INSIGHTS INTO CECROPIN-MEMBRANE INTERACTION MECHANISM GIVEN BY MOLECULAR DYNAMICS SIMULATIONS

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Abstract. Cancer is a leading cause of death worldwide and conventional chemotherapeutic treatments are not always effective, induce resistance and are often associated with serious side effects. One promising alternative to conventional cancer treatment is represented by cationic antimicrobial peptides (AMPs) due to their selectivity for malignant cells and their lack of toxic properties. One such class of peptides is cecropins, originally identified in insects but later isolated also from mammalian tissues. Using these peptides as therapeutic agents requires a detailed understanding of their mechanisms of action. Although AMPs strong binding and selective disruption of bacterial and cancer cell membranes is believed to be favored by the electrostatic attraction between the negatively charged cancer cells and the positively charged AMPs, their exact mechanism of action has not been elucidated yet. Our objective is to better understand the interaction between cecropin P and membrane using molecular dynamics simulations (20ns simulation time) of atomically detailed models of cecropin P in interaction with POPE lipid bilayer. We built three different systems, in which the cecropin helix is initially oriented parallel with the lipid bilayer. In the first two systems electrostatic interactions are favoured, since peptide helix is oriented with positive charges facing negatively charged lipid phosphate groups and the distance between helix axis and phosphate plane is 9A and 6A, respectively. In the third system hydrophobic interactions are favoured, cecropin helix is partially buried in the membrane bilayer, with the apolar side interacting with hydrophobic lipid tails and the cationic face interacting with phosphate groups. Our results indicate highest stability for the system dominated by hydrophobic interactions and peptide induced deformations of lipid bilayer, while the systems based on electrostatic interactions are less stable. These results suggest that peptide penetration in the membrane may be facilitated by the hydrophobic characteristics of the peptide.

Keywords: Anti-cancer peptides, cecropin, membrane, interaction, molecular dynamics

Running title: MD simulations of cecropin-membrane interaction

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INTRODUCTION

Although major advancements have been made in cancer treatment during the past decade, cancer still is one of the most important cause of mortality in the world (1, 2). "Cancer" is actually a general term describing over 100 distinct diseases affecting different tissues and cell types. But one common feature in all cancer forms is abnormal cell growth generated by either inherited or acquired mutations (3, 4). In case of localized tumors, the most successful treatment options are surgery and/or radiotherapy, while metastatic, advanced disease necessitates chemotherapeutic treatment (5, 6). Although chemotherapeutic agents target rapidly dividing cells, such as cancer cells, their toxicity induce damage to healthy cells and tissues (7-10) and generate undesirable, deleterious side effects. Moreover, cancer cells often become resistant to chemotherapy as a result of mutations increasing cell ability to repair DNA damage, increasing expression of drug detoxifying enzymes and drug transporters, altering interactions between drugs and specific targets and defects in the cellular machinery that mediates apoptosis. Therefore, a major advance in cancer treatment would be represented by a new class of anticancer compounds that do not have the toxicity of conventional chemotherapeutic agents and also are unaffected by common chemoresistance mechanisms. As indicated by an increasing number of studies (11, 12), some of the cationic antimicrobial peptides (AMPs) are toxic to bacteria but not to normal mammalian cells, which may suggest that this class of compounds could also exhibit a broad spectrum of cytotoxic activity against cancer cells.

The electrostatic attraction between the negatively charged components of bacterial and cancer cells and the positively charged AMPs is believed to play a major role in the strong binding and selective disruption of bacterial and cancer cell membranes, respectively. However, the selectivity mechanism governing the interaction between some host defense peptides and malignant cells has not been fully elucidated.

The important differences between the composition of cell membranes in malignant cells and normal cells most likely account for the selective interaction of certain AMPs with cancer cells while sparing healthy cells. In this regard, one of the crucial factors determining the selective toxicity of anti-cancer peptides (ACPs) are thought to be the electrostatic interactions between cationic ACPs and anionic components of cancer cells. Typically, cancer cells express on their surface increased levels of anionic molecules such as phosphatidylserine (13, 14) and O-glycosylated mucins (15, 16). Moreover, neoplastic cells have a negative membrane potential which may favour selective interaction with cytotoxic ACPs (17). On the other hand, interaction with untransformed, healthy cells is not favored because their membrane displays a neutral charge conferred by the zwitterionic character of their components.

Several mechanisms have been proposed for the interaction between AMP/ACPs and cell membrane. It is currently accepted that the electrostatic interactions between positively charged peptides and negatively charged membrane

lipids are responsible for the initial steps. A crucial event occurring after the initial interaction is peptide conformational phase transition, in which peptides refold into new conformations capable to penetrate the membrane bilayer, especially α-helix. This facilitates interaction of amphipatic peptides with corresponding regions in the bilayer and deeper penetration in the membrane, thus forming transmembrane pores or non-selective ion channels that negatively affect cell metabolism.

The mechanisms proposed for the peptide-membrane interaction have been described in detail elsewhere (18, 19). Here we will mention only the most important features of each model:

a) The barrel stave model presumes that peptide helices assemble as cylinders in the membrane, forming a barrel of helical peptides with a central lumen (20, 21). The hydrophobic peptide regions align with the lipid core region of the bilayer and the hydrophilic peptide regions form the interior region of the pore.

b) The 'carpet model' describes peptides as being accumulated on the bilayer surface (22) in a carpet-like manner, due to the electrostatic attraction between cationic peptides and anionic phospholipid head groups at numerous sites covering the surface of the membrane. At high peptide concentrations, peptides are oriented parallel to membrane surface and presumably disrupt the lipid bilayer through a detergent-like mechanism, eventually forming micelles (23, 24). At an even higher, critical threshold concentration, the peptides form toroidal transient holes in the membrane, allowing additional peptides to access the membrane. Finally, the membrane disintegrates and forms micelles after disruption of the bilayer curvature (25, 26).

c) The 'toroidal-pore model' describes antimicrobial peptide helices as being inserted into the membrane while at the same time they induce the lipid monolayers to bend continuously through the pore so that the water core is lined by both the inserted peptides and the lipid head groups (27). In forming a toroidal pore, the polar faces of the peptides associate with the polar head groups of the lipids (28). Then the lipids in these openings deviate from the lamellar normal orientation and connect the two membrane leaflets, forming a continuous curve from the top to the bottom similarly to a toroidal hole; the pore is lined by both peptides and lipid head groups, which possibly interact and attenuate the effect of cationic peptide charges. The toroidal model differs from the barrel-stave model as the peptides are always associated with the lipid head groups even when they are perpendicularly inserted in the lipid bilayer (20).

Within the class of AMPs, a special interest is given to cecropins, a family of small peptides initially isolated from insects but also identified in mammalian organisms. In addition to their strong antibacterial activity, cecropins have been found to selectively kill cancer cells, without affecting normal cells (29, 30), thus showing much promise as new anticancer agents. However, using these peptides as therapeutic agents requires a detailed understanding of their mechanisms of action.

Our goal is to better understand the interaction between cecropin P and model membrane bilayer, clarify the role played by electrostatic and hydrophobic interactions and estimate the probability of each of the previously described mechanisms in the case of cecropin P. This would also allow development of novel anticancer agents with improved selectivity and efficiency.

MATERIALS AND METHODS

STRUCTURE MODELING

Secondary structure of cecropin P was predicted using several freely available methods: JPred (31), Porter (32), Prof (33), Psipred (34) and Sable (35). All predictions indicate the presence of a helical structure along the entire sequence, except for the last 4 residues. These results are in agreement with experimental data such as circular dichroism and proton-NMR on cecropin P1 in water solution with 30% propanol, which also indicate a helical structure along the entire peptide sequence (36). Based on these results, we generated a helical structural model of cecropin P, using Modeller 9v8 (37, 38).

SYSTEM PREPARATION

Cecropin P peptide structure was combined with a patch of the lipid bilayer of palmitoyl-oleoyl-phosphatidyl-ethanolamine (POPE) fully hydrated with TIP3P water molecules (39). Each system contains the following components: a) cecropin P having a helical structure; b) POPE lipid bilayer pre-equilibrated in a solvated flexible simulation cell; c) water; d) Na^+ and Cl^- ions in physiological concentration to neutralize the electric charge of the system.

Three different systems were generated, all with the helix axis oriented parallel with the plane of the lipid phosphate atoms, but in different positions as follows:

a) Cec_pope: the distance between helix main axis and phosphate plane is 9Å; helix cationic face is oriented towards the lipids heads and apolar face towards the solvent

b) Cec_pope_near: the distance between helix main axis and phosphate plane is 6Å; helix cationic face is oriented towards the lipids heads and apolar face towards the solvent

c) Cec_pope_R-up: cecropin helix is partially buried in the membrane bilayer, with the apolar side interacting with hydrophobic lipid tails and the cationic face interacting with the negatively charged phosphate groups.

All systems simulated in this study are described in detail in Table 1. A sample of each simulation cell is shown in Figure 2.

All systems were processed according to the following protocol:

a. Hydrogen atoms were added; ionizable residues were in their default protonation state.

b. The system was explicitly solvated using TIP3 water molecules (39).
c. Ions were added in order to maintain electroneutrality
d. Structure file was generated using the psfgen plugin in VMD (40). The total number of water molecules, lipids and ions in all the models is shown in Table 1.

Table 1
Description and nomenclature of simulated systems

	cec_ pope	cec_ pope_near	cec_ pope_R-up
System description	Helix-membrane Distance = 9Å Arg/Lys side chains oriented downwards, towards membrane phosphate groups	Helix-membrane Distance = 6Å Arg/Lys side chains oriented downwards, towards membrane phosphate groups	Arg/Lys side chains are oriented upwards,while the hydrophobic side of the helix is in contact with the apolar lipid environment*
Numbe of atoms	30721	30814	29518
Number of water molecules	5073	5104	4672
Number of lipids in upper layer	62	62	62
Number of lipids in bottom layer	58	58	58
Size of simulation cell (Å)	78 x 45 x 90	78 x 45 x 90	78 x 45 x 90

MOLECULAR DYNAMICS SIMULATIONS

MD simulations were performed in NPT ensemble, using NAMD software package (41, 42) with Charmm force field (43).

RESULTS

STRUCTURAL MODEL OF CECROPIN P PEPTIDE

Cecropin P was modeled as an α-helix, in agreement with experimental results from circular dichroism and proton-NMR experiments and with secondary structure prediction. Resulting helix shows an amphipathic character, as displayed

in Fig 1. Cecropin helix was oriented relatively to the membrane in three different positions, as described in Methods.

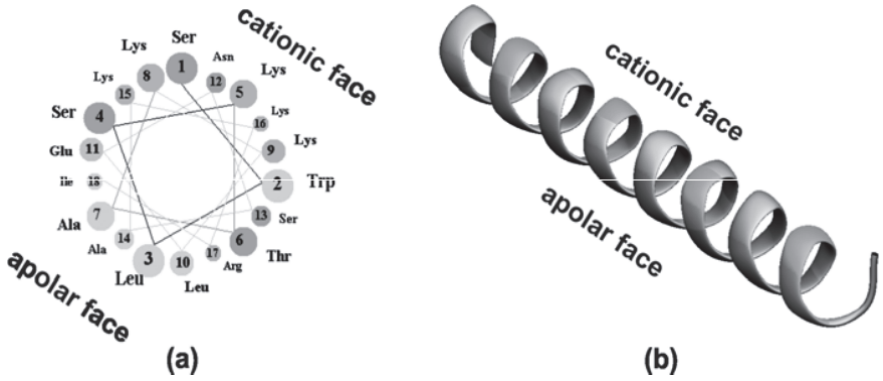

Figure. 1: Helical structure of cecropin P, with a cationic face and an apolar face. (a) helical wheel representation of the sequence; (b) cartoon representation of the structure.

MOLECULAR DYNAMICS SIMULATIONS OF CECROPIN P – MEMBRANE COMPLEX

Each of the previously described systems were subject to MD simulations for 20ns. The configuration of each system at various timesteps during simulation time is shown in Fig. 2.

It is interesting to note that the two systems in which cecropin is initially outside the membrane (systems named cec_pope and cec_pope_near) show a remarcable stability of the helical structure, although the interaction between helix and membrane is highly unstable. In these two systems, although initially the positively charged residues form electrostatic interactions with the negatively charged lipid phosphate groups, these interactions are unstable, since they break after 1.5ns but temporarily re-form after 2ns. Surprisingly, when the peptide helix is closer to membrane in the starting conformation, the peptide-membrane interaction is lost sooner than in the other model where the helix-membrane distance is larger. However, in any of these two systems (named cec_pope and cec_pope_near) with positively charged residues oriented towards membrane phosphate groups, the peptide looses interaction with membrane and changes orientation, with the hydrophilic side facing the solvent.

In contrast with these unstable systems, the third system in which hydrophobic interactions between peptide and membrane are dominant (named cec_pope_R-up), is remarcably stable. The association between peptide and membrane is maintained during the entire simulation. However, peptide helical structure is slightly disrupted in the central highly flexible region (residues Ala12-Ser-Ala14) even from the initial equilibration step. These conformational changes also induce distorsions of the upper lipid bilayer, which may be correlated with the peptide cytotoxic effects.

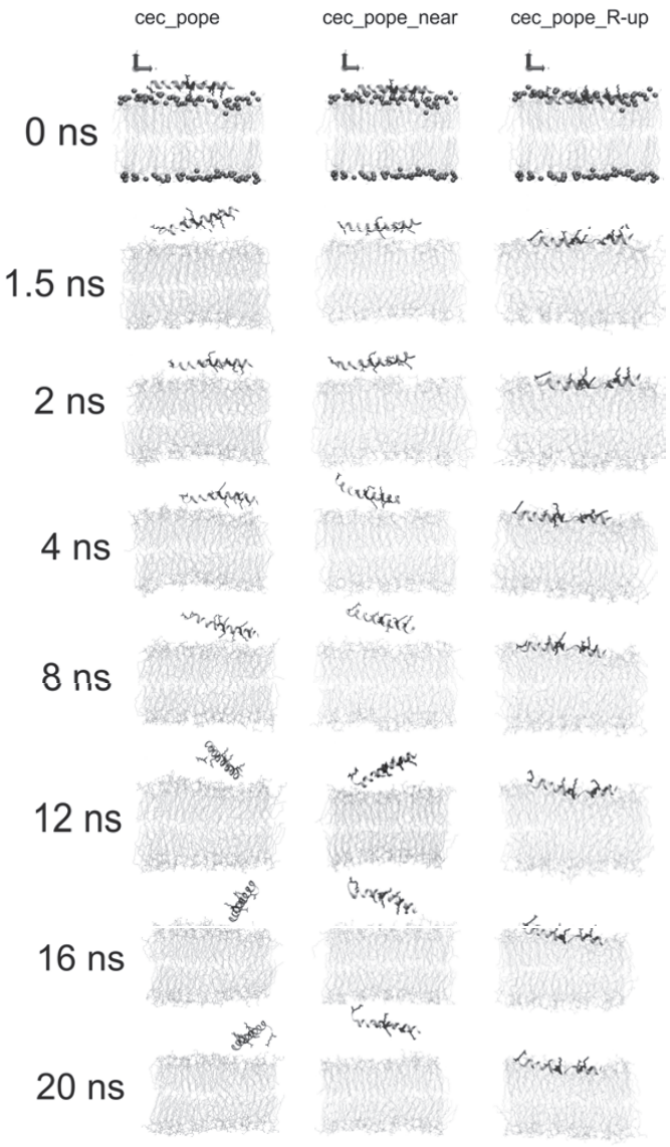

Figure 2: Conformation of each simulated system, at different timesteps (first column). Peptide mainchain is represented as a ribbon, while side-chains of positively charged aminoacids are fully displayed. For simplicity, water molecules are not shown.

DISCUSSION

MD simulations indicate that stability of interaction between cecropin peptide and model POPE membrane is maintained by electrostatic, but also by hydrophobic interactions. Peptide orientation with the hydrophobic side facing the solvent is highly unfavorable, while it is highly favoured the conformation with the apolar helix side buried in the hydrophobic lipid tails and the positively charged side interacting with the lipid phosphate groups. These results support the "carpet" type mechanism.

One possible explanation for the observed effects in the first two simulation experiments is that Cecropin P is subject to multiple forces that induce the reorganization of the molecule relatively to the solvent and the membrane. On one side the solvent needs to interact with the polar side of the molecule (the positively charged side of the helix), but on the other also the negative charged membrane must interact with the positively charged residues. So the molecule is subjected to a ping-pong mechanism between the solvent and the membrane, in dispute being the polar and especially the positively charged residues (Arg/Lys). The molecule rearranges itself so that minimal superficial tension is acquired on the hydrophobic side of the helix. It is not clear whether the main orientation of the molecule is that acquired after 20 ns of simulation or the molecule would change it's orientation relatively to the membrane after a longer time. One possible explanation for the molecule reorientation in the cec_pope_near system is that superficial tension is higher (because there are less molecules of the solvent present between the phosphate negatively charged groups of the membrane and the positively charged side of the helix/because there are more molecules of the solvent interacting with the hydrophobic region of the helix). Thus a higher superficial tension is achieved so the membrane-cecropin P interactions are unstable, the peptide-membrane interaction being lost sooner than in the cec_pope system. Both systems are unstable and eventually the helix-membrane interaction is lost with the peptide changing it's orientation relatively to the membrane.

On the other hand the third system is stable and the interactions between the peptide and the negative face of the membrane are maintained through the entire simulation (20 ns). So the interactions between the hydrophobic tails in the membrane and the hydrophobic side of the helix are very important in the stabilization of the association between the peptide and the membrane. Moreover, the electrostatic interactions with the phosphate negatively charged groups favors the association of the helix with the membrane and the polar residues being exposed both to the polar solvent and to the phosphate groups.

Another important aspect is the region Ala12-Ser-Ala14, this region being disrupted from it's initially helical structure. Ser is a negatively charged (polar) amino acid so this could promote electrostatic repulsions with the negatively charged groups of phosphate. Also the two Ala are hydrophobic, so their association with the hydrophobic tails from the membrane could also count for the disruption of the membrane. This region could act as a promoter in the disruption of the bilayer being the starting point in the membrane disintegration.

CONCLUSIONS AND FUTURE PERSPECTIVES

This study brings a glimpse of light in the pathway for elucidating the mechanism of action of cecropins. Three main ideas are revealed as a result of this experiment. The first one is that the superficial tension could be a critical point for the association between the peptide and the membrane, therefore electrostatic interactions are not sufficient for initiation of the membrane disruption mechanism. The second is that the hydrophobic interactions could be a critical aspect for the initiation of the disruption process of the membrane and the third idea is that the Ala12-Ser-Ala14 region from the helix is rapidly disorganized from the it's helix structure, in contact with membrane, promoting the disorganization of the membrane. Future studies with other types of membranes and with more peptide molecules would bring a major contribution in elucidating the mechanism of action of ACPs.

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