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Research Paper

# Zn(II) and Ni(II) complexes with poly-histidyl peptides derived from a snake venom

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ABSTRACT

The snake venoms are complex mixtures containing many bioactive peptides and proteins; some of them are aimed to protect the snake glands, where the venom is stored, until the latter is inoculated in the victim. In the venom of some vipers of the genus *Atheris*, a set of peptides containing poly-His and poly-Gly segments was recently found. Poly-His peptides are not rare in Nature. Although their exact biological function is most often unknown, one thing is certain: they have good binding properties towards the transition metal ions. As a matter of fact, the imidazole side chain of histidine is one of the groups most frequently involved in metal complexation in the active sites of metallo-enzymes. This is also true for snake-venom metallo-proteases, which contain Zn(II) and Ca(II) ions.

In the present paper, the complex-formation ability of the poly-His-poly-Gly peptide found in the venom of *Atheris squamigera* ($EDDH_9GVG_{10}$-$NH_2$) towards the Zn(II) and Ni(II) ions was investigated by means of thermodynamic and spectroscopic techniques. Two model peptides, derived from the poly-His portion of this peptide but where His residues were alternated with alanines (Ac-EDDAHAHAHAHAG-$NH_2$, and Ac-EDDHAHAHAHAHG-$NH_2$) were also studied, for the sake of comparison. The high affinity of these peptides for the metal ions under investigation was confirmed. In addition, it was demonstrated that the number of His residues in the peptide and their relative position play a main role in the complex-formation ability of the ligand. The very high affinity of $EDDH_9GVG_{10}$-$NH_2$ for Zn(II) can be the key for its role in the inactivation of the venom in the snake glands.



## 1. Introduction

The snake venoms are complex mixtures containing hundreds of toxins [1] from which valuable active ingredients for several drugs have been derived, such as those used for the treatment of cardiovascular diseases, for the relief of pain or for the skin care [2]. The venoms of different snake families have been recently successfully tested for their cytotoxic effect against breast and skin cancer cells [3]. The main enzymes in most snake venoms are metalloproteinases (SVMPs) [4], which are responsible for their haemorrhagic activity. The SVMPs are phylogenetically related to the family of adamalysin (or ADAM) and usually contain calcium and/or zinc ions [1]. The zinc-binding domain is highly conserved throughout the ADAM family and contains three histidine residues separated by three and five amino acids, respectively (-HEXXHXXGXXH-) [5]; the histidines are bound to the zinc ion together with a water molecule in a tetrahedral structure [1].

Since the SVMPs contained in the snake venom can induce severe local and systemic bleeding in the victim, why the venom stored in the snake glands does not damage the snake itself? The advanced hypotheses to answer this question derive from the fact that high concentrations of citrate and peptides with metal-ligand capabilities have been found in venoms and that the pH in the snake gland is about 5. The activity of SVMPs in the snake glands would therefore be inhibited by three synergistic mechanisms: (i) chelation of calcium by citrate anion; (ii) acidic pH and (iii) enzyme inhibition by specific peptides [6]. Once the venom is injected into the victim's blood, its dilution and the physiological pH cancel the inhibitory mechanisms.

It has been shown that the snake venoms disturb the homeostasis of some metal ions of the victim: a significant increase of copper

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levels in serum was observed [7] and it was suggested to use copper/zinc ratio in the serum as a marker of effects of poisoning [8]. On the other hand, it was instead demonstrated that copper can act as an inhibitor against serine proteases and metalloproteinases [9,10], according to a mechanism not yet fully explained.

An investigation on the venoms from snakes of the genus *Atheris* showed that they contain a set of poly-His/poly-Gly peptides (pHpG) [11]. In particular, the peptide $EDDH_9GVG_{10}$, characterized by the presence of nine consecutive His residues, has the ability of neutralizing the haemorrhagic activity of the *Echisocellatus* venom [12].

Besides the venom of snakes, His-rich proteins (HRP) have been found in many living organisms [12–17]. They can play crucial roles in many aspects of the life [18] included the transport of metal ions into/through the organism [19], as it happens for the two zinc-binding proteins AdcA and AdcAII which are critical for zinc feeding in Streptococcus pneumoniae [20]. Several HRPs contain at least one His-tag region [13,17,21,22] which is very effective to bind metal ions and can be exploited for the purification of such proteins through Immobilized Metal Ion Affinity Chromatography (IMAC). The His-tag (usually $His_6$-tag) can be also artificially added to the sequence of the protein [23,24]. In fact, the imidazole nitrogens of histidine side chains are able to form strong coordination bonds with metal cations such as Ni(II), Cu(II), Co(II) or Zn(II), immobilized into the chromatographic stationary phase [25–27].

Our research group has recently studied the binding properties of the protected peptides $EDDH_9GVG_{10}$-$NH_2$ (L3) and Ac-$EDDH_9G$-$NH_2$ [28,29]. The presence of nine histidine residues in a row ($His_9$-tag) makes these fragments very efficient metal chelators, also in comparison to other His-rich peptides. Most interestingly, it was demonstrated the $His_9$-tag acts as a "polymorphic binding site", since different sets of imidazoles can bind the metal ion in different ways thus allowing it to "move along" the poly-His sequence. In addition, the metal binding induces the formation of a regular α-helical structure. A similar behaviour was also found for the Cu(II) complexes with the $His_6$-tag [30,31].

In order to shed more light on the coordination modes of the ($His_9$-tag), the above study was recently extended to two analogues of Ac-$EDDH_9G$-$NH_2$ in which some His residues were mutated to alanines [32]: the number of histidines is reduced and they are no longer consecutive. The first peptide contains four His residues alternating with five Ala residues (Ac-EDDAHAHAHAHAG-$NH_2$, L1) while the second one consists of five His residues alternating with four alanines (Ac-EDDHAHAHAHAHG-$NH_2$, L2). The latter study has demonstrated that the presence of 4 or 5 histidines makes both peptides L1 and L2 excellent ligands for the Cu(II) ion. However, it was confirmed, in agreement with the previous literature [14,33–35], that the total number of histidines in the ligand rules the stability of the formed Cu(II) complexes, also if an equal number of imidazole groups is coordinated to copper. As a matter of fact, a lower number of available histidines reduces the number of possible binding combinations.

It is well known that imidazole side chain of His is a good binding site not only for Cu(II) but also for other biologically relevant metal ions, as Zn(II) and Ni(II). While the latter shares with Cu(II) the capability of displacing the amidic protons of the peptidic backbone [36,37], zinc does not have this capacity but it is able to form stable poly-His complexes [38,39]. In order to complete the survey on the behaviour of pHpG peptides derived from *Atheris Squamigera*, in the present paper a thermodynamic and spectroscopic study on the formation of Ni(II) and Zn(II) complexes with the three peptides Ac-EDDAHAHAHAHAG-$NH_2$ (L1), Ac-EDDHAHAHAHAHG-$NH_2$ (L2) and $EDDH_9GVG_{10}$-$NH_2$ (L3) is described; the results are compared with those previously described in the literature for these ligands with Cu(II) and for other poly-His peptides with Ni(II) and Zn(II).

## 2. Experimental

### 2.1. Materials

The synthesis of peptides Ac-EDDAHAHAHAHAG-$NH_2$ (L1) and Ac-EDDHAHAHAHAHG-$NH_2$ (L2) is describes elsewhere [32]. The C-protected peptide $EDDH_9GVG_{10}$-Am (L3) was purchased from Selleck Chemicals (Huston, TX) (certified purity: 99.35%) and used as received. The purity of the three ligands was potentiometrically checked.

$NiCl_2$ and $ZnCl_2$ were extra pure products (Sigma-Aldrich); their stock solutions were standardized by EDTA titration and periodically checked *via* ICP-MS. The carbonate-free stock solution of KOH (Sigma-Aldrich) was potentiometrically standardized with the primary standard potassium hydrogen phthalate. The HCl stock solution was prepared by diluting concentrated HCl (Sigma-Aldrich) and standardized with standard KOH. All sample solutions were prepared using freshly doubly-distilled water. The ionic strength was adjusted to 0.1 mol $dm^{-3}$ by adding suitable amounts of KCl (Sigma-Aldrich). Grade A glassware was employed throughout.

### 2.2. Potentiometric measurements

Protonation and complex-formation constants were calculated from potentiometric titration curves recorded in the pH range 2.5–10.5; the sample solution volume was 1.5 $cm^3$. The pH-metric titrations were performed with a MOLSPIN pH-meter system equipped with a Russell CMAW711 semi-micro, glass, combination pH electrode, daily calibrated in proton concentration using HCl [40]. KOH was added into the titration cell with a 0.500 $cm^3$ micrometer syringe, previously calibrated. The delay time between two additions of titrant was suitable to guarantee the attainment of equilibrium; the kinetics of complex-formation was fast with zinc but rather slow with nickel, especially in the alkaline pH range. The ligand concentration was always about $5 \cdot 10^{-4}$ mol $dm^{-3}$ and the metal/ligand ratio was 1:1.2. The temperature the sample solution of was kept at 298.2 ± 0.1 K by means of a circulation thermostat. High purity grade argon was gently blown over the test solution in order to maintain an inert atmosphere. Constant-speed magnetic stirring was applied throughout.

The standard potential and the slope of the electrode couple were computed from the calibration titrations by means of Glee [41] or SUPERQUAD [42] programs. The exact concentration of the ligand in each sample solution was determined with the Gran method [43]. The HYPERQUAD program [44] was employed for the calculation of the overall ($\beta_{pqr}$) stability constant, referred to the following equilibrium:

$$pM + qL + rH \rightleftarrows M_pL_qH_r \quad (1)$$

(charges omitted; p is 0 in the case of ligand protonation; r can be negative). Reported $K_a$ values are instead the acid dissociation constants of the corresponding species. The computed standard deviations (referring to random errors only) are shown in parentheses as uncertainties on the last significant figure. The distribution diagrams were computed with the HYSS program [45]. Metal hydrolysis constants were taken from the literature [46]. A $pK_w$ value of 13.77 was used and checked through separate experiments.

The stability constant values of complexes with different stoichiometries and/or protonation degrees cannot be directly compared. The overall metal binding ability can instead be evaluated in a wide pH range by computing and plotting the competition diagrams, starting from the binary speciation models. A solution containing the metal and the two (or more) ligands (or *vice versa*) is simulated, admitting that all the components compete with each