Journal of Inorganic Biochemistry xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

# Influence of the N-terminus acetylation of Semax, a synthetic analog of ACTH(4-10), on copper(II) and zinc(II) coordination and biological properties

Antonio Magrì [a], Giovanni Tabbì [a,*], Alessandro Giuffrida [a], Giuseppe Pappalardo [a], Cristina Satriano [c], Irina Naletova [b,d], Vincenzo G. Nicoletti [b], Francesco Attanasio [a,*]

[a] Istituto di Biostrutture e Bioimmagini, Consiglio Nazionale delle Ricerche (CNR), Via P. Gaifami 18, 95126 Catania, Italy
[b] Dipartimento di Scienze Biomediche, Università degli Studi di Catania, Viale A. Doria 6, 95125 Catania, Italy
[c] Dipartimento di Scienze Chimiche, Università degli Studi di Catania, Viale A. Doria 6, 95125 Catania, Italy
[d] Consorzio Interuniversitario C.I.R.C.S.M.B., Via C. Ulpiani 27, 70125 Bari, Italy

ARTICLE INFO

*Article history:*
Received 31 March 2016
Received in revised form 23 August 2016
Accepted 25 August 2016
Available online xxxx

*Keywords:*
Copper
Zinc
Biological activity
Oxidation
Voltammetry
Stability constants

ABSTRACT

Semax is a heptapeptide (Met-Glu-His-Phe-Pro-Gly-Pro) that encompasses the sequence 4-7 of N-terminal domain of the adrenocorticotropic hormone and a C-terminal Pro-Gly-Pro tripeptide. N-terminal amino group acetylation (Ac-Semax) modulates the chemical and biological properties of parental peptide, modifying the ability of Semax to form complex species with Cu(II) ion. At physiological pH, the main complex species formed by Ac-Semax, $[CuLH_{-2}]^{2-}$, consists in a distorted $CuN_3O$ chromophore with a weak apical interaction of the methionine sulphur. Such a complex differs from the Cu(II)-Semax complex system, which exhibits a $CuN_4$ chromophore. The reduced ligand field affects the $[CuLH_{-2}]^{2-}$ formal redox potential, which is more positive than that of Cu(II)-Semax corresponding species.

In the amino-free form, the resulting complex species is redox-stable and unreactive against ascorbic acid, unlike the acetylated form. Semax acetylation did not protect from Cu(II) induced toxicity on a SH-SY5Y neuroblastoma cell line, thus demonstrating the crucial role played by the free $NH_2$ terminus in the cell protection. Since several brain diseases are associated either to Cu(II) or Zn(II) dyshomeostasis, here we characterized also the complex species formed by Zn(II) with Semax and Ac-Semax. Both peptides were able to form Zn(II) complex species with comparable strength. Confocal microscopy imaging confirmed that peptide group acetylation does not affect the Zn(II) influx in neuroblastoma cells. Moreover, a punctuate distribution of Zn(II) within the cells suggests a preferred subcellular localization that might explain the zinc toxic effect. A future perspective can be the use of Ac-Semax as ionophore in antibody drug conjugates to produce a dysmetallostasis in tumor cells.



## 1. Introduction

Adrenocorticotrophic hormone (ACTH), melanocyte-stimulating hormone (MSH) fragments and synthetic analogues thereof, belong to a class of endogenous regulatory peptides [1,2] with a marked activity on the functions of the central nervous system (CNS). These compounds showed neurotrophic, nootropic and neuroprotective effects [3–5] and can affect human and animal behaviour [6–9]. This action is not associated with the ACTH hormonal activity and likely is the result of a direct action on the CNS [10]. Several studies showed that both behavioural and neurotrophic activity of ACTH are ascribable to the N-terminal part of the ACTH molecule [4], with the fragment ACTH(4-10), which mimic some features of the full-length ACTH molecule, showing the most strong effects on behaviour [11–14]. Several analogues of the ACTH natural peptides, showing more effectiveness than the natural N-terminal ACTH fragments, have been synthesized [15–17].

Semax is an ACTH(4-10) analog peptide that lacks hormonal effects but has marked neurotrophic activity and protease stability [18]. In vitro and in vivo studies demonstrated a high efficacy of Semax in the treatment of cognitive/memory disorders [19] and ACTH-like and anti-inflammatory effects [20–22]. These properties can be explained by taking into account that Semax: i) modulates the vascular endothelial growth factor (VEGF) family gene expression in focal ischemia of rat brain [23]; ii) influences the morphology and proliferative activity of rat brain cells during experimental ischemia [24]; iii) regulates the expression of brain derived neurotrophic factor (BDNF) and of the tyrosine receptor kinase (TrkB) in the rat hippocampus [25].

Transition metal ions are important players involved in neuromodulation/neurotransmission [26,27], neurodegenerative diseases, memory and cognitive processes [28–30]. In particular, it is well known the key role of essential transition metals such as copper(II) and zinc(II) in the neuropathology of Alzheimer disease (AD) [31–33]

* Corresponding authors.
*E-mail addresses:* giovanni.tabbi@cnr.it (G. Tabbì), francesco.attanasio@cnr.it (F. Attanasio).

http://dx.doi.org/10.1016/j.jinorgbio.2016.08.013
0162-0134/

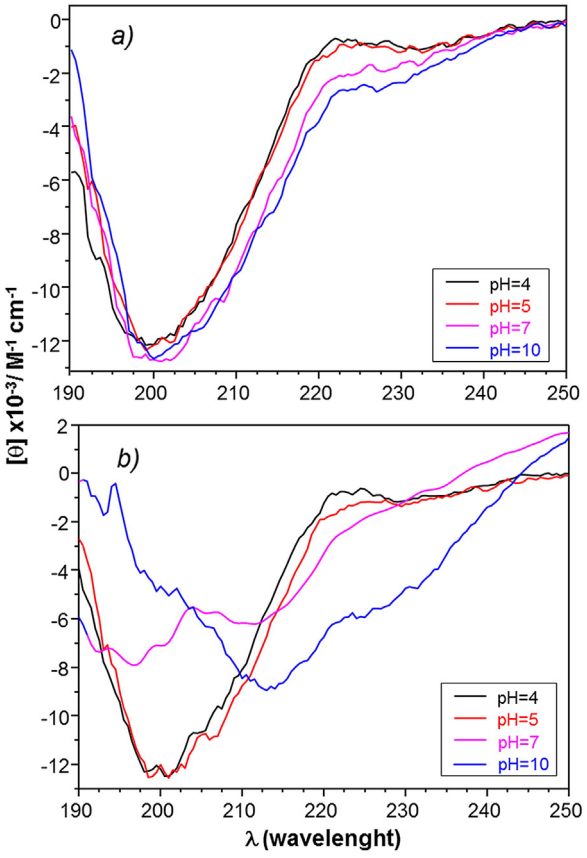


**Fig. 1.** Far-UV CD spectra recorded in aqueous solution at different pH values of a) Ac-Semax and b) Cu(II)-Ac-Semax system. (M:L = 1:1, [L] = $1 \times 10^{-5}$ M).

and their effects on neurotoxicity [34–36] and rupture of the redox-active metal ions homeostasis in the brain and in senile plaques [37, 38]. More recently, metal ions have been suggested to play an essential role in the mechanisms of memory formation [28].

A large body of evidence suggests a cross-talk between copper and zinc dyshomeostasis and dysregulation of expression of proteins related to learning and memory, including nerve growth factor (NGF) and BDNF [39–43]. The mechanisms of interaction of these metal ions with various neurotrophins and their peptide fragments have gained increasing interest [44–47].

N-terminal peptide acetylation is commonly used to make the peptide similar to the protein backbone, especially to study the chelating properties of a specific protein region. For example, proteins like α-synuclein are naturally N-terminal acetylated and this acetylation

**Table 1**
Protonation constants (log β) and pK values for Ac-Semax and Semax peptides (T = 298 K, I = 0.1 M $KNO_3$).[a]

| Species | L = Ac-Semax | L = Semax |
|---|---|---|
| | log β | log β[b] |
| LH | 6.70 (1) | 7.71 |
| $LH_2$ | 11.07(1) | 14.32 |
| $LH_3$ | 14.17(1) | 18.63 |
| $LH_4$ | – | 21.62 |
| pK $NH_2$ | – | 7.71 |
| pK His | 6.70 | 6.61 |
| pK $COO^-$ | 4.37 | 4.31 |
| pK $COO^-$ | 3.09 | 2.99 |

[a] Standard deviations (3σ values) are given in parentheses. Charges are omitted for clarity.
[b] Ref. [49].

**Table 2**
Stability constants (log β), pK values and UV–Vis parameters of copper(II) complexes with Ac-Semax; [Cu] = $1 \times 10^{-3}$ M; [L] = $1.2 \times 10^{-3}$ M; (T = 298 K, I = 0.1 M $KNO_3$).[a]

| Species | log β | UV–Vis λ (nm) (ε, $M^{-1}$ $cm^{-1}$) |
|---|---|---|
| [CuL] | 4.22 (3) | 740 (25) |
| $[CuLH_{-2}]$ | −7.93 (2) | 625 (95) |
| $[CuLH_{-3}]$ | −17.09 (1) | [b] |
| $[CuLH_{-4}]$ | −27.17 (1) | [b] |
| pK(−2/−3) | 9.16 | |
| pK(−3/−4) | 10.48 | |

[a] Standard deviations (3σ values) are given in parentheses. pK(n/m) values reflect the pK value of Cu(II) complexes. Charges are omitted for clarity.
[b] Discussion in the text.

reduces the $Cu^{2+}$ affinity for the N-terminal binding site and modulates the copper mediated aggregation kinetics of the protein [48].

Recently, we demonstrated that Semax possesses a high affinity for Cu(II) ions and a protective ability against metal-induced cell toxicity, suggesting that this peptide might have a role in copper homeostasis [49]. Ac-Semax can be a promising drug to obtain the prolongation of the Semax nootropic effect on the CNS, owing to the higher stability with respect to Semax in biological fluids [50].

In the present work, the copper(II) complexes formed by Ac-Semax are described. The results obtained are compared with those previously reported for Semax [49] in order to discern the effects of acetylation of the N-terminal amino group on copper(II) chelation properties. Moreover, since there is a cross-talk between BDNF and zinc [51], and Semax has a regulatory action on BDNF, the chelation ability towards $Zn^{2+}$ was also characterized for both acetylated and non-acetylated derivatives.

Moreover, the effect of Semax acetylation on the copper-induced cytotoxicity on SH-SY5Y neuroblastoma cell line was also studied in comparison with the previously reported Semax protective activity. Finally, the functional interaction of Zn(II) with both peptides were tested by evaluating the effects on the viability of the SH-SY5Y neuroblastoma cells.

## 2. Experimental

### 2.1. Materials

All reagents and solvents were purchased from commercial sources and used as received unless otherwise noted. Ac-Semax and Semax were purchased from JPT Innovative Peptide Solution (Germany).

### 2.2. Potentiometric titrations

Potentiometric titrations were carried out using a home-assembled fully automated apparatus sets (Metrohm E654 pH-meter, combined micro pH glass electrode, Orion 9103SC, Hamilton digital dispenser, Model 665). A thermostated (298.0 ± 0.2 K) titration cell (2.5 ml) was used and the titrated solutions were maintained under argon atmosphere and the ionic strength used was 0.1 M in $KNO_3$. KOH titrating solutions were added through a Hamilton burette equipped with a 1 $cm^3$ syringe. The ligand concentration used for protonation and complexation with Cu(II) and Zn(II) measurements ranged from 1 to 2 mM and at least four independent titrations were performed. Other experimental details are as reported elsewhere [52]. Protonation and complexation stability constants were obtained from refinement of potentiometric data by using Hyperquad program [53]. Three times standard deviation was used to report the errors on stability constants. The formation reaction equilibria of peptide (Ac-Semax or Semax) with either Cu(II) or Zn(II), and protons, are given in Eq. (1):

$$pM + qH + rL = M_pH_qL_r \quad (1)$$

Please cite this article as: A. Magrì, et al., Influence of the N-terminus acetylation of Semax, a synthetic analog of ACTH(4-10), on copper(II) and zinc(II) coordination and bi..., J. Inorg. Biochem. (2016), http://dx.doi.org/10.1016/j.jinorgbio.2016.08.013