidin Accumulates in Renal Insufficiency. *Clin. Chem. Lab. Med.* **2004**, *42*, 387–389, doi:10.1515/CCLM.2004.069.
34. Klaassen, C.D.; Liu, J.; Diwan, B.A. Metallothionein Protection of Cadmium Toxicity. *Toxicol. Appl. Pharmacol.* **2009**, *238*, 215–220, doi:10.1016/J.TAAP.2009.03.026.
35. Chan, W.; White, P. *Fmoc Solid Phase Peptide Synthesis. Practical Approach*; Oxford University Press: Oxford, 1999; Vol. v. 222; ISBN 9780199637249.
36. Peris-Díaz, M.D.; Guran, R.; Domene, C.; De Los Rios, V.; Zitka, O.; Adam, V.; Krężel, A. An Integrated Mass Spectrometry and Molecular Dynamics Simulations Approach Reveals the Spatial Organization Impact of Metal-Binding Sites on the Stability of Metal-Depleted Metallothionein-2 Species. *J. Am. Chem. Soc.* **2021**, *143*, 16486–16501, doi:10.1021/jacs.1c05495.
37. Kocyła, A.; Pomorski, A.; Krężel, A. Molar Absorption Coefficients and Stability Constants of Metal Complexes of 4-(2-Pyridylazo)Resorcinol (PAR): Revisiting Common Chelating Probe for the Study of Metalloproteins. *J. Inorg. Biochem.* **2015**, *152*, 82–92, doi:10.1016/j.jinorgbio.2015.08.024.
38. Eyer, P.; Worek, F.; Kiderlen, D.; Sinko, G.; Stuglin, A.; Simeon-Rudolf, V.; Reiner, E. Molar Absorption Coefficients for the Reduced Ellman Reagent: Reassessment. *Anal. Biochem.* **2003**, *312*, 224–227, doi:10.1016/S0003-2697(02)00506-7.