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Structural and functional behavior of biologically active monomeric melittin

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Abstract

Melittin is a well-known water-soluble toxic peptide present in bee venom of *Apis mellifera*, capable of interacting with and disrupting cell membranes thus producing many effects on living cells. Additionally, melittin induces activation of phospholipases and calmodulin upon interaction with cellular membranes. The conformation and aggregation state adopted by melittin in solution depends on several factors including the peptide concentration, ionic strength, pH and the nature of the ions in the aqueous medium. Such conformational dependence on the peptide environment gives new insights over the currently available 3D structures of melittin and, ultimately, over its biologically functional unit. Based on crystallographic data, the melittin tetramer has been proposed as its bioactive form. Contrarily to such data, we show in this work the results obtained from molecular dynamics simulations, which clearly indicate that the tetrameric organization of melittin is not stable under biological conditions dissociating after 2.5 ns through a 10 ns trajectory. We found that the tetrameric form of melittin is stable only in conditions of high pH and high peptide concentration in the molecular dynamics simulations. Moreover, when in plasma melittin appears to be a random coil monomer, folding only upon interaction with biological membranes. In summary, these findings elucidate several properties of melittin structure and dynamics, projecting significant implications in the study of its biological function.

Keywords: Melittin; Molecular dynamics; Bee venom; *Apis mellifera*; Peptide folding; Melittin biological unit

1. Introduction

Melittin is a well-known water-soluble toxic peptide present in bee venom of *Apis mellifera*, comprising about 50% of its dry weight. This peptide is able to disrupt membranes, producing many effects on living cells [1,2]. Like other amphiphilic α-helical peptides, melittin has an antibacterial activity, induces voltage-gated channel formation and can also produce micellization of phospholipids bilayers due to its membrane-interacting effect [1,3,4]. However, the major effect of melittin on erythrocytes is to cause lyses, as its binding to cell membranes results in the release of hemoglobin to the extracellular medium [3]. The molecular mechanism underlying melittin interaction with biological membranes and lipid bilayers is not well understood. Moreover, it seems that different molecular mechanisms could generate different actions of the peptide [3]. Interestingly, melittin interaction with certain proteins in the cell, such as phospholipases and calmodulin, thus inducing their functional modulation, has been also described [5–7].

This helical amphipatic peptide consists of 26 amino acid residues, comprising the sequence: GIGAVLKVLTTGLPALIS-WIKRKRQQ, and a total net charge of +5, four of which (KRKR) are in the C-terminal portion. Its conformation and structure has been studied by different approaches, including X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, circular dichroism (CD), and molecular dynamics (MD) simulations [8–16]. Melittin is soluble in both water and methanol, and its monomeric structure is described as shaping like a bent rod due to the presence of a proline residue in position 14 [9]. The peptide's crystallographic structure was deposited in the Protein Data Bank (PDB) under accession number 2MLT [10]. According to this data melittin crystallizes as a dimer,

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and the proposed biologically active unit has been described as a tetramer [10,11]. Contrarily to crystallographic data, biochemical studies show that the helical content and aggregation state adopted by the peptide depends, in a complex way, on several factors including the peptide concentration, ionic strength, pH and the nature of the ions in the aqueous medium [2,8]. Such apparent contradiction between structural and biochemical data has not been explained or correlated to conformational and functional data by any molecular model so far.

A recent publication [17] presented a molecular dynamics simulation of the melittin tetramer as the bioactive form of this peptide. The authors describe the tetrameric form as a 'channel', defined between two melittin dimers, which collapse during a water drying transition. Surprisingly, using the same methodology we were unable to reproduce these computational results. We believe that this is due to the fact that computational models should be built in a way that the system factors that are essential to a precise quantification of the property of interest must be both adequately considered and described, as well as sufficiently sampled [18]. The correct representation of these factors can be inferred by how well a molecular dynamics simulation reproduces known quantities [19]. Regarding melittin description by molecular dynamics, an adequate model should necessarily be capable of reproducing its conformational dependence of pH, salt and peptide concentrations.

In this work we used the crystallographic structure (PDB 2MLT) as the starting point for molecular dynamics simulations of melittin. We investigate the conformation of the peptide on both monomeric and oligomeric forms under different solvents, peptide concentrations, ionic strength and pH conditions. We describe here the conformational equilibrium assumed by the peptide in solution, a result that reproduces and correlates, for the first time, the peptide' structural properties with its biochemical data so far reported.

2. Methods

2.1. Software

Energy minimization calculations, molecular dynamics simulations and trajectory analysis were done using the GROMACS simulation suite [20]. Molecular visualization was done in Swiss PBD viewer (SPDBV) environment [21] and the secondary structure content analyses were performed with PROCHECK [22].

2.2. Molecular dynamics simulations

Melittin in its tetrameric form was retrieved from Protein Data Bank under code 2MLT. This oligomerization state was kept for simulation of the tetramer, the chains AB were isolated for simulation of the dimer, and the chain A was isolated for simulation of the monomeric form of the peptide. The monomeric, dimeric, and tetrameric forms of melittin were further solvated in a cubic box using periodic boundary conditions and using single point charge (SPC) water model [23] or Gromos96 methanol model [24] for water and methanol modeling, respectively. The ionic strength was adjusted by addition of chloride or sulfate ions, while the peptide concentration was obtained by varying the box size. Different protonation states of melittin under pH 7.0 and 11.0 were adjusted manually according to the standard pK_a values of the amino acids side chains. Consequently, at pH 11.0 lysine side chains were deprotonated. The final systems composed by peptide, water and ions comprise up to 28,000 atoms. The Lincs and Settle methods [25,26] were applied to constrain covalent bonds lengths, allowing an integration step of 2 fs after an initial energy minimization using the Steepest Descents algorithm. Electrostatic interactions were calculated with the generalized reaction-field method [27], with Coulomb and Lennard-Jones cut-off adjusted at 16 Å. The simulations were performed under constant-pressure (1 atm) and constant-temperature (310 K), using Gromos96 or OPLSAA force fields. The dielectric constant was treated as $\varepsilon = 1$. The monomeric, dimeric and tetrameric systems were heated slowly, from 50 to 310 K, in steps of 5 ps. At each step the reference temperature was increased by 50 K, allowing a progressive thermalization of the molecular systems. The simulation was then extended up to 10 ns. As several simulations were performed, the total conformational sampling of the peptide was about 0.1 μs.

3. Results and discussion

3.1. Simulations of oligomeric melittin

In order to elucidate the oligomeric organization of melittin in biological solutions we performed unrestrained molecular dynamics simulations of a melittin tetramer in three different conditions: (1) 23.5 mM melittin, 120 mM chloride ions, pH 7.0; (2) 54.6 mM melittin, 273 mM chloride ions, pH 7.0; (3) 23.5 mM melittin, 120 mM chloride ions, pH 11.0. Observing

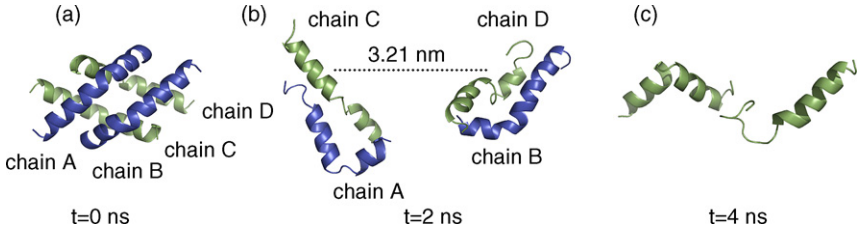

Fig. 1. Molecular dynamics simulation of oligomeric melittin. (a) Melittin crystallographic conformation (PDB 2MLT). (b) Melittin tetrameric conformation after 2 ns of molecular dynamics simulation in 120 mM chloride ions and pH 7.0; the distance in nm between dimers AC and BD is shown in dotted lines. (c) Snapshot of melittin dimeric conformation after 4 ns of molecular dynamics simulation.

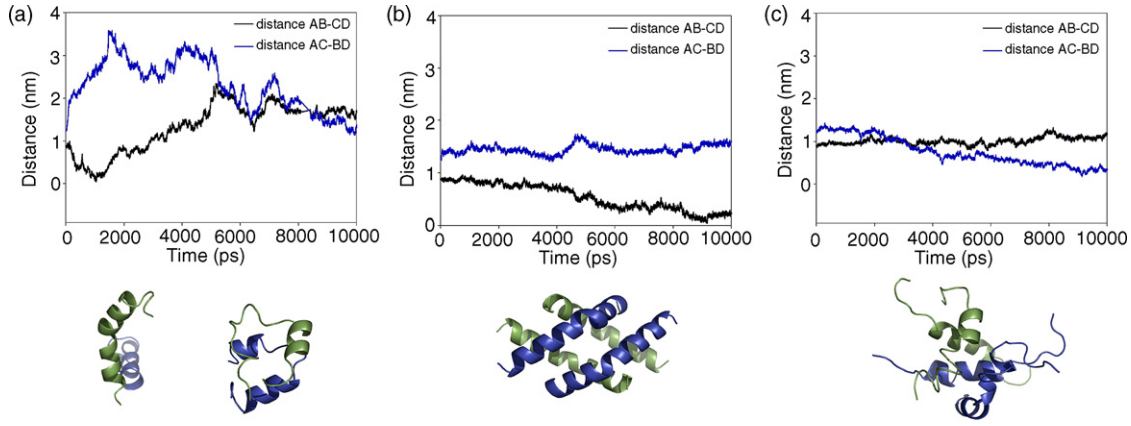

Fig. 2. Plot of the distances between dimers of melittin against molecular dynamics simulation time (upper) and snapshots of tetrameric conformation after 4 ns of molecular dynamics simulations (lower). (a) Condition 1, 23.5 mM melittin, 120 mM Cl^-, pH 7.0, Gromos96 force field. (b) Condition 2, 56.6 mM melittin, 273 mM Cl^-, pH 7.0, Gromos96 force field. (c) Condition 3, 56.6 mM melittin, 273 mM Cl^-, pH 7.0, OPLSAA force field.

the simulation condition closest to the plasmatic medium (condition 1), the monomers that compose the tetrameric structure present a progressive re-orientation, irreversibly splitting into two dimers within 2.5 ns of the trajectory (see Supplementary data). The initial conformation of tetrameric melittin can be observed in Fig. 1a and the resulting tetrameric form unfolded into two dimers (AC and BD) is seen in Fig. 1b. The distances between dimers increases drastically, as shown in Fig. 2a, becoming larger than 3.5 nm after the first 1.5 ns. Such behavior allowed us to conclude that the formation of the 'channel' as proposed in a previous work [17] on this system, seems to be an artifact.

In fact, the stability of the tetrameric form of melittin (and of its 'channel') in unrestrained simulations was obtained only through the modulation of the medium physical–chemical conditions. Simulation under condition 2 presents a 2.3-fold increase in both melittin and salt concentration, while condition 3 presents an increase in pH from 7.0 to 11.0. Under conditions 2 and 3 the initial tetrameric conformation (Fig. 1a) could not be set apart (Fig. 2b) during the trajectories indicating a stable tetrameric form. It must be emphasized that even the lowest melittin concentration (23.5 mM) tested (simulation conditions 1 and 3) is at least eight-fold higher than its concentration in crude bee venom. This observation is of extreme importance regarding any mechanism proposed for explaining the peptide's biological activity, which should take into consideration both the conformation and oligomerization status of the peptide when acting in a physiological medium. As clearly indicated here, a lower plasmatic concentration would induce an even faster unfolding of the melittin tetramer.

Since the previous work on melittin simulation used the OPLSAA force field to describe the tetrameric form as a 'channel' [17] and in order to avoid any force field dependent results, we also simulated the melittin tetramer using such force field under both low (23.5 mM) and high (56.6 mM) peptide concentrations. The conformational behavior of melittin tetramer was very similar in both Gromos96 and OPLSAA force fields, being stable only at high peptide concentrations. However, under OPLSAA a partial unfolding of its constituent peptide units was observed (Fig. 2c). Our data demonstrate that the 'channel' between the dimers in the crystallographic structure remains stable only under extreme and non-physiological conditions (Fig. 2b), being thus inappropriate to attribute a biological role for such melittin structure. In agreement with these observations, a recent work [29] reported that aggregation of melittin in a tetrameric form was only observed in high salt aqueous solutions.

Considering the fact that the melittin tetramer disassembles into two dimers under conditions close to that of a physiological medium, we also performed molecular dynamics simulations of the crystallographic dimeric form of the peptide. The parameters of pH, melittin and salt concentration chosen for such molecular dynamics simulation were the same as the simulation condition 1. Doing so, we observed that the dimeric form of melittin also dissociates in solution, with the distance between monomers changing from 1.3 to 4.5 nm in the first 2.5 ns of the trajectory (Fig. 1c). This result led us to the conclusion that, in plasma, melittin should not be in a tetrameric nor a dimeric oligomerization state, but is probably present as a monomeric peptide.

3.2. Simulations of monomeric melittin

The data obtained by molecular simulations of oligomeric melittin indicate that the aggregation state of the peptide is highly dependent on the environment conditions, i.e. peptide concentration and/or pH. The effect of physical–chemical parameters of the medium on the structure of melittin can be easily noticed in the monomeric form of the peptide. For example, the helical content of melittin increased from 12% to 65% in response to a salt concentration changing from near zero to 1 M NaCl, as reported by others [8,16]. Thus, in addition to the oligomerization state, the peptide secondary structure is also dependent on solution conditions.

Considering the lack of stability of the peptide oligomeric states under 'physiological' conditions and aiming to correlate these data to the peptide's helical content, we investigated the behavior of monomeric melittin under a number of different

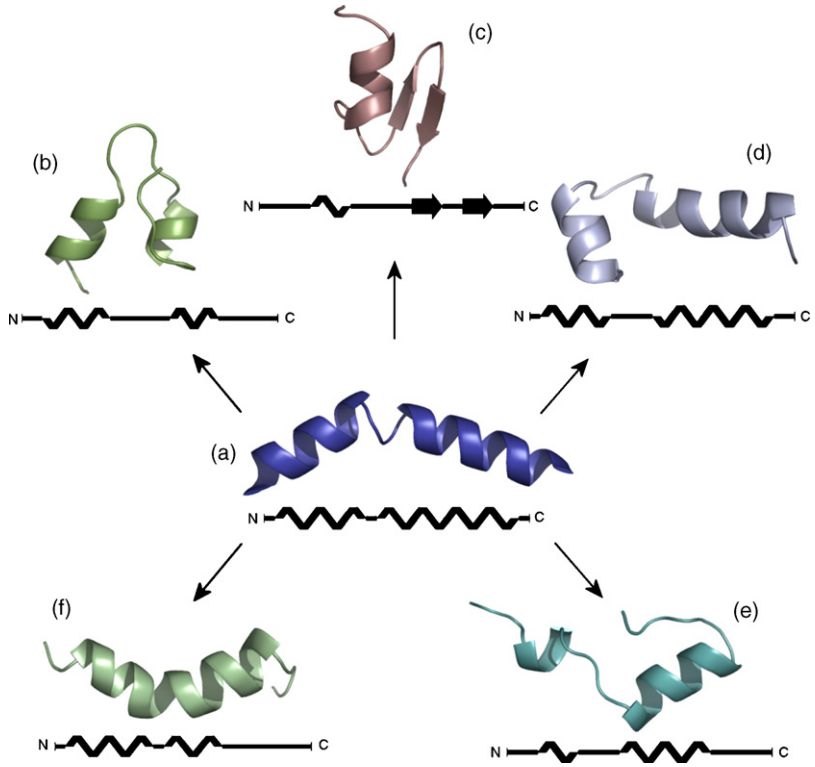

Fig. 3. Monomeric melittin conformations after 10 ns of molecular dynamics simulations. (a) Melittin crystallographic structure (PDB 2MLT). (b) Melittin in water pH 7.0, Cl^- 50 mM. (c) Melittin in water pH 7.0, SO_4^{2-} 50 mM. (d) Melittin in water pH 7.0, Cl^- 1.2 M. (e) Melittin in water pH 7.0, SO_4^{2-} 1.2 M. (f) Melittin in water pH 11.0, SO_4^{2-} 50 mM. Secondary structure prediction by PROCHECK [23] is presented in black.

conditions. An overview of melittin conformations after 10 ns of molecular dynamics simulations under different simulation parameters, is presented in Fig. 3.

In agreement with biochemical observations, the simulations of a melittin monomer in presence of 50 mM chloride ions indicated a significant decrease in its helical character (Fig. 3b), a behavior also observed under an equivalent concentration of sulfate ions (Fig. 3c). On the other hand, the increase in ionic strength, using either chloride or sulfate ions, enhance the helix content of melittin in an ion-nature independent manner (Fig. 3d and e). In addition, a curious behavior is observed at 50 mM SO_4^{2-}, in which the helical character is partially transformed in a β-sheet structure. Such conformational modification is reproducible and can be reverted under higher ionic strength.

The pH has a critical effect on the stabilization of the helical structure of melittin, as reported by other authors [8]. Again, molecular dynamics simulations correctly reproduce experimental data: loss of the helical character is observed when the peptide is exposed to pH 7.0 (Fig. 3c), being the secondary structure almost completely recovered upon increasing the pH medium to 11.0 (Fig. 3f). These data are illustrated in Fig. 4 by

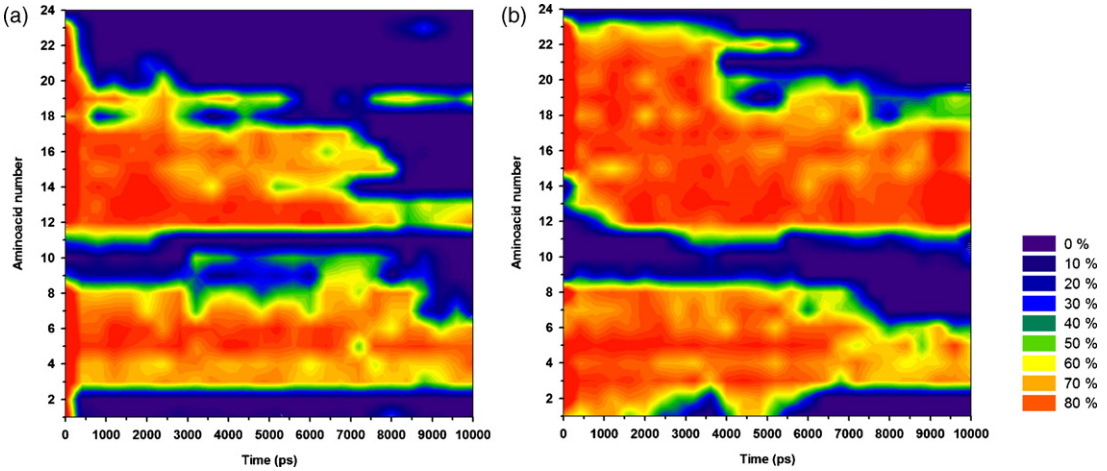

Fig. 4. Ellipticity maps [27] of monomeric melittin as a function of time and aminoacid number. (a) 26.5 mM melittin, 50 mM Cl^-, pH 7.0. (b) 26.5 mM melittin, 1.2 M Cl^-, pH 7.0.

means of the ellipticity parameter [28] as a function of both time and amino acid sequence.

Besides the critical parameters of ionic strength and pH, some solvents, especially methanol, have been considered in the study of melittin structure and conformation. Methanol is an interesting solvent for investigation, since it was the solvent used to obtain the NMR structure of melittin [9], which shows the peptide as a completely folded helix. However, contrarily to this a 10 ns molecular dynamic simulation in presence of methanol presented an unfolded structure on the peptide N-terminal portion (data not shown). Conversely, the C-terminal helix remained stable over the entire simulation. Similar results were obtained in previous work, in which a completely unfolded melittin was seen after a 120 ps molecular dynamics simulation in methanol [15]. Regarding the molecular basis for the difference between NMR and molecular dynamics findings, a possible reason are the conditions in which the peptide structure is simulated. As shown in Fig. 3, the peptide conformation and dynamics is highly determined by the surrounding environment, which is influenced by the procedures used for the peptide purification. For example, residual ions in the purified sample could be the key factor for inducing the differences between folded and unfolded helix, as shown in Fig. 3d being the increase in helical character due to an increase in chloride ions concentration.

The simulations of the monomeric form of the peptide, in a total sampling of about 0.1 μs in this study, suggest a rather simple explanation for the discrepancy so far existing in the literature about the peptide structure and its biochemical properties in solution. As shown here the conformation of melittin can be highly influenced by physical–chemical conditions (Fig. 3), inducing the peptide to adopt a diversity of forms under different media. On the other hand, both crystallographic and NMR structures are usually obtained under artificial aqueous conditions (i.e. different from plasma composition), as has been the case for melittin. As a consequence, the helical structure observed in crystallographic and NMR data for this peptide should be interpreted as a consequence of both high salt and melittin concentrations. In this context, our calculations suggest that in physiological medium such as human plasma, melittin should assume a monomeric form, mostly unfolded.

3.3. Implications to current understanding of melittin biological properties

The melittin conformation responsible for its pore formation capabilities has been a matter of controversy [3,30–32], especially because some authors assume that melittin approaches and binds to membranes in a defined conformation and orientation. In fact, the orientation of the peptide over lipid bilayers has been proposed as a function of the peptide concentration, e.g. at low concentration melittin binds parallel to the membrane surface and as the peptide concentration increases in the medium the binding turns to a perpendicular orientation [32]. The pore model has also been assumed as a toroidal model by neutron diffraction [32], and a previous molecular dynamics simulation study has proposed that even when inserted into lipid bilayers the position of the melittin monomers is not stabilized as a perpendicular tetramer [14]. Besides, the orientation of melittin chains in a pore is enormously different [32] to that proposed by X-ray crystallography [10,11]. Thus, considering the whole of experimental and theoretical data available for melittin structure and dynamics, the peptide appears to fold into a helix conformation only when interacting with highly negative biological surfaces, as membranes of activated platelets or to membrane proteins, as phospholipases. Such charged surfaces would perform a role equivalent to high salt conditions in the peptide folding. Actually it has been experimentally shown [33] that melittin binds to a lipid vesicle as a disordered monomeric peptide form, inserts itself into the lipid membrane, and its concerted folding as a helix is promoted upon the peptide penetration. The characterization of this process at the atomic level was recently shown, with the observation that DMPC bilayers are capable to stabilize the secondary structure of melittin related to the unfolded peptide in aqueous solution [34].

4. Conclusion

In this work we performed molecular dynamics simulation of melittin in three different oligomeric states, i.e. monomeric, dimeric, and tetrameric forms, in order to have a complete overview of the peptide structure, conformation and dynamics according to environmental conditions. Our results show that the tetrameric organization of melittin is not possible in plasma, but only in extreme physical–chemical conditions. We have seen that the crystallographically observed inner channel does not remain stable in solution under physiological conditions and cannot be responsible for the biological properties of melittin.

Additionally, we observed that the peptide is not a perfect helix, but a random structure at pH 7.0 in physiological ionic strength. Our findings are important not only for the understanding of the melittin mechanism of action concerning its lytic activity, but are also relevant for the study of its ability to modulate phospholipases and others physiologically important proteins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmgm.2006.06.006.

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