Effect of ethanol as molecular crowding agent on the conformational dynamics of α-synuclein

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ABSTRACT
The functions of many proteins have been directly connected to their conformational changes. The macromolecular crowding environment inside the cell is known to have a significant impact on the equilibria and transition rates between different conformations of the protein. Here we demonstrate the effect of ethanol as crowders on the conformational dynamics of α-synuclein, a primary component of the fibrillar neuronal inclusions, and known as Lewy bodies that are diagnostic of Parkinson’s disease. We observed the α-synuclein protein to experience stronger crowding effects with an increase in concentration of ethanol, the crowding agent. The findings that we obtained from this simulation study would serve as valuable guides for expected crowding effects on conformational dynamics of α-synuclein.

Keywords: Parkinson’s disease; Macromolecular crowding; presynaptic, aggregation.

1. INTRODUCTION

In the recent past, many research studies have highlighted the importance of the protein dynamics as a valuable platform to understand the association between the structure and function [1-6]. The protein dynamics leads to the sampling of alternative conformations. Because of ligand binding [7] and post-translational modifications like phosphorylation [8], the conformational changes in the protein molecule gets initiated. As a result, the protein molecule adopts different conformations at varying functional states.

From these structures, the conformational changes at atomistic level can be studied. We generally see that biological characterizations of conformational changes in protein have been studied mostly under dilute and lesser densed medium. But the proteins perform their biological functions inside the cell which is highly crowded with macromolecules. For example, the cytoplasm of Escherichia coli contains high concentration of macromolecules (about 300–400 g/l and 30% of the total volume occupancy)[9]. Because of crowding in cell membranes, membrane proteins occupy a similar level of the total surface area [10]. However, the impact of crowding environment in cell on the equilibria and transition rates of diverse conformations of proteins are not understood well. The macromolecular crowding in the cell also likely to alter the energy landscapes of conformational changes in a protein resulting in more compact structures over more open structures [11]. Such effects of crowding have been verified experimentally [8].

Molecular Dynamics (MD) simulations have also been used as a tool to investigate the energy landscapes of a number of proteins in a crowding environment, in the context of either conformational change [12] or folding-unfolding transition [13-15]. In our study, we have investigated the consequences of ethanol as a crowding medium on the conformational dynamics of α-synuclein. We have found the crowding environment to affect the secondary structure content of α-synuclein to a greater extent.

2. MATERIALS AND METHODS

The initial 3-D structure of α-synuclein was taken from Protein Data Bank (PDB). In order to study the effect of different concentrations of ethanol, the crowding agent on the conformational dynamics of α-synuclein, we have employed MD simulation using the explicit solvent model. MD simulations were performed using periodic boundary conditions. In carrying out this experiment, cubic simulation boxes were filled with different proportions of water-ethanol mixtures using Packmol. In all these cases, the protein molecule was placed at the center of the simulation box using Leap module of AmberTools 14 program.

The protein molecules are then overlaid by equilibrated triple point charge (TIP3P) boxes in order to solvate the molecule of interest in the respective cubic simulation boxes. In addition, positively charged Na+ counter-ions were added into the system to neutralize the negative charge on the protein molecules. The volume occupied by ethanol was set up to about 0%, 5%, 10%, 20%, 50% and 100% of total volume. To study the structural dynamics of intrinsically disordered proteins (IDPs), MD simulations have been extensively in use. The AMBER14 package was used to perform MD simulation while protein and water molecules are described by parameters from ff99SB force field and TIP3P water molecules in the system. In each system, the charge of the protein was neutralized by adding Na+/Cl- counter ions. An isobaric–isothermal ensemble was applied using Langevin dynamics [16] along with Berendsen- thermostat [17] for temperature control. The system was subjected to one stage minimization to ensure the stability of the structure. The integration time step was set to 1 fs. To further take the system to
room temperature, heating was gradually performed to bring the
temperature of the system to 298 K over a time of 10 ps. To ensure
the equilibration of the system, pressure, density, temperature, root
mean square deviation (RMSD), potential energy, kinetic energy
and total energy of the initial structure of α-synuclein were plotted
as a function of simulation time. The trajectories were collected
and visualized by VMD[18] package after intervals of 10ns for a
total MD run of 50ns and analysed using cpptraj [19] program
from AMBER tools.

3. RESULTS

In order to compare the conformational dynamics of α-
synuclein in different concentrations of ethanol, we carried out all
atom molecular dynamics simulation using the PMEMD module
of AMBER14 software package with ff99SB force field.

Our results demonstrate that α-synuclein folds in a
multiphasic manner in the presence of ethanol as a crowding
agent. We noticed the folding pathway of α-synuclein to vary with
the concentration of ethanol. Although the mechanism of folding
changes with the concentration of ethanol, yet it was characterized
by a common first stage that actually leads to the partially folded
intermediate [20-24]. The nature of the solvent is responsible to
decide for the subsequent fate of this intermediate. It has been
seen that the higher concentrations of ethanol gave rise to an α-
helical conformation. These observations infer that, depending on
the environment, the partially folded intermediate may undergo
self-association to form dimers, soluble oligomers or amorphous
aggregates and fibrils. We also observed that α-synuclein in higher
concentrations of ethanol revealed significant ordered secondary
structure. The capacity of concentrated organic solvent inducing
the structural changes in the native globular proteins have been
reported. Typically, alcohol-induced denaturation of globular
proteins is accompanied by a characteristic increase in α-helix
content [25-38]. Much less is currently known about the behaviour
of natively unfolded proteins in water/organic mixtures [39-41];
however, one would expect similar effects. The structural
transformations and oligomerization of α-synuclein in simple
alcohols were driven by the increase in solvent hydrophobicity.
These results, therefore, exclude contributions from specific
protein alcohol interactions, indicating that water/alcohol mixtures
might be useful models for the effect of hydrophobic membrane
surfaces (the membrane field effect) on the conformation of α-
synuclein and other natively unfolded proteins.

3.1. Conformational dynamics of α-synuclein in different
concentrations of crowding agent.

To study the conformational dynamics of α-synuclein in
different concentrations of ethanol, the crowding agent, we have
analyzed the RMSDs of the Cα atoms, radius of gyration (Rg),
Root Mean Square Fluctuation (RMSF) and Solvent Accessible
Surface Area (SASA) over the course of simulation time as shown
in Figure 1A, 1B, 1C and 1D. Assessment of the structural drift
was carried out by analyzing the Cα atom RMSDs. We observed
the RMSD profile in each case of the protein to be varying
(Figure 1A). The backbone RMSD of the protein attained almost
stable conformation from the initial stage in case of 100% ethanol,
whereas in case of 0%, 5%, 10%, 20%, 50% ethanol the structure
had undergone conformational changes through the initial time
period of around 4.5ns, 4.5ns, 4ns, 2.5ns and 2ns respectively, and
then reached equilibration. RMSD value of α-synuclein in 100%
ethanol corresponds to 7.5 Å, 50% ethanol is 11 Å, 20% ethanol is
13 Å, 10% ethanol is 15 Å, 5% ethanol is 19 Å and 0% ethanol is
21 Å. From these RMSD analyses we can infer that α-synuclein
reaches equilibration very quickly with increase in concentration
of crowding. The stability of the protein is due to its ability to
retain its native α-helical conformation even at higher
concentration of ethanol.

Figure 1B shows the radius of gyration analysis of α-synuclein
protein as a function of time. In the simulation carried out, the
radius of gyration oscillated to a greater degree before 2 ns, further
confirming that the peptide structure remained stable after 2 ns
from the simulation being initiated. From the Rg plots, we can see
that with an increase in ethanol concentration, the structure of α-
synuclein was found to be less compact. The size of the molecule
was bigger in a higher concentration of ethanol. α-synuclein in
100% ethanol depicts the highest Rg value as observed from the
plot. So, it can be inferred that α-synuclein retains its structure
having more helical content in the higher crowding environment
that makes it lesser prone to aggregation. Thus, it is evident that
the aggregation propensities of α-synuclein decreases with
increasing proportions of ethanol. To obtain information on local
structural flexibility, thermal stability and heterogeneity of
macromolecules, root mean-square fluctuations (RMSF) of α-
synuclein were studied (Figure 1C). RMSF values obtained for the
backbone Cα atom in different concentrations of ethanol were
calculated from the corresponding MD simulation trajectories and
were plotted against their residue numbers. It can be inferred from
the plot that fluctuation in conformational dynamics of α-
synuclein was found to decrease with an increase in ethanol
concentration. In order to get information regarding the buried and
exposed area present in the protein structure, SASA analysis was
carried out (Figure 1D). The overall solvent accessible area of the
protein molecule was analyzed in different concentrations of
ethanol.

From Figure 1D, it can be seen that α-synuclein in 0%
ethanol has higher SASA values while in 100% ethanol the SASA
value was found to decrease. The SASA of α-synuclein decreases
with an increase in the ethanol concentration.

3.2. Secondary Structure Analysis of α-synuclein in different
concentrations of crowding agent.

The secondary structure analysis was carried out for α-
synuclein in the crowding medium using the Kabsch and Sander
algorithm incorporated in their Dictionary of Secondary Structure
for Protein (DSSP) program [42]. The probability score graph results were in good agreement with the assessment that crowding supports in retaining the native structure of α-synuclein. (Figure 2A). From the graph we can observe that most of the residues retained their α-helical conformation in presence of 100% ethanol.

![Figure 2. (A) Probability score of secondary structure for each residue in α-synuclein in 0%, 5%, 10%, 20%, 50% and 100% ethanol concentration.](image)

The plot shows the secondary structural variation of each residue during the course of simulation time. In each case, the alpha helical portion of the structure was increased with an increase in concentration of ethanol, the crowding medium. The protein in 100% ethanol tends to have higher helical content in comparison to other proportions. So, the existence and rapid changes in structural dynamics of α-synuclein in crowding media were clearly visible from secondary structure analysis. We also calculated the percentage of individual secondary structure content in α-synuclein across all conformations using YASARA software [43] that were sampled during the production job of trajectories and the results were summarized in Table 1.

From Table 1, we observed that α-synuclein in 100% ethanol contains a higher amount of α-helix than the other systems. So, these observations support that higher helical conformation in α-synuclein which is predominant in case of 100% ethanol, to be actually responsible for preventing fibrillation process as this structural characteristic feature would induce a similar conformation that restricts fibrillation as proposed earlier. Figure 2B shows the classification of the trajectories in terms of secondary-structure elements obtained by the software tool DSSP which assigns secondary structures to the amino acids of a protein, by identifying the intra-backbone hydrogen bonds of the protein. From the plot we can see the stability (or de-stability) of secondary structure elements as a function of time. Examination of Figure 2B shows that the main features of the α -helix structure were largely retained in the case of 100% ethanol as compared to other systems.

4. CONCLUSIONS

In this work, the effect of molecular crowding on the conformational dynamics of α-synuclein was studied. The

| Concentration of Ethanol | α-Helix | Sheet | Turn | Coil | 3α helix | α-helix |
|--------------------------|---------|-------|------|------|----------|---------|
| 0%                       | 4.3%    | 0.0%  | 34.3%| 61.4%| 0.0%     | 0.0%    |
| 5%                       | 8.6%    | 0.0%  | 37.1%| 54.3%| 0.0%     | 0.0%    |
| 10%                      | 9.0%    | 0.0%  | 30.0%| 60.0%| 0.0%     | 0.0%    |
| 20%                      | 22.1%   | 0.0%  | 6.0% | 30.0%| 0.0%     | 0.0%    |
| 50%                      | 20.0%   | 0.0%  | 2.1% | 30.0%| 0.0%     | 0.0%    |
| 100%                     | 55.0%   | 0.0%  | 5.7% | 39.3%| 0.0%     | 0.0%    |

3.3. Conformers of α-synuclein at different concentrations of ethanol.

In Figure 3, we can see the snapshots of α-synuclein at 0%, 5%, 10%, 20%, 50% and 100% concentration of ethanol. We observed most of the residues to be in α-helical conformation with increasing concentration of ethanol.

3.4. Analysis of Diffusion Coefficient.

The values of diffusion coefficient for α-synuclein in different concentrations of ethanol were summarized in the Table 2.

| Concentrations of Ethanol | Diffusion Coefficient (x10^-5 cm²/s) |
|---------------------------|-------------------------------------|
| 0% Ethanol                | 0.3829                              |
| 5% Ethanol                | 0.3703                              |
| 10% Ethanol               | 0.2516                              |
| 20% Ethanol               | 0.2148                              |
| 50% Ethanol               | 0.2699                              |
| 100% Ethanol              | 0.4446                              |

Diffusion coefficient values tends to decrease with increasing concentration of ethanol but again at higher concentrations the corresponding values increase. This is so because of the shifts in the dielectric medium and structural changes of the molecule. In the beginning, the structure of α-synuclein was more compact and its diffusion was affected by the presence of water molecules. But, gradually with an increase in ethanol concentration, the intermolecular interaction between water and α-synuclein decreases. In 50% and 100% ethanol, the number of water molecules eventually decreases and becomes negligible, for which the attraction between the water molecules and the protein diminishes and thus the diffusion coefficient value escalates. This facilitates the sudden change in the pattern of the diffusion coefficient values with respect to increasing concentrations of ethanol.
ethanol, the crowding agent. We also noticed the solvent accessible surface area of α-synuclein protein to decrease and the 3-D structure to become less compact at higher concentration of ethanol, which explains its decreasing tendency towards aggregation. Diffusion coefficient of α-synuclein was found to be dependent on concentration of the ethanol, the crowding agent. With an increase in concentration of ethanol, the value of diffusion coefficient decreases initially but again increases at higher concentrations due to the change in dielectric medium, intermolecular interactions and structural changes in α-synuclein. The intermolecular interactions between the solvent water molecules and α-synuclein protein were found to decrease with an increase in concentration of ethanol. Our results show that along with excluded volume effect, the co-solute properties of crowded intracellular environment need to be considered to understand α-synuclein dynamics in cells.

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