Molecular mechanism of pore creation in bacterial membranes by amyloid proteins

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Abstract. This study explores the mechanism of pore creation in cellular membranes by MccE92 bacterial proteins. The results of this study are then compared with the mechanism of alpha-synuclein (aS)-based pore formation in mammalian cells, and its role in Parkinson’s disease.

1. Introduction
The process of self-organization of proteins and peptides into long fibrils is historically called amyloidogenesis, and the proteins/peptides capable of organizing into such structures are often called amyloids. The process of fibril formation was found to be responsible for a number of neurodegenerative diseases, such as Parkinson’s, Alzheimer’s, Huntington’s, prion diseases, etc. [1]. Lashuel et al. [1, 6] pointed out that the toxicity observed during amyloidogenesis does not arise from the formation of long amyloid fibrils, but instead, by intermediate ring-like oligomers. It is presumed that these amyloid proteins have a physiological role in mammalian cells, but the question of why these toxic fibrils are created by the mammalian cells is still unanswered.

Recently scientists found that bacterial cells create amyloid-like fibrils that have very specific functions in supporting bacterial survival and development. For example, the bacterium Escherichia coli and some other Gram-negative bacteria create functional amyloid fibers called curli. These curli support bacterial binding to other cells and the organization of biofilms [2]. It is noteworthy that the expression of the curli-creating proteins is controlled genetically with the appropriate feedbacks to proceed in proper time of bacteria life. Other bacteria use a fibril formation process to prevent growth of other bacteria in their vicinity. The protein Microcin E492 (MccE92) produced by Klebsiella pneumoniae self-assembles to create cytotoxic pores in neighboring cells [6], and it also self-assembles into amyloid fibrils. Bian et al. [5] showed that cytotoxic pore formation in bacteria is related to the “protofibril” stage of oligomerization, while the MccE92 protein loses most of its cytotoxicity when it oligomerizes into long fibrils [3, 4]. Thus, as noted by Lashuel et al. [7], there is a striking analogy between the bacterial and mammalian proteins that form cytotoxic protofibrils and large fibrils.

In view of the phenomenological similarity between these bacterial and mammalian proteins, the comparative study of these proteins may help us better understand the mechanism of pore formation in both mammalian and bacterial cell membranes. Accordingly, this study was undertaken to explore the mechanism of pore creation in cellular membranes by MccE92 bacterial proteins. The results of this study
are then compared with the mechanism of alpha-synuclein (aS)-based pore formation in mammalian cells, and its role in Parkinson’s disease [7, 14, 15].

2. Methods
Both MccE92 and aS exist in a numerous conformations in solution, and undergo rapid changes among these various forms. Annular structures were found in a number of amyloid systems [1] and these are thought to be responsible for pore formation of the cell membrane. These structures cause cytotoxicity by permitting flow of metal ions across the cell membrane. The number of events is consistent with such pore formation and we use the multi-program, multi-data modeling approach to address all of these steps. By simulating the time course of conformational changes of MccE92 and aS, it is possible to predict the tendency of these proteins to form oligomers in solution and on the surface of a phospholipid bilayer, and their tendency to aggregate into annular structures in both environments.

2.1. Homology modeling
The structure model of MccE92 was based on its 29–30% sequence homology with the redox-sensing transcriptional repressor rex (Pdb ID 1XCB_G). The homology template structure was selected by our protein structure prediction server HMM-SPECTR [8].

2.2. Molecular dynamics
We used two types of molecular dynamics (MD) simulations: first, MD of MccE92 in a water box; second, MD of the MccE92 oligomers on the POPC membrane. Once MccE92 protein is expressed by the bacteria, it travels toward the neighbor bacteria and penetrates the cell membrane toward the cytoplasm. After entering the cell, the MccE92 molecule proceeds to form amyloid-like fibrils [2]. This process goes through the stage of protofibril formation with self-organization into ring-like oligomers. These oligomers are created after various times of MccE92 penetration into the cell. Our simulations of MccE92 in water model this situation — after MccE92 exists for some time in solution, it undergoes conformational changes and then docks to the membrane.

We used periodic boundary conditions at constant pressure (1 atm) and temperature (300 K). The NAMD molecular dynamics program [9] version 2.5 was used with the CHARMM27 force-field parameters [10], cutoff 12 Å, to simulate behavior of an aS molecule in water in normal conditions. The temperature was maintained at 300 K by means of Langevin dynamics using a damping coefficient of 1/ps. A constant pressure (1 atm) was employed as implemented in the NAMD software package. Initial coordinates were taken from a previously equilibrated 500 ps system. Data for analysis were taken between 50 ps and 5 ns of the simulation. MD simulations of a single MccE92 molecule (first type of MD) were conducted in a water box with the shortest distance between the protein molecule and the box walls 30Å. The system contained more than 200,000 atoms of water. BlueGene computers at Argonne National Laboratory and San Diego Supercomputer Center were used for these simulations.

For the second type of simulation, we used 1-palmitoyl 2-oleoyl phosphatidylcholine (POPC) membrane model 180 x 180 Å. The heptamer was constructed with MccE92 molecules (3 ns conformers of single molecule MD) using minimum energy docking, taking into consideration the predicted membranephilic surfaces of proteins. Then the multimer was positioned on the surface of the membrane. The system was inserted in the water box with the following geometrical parameters: the shortest distance between the protein molecule and the box walls 25 Å, the shortest distance between the membrane molecules (on the surfaces not contacting proteins) and the box walls 10 Å. Both MD simulations were conducted in four steps: initially the system of protein and water molecules (or the system of protein, membrane and water) was minimized for 10,000 iterations; then the system was heated in 0.1° increments and equilibrated for 10 ps; then the molecular dynamics simulation was conducted; data for analysis were taken between 50 ps and 5.0 ns of the simulation.
2.3. Membrane-contacting surface of proteins prediction

During their existence in solution, different conformers dock to the cell membrane and organize the membrane-bound aggregates. To study these aggregates’ formation, we first had to find which surfaces of the protein are in contact with the membrane and which are free to be in contact with other MccE92 proteins during the aggregation process. To elucidate the possible surface of MccE92 protein that will most probably contact the membrane, we used the program MAPAS [11]. The MAPAS algorithm involves deconvoluting the protein surface into a set of planes, and then selects the planes that most probably will create strong membrane contacts. This program was applied to the 3 ns MD conformer of MccE92 identified as having the best combination of membrane-contacting parameters.

The MAPAS program uses three scoring parameters: membranophilic residues score (MRS), membranophilic area score (MAS), and coefficient of membranophilic asymmetry (Kmpfa).

MRS is calculated in the following way:

\[
MRS = \left( \sum w(Nu_i \cdot Su_i/Su_{imax}) \right) + \left( \sum (W \cdot Nc_i) / (Nu_i + Nc_i) \right)
\]

where W is the membrane disengagement score [11] for each amino acid (called the “membranophilicity” of the residue); Nu_i is the number of uncharged residues in the plane; Nc_i is the number of charged residues in the plane; Su_i is the solvent accessible surface of the uncharged residue that is included in the plane; Su_{imax} is the maximum solvent accessible surface of the uncharged residue that is included to the plane; and Nc_i is the number of charged residues in the plane.

MAS is calculated the following way:

\[
MAS = \frac{Stop(plane)}{Sall(plane)}
\]

where Stop(plane) is the solvent-accessible surface of a selected number of the residues having top membranophilicity in the best putative membranophilic plane; and Sall(plane) is the solvent-accessible surface of all residues in this plane.

Kmpfa is calculated the following way:

\[
Kmpfa = \frac{(MAS)}{(Stop(protein)/Sall(protein))}
\]

where Stop(protein) is the solvent-accessible surface of a selected number of the top membranophilic residues of the entire protein, and Sall(protein) is the solvent-accessible surface of all residues of the protein. (See more descriptions of these parameters and prediction strategy in [11] and its web supplements.)

2.4. Molecular docking

We conducted theoretical docking of MccE92 molecules during their aggregation. The MD conformers of MccE92 that have the best membrane-contacting scores were selected for docking. Docking was conducted using the program Hex [12, 15]. The final docking refinement was done with the InsightII program (Accelrys, 2007) using the 1 Å grid with the cutoff 14 Å. Only the docking complexes with the vDW energy of interaction less than –10 kcal/mole were selected for further consideration. The lowest energy two-molecular complexes where both molecules contact the same membrane surface by the predicted membrane-contacting amino acids were selected for further docking toward the final aggregate configuration.

3. Results

Microcins belong to a family of proteins produced and excreted by some enterobacteria including E. coli and K. pneumoniae [13]. One of the best studied proteins from this family, Microcin E492 (MccE92), obtained this name because it is produced by the RUC492 strain of K. pneumoniae. It has been shown that this protein creates pores in the bacteria cell membranes [2] and artificial lipid bilayers [13]. This protein has antibacterial activity as a result of its ability to organize into membrane-disrupting pores [2].

We used MD simulations to study the process of aggregation and membrane penetration of MccE92. Our simulations suggest that MccE92 is an unstructured protein that can have a significant number of conformational states. In our calculations we explored the set of conformations that MccE92 samples in 5 ns MD simulation runs in a water box; structures observed ranged from more globular forms with mostly helical N-terminal parts to more extended conformations. To select conformers that have the greatest probability of binding to the membrane (to start the process of aggregation and penetration), we
assessed the membranephilicity score, i.e., finding which conformer would have the best membrane contact and the specific region of membrane contact on the surface of this protein.

Figure 1 shows the MccE92 conformers of the 5 ns MD run. The overall conformation of the protein changes quite significantly during the simulations. The residues creating the surface of the protein and the residues that are involved in the intramolecular interactions are changing during this process. These changes actually define the further destiny of these molecules. If the membranephilic residues prevail on the surface, the protein will most likely bind to the membrane and became a potential candidate for further aggregation. If the membranephilic residues are not exposed enough, the protein will remain in solution and will not bind to the membrane (at least until its conformation changes significantly during its life span).

Figure 2A shows the evolution of the secondary structure of this protein. One can see that the secondary structure changes familiar to us from previous studies of alpha-synuclein also happen to the MccE92 protein during MD simulations. We see the evolution of the alpha-helical part of the secondary structure to the pi-helical structure (figures 2B and 2C). Transforming the alpha-helical parts of the molecule to pi-helical parts is a very important process. Pi-helices have been shown to be involved in a number of membrane contacts and usually serve a specific purpose in proteins. Further evolution of pi-helices is toward the unfolded turns and similar elements of the secondary structure with possible farther transformation to beta strands.

All of the conformers were then submitted to our membrane-contacting prediction program package MAPAS [11]. This program makes it possible to sort the conformers by their membranephilicity. Some of the prediction results are shown in Table 1. One can see that during MD, the membranephilic score (figure 3) actually increases at the time point of 3 ns. This observation supports the increased probability
Figure 2. (A) Evolution of the secondary structure of Mcc during MD. (B) Evolution of the alpha-helical and (C) pi-helical secondary structure of Mcc during MD. Transformation of alpha-helix to pi-helix causes the increase of membrane-contacting properties of the protein and eventually supports creation of the ring-like structures of protein aggregates with further pore creation.
of the molecule becoming membrane-contacting. The study of evolution of the secondary structure of MccE92 during MD sheds light on the possible mechanism of influence of the conformational changes to the membrane-contacting properties of the protein.

| Time (ns) | members | MRS  | MAS  | Kmpha |
|-----------|---------|------|------|-------|
| Initial   | 12      | 2.08 | 33.65| 1.82  |
| 0.5       | 9       | 1.70 | 30.36| 1.67  |
| 1.0       | 12      | 2.18 | 40.77| 2.23  |
| 1.5       | 18      | 2.14 | 33.91| 1.80  |
| 2.0       | 12      | 1.74 | 38.32| 2.06  |
| 3.0       | 14      | 1.85 | 44.83| 2.27  |
| 3.5       | 10      | 1.53 | 42.17| 2.33  |

**Figure 3.** MAPAS selected membrane-contacting surfaces of Microcine E92 (their scoring parameters are presented in table 1). Conformer 3 ns has the best combination of the scoring parameters.
Consecutive docking steps of MccE92 were conducted until the free energy of multimer formation did not decrease further. Figure 4 shows the structure of a final heptameric aggregate of MccE92 on the membrane surface on the initial stage of simulation.

Figure 4. Position of MccE92 on the membrane at the first 200 ps of MD.

The development mechanism of these oligomeric structures is very similar to the mechanism of oligomerization of another unstructured protein, alpha-synuclein (known to be involved in Parkinson’s disease). This mechanism is explicitly elucidated in [14, 15]. The final MD simulation is done with the ring-like oligomer situated on the membrane in a water box with a small additional force toward the plane of the membrane to accelerate the possible penetration.
If the conformation of a protein is not suitable for penetration, such a force would not cause any significant movement of the complex to the membrane; on the other hand, when the configuration and orientation of the complex vs the membrane are appropriate, the force will just accelerate the already occurring penetration, so we can use less computational time for modeling. Figure 5 shows penetration of the MccE92 heptamer into the membrane at the 4 ns stage of MD. These results strongly support our concept of the molecular mechanism of pore formation by bacterial proteins. MccE92 can form the ring-like oligomers that can penetrate the membrane and create the transmembrane pores. Our further experiments show that MccE92 eventually penetrates to the other side of the membrane, creating a transmembrane pore.

Figure 5. Position of MccE92 on the membrane at 4 ns of MD.
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References

[1] Lashuel H A and Lansbuty P T 2006 Are amyloid diseases caused by protein aggregates that mimic bacterial pore-forming toxins? *Quarterly Reviews of Biophysics* 39:1676-201
[2] Hammer N D, Wang X, McGuffie, B A, Chapman M R 2008 Amyloids: Friend or Fue *J Alzheimer’s Disease* 13:407-419
[3] de Lorenzo V, Martinez J L and Asensio C 1984 Microcin mediated interactions between Klebsiella pneumoniae and Escherichia coli strains, *J Gen Microbiol* 130:391–400
[4] de Lorenzo V 1985 Factors affecting microcin E492 production *J Antibiot (Tokyo)* 38, 340–345
[5] Bieler S, Estrada L, Lagos R, et al. 2005 Amyloid formation modulates the biological activity of a bacterial protein *J Biol Chem* 280:26880–26885
[6] Lagos R., Wilkens M., Vergara C., Cecchi X. and Monasterio O.1993. Microcin E492 forms ion channels in phospholipid bilayer membranes. *FEBS Lett*. 321:145–148.
[7] Lashuel H A, Petre B M, Wall J, et al. 2002 Alpha-synuclein, especially the Parkinson’s disease-associated mutants, forms pore-like annular and tubular protofibrils. *J Mol Bio* 322:1089-1102
[8] Tsigelny I, Sharikov Y, Ten Eyck L F 2002 Hidden Markov models-based system (HMMSPECTR) for detecting structural homologies on the basis of sequential information Protein Eng. 2002 15:347-52
[9] Kalé L, Skeel R, Bhandarkar M, at al, 1999 NAMD2: Greater scalability for parallel molecular dynamics *J Comp Physics*, 151:282-312
[10] Feller S, MacKerell A 2000 An improved empirical potential energy function for molecular simulations of phospholipids *J Phys Chem B*. 104:7510-5751
[11] Sharikov Y, Walker RC, Greenberg J, Kouznetsova V, Nigam S, Miller MA, Masliah E, Tsigelny I 2008MAPAS: a tool for predicting membrane-contacting protein surfaces. *Nature|Methods*, 5:119
[12] Ritchie D W and Kemp G J L Protein docking using spherical polar Fourier correlations 2000 Proteins 39:178-194.
[13] Barnhart M M and Chapman M R 2006 Curli biogenesis and function *Annu Rev Microbiol* 60 :131–147.
[14] Tsigelny I F, Bar-On P, Sharikov Y et al. 2007 Dynamics of alpha-synuclein aggregation and inhibition of pore-like oligomer development by beta-synuclein. *FEBS J*. 274:1862-1877
[15] Tsigelny IF, Sharikov Y, Miller MA, Masliah E. 2008 Mechanism of alpha-synuclein oligomerization and membrane interaction: theoretical approach to unstructured proteins studies. *Nanomedicine* 2008 4:350-357