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The Role of Histidine in a Copper-Specific Metallothionein

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Abstract. Metallothioneins achieve metal binding specificity by modulation of their amino acid sequences through evolution. Non-coordinating residues seem to play a key role in this function, and among them histidine may be of particular importance. Here we report how this residue regulates Cu^{I} binding to a highly copper specific isoform, the CuMT of the snail *Helix pomatia*, by analysis of the recombinant complexes yielded by a constructed mutant where this residue has been changed to an alanine.

Introduction

Metallothioneins (MTs) constitute a universal family of cysteine-rich, low-molecular-weight proteins with a high metal-binding capacity. They have been related to multiple biological processes, among which homeostasis of essential metal ions (Zn^{II} and Cu^{I}), and protection against toxic metal ions (i.e. Cd^{II}, Pb^{II}) appear as most relevant.[1] Almost all kinds of organisms contain multiple MT isoforms, normally homologous proteins that exhibit either similar or highly different preferences for divalent vs. Monovalent metal ion binding.[2,3] To date, there is no information about the molecular basis that determines when an MT peptide will yield well-folded, stable complexes with a determined metal ion, although this is a keystone to understand MT structure/function relationships, their physiological function, and their differentiation pattern through evolution. The best-known paradigm of highly similar MT peptides with fully conserved coordinating Cys residues, but nevertheless exhibiting opposite metal preferences, is offered by the Roman snail (*Helix pomatia*) MT system.

This pulmonate mollusk synthesizes two MT isoforms, which show clear and distinct metal specificity: the Cd-specific isoform, HpCdMT, involved in Cd binding and detoxification within the digestive tissues of the snail,[4] and the Cu-isoform, HpCuMT, which is only expressed in the rhogocyte cell type, where it seems to play a role associated with hemocyanin synthesis.[5] In a previous work, we showed how the two *H. pomatia* MT isoforms achieved their metal binding specificity by amino acid sequence diversification.[6] Since the two HpMTs exhibit a full conservation of the coordinating Cys positions (Table 1), it remains clear that the intercalating residues must be those that determine their intrinsic and extremely distinct metal binding behavior towards divalent (Zn^{II} and Cd^{II}) or monovalent (Cu^{I}) metal ions. Therefore, it is crucial to ascertain the role of the non-Cys residues of MT in metal ion coordination, and among them we centered our attention on the His present at position 38 of the Cu-specific HpMT peptide. Histidine contribution to metal-MT coordination is currently well established,[3,7–9] after being first reported in the cyanobacterial SmtA[10] and the wheat E_c-1 proteins.[11]

However, both these cases, and others involving animal MTs (*C.elegans* MT1[12] and chicken MT[13]), implicate divalent metal ion coordination and MTs that have the character of Zn-thioneins.[3] No data are available regarding the possible role of His in Cu-thioneins. Significantly, neither spectrometric and spectroscopic studies,[14] nor the 3D structure determination[15] showed any influence of the His present in Cup1 in Cu-coordination. Thus, we consider especially relevant the current study. Here, the role of the unique His residue of the HpCuMT isoform (Table 1) has been tested through the analysis of the in vivo metal binding properties of a recombinantly synthesized HpCuMTAla mutant, and it has been compared with the corresponding HpCuMT wild type isoform. The results obtained suggest that the His residue in the HpCuMT peptide could be related with the facility to release Cu^{+} at certain physiological conditions in snail rhogocytes.

Results and Discussion

DNA sequencing of the HpCuMTAla coding construct confirmed the presence of the His/Ala codon substitution,

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SHORT COMMUNICATION

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Table 1. Sequence alignment of the (1) *H. pomatia* HpCdMT, (2) HpCuMT and (3) HpCuMTAla mutant (His38 → Ala). (*Note*: the initial GS residues are the consequence of the recombinant construct used for protein synthesis).

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1) GSGKGKGEKCTSACRSEPCQCGSKCQCGEGCTCAACKTCNCTSDGCKCGKECTGPDSCKCGSSCSCK
2) GS..GRGKNCGGACNSNPCSCGNDCKCGAGCNCDRCSSCHCSNDDCKCGSQCTGSGSCKCGSACGCK
3) GS..GRGKNCGGACNSNPCSCGNDCKCGAGCNCDRCSSCACSNDDCKCGSQCTGSGSCKCGSACGCK
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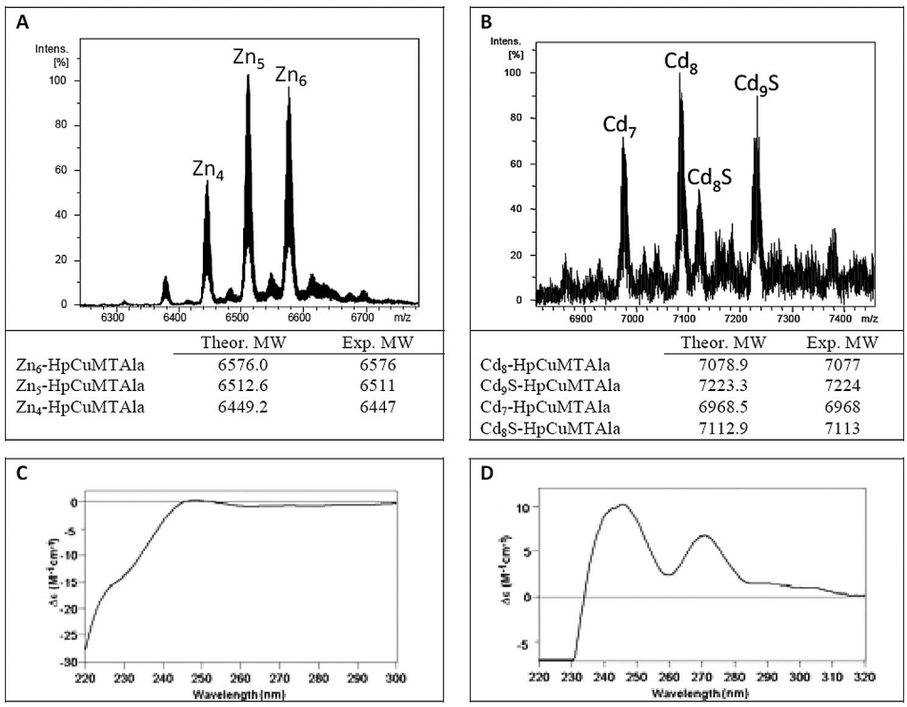
and SDS-PAGE gels of total protein extracts from pGEX*HpCuMTAla*-transformed BL-21 *E. coli* cells showed the presence of a band of the expected size for the recombinant protein. Homogeneous metal-HpCuMTAla preparations (final concentrations of 2.00×10^{-4} M, 1.69×10^{-4} M and 0.75(regular)/0.64(low) $\times$ 10^{-4} M for Zn-, Cd- and Cu complexes, respectively) were obtained from 5-L cultures. Acidification of the Zn-HpCuMTAla preparation yielded the corresponding apo-form, with a molecular mass of 6194.2 Da, in accordance with the calculated theoretical value (6195.6). This confirmed both the identity and the integrity of the recombinant HpCuMTAla polypeptide (Table 1).

First, the HpCuMTAla metal binding ability for the noncognate metal ions (i.e. Zn^{II} and Cd^{II}), was analyzed. Synthesis in Zn-enriched cultures gave rise to several Zn-containing species, with major Zn_6- and Zn_5-HpCuMTAla complexes, which suggested a low in vivo preference for Zn^{II} coordination (Figure 1A). The CD spectrum of these preparations was also indicative of a poor folding degree (Figure 1C). Since these results were practically coincident with those described for HpCuMT,[6] it was concluded that the His38 residue did not play a major role in Zn-coordination.

A similar situation was found for the Cd^{II} binding features. In this case, the synthesis of HpCuMTAla in Cd^{II}-rich media always rendered highly heterogeneous preparations, including both sulfide-devoid and sulfide-containing complexes. The major species recovered were, Cd_7-, Cd_8- and Cd_9S-HpCuMTAla, which is again coincident with those rendered by HpCuMT (Figure 1B). The CD of this sample (Figure 1D) fully corroborated the presence of Cd_xS-HpCuMTAla species, with absorptions at ca. 250 nm corresponding to the Cd-SCys chromophores, and the signals at ca. 280 nm associated with the presence of sulfide ligands bound to Cd^{2+}.[16] Again, as the absence of His38 in the mutant form did not apparently alter the Cd^{II} coordination abilities of the HpCuMT isoform, it can be proposed that His does not play a significant role in the divalent metal ion binding to this copper-specific MT form.

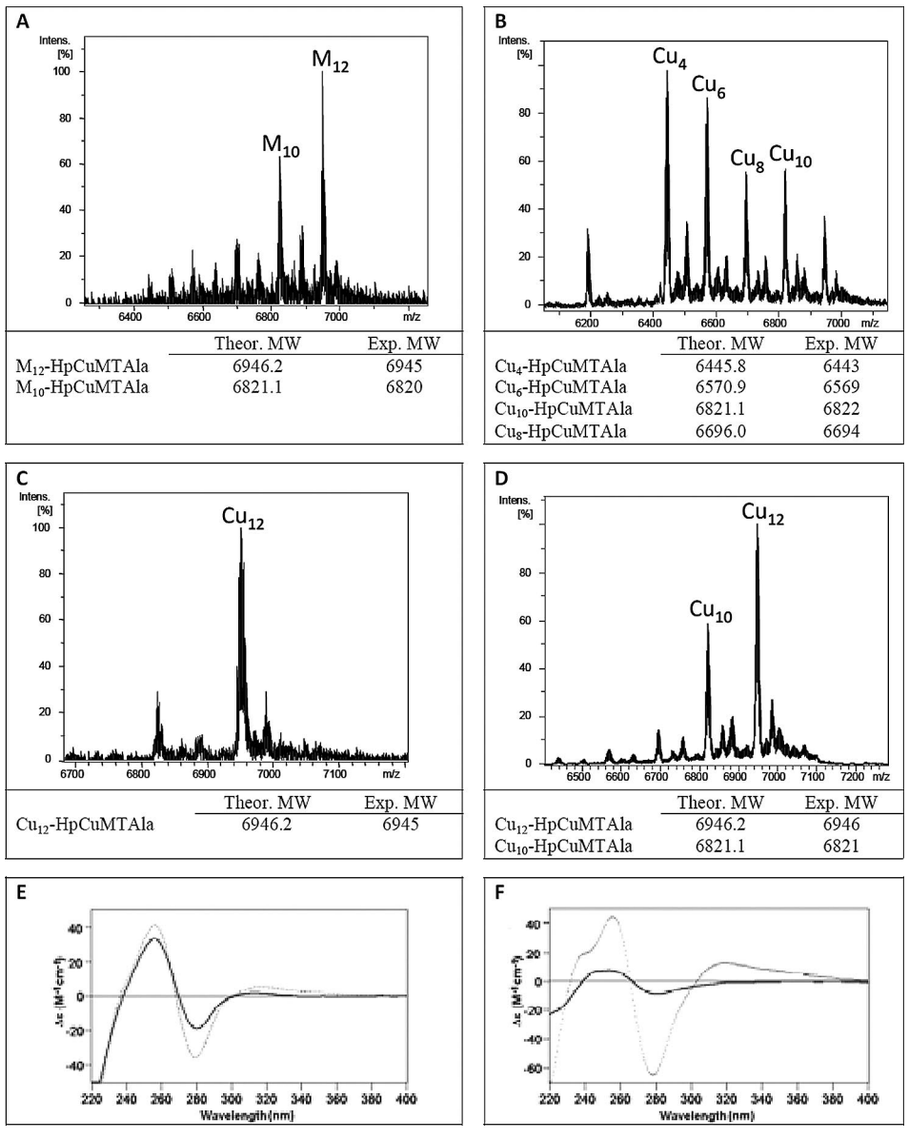
On the other hand, the synthesis of HpCuMTAla in Cu-supplemented cultures, grown either at regular or low aeration conditions (determining regular and high cell copper content, respectively) revealed interesting differences between the mutated and wild-type isoforms. Under normal aeration, Cu-HpCuMTAla is synthesized as a major heterometallic M_{12}-, followed by M_{10}-HpCuMTAla complexes (M = Zn or Cu).



	Theor. MW	Exp. MW
Zn_6-HpCuMTAla	6576.0	6576
Zn_5-HpCuMTAla	6512.6	6511
Zn_4-HpCuMTAla	6449.2	6447

	Theor. MW	Exp. MW
Cd_8-HpCuMTAla	7078.9	7077
Cd_9S-HpCuMTAla	7223.3	7224
Cd_7-HpCuMTAla	6968.5	6968
Cd_8S-HpCuMTAla	7112.9	7113

Figure 1. Deconvoluted ESI-MS spectra, recorded at pH 7.0, of the recombinant preparations of HpCuMTAla in (A) Zn- and (B) Cd-enriched media. The error associated to the experimental MW values was always lower than 1%. CD spectra of the in vivo (C) Zn-HpCuMTAla and (D) Cd-HpCuMTAla preparations.



	Theor. MW	Exp. MW
M_{12}-HpCuMTAla	6946.2	6945
M_{10}-HpCuMTAla	6821.1	6820

	Theor. MW	Exp. MW
Cu_4-HpCuMTAla	6445.8	6443
Cu_6-HpCuMTAla	6570.9	6569
Cu_{10}-HpCuMTAla	6821.1	6822
Cu_8-HpCuMTAla	6696.0	6694

	Theor. MW	Exp. MW
Cu_{12}-HpCuMTAla	6946.2	6945

	Theor. MW	Exp. MW
Cu_{12}-HpCuMTAla	6946.2	6946
Cu_{10}-HpCuMTAla	6821.1	6821

Figure 2. Deconvoluted ESI-MS spectra of the recombinant preparations of HpCuMTAla in Cu-enriched media under (A, B) regular or (C, D) low aeration conditions at (A, C) pH 7.0 and (B, D) pH 2.4. M stands for Zn+Cu, owing to the ESI-MS difficulties for discriminating between these two metal ions. The error associated with the experimental MW values was always lower than 1 %. CD spectra of the (E) Cu-HpCuMTAla and (F) Cu-HpCuMT preparations obtained at regular (solid) and low (doted) aeration conditions.

This is evidenced by the ESI-MS spectra of the corresponding preparations run at neutral and acidic pH (Figure 2A and Figure 2B, respectively) and the fact that ICP-AES measurements clearly indicated the presence of zinc in the sample (7Cu: 3Zn). The ESI-MS analysis at pH 2.4, which causes the loss of all Zn^{II} ions, but not regularly of Cu^{I}, revealed two peaks, corresponding to Cu_4- (major) and Cu_6-HpCuMTAla (minor) complexes. The most straightforward explanation for these results is that the M_{12}- and M_{10} complexes observed at neutral pH correspond to a mixture of hetero Cu_4Zn_x- and Cu_6Zn_y-HpCuMTAla species.

However, when the same MT peptide is synthesized under low aeration (i.e. high intracellular Cu), it folds into homonuclear, almost unique Cu_{12}-HpCuMTAla complexes, as revealed by the ESI-MS spectra (Figure 2C and Figure 2D) and the ICP-AES results that only report the presence of copper. The appearance of a second peak for Cu_{10}-HpCuMTAla in the acidic ESI-MS spectra is therefore probably explained if it is assumed that two of the Cu^{I} ions in the Cu_{12}- complex are bound too loosely to support the ionization conditions. CD spectra of the Cu preparations of the HpCuMT wild type and mutated forms (Figure 2E and Figure 2F) confirm the higher capacity of the Ala mutant to yield well folded complexes both at normal and high copper concentrations, while the wild type form only achieves this level of structuration when copper is high. It is worth remembering that under normal oxygenation of the cul-

tures, the wild-type HpCuMT produced a mixture of heterometallic M_{10}-, M_8-, M_6- and M_5- species, with a global equimolar Zn:Cu ratio, while under low aeration, it also rendered an almost unique Cu_{12}-HpCuMT species. Both Cu_{12}- species do seem to have the same stability at neutral pH. However that of the wild-type exhibited a higher instability than the mutant at the acidic ESI-MS conditions, yielding a Cu_{10}-HpCuMT peak (Figure S1, Supporting Information). Regardless of the reasons provoking these behaviors, it can be concluded that when coordinating Cu^I at normal conditions, HpCuMTAla folds into complexes with a higher Cu^I content than HpCuMT, and it forms more stable homonuclear Cu^I complexes where the Cu^I ions are more tightly bound.

Conclusions

All the results obtained suggest that, while the absence of His in HpCuMT has no effect on divalent metal ion coordination, it increases the Cu-binding abilities of this isoform. Thus, it is reasonable to postulate that the presence of His in HpCuMT could confer it with an optimal ability for copper binding as well as for copper release, a plasticity that would be essential for a putative role of HpCuMT in the transference of Cu^I to biomolecules that require it, as repeatedly hypothesized for haemocyanin synthesis in snail roghocytes.[5] Our data show that the metal binding properties of an MT peptide can be significantly changed by a mutation of one single amino acid, and support a role of His38 in the HpCuMT protein, probably enabling a controlled Cu^I release from the fully loaded Cu-HpCuMT complexes. There is no doubt that structural characterization of the Cu-HpCuMT complexes, which is under way, will significantly shed light on this singularity.

Experimental Section

Construction and Cloning of the cDNA Encoding the HpCuMTAla Site-directed Mutant

The site-directed His38Ala HpCuMT mutant (HpCuMTAla) was constructed by a two-step mutagenic PCR amplification using the wild-type HpCuMT cDNA as template.[6]

In the first step, the forward primer (5'TGCAGTTCTTGCGCTTGTTCCAAT 3') contained the mutated codon, and the reverse primer was the downstream primer (5'AGGCGTCGACTTGTCGTTTATTTGCAG 3') previously used for HpCuMT cloning.[6] PCR amplifications (x35) were performed following the cycle: 94 °C 30 s, 55 °C 30 s, and 72 °C 30 s, using Deep Vent (New England Biolabs) thermostable DNA polymerase. After purification, the resulting product was used as reverse megaprimer for the second PCR step, together with the forward primer previously used for HpCuMT amplification (5'ACAGGATCCGGACGAGGAAAGAACTGC 3'). In the final amplification product, the desired mutation had been introduced, and the flanking restriction sites (*Bam*HI and *Sal*I) allowed the in-frame cloning in the pGEX-4T-1 (Amersham GE Healthcare Bio-Sciences AB, Uppsala, Sweden) *E. coli* expression vector. All the DNA constructs were confirmed by automatic DNA sequencing (ABI 370, Perkin–Elmer Life Sciences), using BigDye Terminator (Applied Biosystems). DH5α was the *E. coli* host strain used for cloning and sequencing purposes, and thereafter, the expression plasmids were transformed into the *E. coli* protease-deficient strain BL21 for protein expression. To this end, *E. coli* LB cultures with 100 mg·mL^{-1} ampicillin were supplemented with: 300 μM $ZnCl_2$, $CdCl_2$, or 500 μM $CuSO_4$. Cu cultures were performed under two aeration conditions (regular and low) as described elsewhere.[17]

Purification and Analysis of the Metal-HpCuMTAla Complexes

All the metal-MT complex purifications were performed as reported in Ref. [6]. Metal-MT complexes were analyzed by ICP-AES, CD spectroscopy and ESI-TOF MS, as detailed in Ref. [8].

Supporting Information (see footnote on the first page of this article): Deconvoluted ESI-MS spectra of the recombinant preparations of HpCuMT in Cu-enriched media.

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