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Copper(II) complexes of neurokinin A with point mutation (S5A) and products of copper-catalyzed oxidation; role of serine residue in peptides containing neurokinin A sequence

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ABSTRACT

A potentiometric, spectroscopic (UV-visible, CD and EPR) and electrospray ionization mass spectrometric (ESI-MS) study of Cu(II) binding to the neurokinin A with point mutation (S5A) (ANKA), His-Lys-Thr-Asp-Ala^5-Phe-Val-Gly-Leu-Met-NH_2 and its N-acethyl derivative (Ac-ANKA), Ac-His-Lys-Thr-Asp-Ala^5-Phe-Val-Gly-Leu-Met-NH_2 were carried out. For the ANKA and Ac-ANKA the additional deprotonation was not observed. It suggests that for the tachykinin peptides with C-terminal sequence of neurokinin A for the additional deprotonation the presence of the serine residue is necessary. For the Cu(II)-ANKA 1:2 system at physiological pH 7.4 the CuH_2L_2 species is present with histamine-like 4 N, $2\times\{NH_2,N_{Im}\}$ coordination mode. With increasing pH the deprotonation and coordination of amide nitrogen atoms occur and the $CuH_{-2}L$, $CuH_{-3}L$ complexes are formed. In pH range 4.5 – 9.5 the dimeric Cu_2HL_2, Cu_2L_2 and $Cu_2H_{-1}L_2$ species in solution are also present. To elucidate the products of the copper(II)- catalyzed oxidation of the ANKA and Ac-ANKA, the liquid chromatography-mass spectrometry (LC-MS) method and Cu(II)/hydrogen peroxide as a model oxidizing system were employed.
In the presence of hydrogen peroxide with 1:1 peptide-H_2O_2 molar ratio for both peptides the oxidation of the methionine residue to methionine sulfoxide was observed. For the Cu(II)-peptide-hydrogen peroxide in 1:2:2 molar ratio systems oxidations of the histidine residues to 2-oxo-histidines and methionine sulfoxide to methionine sulfone were detected.

1. Introduction

Tachykinins constitute a family of multifunctional neuropeptides whose signaling mechanisms seem to be partially conserved through evolution [1–5]. Although the tachykinin peptides display only limited sequence identities when comparing invertebrates and mammals, their G-protein-coupled receptors (GPCRs) display more striking similarities, suggesting ancestral relationships [4,5]. The three principal mammalian tachykinins, substance P, neurokinin A and neurokinin B, are processed from two precursors, preprotachykinin A and B and they act with preferential affinities on three different GPCRs, NK1 – NK3 [5]. Tachykinins are a family of biologically active peptides distributed in the central and peripheral nervous system. Tachykinins elicit a wide and complex array of biological responses, such as the stimulation of extravascular smooth muscle, powerful vasodilation, hypertensive action, activation of immune system, regulation of pain transmission, and neurogenic inflammation [6–8]. The wide range of physiological activity of tachykinins has been attributed to the lack of specificity of tachykinins for a particular receptor type [9].

Neurokinin A (NKA) is a decapeptide found in mammalian neuronal tissue, with the sequence His-Lys-Thr-Asp-Ser^5-Phe-Val-Gly-Leu-Met-NH_2. It was first isolated from the porcine spinal cord [10]. In water NKA prefers to be in an extended chain conformation whereas a helical conformation is induced in the central core and the C-terminal region (D^4 – M^{10}) of the peptide in the presence of perdeuterated dodecylphosphocholine (DPC) micelles, a membrane model system [11]. The results of the structure – activity studies performed by Rovero and collegues and those of other workers have indicated the Asp^4 and Phe^6 of NKA are important for activation of the NK2 receptor [12–15]. Results of analogues of neurokinin A mediating contraction of rat uterus suggest that the His at position one of NKA is of little or no importance in contractile activity, while substitution or truncation of Lys at position two or substitution of Thr at position three causes a decrease in potency [16].

In rats, neurokinin A has been detected across brain regions [17] and some effects on motor behaviour and nociception have been demonstrated [18,19]. It has been suggested that neurokinin A may have a neurotransmitter/neuromodulator role in the substantia nigra

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of rats [20]. The measurement of cerebrospinal fluid (CSF) neurokinin A concentration, in a larger sample of patients, together with brain tissue studies of this neuropeptide in Parkinson's disease, may demonstrate a significant neurokinin A reduction in Parkinsonian patients and indicate the involvement of neurokinin A – containing neurons in this disease [21].

Oxidative stress is considered a major contributor to the pathogenesis of a number of pathological processes leading to atherosclerosis, inflammatory conditions, multiple system atrophy and several neurodegenerative diseases [22–24]. The reactive oxygen species (ROS) are formed in biological systems by both enzymatic and metal-catalyzed oxidation (MCO) reactions [25,26]. A feature of metal-catalyzed oxidations is the site-specific nature of the reaction, i.e. specific amino acid residues located at the metal-binding sites are generally altered [27]. Therefore, the knowledge of the products of metal-catalyzed oxidation may suggest the metal-binding sites [28–30]. Most susceptible to metal-catalyzed oxidation are His, Met, Cys [27,31] because of their ability to form complexes with metals such as Cu(II) or Fe(III). The reactive oxygen species are generated, and oxidation of specific amino acid residues occurs in what is reffered to as a "caged" process [32]. The MCO reaction involves reduction of Fe(III) or Cu(II) by a suitable electron donor such as NADH, NADPH, mercaptane, or ascorbate. Fe(II) or Cu(I) ions bound to specific metal-binding sites on proteins react with H_2O_2 to generate $^{\cdot}OH$ [33,34]. The mechanism of generation for the active species from Cu(II)/peptide/ H_2O_2 system has been considered (reactions (1) – (3)) [35,36].

$$\text{Peptide–Cu(II)} + H_2O_2 \rightarrow \text{peptide–Cu(I)} + O_2^{-\cdot} + 2H^+ \quad (1)$$

$$\text{Peptide–Cu(II)} + +O_2^{-\cdot} \rightarrow \text{peptide–Cu(I)} + O_2 \quad (2)$$

$$\text{Peptide–Cu(I)} + H_2O_2 \rightarrow \text{peptide–Cu(II)} + {}^{\cdot}OH + OH^- \quad (3)$$

The present paper reports the results of combined spectroscopic and potentiometric studies on the copper(II) complexes of the modified neurokinin A and its analogue with N-terminal amine protected group by acethylation. The peptides involved in the study are: neurokinin A (ANKA) HKTDA^{5}FVGLM-NH_2 and Ac-neurokinin A (Ac-ANKA) Ac-HKTDA^{5}FVGLM-NH_2, both peptides contain point mutation (S5A). Our studies on the acid-base properties and coordination abilities towards to copper(II) of the neurokinin A [37], neuropeptide gamma and its fragments [38–40], and neuropeptide K fragments [41] (all peptides contain the neurokinin sequence in their C-terminal), clearly indicate the presence of additional deprotonation of the ligands studied. The CID MS/MS analysis (collision-induced dissociation tandem mass spectrometry analysis) of the neurokinin A [30] indicates that Ser^5 may be particularly responsible for this additional deprotonation. To support this suggestion we synthesized the neurokinin A and its N-acetyl derivative with point mutation (S5A). The acid-base properties of these ligands were determined by potentiometric studies. The metal-catalyzed oxidation of the ANKA and Ac-ANKA by the Cu(II)/ H_2O_2 system demonstrate the relationship between the binding sites of copper(II) ions and the oxidation products of the ligands studied.

2. Material and methods

2.1. Synthesis of the peptides

Synthesis of ANKA with point mutation (S5A), His-Lys-Thr-Asp-Ala^5-Phe-Val-Gly-Leu-Met-NH_2, and its acethyl derivative (Ac-ANKA) peptides were carried out using Millipore 9050 peptide synthesizer and continuous-flow methodology [42–44]. A polystyrene/polyethylene glycol copolymer resin (TentaGel R RAM, Rapp Polymere) was used as a solid support. The peptidyl-resin was divided into two portions; one of them was subjected to 1 M acetylimidazole in DMF to acetylate the N-terminal amino group of the peptide and produce Ac-ANKA. Both peptides were cleaved from the resin and deprotected by treatment with a mixture containing 94.0% of trifluoroacetic acid (TFA), 2.5% of H_2O, 2.0% of triisopropylsilane (TIS) and 1.5% of 1,2-ethanedithiol (EDT). The cleavage reaction was carried out for 1.5 h at room temperature.

The crude peptides were purified by reversed-phase (RP) HPLC using a Luna C_8 semi-preparative column (21.2×250 mm, 100 Å, 5 μm, Phenomenex). The purity of the peptides was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and analytical RP-HPLC using a C_8 Kromasil column (4.6×250 mm, 5 μm) and 30 min. linear gradient of 5-80% acetonitrile (ACN) in 0.1% aqueous trifluoroacetic acid as a mobile phase.

Analytical data were as follows: ANKA: $R_T = 15.4$ min, MS: $[L]^+ = 1117.3$, calc. 1117.3; Ac-ANKA: $R_T = 15.6$ min, MS: $[L]^{+\cdot} = 1159.3$, calc. 1159.4.

The purity of the peptides was checked and the exact concentration of their stock solutions was determined by the Gran method [45].

2.2. Potentiometric measurements

Stability constants for proton and Cu(II) complexes were calculated from pH-metric titrations carried out in an argon atmosphere at 298 K using a total volume of 1.9 – 2 ml. Alkali was added from a 0.250 ml micrometer syringe which was calibrated by both weight titration and the titration of standard materials. Experimental details: ligand concentration 1.5×10^{-3} M, metal-to-ligand molar ratio 1:2; ionic strength 0.10 M (KNO_3); $Cu(NO_3)_2$ used as the source of the metal ions; pH-metric titration on a MOLSPIN pH-meter system using a Russell CMAW 711 semimicro combined electrode calibrated in concentration using HNO_3 [46] , number of titrations = 2; method of calculation SUPERQUAD [47]. The samples were titrated in the pH region 2.5 – 10.5. Standard deviations (values) quoted were computed by SUPERQUAD and refer to random errors only. They are, however, a good indication of the importance of the particular species involved in the equilibria. The precipitation for the Cu(II)-ANKA 1:1.1 system was observed, therefore, the 1:2 metal-to-ligand molar ratio was investigated. However, the Cu(II)-Ac-ANKA system could not be studied because of precipitation for 1:1, 1:2 and 1:higher metal-to-ligand molar ratios.

2.3. Spectroscopic measurements

Solutions were of similar concentrations to those used in potentiometric studies. Absorption spectra (UV-visible) were recorded on a Cary 50 "Varian" spectrophotometer in the 850 – 300 nm range. Circular dichroism (CD) spectra were recorded on a Jasco J-715 spectropolarimeter in the 750 – 250 nm range. The values of $\Delta\varepsilon$ (i.e., ε_l - ε_r) and ε were calculated at the maximum concentration of the particular species obtained from potentiometric data. Electron paramagnetic resonance (EPR) spectra were performed in an ethylene glycol-water (1:2, v/v) solution at 77 K on a Bruker ESP 300E spectrometer equipped with the ER 035 M Bruker NMR gaussmeter and the HP 5350B Hewlett-Packard microwave frequency counter at the X-band frequency (~9.45 GHz). The spectra were analyzed by using Bruker's WIN-EPR SimFonia software, version 1.25. Copper(II) stock solution was prepared from $Cu(NO_3)_2\times3\ H_2O$.

2.4. ESI-MS measurement

The mass spectra were obtained on a Bruker MicrOTOF-Q spectrometer (Bruker Daltonik, Bremen, Germany), equipped with Apollo II electrospray ionization source. The mass spectrometer was operated in