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The binding of Cu(II) by the peptide with β-Asp located in non-coordinating site – Solution and structural studies

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ABSTRACT

In the present study, the coordination abilities of Ac-TLEGTKKGHKLHLβDY-NH_2 peptide (the analogue of SPARC 114–128 fragment containing β-Asp^{127} residue) are discussed. The analysis is provided based on the results of potentiometric and spectroscopic measurements supported by quantum-chemical calculations. Presented results clearly show that the β-Asp amino acid residue may influence the efficiency of metal ion binding despite the fact that it is not directly involved in metal ion binding. Moreover, in order to further characterize experimentally observed species, we performed quantum-chemical calculations for structures mimicking SPARC 114–128 fragment as a step towards a better understanding of structural and energetical aspects related to the coordination abilities of the analogue of SPARC fragment.

1. Introduction

SPARC (secreted protein which is acidic and rich in cysteine), also known as osteonectin, BM-40 or 43 kDa protein, is a glycoprotein produced by a variety of cells types [1] and plays a complex role in carcinogenesis. SPARC is a transiently expressed extracellular matrix-binding protein that alters cell shape and regulates endothelial cell proliferation *in vitro* [2,3]. SPARC protein is also known for its ability to inhibit angiogenesis [4], while peptide fragments derived from the protein are able to promote apoptosis *in vitro* and *in vivo* [5]. Smaller peptide analogues have greater chemosensitizing and tumor-regressing properties compared to the native protein [5,6]. Both positive and negative associations between high SPARC level and tumors were reported [7–10]. They are effects of differences in the biological activities of the various proteolytic products of SPARC cleavage.

Structurally, SPARC contains three distinct domains which contribute to its biological properties: N-terminus (NT-residues 1–52 after a 17-amino acid signal sequence), follistatin-like (FS-comprise residues 53–137) and the extracellular C-terminus (EC-residues 138–286).

N-terminal domain, an acidic region rich in Asp and Glu, is able to bind 5–8 calcium ions [11] and can interact with hydroxyapatite [12]. This part of the protein is involved in the mineralization of cartilage and bone [13]. This region is also identified as the region with its chemosensitizing properties. This domain of SPARC and its 51-aa peptide are highly efficacious in modulating and enhancing apoptosis, thereby conferring greater chemosensitivity to resistant tumors [6].

The second domain of SPARC protein – follistatin-like (FS) domain – is rich in Cys residues. All the Cys are disulfide-bonded, and with an N-linked complex carbohydrate at Asn99. FS contains two bioactive peptide sequences (comprised of residues 54–73 and 113–130), both containing copper binding sites. [14] The second one with the sequence Lys-Gly-His-Lys (residues 120–123) opposes the activity of the full-length protein by stimulating angiogenesis *in vitro* and *in vivo* [15]. The presence of the His in position 122 is able to stimulate cell proliferation and angiogenesis [2,16].

The extracellular α-helical domain (EC), poses high affinity to collagens and calcium ions [17,18] and may have anti-angiogenic properties [19–21]. An endogenous protease cleavage site in EC is located at the Leu–Leu bond in position 197/198 in the α-helical region [17,22].

β-Peptides, which differ from natural peptides/proteins by additional CH_2 group in the amino acid backbone are used in medicine. Their chemical structure allows them to mimic or improve the effects of some medications based on α-peptides so they have potential as pharmaceuticals [23,24]. The additional carbon atom in the backbone allows to display high folding properties [23,25,26]. β-Peptides exhibit increased resistance to proteases *in vivo* and *in vitro* which makes them good candidates for biomedical applications. According to a complementary properties of

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α- and β-amino acids α/β-mixed peptides are in high interest for bioinorganic and biomedical fields [27].

To check the influence of insertion of one β-amino acid residue (β-Asp) into $SPARC_{114-128}$ sequence, Ac-TLEGTKKGHKLHLβDY-NH_2 peptide, (Fig. 1) was synthesized and potentiometric and spectroscopic studies were performed.

2. Experimental

2.1. Synthesis

Peptide was synthesized by the automated Fmoc solid-phase peptide synthesis method on the Fmoc-Tyr(tBu)-Rink MBHA resin (0.69 mM/g, Iris Biotech GmbH). Synthesis was performed using Activo P11 peptide synthesizer (Activotec). Fmoc protecting groups were removed by 25% piperidine in dimethylformamide. Subsequent Fmoc-protected amino acids (3 eq) were attached by using 3 eq TCTU (O-(6-Chlorobenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate) as a coupling reagent in the presence of N-hydroxybenzotriazole (3 eq) and diisopropylethylamine (6 eq) for 90 min. at room temperature. Acetylation of the N-terminal amine group was performed on the resin by 1:1 mixture of acetic anhydride and 0.4 M N-methylmorpholine in dimethylformamide. Final cleavage of the peptide was achieved by "Reagent K" (81.5% trifluoroacetic acid, 5% phenol, 5% thioanisole, 5% water, 2.5% ethanedithiol, 1% triisopropylsilane) in 2 h at room temperature. Crude peptide was precipitated by cold diethylether, washed with ether, dissolved in water and lyophilized.

Peptide was purified by semipreparative HPLC using Varian ProStar apparatus equipped with TOSOH Bioscience C18 column (300 × 21.5 mm, 10 μm beads) and 220 nm/254 nm dual-wavelength UV detector. Water–acetonitrile gradient containing 0.1% TFA at a flow rate of 7 ml/min. was used for the elution. Final purity of the lyophilized peptide was >95% by analytical HPLC (Thermo Separation Product; column: Vydac Protein RP C18, 250 × 4.6 mm, 5 μm; linear gradient from 0% to 100% B in A in 60 min., solvent A – 0.1% TFA in water, solvent B – 0.1% TFA in 80% acetonitrile: water solution, UV detection at 220 nm).

Chemical identity of the peptide was confirmed by high resolution ESI-MS on a Bruker apex FT-ICR mass spectrometer. Analytical data of the peptide are given in Table 1.

2.2. Potentiometric measurements

Stability constants both for protons and Cu(II) complexes were calculated from three titrations carried out over the pH range 3–11 at 25 °C using a total volume of 1.5 cm^3. The purities and exact concentration of the solutions of the ligands were determined by the method of Gran [28]. NaOH was added from 0.250 cm^3 micrometer syringe which was calibrated by both weight titration and the titration of standard materials. The metal ion concentration was 1×10^{-3} mol/dm^3 and the metal to ligand ratio was 1:1.5. The pH-metric titrations were performed at 25 °C in 0.1 mol/dm^3 KCl on a Molspin pH-meter system using semi-combined electrode calibrated in hydrogen ion concentrations using HCl [29]. The Superquad titration program was used for stability constant calculations [30]. Standard deviations was computed by Superquad and refer to random errors only. They are, however, a good indication of the importance of a particular species in the equilibrium.

2.3. Spectroscopic measurements

Solutions were of similar concentrations to those used in the potentiometric studies.

UV–Vis spectra were recorded on a Perkin-Elmer Lambda Bio 20 spectrophotometer in the 800–200 nm range. Circular dichroism (CD) spectra were recorded on a Jasco J-715 spectropolarimeter in the 800–235 nm range. The values of $\Delta\varepsilon$ (*i.e.* ε_1 - ε_2) and ε were calculated at the maximum concentration of the particular species obtained from the potentiometric data.

Electron paramagnetic resonance (EPR) spectra were measured on a Bruker ESP 300E spectrometer at X-band frequency (9.4 GHz) in liquid nitrogen. The 30% ethylene glycol was used as cryoprotectant. The EPR parameters were obtained at the maximum concentration of the particular species.

2.4. Molecular modeling study

The conformational analysis of proposed complexes was carried out using PM6 method which is known to be a quite successful approach in determining structures of bioinorganic complexes with transition metals [31,32]. As it was also shown, for many properties (including equilibrium geometries or electric-dipole properties) the PM6 method improves upon PM3 method. Harmonic frequency analysis was performed to verify that each structure corresponds to a stationary point on the potential energy surface. Furthermore, all computations were performed with the inclusion of solvent effects (water) using polarizable continuum model (PCM) [33–37]. Among all optimized structures, only the lowest energy conformers for each species are described throughout the manuscript. All the calculations described above were performed within unrestricted formalism using the GAUSSIAN 09 suite of programs [38].

3. Results and discussion

Ac-TLEGTKKGHKLHLβDY-NH_2 peptide shown in Fig. 1 is a ligand with 8 protonation sites. Protonation constants for all protonated residues are collected in Table 2. Three first protonation

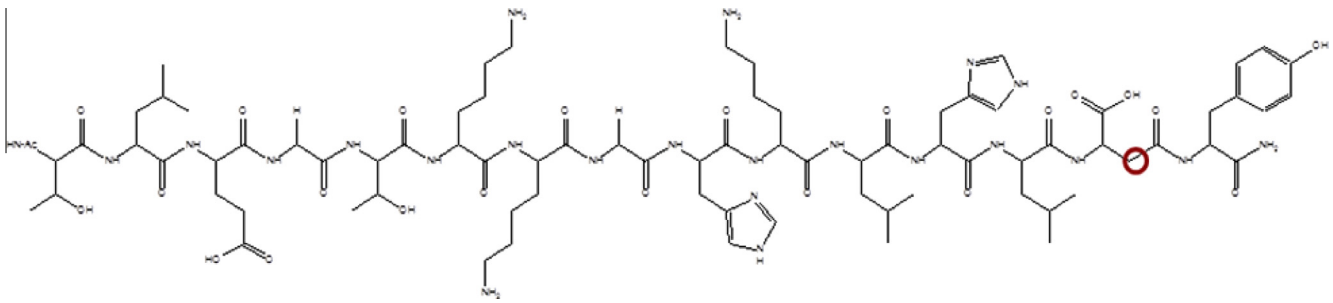

Fig. 1. A primary structure of Ac-TLEGTKKGHKLHLβDY-NH_2 peptide with additional CH_2 group in β-Asp amino acid residue marked by the circle.