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Modelling of a metal-containing hepcidin

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Abstract

Hepcidin was originally identified as a liver-expressed antimicrobial peptide but further studies have shown that it also has a key role in iron homeostasis. The NMR structure of the synthetic peptides reveal a distorted beta-sheet containing 4 disulphide bridges, with an unusual vicinal disulphide bridge which has been suggested to be functionally significant. In this study, we report the presence of co-purified iron with the urine-purified 20 and 25 residue hepcidins. Since the published structure does not allow metal binding, the interaction of hepcidin with metals was investigated for other possible structural conformations by threading its primary sequence onto existing 3D folds. Several alignments were obtained and the best scores were used to build a 3D model of hepcidin containing one atom of iron. The new 3D structure, that contains only reduced Cys residues, is completely different from the solved structure of the synthetic peptide. Although the model presented here shows only one metal bound to the peptide, the binding of several metal atoms cannot be excluded from such a short flexible peptide. The co-purification of iron with both peptides, together with our 3D model, suggest a conformational polymorphism for hepcidin, reminiscent of the iron regulatory proteins IRPs.

Introduction

Hepcidin is a peptide predominantly expressed in the liver which was first purified from human blood and urine as an antimicrobial peptide (Krause *et al.* 2000; Park *et al.* 2001). Further studies have shown that over-expression of hepcidin results in iron-deficiency anemia in mice and that its levels were inversely proportional to the iron content in their food (Pigeon *et al.* 2001; Nicolas *et al.* 2002). These observations support the role of hepcidin as a signal that limits intestinal iron absorption. Hepcidin expression is decreased in hemochromatosis patients and is also affected by hypoxia and inflammation suggesting a key role in iron homeostasis under various patho-physio logical conditions (Roetto *et al.* 2003, 2004; Ganz 2004). Recently, hepcidin has been shown to bind to the iron exporter ferroportin, leading to decreased export of cellular iron. Identification of such a hepcidin receptor defined its role as a hormone which, by controlling the concentration of ferroportin on the cell surface, can limit the release of iron from the intestine and macrophages (Nemeth *et al.* 2004).

The mature peptide is found under two predominant forms of 20 and 25 residues, respectively, and the structure of both synthetic peptides has been determined by NMR spectroscopy (Hunter *et al.* 2002). These small cysteine-rich peptides form a distorted beta-sheet, containing 4 S–S bridges, with an unusual vicinal disulphide bridge which has been suggested to be functionally significant. Both peptides exhibit an overall amphipathic structure with six of the eight cysteine residues involved in maintaining inter-strand connectivity. It has to be noted that such beta-sheet structure was not expected from the circular dichroism (CD) analysis spectra obtained with the native peptide and published by Park *et al.*, which

suggest a high content of random coil structure (Park *et al*. 2001). Diffusion studies indicate that hepcidin-20 exists as a monomer in solution, whereas hepcidin-25 readily aggregates, a conformation that seems to involve the first five residues at the N-terminus (Hunter *et al*. 2002). Although cysteine-rich antimicrobial peptides are abundant in animal and plant tissues involved in host defence, no close sequence similarity has been described with other known peptides.

In the present study, following the co-purification of iron with human hepcidin purified from urine, bioinformatics studies were carried out in an attempt to dock metal atoms in the hepcidin molecule. Since docking of such atoms in the solved structure of the synthetic peptide was found to be impossible, a search for structure similarities with other known structures was undertaken. A structure/sequence alignment was obtained between hepcidin and the ubiquitous cysteine-rich metal-binding protein family, the metallothionein (MT) protein family. Although the biological function of MTs is still to be established, MTs have been suggested to be involved in the detoxification of heavy metals to protect tissues against various forms of oxidative injury and to have the activity of a reactive oxygen scavenger (Sato *et al*. 1992; Sato & Bremner 1993; Sato & Kondoh 2002). Based on sequence alignment with the α domain of the mouse methallothionein 1 (MT1), a 3D model of hepcidin containing metal was built. The new 3D structure is completely different from the published solved structure of the synthetic peptide, suggesting a conformational polymorphism for hepcidin, reminiscent of the iron regulatory proteins IRP1 and 2.

Materials and methods

Hepcidin analysis

The human hepcidin peptides of 20 and 25 residues, Hepc20 and Hepc25 respectively, were a gift from Dr. Zoller from Addenbrooke's Hospital, Cambridge. The peptides were extracted from pooled urine samples and were purified following the protocol described by Park *et al*. (2001). The iron content analysis was determined by inductively coupled plasma-mass spectrometry (ICP-MS) using a Perkin Elmer Elan mass spectrometer at the Mass Spectrometry Facility based in the School of Health and Life Sciences at King's College London.

Structure/sequence alignment and model building

The sequence of the mature Hepc25 was submitted to the 3D-PSSM Web Server V 2.6.0, from Imperial College London, a fast, web-based method for protein fold recognition using 1D and 3D sequence profiles coupled with secondary structure and solvation potential information to detect remote homology relationships with known structures (Kelley *et al*. 2000). Preliminary structural analysis on the Hepc25 sequence was performed using fold recognition methods, 3D-PSSM and THREADER, to predict protein fold groups to which hepcidin may belong. The template structure was displayed using the molecular modelling programme INSIGHTII (Molecular Simulations, Inc., Cambridge, UK) and a model of Hepc20 was constructed using the HOMOLOGY modelling programme which is part of the INSIGHTII suite of programmes. All modelling was carried out using Silicon Graphics 'Octane' and 'Indigo' workstations (Silicon Graphics Ltd., Reading, UK). The hepcidin sequence was aligned with template sequences according to the information provided by the fold recognition programmes. The extents of structurally conserved regions (SCR) were carefully examined. Minor realignments were made to ensure correct positioning of conserved residues, in particular disulphide-forming cysteines and core hydrophobics. Co-ordinates for the SCR of hepcidin were assigned directly from the template molecule. Co-ordinates for external loop regions on hepcidin, which were not homologous to the template molecule, were obtained using the conformational search programme GENLOOPS within the INSIGHT programme suite. Loops were selected on the basis of broadly acceptable stereo-chemical parameters: allowable *phi* and *psi* angles on a Ramachandran plot, the alignment of Cα—Cβ bonds at splice points and, in particular, on minimal steric interaction with adjacent loop or SCR residues. The whole model was then examined for steric overlaps. Attempts were made to ease any overlap by replacing the existing side-chain rotamer with alternatives from the rotamer library in HOMOLOGY. Energy minimization was performed using the SwissPDBviewer programme.

Results and discussion

The ICP-MS analysis of urine-purified 20 and 25 residue human hepcidins revealed co-purification of iron with the peptides, with a stochiometry of one atom of iron for 3 peptides (Table 1). The published structures of the synthetic peptides show fully-oxidized peptides with 4 disulphide bridges that do not allow metal binding. In order to explore the possible interaction of hepcidin with iron and other metals, the peptide sequence was investigated for other possible structural conformations by threading its primary sequence onto every defined 3D fold. The best scores, following the solved 3D structure of synthetic Hepc20 and Hepc25, were obtained for several members of the metallothionein (MT) superfamily, with 40% identity for the α-domain of mouse metallothionein1 (MT1) (accession number 1DFS in the PDB database). The highly conserved distribution of cysteine-containing motifs in the α-domain of MTs shows a high degree of identity with the hepcidin sequence (Figure 1). MTs are low-molecular-weight proteins composed of two homologous domains, the C-terminal α-domain and the N-terminal β-domain, which are responsible for metal binding via S-Cys donor functions (Henkel & Krebs 2004). They have been proposed to play a role in heavy metal detoxification, metal metabolism regulation and radical scavenging action (Sato & Kondoh 2002) with a low affinity for Zn and a high affinity for toxic metals such as Cd and Hg.

Using the obtained primary structure alignment (Figure 2), a 3D-model of Hepc20 was built using INSIGHTII (Figure 3). Based on the conserved cysteine residues between both molecules, and also their metal connectivities in MT1 (Figure 3), one metal atom was built into the hepcidin model. As for MT1, the new model is mainly made of random coil with a lack of defined

Table 1. ICP-MS analysis.

C1	Fe/μM (based on 54 Fe) < 0.1	Fe/Hepc NA
Hepc20 (1.8 μM)	0.59	0.33
Hepc25 (1.8 μM)	0.57	0.33

C1: control, elution buffer (5% acetic acid); Hepc20: 5 μg of hepcidin 2; Hepc25: 5 μg of hepcidin 25; Fe/Hepc: stochiometry of atom of iron per molecule of peptide.

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Avian          -CCXCC---.CXXC---CXC-------CXCC-
Mammalian      -CCXCC---.CXXC---CXC----.--CXCC-
Piscine        -CCXCC---.CXXC---CXC----C--.XCC-
Rat MT-III     -CCXCC---.CXXC---CXC---X---CXCC-
HEPC_HUMAN    C-CCXCC----CXXC...CX-
HEPC_CANIS    C-CCXCC----CXXC...CX-
HEPC_PIG      C-CCXCC----CXXC...CX-
HEPC_ORANG    Y-CCXCC----CXXC...CX-
HEPC2_MOUSE   C-CCXCC----CXXC...CX-
HEPC_MOUSE    C-CCXCC----CXXC...CX-
HEPC_RAT      C-CCXCC----CXXC...CX-
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Figure 1. Schematic representation of the consensus arrangement of cysteine residues of the α-domain of MT-I and MT-II isoforms from avian, mammalian and piscine species and rat MT-III aligned with mammalian hepcidin sequences (C): cysteine residue, (X) residues of which identity is not thought to be critical but connecting cysteines; (–): any amino acid residue not critical for metal binding; (.): gap; HEPC_HUMAN: human hepcidin, HEPC_CANIS: dog hepcidin; HEPC_PIG: porcine hepcidin; HEPC_ORANG: orangutan hepcidin; HEPC_-MOUSE and HEPC2_MOUSE: hepcidin 1 and 2 from the mouse respectively; HEPC_RAT: rat hepcidin.

regular secondary structure. Such secondary structure is more consistent with the published CD spectra obtained with the native peptides (Park *et al.* 2001) than the solved structure of the synthetic peptides. The α-domain and the β-domain of MT1 have been found to contain a tetranuclear and a trinuclear metal cluster, respectively, but due to the absence in the hepcidin sequence of the last 3 cysteine residues present in MT1, our model shows only one metal atom. However, the binding of two or three metal atoms cannot be excluded since the flexibility of such a short peptide might allow for another cysteine residue, for example C82, to compensate for such a loss. Based on the MT structure, the cysteine distribution in hepcidin seems to be a good candidate for iron binding but other residues such as His62, Cys66, His74 and Met80 might also participate in such an interaction with metals in Hepc25.

The sequence homology between MT1 and hepcidin supports further the metal-binding capacity of hepcidin. In MTs, the metal binding is mediated through the abundant cysteine residues, which are clustered in motifs, with a highly conserved arrangement of the cysteine residues throughout vertebrate MTs (Figure 2). The non-conservation of the intervening residues between cysteine residues suggests that they have a limited role in cluster formation and that the overall fold of the peptide is determined by the cysteine arrangement and not by the intervening residues (Kille *et al.* 1994). In such a model, the lack of

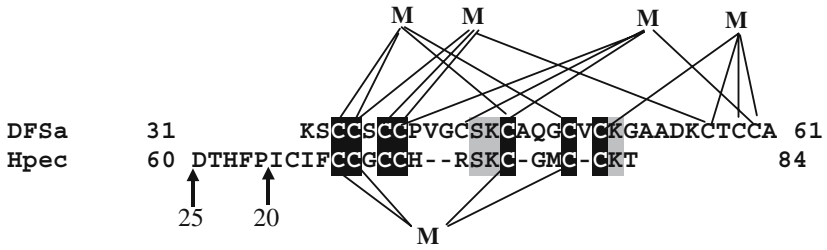

Figure 2. Primary sequence alignment of the α-domain of the mouse metallothionein (1DFSa) with the hepcidin mature peptide sequence showing metal connectivities DFSa: mouse Methallothionein-1, α-domain; Hepc: human hepcidin; arrows show the N-terminus of each peptide Hepc20 and Hepc25. Conserved Cys residues are white on black and other conserved residues are black on grey; M: bound metal.

homology between intervening residues in the alignment between hepcidin and MT1 is less critical. The lysine residues adjacent to some of the cysteine residues in MT1, two of which are conserved in hepcidin, have been proposed to play a role in the deprotonation of the thiol group, promoting interaction with the metals in MT1 (Kille *et al*. 1994). Such a role could also be played in hepcidin, not only by both lysine residues but also by the histidine and arginine residues.

Although MTs have been shown to bind principally toxic metals, copper and zinc, their ability to bind iron has been shown with rabbit MT which was found to bind seven Fe(II) ions, each tetrahedrally coordinated by thiolate sulphur (Good & Vasak 1986). Heterometallic clusters (Fe/Cd) have also been described. The early discovery of MT in equine renal tissue prompted the proposal that MT was involved in intracellular iron metabolism (Kagi & Vallee 1961). Interestingly, the binding of Cu(I) atom to hepcidin was reported by Zoller *et al*. after mass spectroscopy (MS) analysis of urine-purified human hepcidin and presented at the International Bioiron Meeting in Prague this year. A series of multiple species suggested Cu(I) clusters of 1, 2 and 3 atoms bound to hepcidin. In addition, by analogy with MT1, which has been shown to bind hetero-metallic clusters, these four species, apohepcidin and the three metallic complexes, could correspond to hepcidin bound to hetero-clusters. Depending on the oxidation state of the peptide, different combinations of iron or copper atoms could be assigned to each of the 4 peaks obtained. The preferential binding of Cu(I), trigonally co-ordinated in the N-terminal domain of MT, is compatible with results obtained by Zoller *et al*. The analogy with MT suggests that iron and copper bound to hepcidin might be

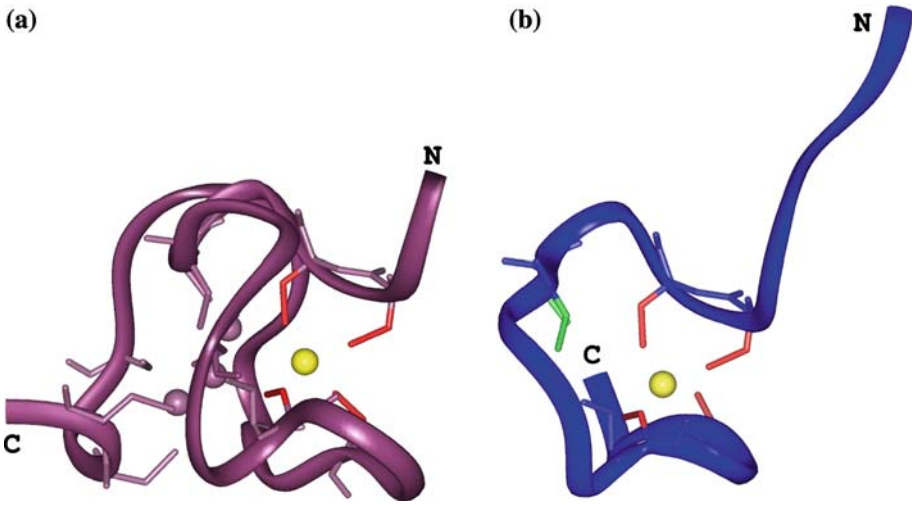

Figure 3. 3D-Model Structure of hepc20 with one metal atom. (a) 1DFSa, (b) Hepc20; only side chains of Cys residues are represented; N and C indicate the N-terminus and the C-terminus respectively.

bound as Fe(II) and Cu(I) although this has yet to be confirmed. In an attempt to repeat with iron Dr. Zoller experiment of binding copper to native hepcidin, the synthetic peptide was reduced and incubated with iron. Preliminary results did not show any bound metal detected by mass spectrometry (data not shown). The non-binding of iron could be due to either a different reactivity of the synthetic peptide or an irreversible oxidized conformation.

In our search for sequence similarities with other proteins, another alignment, although with lower homology, was identified between hepcidin and IRP2 (Figure 4). Interestingly, the part of IRP2 that aligns with hepcidin is the domain which is not present in IRP1 and which has been proposed to contain an iron-binding site (Kang *et al*. 2003). Although the introduction of an iron-sulphur cluster is involved in the IRP1 mechanism, the degradation domain specific to IRP2 has been proposed to contain the three thiolate anions necessary to ligate the iron cation. It is interesting to note that the three cysteine residues forming the binding site in IRP2 are conserved in this alignment with the hepcidin sequence.

Regarding the quantitative aspect of our findings, several interpretations can account for the unusual stochiometry of 1 atom of iron for 3 molecules of peptide. Since a complex of three molecules of peptides connected through an atom of iron, which was not identified in MS analysis, with an unequal distribution of S–Fe bonds, seems quite improbable, such a stochiometry relates probably to the presence of a mixture of holo- and apo-peptides. Since the peptide was purified from pooled urine samples, it might be difficult to find physiological significance to such ratio. In addition, with the different combinations of metallic clusters of 1–3 atoms, it would be difficult to determine the proportion of each species. Furthermore, a complete analysis of metal content could reveal the presence of other metals such as zinc and copper, which would increase the proportion of holo-peptide. As the peptides were purified from urine, one could suspect that their function has already been fulfilled and that some ligands could have been lost. Such a partial loss of ligands could also be the consequence of the harsh acidic conditions used for purification.

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IRP-2   163 PKKLP-CRGQTTCRGSCDSGELCRNSGT 189
HEPC25   60 DTHFPIC---IFCCGCCHRSKCGMCCKT  84
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Figure 4. Partial sequence alignment of IRP2 with hepc25 Identities between the hypothetical iron binding site of IRP2 (K160, K165, C168, C174, C178) and the hepcidin sequence are in white over black and homologies are in black over grey.

Without more quantitative analysis, one can only speculate on the qualitative analysis of binding of iron or other metals to hepcidin to their physiological function. Since our hepcidin sample was purified from urine, one of the first functions to come to mind could be to eliminate excess iron or other metals, a mopping function similar to the one identified for MT1. Although such a function would be in agreement with the role of hepcidin as a limiting factor for excess iron, both concentrations of peptide and bound iron seem to be very low for this to be its primary function. Alternative functions could be related to its identified interaction with ferroportin (Nemeth *et al*. 2004). After binding to hepcidin, ferroportin has been shown to be internalized and degraded, resulting in a decreased export of cellular iron. Since hepcidin is still found in urine, either not all hepcidin is involved in such an interaction with ferroportin, or hepcidin is not degraded after internalization, but instead is secreted back into the bloodstream to be excreted or perform a further function. Alternatively, a physiological significance of the presence of both ferric and non-ferric forms of hepcidin, could relate to its suggested function as the main iron metabolism regulator, as substantiated by its structure alignment with IRP2. In IRPs, the binding of iron to both molecules seems to determine their fate in order to regulate iron homeostasis (Pantopoulos 2004). A similar mechanism could be proposed for hepcidin, where its binding to iron atoms would be dependent upon the level of iron available in the cell. Since the presence of a leader sequence in the pre-propeptide indicates that the synthesis and maturation of hepcidin are occurring in the endoplasmic reticulum (ER), this organelle might be at the heart of such a regulation. As with IRPs, a similar situation with two conformations and two fates could be possible for hepcidin. When the level of iron is low in the ER, the lack of binding to the pro-peptide, prohepcidin, prevents its maturation into an active peptide, whereas only a higher level of iron would permit its maturation and therefore its function which is to decrease the assimilation of iron. It is worthy of note that such a requirement for metal binding in

the ER in addition to the complex process of maturation, in order to obtain the active peptide Hepc25, could explain the difficulties encountered in chemical synthesis of the 25-mer peptide in the absence of any metal.

The actual function of limiting iron-uptake in the body could occur not only at the protein level as described with ferroportin (Nemeth *et al.* 2004), but also at the transcriptional or post-transcrip tional level, either as a transcription factor, or as an IRP in the translational regulation of mRNAs. Ferroportin expression regulation has been proposed to involve both transcriptional and translational mechanisms (Liu *et al.* 2002). Participation of hepcidin in such a mechanism could explain the hepcidin-mediated regulatory interference with ferroportin expression described by Mok *et al.* (2004) which suggests translational regulation of ferroportin by hepcidin.

Although such mechanism might seem unlikely due to the different expression patterns described for the two genes, with ferroportin being most strongly expressed in enterocytes and macrophages, whereas hepcidin is mainly expressed in the liver, the expression pattern of hepcidin has not been fully investigated yet. In mice, expression was observed predominantly in the liver but also weakly in stomach, intestine, colon, lungs, heart, and thymus (Park *et al.* 2001). In human, mRNA expression was also found in the adult and fetal liver and to a lesser extent in adult left atrium of heart, fetal heart, and adult spinal cord. Recent studies have indicated that hepcidin is also produced as an intrinsic peptide in the tubular cells of the mammalian kidney (Kulaksiz *et al.* 2005). These results suggest that further studies might reveal a different distribution of hepcidin expression. In addition, the described interaction of hepcidin with ferroportin suggests an internalization of the peptide. If the peptide were not degraded, such an event would imply the presence of hepcidin inside enterocytes. The same mechanism could be described in macrophages. Ferroportin has been shown to be expressed in vesicular compartments that can reach the plasma membrane where hepcidin has been shown to induce rapid internalization and degradation of the macrophage iron exporter (Delaby *et al.* 2005) suggesting post-transcriptional down-regulation of ferroportin by hepcidin in these cells. A particularly stable hepcidin would also compensate for a low level of expression.

Although this is only one of several possible scenarios, the binding of iron and other metals to hepcidin opens the door to new mechanisms for its role as the iron-metabolism hormone regulator and could explain numerous puzzling phenotypes.

In summary, iron was found to bind hepcidin and a new 3D model has been built, which demonstrates how hepcidin could bind to iron and other metals. The new model is different from the solved structure of the synthetic peptide, but more consistent with previously published CD spectra of the native peptide. In addition, it is a common feature for peptides resulting from the processing of a pro-peptide precursor to require the presence of the pro-sequence to achieve their correct folding and interaction between cysteine residues, as observed with insulin synthesis. This questions whether the 3D structure obtained with the synthetic hepcidin is representative of that of the corresponding native peptide. The presence of bound iron in hepcidin and the possibility of both holo- and apo-forms is a common characteristic of the majority of proteins involved in iron metabolism. Such a conformational polymorphism is reminiscent of IRPs and suggests a regulatory mechanism for iron uptake as part of its role as a regulator of iron homeostasis. Our results are consistent with the recent findings of Gerardi *et al.* (2005) which describes recombinant human hepcidin expressed in *E. coli* as an iron containing protein. A mechanism of iron-dependent conformation in the ER could be the key to iron homeostasis. It is only the tip of the iceberg and many questions remain. Despite the limited amount of native peptide available and the difficulties encountered in its purification, such differences observed with the synthetic peptide suggests that it is on the native peptide that further study should be done in order to elucidate the regulatory role and mechanism of hepcidin.

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