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BEYOND THE METAL BINDING ABILITY OF ZinT

The bacterial periplasmic protein ZinT is primarily involved in Zn^{2+} cellular transport. Since metal acquisition is a crucial aspect of infections, understanding the thermodynamics and coordination chemistry of the metal-ZinT interaction can help designing novel antimicrobial strategies. In this work the metal binding sites of ZinT from* Escherichia coli *and* Salmonella enterica *have been characterized.

Transition metal ions are undoubtedly important factors for the onset and progression of infectious diseases, therefore understanding the dynamics behind metal acquisition processes at the host/pathogen interface can be crucial to direct the rational design of new antimicrobials [1]. Due to the absence of homologue systems in eukaryotic cells, the mechanism of Zn^{2+} assimilation by the bacterial ZinT/ZnuABC transporter is a promising drug-target for specific and selective treatments. ZinT is a protein able to shuttle the metal from the periplasm to the ZnuABC transporter under zinc-limited conditions. There is a general consensus that the three histidine residues H167, H176 and H178 constitute an efficient zinc binding site, together with the evolutionarily conserved *N*-terminal fragment ^{24}HXHHXH^{24} [2]. In order to characterize these unstructured metal binding sites, we studied the corresponding peptides (protected at the amino- and carboxyl- termini by acetylation, Ac, and amidation, Am, respectively), since they can serve as models to simulate the coordination and transport of Zn^{2+} and Cu^{2+} - two endogenous and competing metal ions - within the native protein. The peptides included in this work correspond to the 24-29 and 166-178 amino acid sequences of ZinT from *Escherichia coli* (*Ec*ZinT) and *Salmonella enterica* (*Se*ZinT): L1=Ac-HGHHSH-Am (*Ec*ZinT), L2=Ac-HGHHAH-Am (*Se*ZinT), L3=Ac-DHIIAPRKSSH-FH-Am (*Ec*ZinT) and L4=Ac-DHIIAPRKSAH-FH-Am (*Se*ZinT).

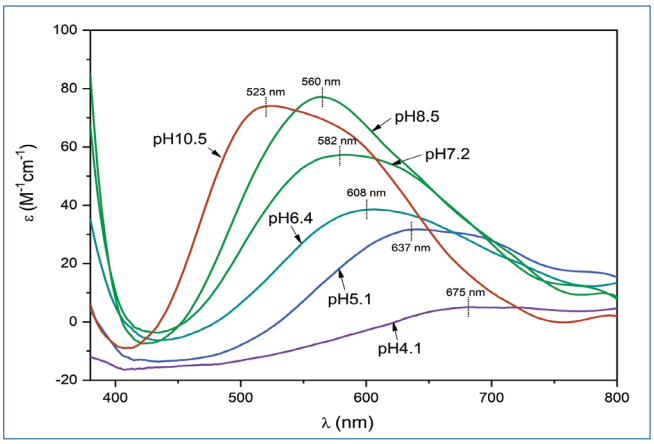

Fig. 1 - Vis absorption spectra of Cu^{2+}/L1 complexes; M:L molar ratio 0.9:1; $C_M = 0.45 \cdot 10^{-3}$ M, optical path 1 cm

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Cu^{2+}				
	L1	L2	L3	L4
$log\beta_{112}$	19.72(8)	19.4(1)	22.26(3)	22.53(4)
$log\beta_{111}$	15.27(3)	14.93(4)	16.94(4)	17.13(5)
$log\beta_{110}$	9.00(7)	8.56(8)	11.19(4)	11.45(5)
$log\beta_{11-1}$	1.85(8)	1.32(9)	5.00(4)	5.10(4)
$log\beta_{11-2}$	-5.46(7)	-6.06(8)	-4.03(7)	-4.54(6)
$log\beta_{11-3}$	-14.78(8)	-15.59(9)	-14.20(7)	-14.90(5)

Zn^{2+}				
	L1	L2	L3	L4
$log\beta_{112}$	-	17.3(1)	20.04(7)	20.17(5)
$log\beta_{111}$	11.85(7)	11.5(1)	13.76(5)	13.70(5)
$log\beta_{110}$	5.5(1)	5.5(1)	7.25(3)	7.09(2)
$log\beta_{11-1}$	-0.82(7)	-0.57(3)	-	-

Tab. 1 - Equilibrium constants for Cu^{2+} and Zn^{2+} complexes at T = 298 K and I = 0.1 M. Standard deviations on the last significant figure are in parenthesis. The overall thermodynamic constant β_{pqr} corresponds to the following generic equilibrium: $pM + qL + rH \rightleftharpoons M_pL_qH_r$, where M=metal, L=ligand, H=proton

The required parameters for the characterization of simultaneous complex-formation equilibria in solution are contained in the so-called "speciation model", which provides the stoichiometry of the formed species and their formation constants at a given temperature (*T*) and ionic strength (*I*). These equilibria are related to the ligand acid-base behaviour, and thus to the amino acid side chain properties. In L1 and L2 there are four protonable sites corresponding to the four histidines, while in L3 and L4 there are three histidines, one aspartic acid and one lysine (the arginine remains protonated in the pH range here employed). Hence, by means of potentiometric titrations, the thermodynamic constants for protonation equilibria and metal complex formation have been determined (Tab. 1). Furthermore, spectroscopic techniques, like UV-Vis, circular dichroism (CD) and EPR, provide a complementary study to understand the most probable coordination modes of the formed Cu^{2+} complexes. On the contrary, Zn^{2+} complexes are spectroscopically silent and their formation in solution has been studied by titrimetric techniques and mass spectrometry only.

Increasing the pH value, the visible absorption spectra of all the investigated Cu^{2+}/ligand systems shift towards shorter wavelengths (Fig. 1); this behaviour typically indicates an increasing number of the coordinated nitrogen atoms, to form complexes where up to 4 histidines are coordinated to the Cu^{2+} ion, with a distorted octahedral geometry [3]. The aspartic acid of L3 and L4 may also participate in complexation under the most acidic conditions (Fig. 2). A significant optical activity of copper complexes is not evident until physiological pH, when the backbone amides (up to 3) begin to gradually substitute the histidines in the equatorial coordination plane, increasing the square-planar character of the Cu^{2+} complex [4].

In the case of zinc complexes, a tetrahedral coordination geometry is instead likely expected, with 3 His residues coordinated to the metal ion and the fourth position occupied by one oxygen atom (carboxylic O- or water molecule) (Fig. 2). The formation of a pentacoordinate system (4N, 1O) with a distorted pyramidal arrangement has been also suggested for Zn^{2+}/L1 and Zn^{2+}/L2 complexes above neutral pH.

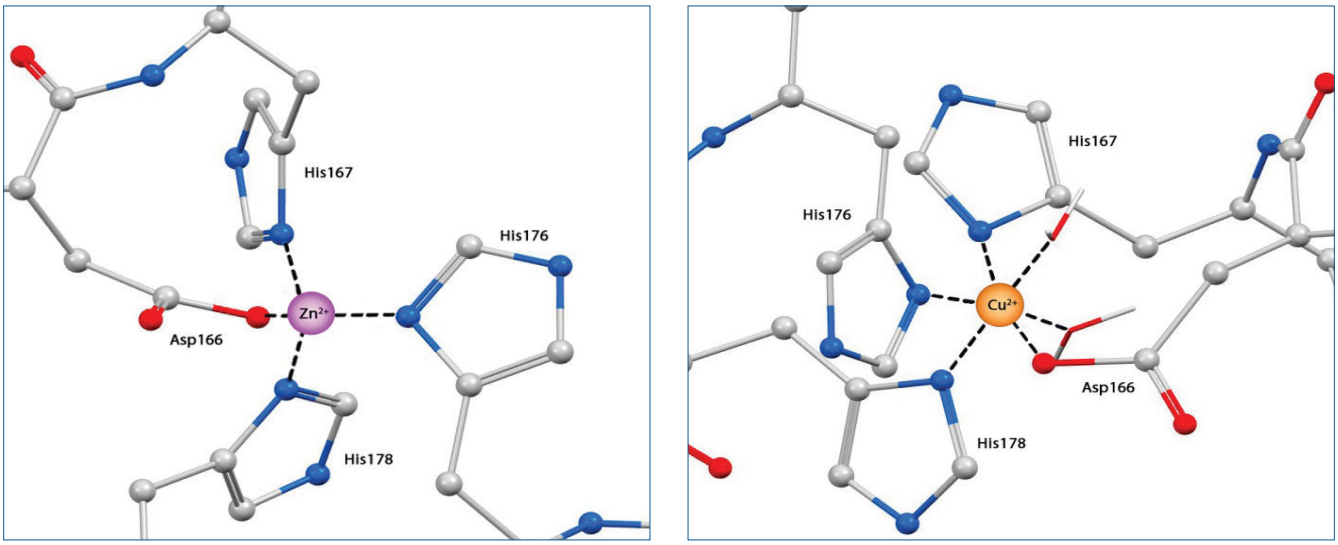

Fig. 2 - Proposed coordination sphere for Zn^{2+} (left) and Cu^{2+} (right) complexes with L3 and L4 at acidic pH. Explicit hydrogen atoms are omitted for clarity

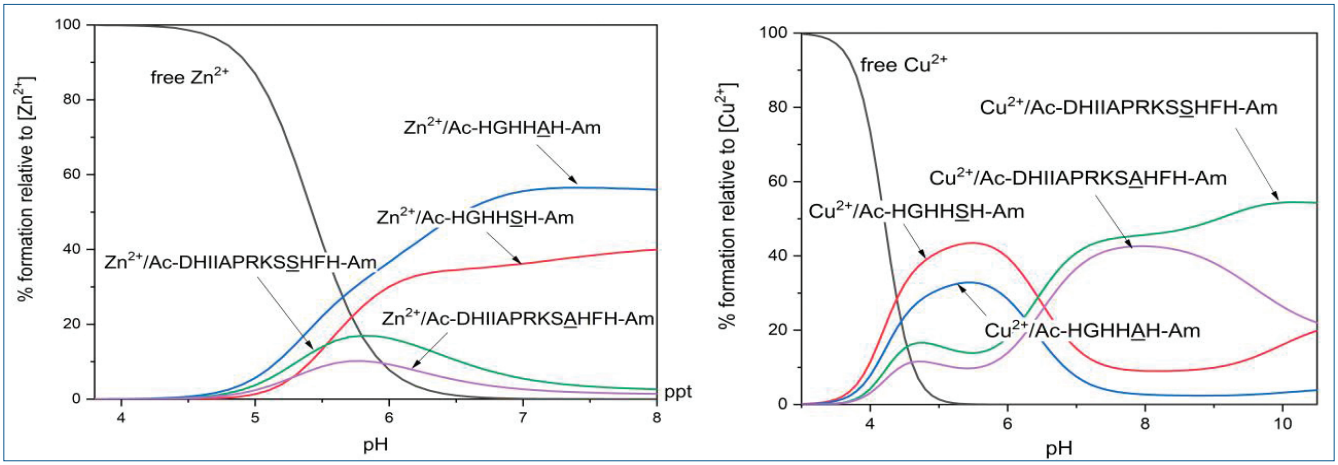

Fig. 3 - Competition plots for a solution containing equimolar concentration ($1 \cdot 10^{-3}$ M) of metal ion, L1, L2, L3 and L4. Metal ion = Zn^{2+} (left); Cu^{2+} (right)

Further interesting information about metal complex stabilities can be obtained comparing the studied systems through appropriate competition plots. These diagrams represent a simulation of solutions containing equimolar concentrations of the metal and the chosen ligands, admitting the formation of only the binary complexes described in the speciation models. The efficacy observed for HGHHXH in zinc chelation (Fig. 3) confirms the general assumption that a higher number of histidine residues favours the metal complexation [5]. Analogously, L1 and L2 provide a high number of vicinal anchoring sites to favour the first steps of copper complexation. Nonetheless under alkaline conditions, where backbone amides substitute His residues in the Cu^{2+} coordination sphere, L3 and L4 become the best ligands.

These results are in line with the hypothesised role of the HGHHXH loop as metal scavenger capable of recovering and stabilizing the metal ion from the surrounding environment, before delivering it to ZinT canonical binding site (L3 and L4 fragments) for storage or transfer to other proteins. From this perspective, a comparison between the metal binding affinity of ZinT and ZnuA proteins can be useful to evaluate the mechanism of zinc transfer to the ZnuABC importer. In fact, *Se*ZinT is supposed to interact with ZnuA, forming a binary complex in the presence of zinc ions and likely transferring the metal to the partner protein [6]. This process can be successfully achieved only if the metal binding sites of ZnuA have a higher affinity for Zn^{2+} than ZinT, and this is the case highlighted by our thermodynamic results; in fact, the His-rich loop of ZnuA from *E. coli* (-MKSIHGDDDDHDHAEKSDEDHHHGDFNMHLW-) [7] turned out to be a more efficient zinc binding site than the ZinT fragments studied in this work [8]. On the contrary, based on our results, the metal transfer process cannot occur in the case of Cu^{2+}.

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Al di là della semplice capacità di legare i metalli di ZinT

La proteina batterica periplasmica ZinT è coinvolta principalmente nel trasporto cellulare di Zn^{2+}. Poiché l'acquisizione dei metalli è un aspetto cruciale delle infezioni, comprendere la termodinamica e la chimica di coordinazione dell'interazione metallo-ZinT può aiutare a sviluppare nuove strategie antimicrobiche. In questo lavoro sono stati caratterizzati i siti di legame metallico di ZinT in *Escherichia coli* e *Salmonella enterica*.