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# A minimalist chemical model of matrix metalloproteinases — Can small peptides mimic the more rigid metal binding sites of proteins?

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ABSTRACT

In order to mimic the active center of matrix metalloproteinases (MMPs), we synthesized a pentadecapeptide (Ac-KAHEFGHSLGLDHSK-$NH_2$) corresponding to the catalytic zinc(II) binding site of human MMP-13. The multi-domain structural organization of MMPs fundamentally determines their metal binding affinity, catalytic activity and selectivity. Our potentiometric, UV-visible, CD, EPR, NMR, mass spectrometric and kinetic studies are aimed to explore the usefulness of such flexible peptides to mimic the more rigid metal binding sites of proteins, to examine the intrinsic metal binding properties of this naked sequence, as well as to contribute to the development of a minimalist, peptide-based chemical model of MMPs, including the catalytic properties. Since the multiimidazole environment is also characteristic for copper(II), and recently copper(II) containing variants of MMPs have been identified, we also studied the copper(II) complexes of the above peptide. Around pH 6–7 the peptide, similarly to MMPs, offers a $\{3N_{im}\}$ coordination binding site for both zinc(II) and copper(II). In the case of copper(II), the formation of amide coordinated species at higher pH abolished the analogy with the copper(II) containing MMP variant. On the other hand, the zinc(II)-peptide system mimics some basic features of the MMP active sites: the main species around pH 7 ($ZnH_2$**L**) possesses a $\{3N_{im},H_2O\}$ coordination environment, the deprotonation of the zinc-bound water takes place near the physiological pH, it forms relatively stable ternary complexes with hydroxamic acids, and the species $ZnH_2$**L**(OH) and $ZnH_2$**L**$(OH)_2$ have notable hydrolytic activity between pH 7 and 9.



## 1. Introduction

The metal binding side chains in metalloproteins are generally far away from each other in the primary sequences, and they are often separated by more than a hundred amino acids. Such metal binding sites are obviously difficult to mimic by small peptides. However, a number of proteins possess relatively short histidine-rich sequences with strong metal binding ability, which substantially contributes to the function of the given macromolecule [1–18]. Beside the well known human serum albumin (HSA) [1], probably the prion proteins (PrP) [2] are the most studied examples of such sequences. Studies on the metal ion binding of peptides related to the N-terminal of HSA [3–5], and those mimicking the octarepeat region of PrP [6–8] demonstrated the usefulness of such studies. Besides, several peptides copying the putative metal binding sequences of *e.g.* human α-synuclein [9], human endostatin [10], bacterial superoxide dismutases [11,12] and *E. coli* SlyD protein [13] have been studied to uncover fine functional details of the corresponding proteins. However, these metal binding sequences are part of less structured regions, frequently at the N-terminus, which may allow closer analogy in the metal binding properties of model peptides and native proteins.

Sequences within the polypeptide chains are generally more rigid due to the tertiary structure of the proteins, which provides a preorganized binding site for the metal ion(s) in metalloproteins. In spite of the short sequences, such preorganization obviously reduces the analogy with the more flexible oligopeptides. Nevertheless, a number of studies are reported in the literature dealing with short in-chain sequences as putative metal binding sites of *e.g.* amyloid precursor protein [14], the repeat sequences of Cap43 [15] and histidine-rich (glyco)proteins [16], the histone H2A protein [17] or the metal-transport protein IRT1 [18]. In spite of the limited analogy between such peptides and the native proteins, in the absence of structural information such studies are of great value, since even the identification of putative metal binding sites may improve our knowledge of the function of the given proteins.

In this context it would be interesting to examine such peptide–protein analogy from a reverse point of view, *i.e.* studying a relatively short, flexible peptide corresponding to the metal binding site of a structurally well characterized metalloprotein. In this way one

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would explore the usefulness of small peptides, lacking the structural stabilization determined by the tertiary structure, to mimic preorganized metal binding sites of proteins. To this end, the consensus catalytic metal binding sequence of matrix metalloproteinases (MMPs) is well suited. These enzymes are responsible for remodeling of connective tissues, and are essential for *e.g.* embryonic development, cell growth, proliferation, and wound repair. MMPs are also implicated in a number of pathological processes, such as arthritis, cardiovascular and neurological diseases, tumor cell invasion and metastasis. Consequently, these enzymes are important therapeutic targets for the treatment of the above diseases [19]. Matrix metalloproteinases contain two zinc ions, one has a catalytic role, whereas the other has a structural role. The N-terminally located catalytic domain of MMPs are characterized by a well conserved sequence with a histidine-triad (HExxHxxGxxH) which coordinates the catalytic zinc ion and contains the glutamate residue that is also critical for catalysis. The catalytic zinc is coordinated by three imidazole nitrogens and a water molecule [20].

In this study we report the coordination properties of a pentadecapeptide (Ac-KAHEFGHSLGLDHSK-$NH_2$, **L**) corresponding to the catalytic zinc(II) binding site (197–213) of human MMP-13 [21], except the first position where the valine has been replaced by lysine in order to increase the water solubility of the peptide and its metal complexes. Our potentiometric, UV-visible, CD, EPR, NMR, ESI-MS (electron spray ionization mass spectrometry) and kinetic studies are aimed to explore the usefulness of such flexible peptides to mimic the more rigid metal binding sites of proteins, to examine the intrinsic metal binding properties of this naked sequence, as well as to contribute to the development of a minimalist, peptide-based chemical model of MMPs, including the catalytic properties and the binding to hydroxamic acids, as model of MMP inhibitors [22]. Since the multiimidazole environment is also characteristic for copper(II), and recently copper(II) containing variants of MMPs have been identified in *Volvox carteri* [23], for comparative purposes we also studied the copper(II) complexes of the peptide.

## 2. Materials and methods

Copper(II) and zinc(II) chloride solutions were prepared from analytically pure compounds (Fluka) and standardized complexometrically. pH-metric titrations were performed with a 0.1 M NaOH (Fluka) standard solution. Fmoc-amino acids, 2-(1H-benzotriazole-1-yl)-1, 1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and N-hydro xybenzotriazole (HOBt) were purchased from Novabiochem. N,N-diisopropylethylamine (DiPEA), diethylether (Sigma), triisopropylsilane, piperidine (Aldrich), pyridine (Merck), acetic anhydride (Fluka), trifluoroacetic acid (Riedel-de Haën), N,N-dimethyl-formamide (DMF), dichloromethane, methanol (Molar Chemicals), and acetonitrile (Scharlau) were analytically pure chemicals and used without further purification.

### 2.1. Synthesis of Ac-KAHEFGHSLGLDHSK-$NH_2$ peptide (L)

The peptide was prepared by solid phase peptide synthesis using the Fmoc methodology (Fmoc = 9-fluorenylmethoxycarbonyl). Tentagel S RAM 0.25 mmol/g (Iris Biotech GmbH) was used as a solid support. The amino acid building blocks were applied in 4-fold excess over the capacity of the resin. The amino acid residues were coupled to each other (and to the resin) by applying HBTU (4 eq./building block), HOBt (4 eq./building block) and N,N-diisopropylethylamine (8 eq./building block) in DMF. The Fmoc-protecting groups were removed by using a solution of 20% piperidine in DMF. The usual coupling reaction time was 1 h. The attachment of each amino acid residue was monitored by Kaiser-test [24] and by the detection of the cleaved Fmoc group at 290 nm in DMF. In the case of successful coupling, the few remaining free terminal amino groups were acetylated with the mixture of acetic anhydride, dichloromethane and diisopropylethylamine (10–80–10%). After the last coupling step the resin was rinsed by dichloromethane and methanol, and then it was dried. Cleavage of the peptide from the resin was performed using a mixture of TFA (trifluoroacetic acid), $H_2O$ and triisopropylsilane (95–2.5–2.5%). The peptide was precipitated in diethyl ether, re-dissolved in water and freeze-dried. The crude product was purified by HPLC (Shimadzu LC-20) using a Supelco Discovery BIO Wide Pore C18 (250 × 10 mm, 5 μm) semi-preparative column. The compound was eluted by using the mixtures of water containing 0.1% TFA and acetonitrile (Eluent A: 99.9% $H_2O$, 0.1% TFA, Eluent B: 99.9% $CH_3CN$, 0.1% TFA) with a 3 mL/min flow rate and applying the following gradient program: 0–5 min 12% B; 5–17 min 12–14% B (linear gradient); 17–25 min 14–18% B (linear gradient); 25–40 min 18–70% B (linear gradient); 40–45 min 70–12% B (linear gradient); ($R_t$ = 13.4 min). After purification the ligand was obtained as a trifluoroacetate salt, the yield was 53.0%. The HPLC chromatogram of the purified peptide is depicted in Fig. S1 (see Supplementary Data).

The peptide was identified by ESI-MS: m/z = 852.45 $[M + 2H]^{2+}$ and m/z = 568.63 $[M + 3H]^{3+}$. The calculated monoisotopic molecular mass is: 1702.85 Da. The purity was also confirmed by potentiometry and NMR spectroscopy.

### 2.2. Potentiometric measurements

The protonation and coordination equilibria were investigated by potentiometric titrations in aqueous solution ($I$ = 0.1 M NaCl, and $T$ = 298.0 ± 0.1 K) under argon atmosphere, using an automatic titration set including a PC controlled Dosimat 665 (Metrohm) autoburette and an Orion 710A precision digital pH-meter. The Metrohm Micro pH glass electrode (125 mm) was calibrated [25] *via* the modified Nernst equation:

$$E = E_0 + K\cdot\log\left[H^+\right] + J_H\cdot\left[H^+\right] + \frac{J_{OH}\cdot K_w}{[H^+]}$$

where $J_H$ and $J_{OH}$ are fitting parameters in acidic and alkaline media for the correction of experimental errors, mainly due to the liquid junction and to the alkaline and acidic errors of the glass electrode; $K_w = 10^{-13.75}$ $M^2$ is the autoprotolysis constant of water [26]. The parameters were calculated by the non-linear least squares method. The complex formation was characterized by the following general equilibrium process:

$$pM + qH + rL + sB \overset{\beta_{M_pH_qL_rB_s}}{\rightleftharpoons} M_pH_qL_rB_s$$

$$\beta_{M_pH_qL_rB_s} = \frac{\left[M_pH_qL_rB_s\right]}{[M]^p[H]^q[L]^r[B]^s}$$

where M denotes the metal ion and L and B the non-protonated peptide and benzohydroxamic acid molecules, respectively. Charges are omitted for simplicity, but can be easily calculated taking into account the composition of the fully protonated ligands ($H_7L^{5+}$ and HB). The corresponding formation constants ($\beta_{MpHqLrBs} \equiv \beta_{pqrs}$) were calculated using the PSEQUAD computer program [27].

The protonation constants were determined from 4 independent titrations (90 data points per titration), with peptide concentration 1–1.5 × $10^{-3}$ M. The complex formation constants were evaluated from 7 to 9 independent titrations (70–90 data points per titration) in case of the zinc(II) and copper(II) containing systems, respectively. The metal-to-ligand ratios were 2:1, 1:1 and 1:2 (in case of zinc(II) precipitation occurred above pH 7 at twofold metal ion excess). The metal ion concentrations varied between 0.6 and 2.9 × $10^{-3}$ M. The titrations were performed between pH 2.6 and 11.4, but due to the rather low concentrations of the peptide ($-\log c_{peptide}$ = 2.5–3.2),