



Probing the binding selected metal ions and biologically active substances to the antimicrobial peptide LL-37 using DSC, ITC measurements and calculations

Joanna Makowska¹ · Dariusz Wyrzykowski¹ · Elżbieta Kamysz¹ · Aleksandra Tesmar¹ · Wojciech Kamysz² · Lech Chmurzyński¹

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Abstract

Differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC) techniques have been used to describe physicochemical properties of polypeptide LL-37. LL-37 is a 37-residue, amphipathic, helical peptide, the only cathelicidin-derived antimicrobial peptide found in the human organism. Thermal stability of the LL-37 peptide in a water solution was measured by DSC over the 288.15–333.15 K range. Furthermore, the ITC method supported by theoretical calculations (peptide–ligand docking) were used to study the interactions between LL-37 and some divalent metal ions, namely Cu^{2+} , Zn^{2+} , and Ni^{2+} ions as well as acetylsalicylic acid, ascorbic acid, and caffeine molecules. It has been proven that under experimental conditions, the LL-37 peptide reveals affinity only toward Cu^{2+} and Zn^{2+} ions. The stoichiometry, conditional binding constants, $\log K_{\text{ITC}}$, and thermodynamic parameters ($\Delta_{\text{ITC}}G$, $\Delta_{\text{ITC}}H$, $T\Delta_{\text{ITC}}S$) of the resulting Cu(II) and Zn(II) complexes were determined and discussed.

Keywords ITC · VP-DSC · LL-37 · Metal ions · Coordination properties

Introduction

The LL-37 (sequence LLGDFFRKSKEKIGKEFKRIVQR IKDFLRNLVPRTES) is a peptide found in human saliva. It plays a very important role in the organism [1, 2]. It is produced in a fairly high concentration at the site of infection or skin injury [3]. It works antibacterially, chemotactically and also participates in repair activities. In the amniotic fluid and in the skin of a newly born child, it acts as a protective device and also constitutes an innate antimicrobial barrier [4]. It was also shown that a deficiency of human cathelicidines may lead to disease symptoms, for example, allergies atopical skin [5, 6] or Kostman's disease, which is a genetic disease diagnosed in

infants characterized by inhibition of maturation of actinomycetes to myelocytes [7, 8]. Lack of LL-37 in saliva may be a reflection of the presence and composition of granulocytes and thus constitute a very good indicator for this disease.

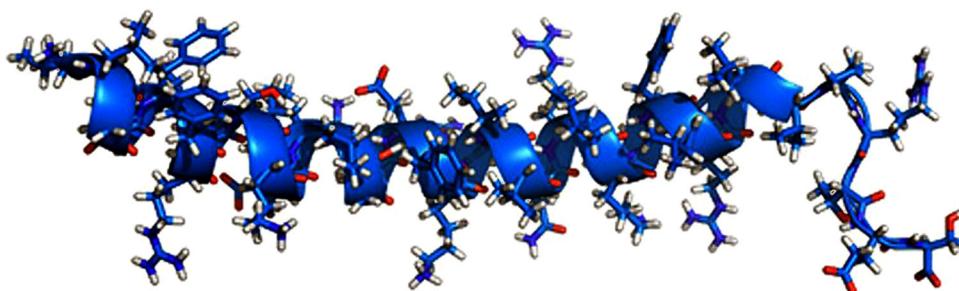
Human cathelicidin LL-37 is the only member of human cathelicidin which has been described so far in the literature. Studies by using infrared spectroscopy (IR) and nuclear magnetic resonance (NMR) spectroscopy have shown that LL-37 is a cationic peptide [9, 10] and in aqueous media has no defined conformation. It probably exists in the conformational equilibrium in water. However, the NMR study of LL-37 in micelles of the sodium dodecyl sulfate (SDS) shows that this peptide obtains in this solution a helical structure [11] (Fig. 1). This indicates a large dependence of the conformation shape of LL-37 on pH value and the surrounding environment. To be able to properly perform polypeptides and proteins functions, each of them must adopt a unique structure (*native structure*). Errors in the formation of this unique structure lead very often to many serious illnesses. Undesirable interactions of

✉ Joanna Makowska
joanna.makowska@ug.edu.pl

¹ Faculty of Chemistry, University of Gdańsk, ul. Wita Stwosza 63, 80-308 Gdańsk, Poland

² Faculty of Pharmacy, Medical University of Gdańsk, Al. Gen. J. Hallera 107, 80-416 Gdańsk, Poland

Fig. 1 NMR structure of LL-37 in SDS (sodium dodecyl sulfate) micelles [11]



proteins and peptides with other molecules or bigger systems may lead to their dysfunction.

It has been proven that LL-37 plays an important role in the human body. However, the interactions of the LL-37 peptide with medical drugs as well as metal ions have not been studied so far. For this reason, it seemed worthwhile to carry out research on just this subject. This contribution initiates a series of efforts confined to the study of the interactions of LL-37 with the commonly used substances, i.e., acetylsalicylic acid (aspirin) and ascorbic acid (vitamin C) as well as caffeine presented in coffee, tea and also energy drinks. Furthermore, the interactions of LL-37 with some biologically relevant metal ions, i.e., Cu^{2+} and Zn^{2+} have also been investigated. Finally, due to the contact of human body with nickel in view of the orthodontic braces or popular piercing in tongues, the possible affinity of LL-37 toward Ni^{2+} ions has been studied. The obtained results can provide some insight into physicochemical properties of the antimicrobial LL-37 peptide which can affect the biological activity of the investigated peptide.

In the presented work, two complementary techniques, namely differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC), have been employed to study physicochemical properties of the LL-37 peptide. These techniques are generally used for the characterization of the peptides in aqueous solution [12–14].

A thermal stability of the LL-37 peptide in water was measured by differential scanning calorimetry (DSC). The investigating thermal stability in water as a solvent without SDS was intended to test the stability of the system before it bound to the cell membrane. This LL-37 system adopts the α -helix conformation in contact with the cell membrane. If, however, its shape is disturbed earlier (even before it interacts with the membrane), because of the influence of external factors (e.g., temperature) or due to its strong connection with another system, its function is impaired. Therefore, in the next step, the isothermal titration calorimetry (ITC) and theoretical calculations were used to study the interactions between LL-37 and the chosen systems.

Experimental

Materials and methods

Materials

All reagents, namely acetylsalicylic acid, caffeine, ascorbic acid, 2-(*N*-morpholino)ethanesulfonic acid hydrate (Mes $\geq 99\%$) and copper(II) nitrate hemi(pentahydrate) ($\text{Cu}(\text{NO}_3)_2 \cdot 2.5\text{H}_2\text{O}$, $\geq 99.99\%$), were purchased from Sigma-Aldrich Chemical Corp. and used as obtained. The doubly distilled water with a conductivity not exceeding $0.18 \mu\text{S cm}^{-1}$ was used for the preparation of the Mes buffer. The pH of the buffer solution was adjusted to 6.0 with 0.1 M NaOH. The stock solutions of the LL-37 peptide (1.0×10^{-3} M) and Cu^{2+} ions (0.1×10^{-3} M) were prepared by dissolving an appropriate amount of the substance in 20 mM Mes buffer solution.

The polypeptide LL-37 was synthesized manually with the solid-phase method using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on a 2-chlorotrityl chloride resin ($1.6 \text{ mmol Cl}^- \text{ g}^{-1}$ resin, 100–200 mesh) [15]. The following amino acids side-chain protecting groups were used: Trt (for Asn, Gln), tBu (for Ser, Thr), Boc for Lys, Pbf for Arg and OtBu (for Glu, Asp). All *N*^z-Fmoc-protected amino acids, the coupling reagents and the resin were obtained from Iris Biotech GmbH (Marktredwitz, Germany). Other reagents and solvents were provided by Merck (Darmstadt, Germany).

The attachment of the first amino acid residue to the 2-chlorotrityl chloride support was performed according to Barlos et al. [16] with a loading of 0.6 mmol g^{-1} . Peptide chain was elongated through consecutive cycles of deprotection and coupling as described previously [17]. After completing the syntheses, the peptide was cleaved from the resin simultaneously with the side-chain protection groups in a one-step procedure, using a trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/phenol/ H_2O (94:2:2:2; v/v/v) mixture for 2 h. The cleaved peptide was precipitated with cold diethyl ether and lyophilized.

The crude peptide was purified using reverse-phase high-performance liquid chromatography (RP HPLC) on a

Phenomenex Gemini-NX C18 (21.20 × 100 mm, particle size 5.0 μm, pore size 110 Å) and using a UV detector. The following solvent systems were used: [A] 0.1% TFA in water and [B] 0.1% TFA in acetonitrile. A linear gradient from 10% to 65% of [B] in 60 min at a flow rate of 10 mL min⁻¹ and monitoring at 214 nm was employed. The purity of the peptide was checked on a Varian ProStar HPLC system (Varian Inc., Palo Alto, CA, USA) controlled by a Galaxie Chromatography Data System using Phenomenex, Luna[®] C18(2) column (3 × 100 mm, 5 μm, 100 Å). A linear gradient from 10 to 100% of [B] in 10 min at flow rate 1.2 mL min⁻¹ and monitoring at 214 nm was applied. Fractions containing the pure peptide were pooled and lyophilized. The peptide LL37 was analyzed using electrospray ionization mass spectrometry (ESI-MS). The values of the measured ions were as expected.

Differential scanning calorimetry (VP-DSC)

Calorimetric measurements were carried out with a VP-DSC microcalorimeter (MicroCal. Inc., Malvern, Northampton, MA, USA), the differential scanning calorimeter for the study of samples in solution, at a scanning rate 1.5 K min⁻¹. Scans were obtained in water at the peptide concentration of ~ 1 mmol L⁻³. The cell volume was 0.5 mL. Results from DSC measurements were analyzed with the Origin 7.0 software from MicroCal using the routines of the software provided with the instrument [18]. The reversibility of the transition was checked by cooling and reheating the same sample. These measurements were recorded three times. The quantity measured by DSC is the difference between the heat capacity of the water-peptide solution and that of pure water. To perform a DSC measurement of protein unfolding, the reference cell was filled with water, and the sample cell was filled with the LL-37 polypeptide solutions. When a polypeptide unfolds during DSC measurements, the absorption of heat that occurs causes a temperature difference (ΔT) between the cells. The reference (water) and sample solutions (LL-37) were equilibrated with dissolved air before being introduced into the cells. Five minutes of vacuum treatment was required to degas all of the samples. The first step in calibration was to carry out the water/water scans with the run parameters exactly the same as for the comparative scans when the polypeptide is present in the sample cell. A pre-scan thermostat period of 15 min was used as recommended [19].

Isothermal titration calorimetry (ITC)

All isothermal titration calorimetry (ITC) experiments were performed at 298.15 K using an AutoITC isothermal

titration calorimeter (MicroCal, Malvern Panalytical) with a 1.4491-mL sample and the reference cells. The reference cell contained the distilled water. All details of the measuring devices and the experimental setup were described previously [20, 21]. The experiment consisted of injecting of 10.02 μL (29 injections, 2 μL for the first injection only) of *ca* 1 mM buffered solution of LL-37 into the reaction cell which initially contained *ca* 0.1 mM buffered solution of the Cu²⁺ salt. A background titration, consisting of an identical titrant solution but with the buffer solution in the reaction cell only, was removed from each experimental titration to account for the heat of dilution. All the solutions were degassed prior to the titration. The titrant was injected at 3.5-min intervals to ensure that the titration peak returned to the baseline before the next injection. Each injection lasted 20 s. For sake of homogeneous mixing in the cell, the stirrer speed was kept constant at 300 rpm. A calibration of the AutoITC calorimeter was carried out electrically by using electrically generated heat pulses. The CaCl₂—EDTA titration was performed to check the apparatus, and the results (*n*—stoichiometry, *K*, ΔH) were compared with those obtained for the same samples (a test kit) at MicroCal Malvern Panalytical.

Docking of active biologically compounds in LL-37

Docking was performed with use of the MTiOpenScreen [22], a service to dock small compounds [23] and by using a portal for bioinformatics analyses, the Mobyly Portal [24, 25]. In the Mobyly Portal, the MTiAutoDock is hosted, the service which is dedicated to small molecule docking and chemical library virtual screening. In our consideration, the blind docking was performed by using AutoDock [26, 27]. AutoDock is one of the programs which are able to predict protein–ligand complex structures with reasonable accuracy and speed. The Lamarckian genetic algorithm (LGA) [23] as implemented in AutoDock 4.2.6 is used to generate orientations/conformations of the compound.

Results and discussion

Peptide synthesis and purification

LL37 was synthesized with solid-phase peptide synthesis. The purity of the peptide after purification was above 98%, as determined by analytical HPLC (retention time 6.0 min. in linear gradient from 10 to 100% of [B] in 10 min at flow rate 1.2 mL min⁻¹ with Phenomenex, Luna[®] C18(2)-column). The identity of LL37 was confirmed by ESI-MS (positive scan, range 100–1250 Da): $m/z = 1123.6$ ($[M + 4H]^{4+}$, calc), $m/z = 1123.29$ ($[M + 4H]^{4+}$, found),

$m/z = 899.1$ ($[M + 5H]^{5+}$, calc), $m/z = 898.43$ ($[M + 5H]^{5+}$, found), $m/z = 749.4$ ($[M + 6H]^{6+}$, calc), $m/z = 748.75$ ($[M + 6H]^{6+}$, found), $m/z = 642.5$ ($[M + 7H]^{7+}$, calc), $m/z = 642.24$ ($[M + 7H]^{7+}$, found), $m/z = 562.3$ ($[M + 8H]^{8+}$, calc), $m/z = 562.16$ ($[M + 8H]^{8+}$, found).

Thermal stability (VP-DSC)

To determine if a conformational transition occurs in the LL-37 peptide, we performed DSC measurements in water. The measurement was carried out in the range of temperature 288.15–333.15 K, with the speed of scanning 90 K h⁻¹. A multiple heating and cooling processes were carried out to check the reversibility of the process. The VP-DSC measurement allowed to determine the calorimetric curve of the phase change for the investigated compound, which is shown in Fig. 2.

Figure 2 shows that the conformational reorganization of LL-37 takes place in the temperature range from ~ 300.15 to 323.15 K. The calorimetric curve (Fig. 2) is not characterized by a sharp peak that would indicate an unambiguous phase transition. The maximum point for the curve is at about 310.65 K, which proves that at this temperature, in the solution, there are 50% of ordered (folded) forms and 50% of unordered (unfolded) forms of the tested protein. It follows that cathelicidin LL-37 has biological activity up to 311.15 K. The enthalpy change (ΔH) of the unfolding process is positive, which means that the reaction is endothermic and involves the reaction of breaking the bonds. The numerical value of the enthalpy change for the observed reaction is the integrated area

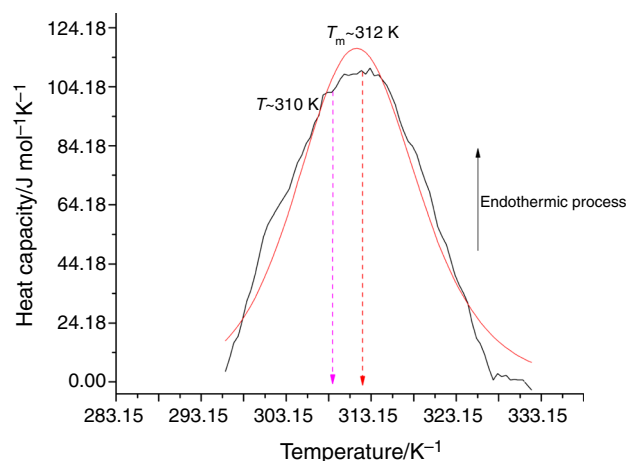


Fig. 2 The heat capacity curves of a polypeptide LL-37 determined by DSC (black line) and the fit to the multi-stage transformation model curve (red line). The pink arrow shows the value of temperature of a healthy organism, and the red arrow indicates value of T_m (the maximum temperature of the conformational transition of LL-37). (Color figure online)

under the peak of the DSC curve. The black curve in Fig. 2 indicates the experimental curve, while the red curve is the so-called the theoretical curve (fit) determined on the basis of a mathematical model assuming a multi-stage transformation for LL-37 (MN2State). The adjustment of the experimental curve to the mathematical model assuming a two-stage transformation (M2S) (folded state \leftrightarrow unfolded state) without intermediate states, did not give good results. It follows that the transition of LL-37 into a state of disorder due to temperature is associated with transient states. As mentioned above, the measurement was carried out in the mode: three times heating and three times cooling of the sample, which allowed to determine that the process of conformational transition of cathelicidin LL-37 is irreversible.

Isothermal titration calorimetry (ITC)

Isothermal titration calorimetry (ITC) method was used to study the affinity of the LL-37 peptide toward some divalent metal ions, namely Cu²⁺, Zn²⁺, and Ni²⁺ ions as well as acetylsalicylic acid, ascorbic acid and caffeine molecules. Based on calorimetric data, it has been found that only Cu²⁺ and Zn²⁺ ions bind to LL-37 under the experimental conditions.

The stoichiometry, binding constants, $\log K_{ITC}$, and binding enthalpy, $\Delta_{ITC}H$, of the peptide–metal interactions were obtained from ITC experiments by fitting isotherms (using nonlinear least-squares procedures) to a model that assumes a single set of identical binding sites. The free energy of binding, ΔG_{ITC} , and entropy change, $T\Delta S_{ITC}$, were calculated using the standard thermodynamic relationship: $\Delta G_{ITC} = -RT \ln K_{ITC} = \Delta H_{ITC} - T\Delta S_{ITC}$. The calculated thermodynamic parameters (K_{ITC} , $\Delta_{ITC}H$, $\Delta_{ITC}G$, and $T\Delta_{ITC}S$) are so-called the conditional parameters as their values depend on the pH of the solution as well as the kind of the buffer used [28]. Representative binding isotherms for the LL-37/Cu²⁺ and LL-37/Zn²⁺ interactions are shown in Fig. 3, whereas conditional parameters are summarized in Table 1.

In the conditions under study, both ions (Cu²⁺ and Zn²⁺) form the complexes of the stoichiometry LL-37 to Cu²⁺ (or Zn²⁺) ca. 3:2. The formation of the complexes is an entropy-driven process ($|\Delta H| < |T\Delta S|$). It is interesting to note that although Cu²⁺ ($A_{Cu^{2+}} = 20.29$ eV) exhibits higher the electron affinity than Zn²⁺ ($A_{Zn^{2+}} = 17.96$ eV) [29], the binding enthalpy of the LL-37/Zn²⁺ complex is less positive than that of the corresponding LL-37/Cu²⁺ complex. Furthermore, the binding constants of the Cu²⁺ and Zn²⁺ complexes do not follow the Irving–Williams order for complex stabilities (Table 1) [30, 31]. This phenomenon can be explained by the fact that the experimental values of $\Delta_{ITC}H$ are the sum of all energetic effects

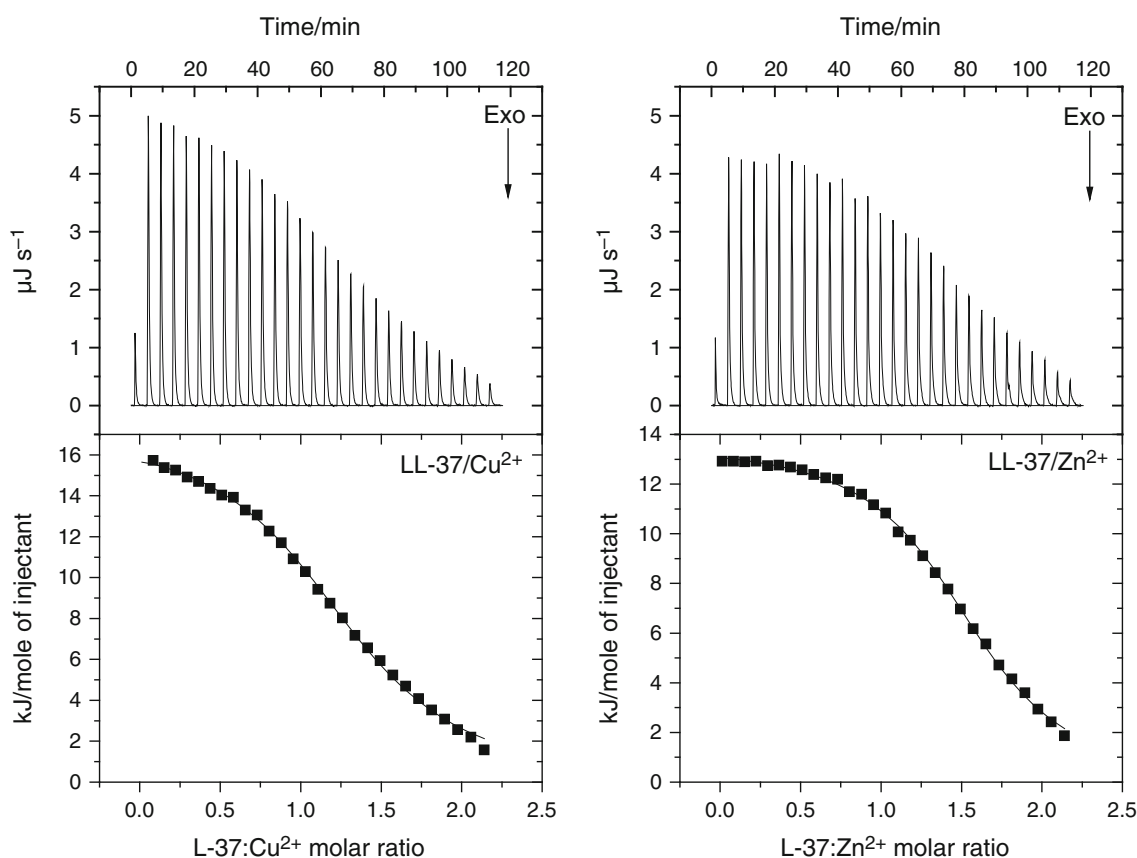


Fig. 3 Calorimetric titration isotherms of the binding interaction between LL-37 and Cu^{2+} (left) and Zn^{2+} (right) in 20-mM Mes buffer (pH 6, $T = 298.15 \text{ K}$)

Table 1 The thermodynamic parameters of Cu^{2+} and Zn^{2+} binding to LL-37 in 20-mM Mes buffer (pH 6, $T = 298.15 \text{ K}$)

Parameter	LL-37/ Cu^{2+}	LL-37/ Zn^{2+}
$\log K_{\text{ITC}}$	4.23 (± 0.02)	5.19 (± 0.01)
$\Delta_{\text{ITC}}H/\text{kJ mol}^{-1}$	17.07 (± 0.13)	13.60 (± 0.04)
$T\Delta_{\text{ITC}}S/\text{kJ mol}^{-1}$	45.19	43.22
$\Delta_{\text{ITC}}G/\text{kJ mol}^{-1}$	- 28.12 (± 0.13)	- 29.62 (± 0.08)

generated during ITC measurements. It has previously been reported that the Mes buffer does not reveal an affinity toward the copper(II) ions [32, 33]. A different situation is observed for zinc ions where the Mes buffer component (a conjugate base) competes with the LL-37 peptide for the Zn^{2+} ion [34]. For these reasons, the heat effects that are not connected directly with the peptide–metal interaction (e.g., metal–buffer interaction) affect the obtained thermodynamic parameters. On the other hand, it can also be supposed that the difference in binding enthalpies of the investigated complexes results from the difference in donor

properties of atoms of side-chain residues of the LL-37 peptide engaged in the metal ion binding.

Docking of active biologically compounds in LL-37

The theoretical method which we have been using in our study allows a fairly rational description of possible interactions of peptides/proteins only with small chemical compounds molecule. Unfortunately, this method is not designed to calculate the interaction between peptides/proteins and metal ions; therefore, we have not tested the interaction of LL-37 with metal ions.

The process of docking of the aspirin molecule with LL-37 has shown no specific interactions between these two systems. During the study, acetylsalicylic acid was found around the C-terminus of the peptide strand but at a very long distance (14–40 Å). This result confirmed the observations of the ITC experiment. The process of docking of the ascorbic acid (vitamin C) molecule with LL-37 has shown some possible interactions. Ascorbic acid molecule approached to the area mostly surrounded by Gly9, Tyr10, His13 residues of the peptide LL-37 at a distance of about 5 Å. However, many of the ascorbic acid molecules also

occurred around Lys14, Glu15 residues. In general, the system was very dynamic and the result of the study did not indicate any unequivocal interactions in a specific region; hence, it was unlikely that this nonspecific interaction could be observed experimentally. However, it can be concluded that ascorbic acid has a better affinity for LL-37 than acetylsalicylic acid molecules. In the case of tests with caffeine, the ligand turned out to be very unspecific system. During the process, the caffeine molecule approached in many areas of LL-37, for a distance of about 5 Å (Asp4, Phe6, Lys8 and Arg19, Arg23, Phe27) which proves the dynamics and high unspecificity of the possible process of interactions of LL-37 with caffeine.

Conclusions

The DSC technique has successfully been used to characterize the thermal stability of the LL-37 peptide in a solution. It has been proven that the investigated peptide is stable up to ~ 311 K and does not aggregate.

In water as a solvent without the addition of SDS solution, the LL-37 structure is not rigid, and in the aqueous solution, it exists in the state of conformational equilibrium. It could be concluded that no significant folding–unfolding transition occurs for the LL-37 peptide in water and it occurs as a mixture of interconverting conformations in a dynamic equilibrium with populations of conformations varying with temperature.

In this paper, we demonstrate that the LL-37 peptide has the highest affinity for binding Cu(II) and Zn(II) metal ions (among all selected systems for our research) before binding to the cell membrane, i.e., before obtaining the helical stable conformation. The obtained stoichiometry for complexes LL-37–Cu²⁺ (or Zn²⁺) is 3:2. It should be noted that because of an occurrence of competitive interactions in LL-37/Zn²⁺ system the binding constant of the LL-37/Zn²⁺ complex is higher than that of the corresponding LL-37/Cu²⁺ complex which is untypical with the Irving–Williams order for complex stabilities. Moreover, the difference in binding enthalpies of the investigated complexes results probably from the difference in donor properties of atoms of side-chain residues of the LL-37 peptide engaged in the metal ion binding.

The theoretical calculations showed that the LL-37 conformation has quite compact and simultaneously very mobile structure. Thus, the investigated ligands had little chance of binding to the LL-37 system permanently. On the other hand, the some interactions of LL-37 with caffeine and vitamin C were noticed. However, both systems are very dynamic and strong interactions are not generated. For this reason, no binding interactions were observed in the ITC curves for the above-mentioned compounds.

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