TEMPERATURE DEPENDENT AGGREGATION MECHANISM AND PATHWAY OF LYSOZYME: BY ALL ATOM AND COARSE GRAINED MOLECULAR DYNAMICS SIMULATION

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ABSTRACT:

Aggregation of protein causes various diseases including Alzheimer's disease, Parkinson's disease, and type II diabetes. It was found that aggregation of protein depends on many factors like temperature, pH, salt type, salt concentration, ionic strength, protein concentration, co solutes. Here we have tried to capture the aggregation mechanism and pathway of hen egg white lysozyme using molecular dynamics simulations at two different temperatures; 300 K and 340 K. Along with the all atom simulations to get the atomistic details of aggregation mechanism, we have used coarse grained simulation with MARTINI force field to monitor the aggregation for longer duration. Our results suggest that due to the aggregation, changes in the conformation of lysozyme are more at 340 K than at 300 K. The change in the conformation of the lysozyme at 300K is mainly due to aggregation where at 340 K change in conformation of lysozyme is due to both aggregation and temperature. Also, a more compact aggregated system is formed at 340 K.

1. INTRODUCTION:

The phenomenon of protein aggregation is part of the normal functioning of cell as well as has some negative consequence via its role in neurodegenerative diseases[1] such as amyloidoses, including ALS, Alzheimer's[2], Parkinson's[3] and Prion[4], Huntington's[5] respectively. Protein aggregation may pass through different pathways[6]. (i) Aggregation through unfolding intermediate and unfolded states[7]: the physical aggregation process suggests that the protein folding or unfolding intermediates are precursor of the aggregation process. At normal conditions, native proteins are in equilibrium with a small amount of intermediates, which are further in equilibrium with the completely unfolded or denatured states. (ii) Aggregation through self association[8]: proteins may aggregate from the native state without going through any intermediate state by simply electrostatics or both electrostatics and hydrophobic interactions depending on the conditions. (iii) Aggregation through chemical linkage[9]: it can occur through the cross links between proteins chains, leading to aggregation mainly by the intermolecular disulfide formation or exchange. (iv) Aggregation through chemical degradation[10]: chemical degradation includes deamidation, oxidation, isomerisation, clipping/fragmentation may lead to important changes in the higher order structure and aggregation may happen.

The model protein of our work is hen egg white lysozyme. It is found in tears, saliva, sweat, and other body fluids[11]. It is also found in other mucosal linings, such as the nasal cavity. Because of its relatively simple structure and small size[12], hen egg white lysozyme is one of the best models to study protein unfolding and aggregation[13]. Due to high interfacial reactivity and remarkable physiochemical properties, lysozyme has been widely used in the recent years in multiple applications.

Major factors that determine whether a protein will aggregate, and the extent and rate of the aggregation are found to depend strongly on the properties of a protein's environment, such as temperature[14,15], pH[16], salt type[14,15], salt concentration[14,15], ionic strength[15], the concentration of the protein[15], co solutes[14] (e.g. Denaturants such as urea[16,17], other chaotropes or kosmotropes including osmolytes[18] and ligands that interact selectively with either the native or non-native conformations of the protein or the aggregated form), preservatives[14], and surfactants[14] as well as the relative intrinsic thermodynamic stability of the native state.

Computational tools[19-21] are progressively being used to figure out the protein aggregation issue[11,22], providing insight into amyloid structures and aggregation mechanisms. To understand the protein aggregation molecular dynamic simulation method offers a unique possibility to investigate the phenomenon because computational studies validated by experiments could provide more detailed information about the onset of aggregation mechanism and pathway[11,23,24]. The key challenges in simulating the aggregation pathway are the associated time and length scales. In order to simulate the aggregation process from monomers to large aggregates, coarse-grained models[18,25-27] is one of the efficient method[28-30].

In this work our purpose was to provide a fundamental understanding of the mechanisms and path by which lysozyme aggregate through monitoring the atomistic details by atomistic and coarse grained simulation and what role the temperature play in lysozyme aggregation. We have selected two different temperatures, one above the melting temperature of lysozyme i.e. 340 K[31] and another at room temperature i.e. 300 K temperature[32]. Our aim was to observe whether at both the temperatures protein follows the same unfolding and/or aggregation pathway or passes through different pathways. Moreover, according to experimental results aggregation of lysozymes is not specific[33], hence an understanding about the mechanism is necessary.

We have analyzed the structural properties of proteins to know how the aggregation affects on the structure of proteins. We found that aggregation of proteins was more above the denaturation temperature of lysozyme and changes in conformation of protein at 300 K was mainly due to aggregation where above the denaturation temperature the changes in conformation of protein was due to both aggregation and temperature.

2. Methods:

2.1 System preparation (atomistic):

X-ray crystallographic structure of lysozyme 5b1f was obtained from protein data bank (http://www.rcsb.org/pdb), which contain 129 amino acid. The system was prepared in CHARMM34 using all atom CHARMM22[34] force field. The two proteins were solvated in a box of length 73 Å * 72 Å * 72Å containing 13824 number of TIP3 water molecules .The two proteins were inserted into the water box diagonally with separation of about 40 Å from each other and to neutralize the system 16 chloride ions[32] were added. Waters which are within 2.6 Å of protein were removed. The atomistic simulations were performed using NAMD_2.7[35] packages with the standard CHARMM22 force field. We carried out two simulations, one at 340 K and another at 300 K. Trajectory analysis was done in CHARMM34 by using CHARMM force field.

2.2 Simulation protocol (atomistic):

All MD simulation were carried out under the isobaric-isothermal (NPT) ensemble with imposed 3D periodic boundary conditions. A time step of 2 fs was used to integrate the equation of motion. The temperature was maintained for the simulation using Langevin dynamics[36], while the pressure was kept constant at 1 bar using a Nose-Hoover-Langevin piston[37] .The smooth particle mesh Ewald method[38] was used to calculate long range electrostatic calculations. Short

range interactions were cutoff at 10Å. All bond lengths involving hydrogen atoms were held fixed using the RATTLE[39] and SATTLE[40] algorithm.

2.3 System preparation (coarse grained):

Two copies of lysozyme proteins were taken and inserted in the water box separating from each other by 5 nm. The whole system was prepared using GROMACS 5.07[41]. One system was simulated at 300K and another at 340 K by GROMACS 5.07 using martini2.2 force field[42]. This was followed by a short energy minimization to release the stress of the system and subsequently the system was equilibrated at NPT ensemble condition. The number of water beads was 11477 and the box size was 11.248nm x 11.248nm x 11.248nm. System was allowed to maintain a minimum distance of 1.5 nm from the protein to any box edge and used a larger van der Waals distance of 0.21 nm during solvation to avoid clashes.

2.4 Simulation protocol (coarse grained):

We have performed two simulations; one at 340K and another at 300K each for 10 μs at an integration time step of 20 fs[43]. The target temperature was kept constant using Velocity-rescale thermostat[44] with 1.0 ps relaxation time. The pressure was kept constant in all direction using Parrinelo-Rahman barostat[45] with 1.0 ps relaxation time. The system was subjected to periodic boundary condition in all direction. Analysis was done using the GROMACS 5.07 analysis tool and VMD 1.9.1 and snapshots were generated using VMD[46].

3. RESULTS:

In order to explain the aggregation phenomenon for a longer time scale, we have performed coarse grained simulation and to get the atomistic details we have done all atom molecular dynamics simulation. We have done the parallel simulation for both coarse grained and all atom MD simulation and from these both simulations we get similar results.

3.1 Inter-protein distance:

For coarse grained system initially, the centre of mass of the two monomers of lysozyme was separated by a distance of 5 nm and the simulations were run at 300 and 340 K. At both temperatures, it was found that aggregates were formed nearly within 300 ns. At the end of 10 μs, the average distances between the centres of masses were 2.18±0.04 nm and 1.89±0.04 nm at 300 and 340 K respectively. These distances were achieved within ~1.4 μs and ~2.0 μs of simulation time and remained almost constant till the end of the simulation (as shown in Figure 1). In case of all atom molecular dynamics simulation, the plot of center of geometry of two proteins versus simulation time, it was noticed that two proteins comes close to each other within first 40 ns (S1: a, plot is given in the supporting information). The distance between the centers of geometry of two proteins after 120 ns and at 300 K was nearly 30 Å and at 340 K it was almost 32 Å. Till 500 ns they were in aggregated form as seen from the snapshots taken using VMD (S1: b , snapshots are given in the supporting information).

3.2 Structural properties: In order to assess the changes in structural characteristics and stability of proteins due to aggregation, geometrical properties such as RMSD (Root Mean Square Deviation), Rg (radius of gyration), and SASA (solvent accessible surface area) were calculated over MD trajectories. On calculating the RMSD of the backbone of the two monomers at the two different temperatures, we found that the change in RMSD is more at 340 K temperature (Figure 2: b) than at 300 K (Figure 2: a). Similar trends in RMSD were obtained from all atom systems (plots are given in the supporting information Figure: S2: a and S2: b). Averaged over the last 0.1 μs simulations of coarse grained simulation, the RMSD values of protein1 and protein2 were 0.71±0.02 nm and 0.84±0.03 nm at 300 K and were 1.10±0.04 and

1.09±0.04 nm at 340 K. The Rg is the root-mean-squared distance of all the atoms in the molecule from the protein center of mass which is highly dependent on the molecular shape[47]. Rg analysis showed a similar kind of fluctuation (Figure 2: c and 2: d). For the last 0.1 μs of the coarse grained simulation time (Table 1), average Rg values of protein1 and protein2 became 1.41±0.01 nm and 1.43±0.01 nm at 300 K respectively whereas at 340 K, the values were 1.42±0.02 and 1.45±0.02 nm for protein1 and protein2 respectively. Alteration in structural geometry was further validated by SASA analysis and Lee and Richards's method[48] was utilized to mimic the water in the system. From the coarse grained simulations (Figure 2: e and 2: f) we found that for the last 0.1 μs of simulation time, average SASA of two lysozymes were 88.25±2.15 and 88.78±2.28 nm² at 300 K and 92.82±2.82 and 94.71±2.65 nm² at 340 K temperature respectively. Moreover our all atom simulation results for Rg and SASA monitored against time followed a similar trend. (Figure: S2: c, S2: d, S2: e and S2: f, of supporting information)

For coarse grained system at 340 K it was observed that a large fluctuation in Rg and SASA was observed between 1 to 3 μs but after that again the changes became almost constant. Where at 300K the changes in Rg and SASA value was less (Figure S3: a and S3: b, Plots are in the supporting information). For all atom system it was noticed that at 340 K both Rg and SASA values became constant after 250 ns and at 300 K these values became constant after 200 ns (Figure S2: c, S2: d, S2: e and S2: f, plots are in the supporting information). The stability of the protein was further evaluated in terms of fraction of alpha carbon contacts[49], intra protein hydrogen bonding, protein-solvent hydrogen bonding and secondary structure (Figure 3) over the all atom MD trajectories at both temperatures.

3.3 Non specific aggregation:

From coarse grained simulation we analyzed both the intra- and inter-protein residue contact pairs which were calculated at 1 μs and 10 μs respectively at a cut off 10 Å[50]. In Figure 4, the black circle represents the interfacial residues of protein and it was found that at both temperatures it was different. In the native protein the intra protein hydrophobic contact pairs (Figure 5, represented by red circle) were Ala42-Leu83, Ala42-Ala82, Ile55-Ala90, Leu56-Ala90, Ile58-Ala90, Ile55-Leu84, Ile55-Leu83, Leu56-Leu83, Ile56-Ala107, Ile56-Val109, and Leu56-Trp108. At 340 K, within first 1 μs of simulation, in both monomers all these contact pairs disappeared. Whereas, at 300 K new hydrophobic contact pairs were generated along with some native hydrophobic contact pairs. Some of them are Ile55-Met105, Leu56-Met105, Leu56-Val99, Ile58-Val99, Leu56-Val99, Leu56-Ala95, Ala42-Ala90, Ala42-Leu84 and the reappearing native hydrophobic contact pairs were Ala42-Leu83, Ala42-Ala82. Contact map analysis clearly indicated that the contacts which were present between the helices of the protein disappeared and regenerated at 300 K. Loss of native intra-protein contacts and formation of new contacts continued throughout the simulation at both the temperatures.

We also assessed the formation of inter-protein contacts that are formed once the two monomers came close to each other. Figure S4 of supporting information shows formation of such contacts as a function of time. It was seen that at initial stages of simulation, number of inter protein contact increases at a faster rate at 340 K rather than at 300 K. At the end of 10 μs the numbers of inter protein contacts were 35 and 71 at 300 K and 340 K respectively.

Salt bridges are by far the most essential specific interactions in biological acceptance processes. Salt bridges between two proteins were analysed for all atom system by using VMD (N and O distance was 3.2Å). At 100 ns salt bridges were found between these pairs Asp87-Arg112, Lys97- Asp101, and Arg68- Asp48 at 300 K. At 340 K Asp18- Lys13, Glu7- Arg45

pairs were found. However after 500 ns no salt bridge pairs were found at 300 K, but at 340 K these pairs were found Arg128- Asp66, Arg5- Asp66, Arg5- Asp48, Asp52- Arg125, Asp119- Arg73, Lys1- Asp48, and Glu7- Arg45. From this data it was noticed that salt bridge pairs at 300 K and 340 K were not same, even when we compared number of salt bridge pairs at 100 ns and 500 ns it was observed that the number of pairs also changed. Taking the salt bridge pairs information obtained from all atom system at 100 ns at both temperatures into account, we calculated the distance between the two residues of salt bridge pairs (mentioned above) in coarse grained system and the distance between the salt bridges pairs were varying in most of the cases at both temperatures. Figure 6 represented that throughout the simulation, the change in the distance between two residues (residue 97 of protein1 and residue 101 of protein2) of salt bridge pairs was between 0.5-0.6 nm. It indicated that the salt bridge has formed between this pair was stable[51]. The distance between residue 87 of protein1 and residue 121 of protein2 was never less than 1 nm. So there was no formation of salt bridge between these two residues. Between residue 18 of protein1 and residue 13 of protein2, it was noticed that probability of distribution of distance was mainly found between 1 to 4 nm which signified that the formation of salt bridge between these two pairs was not stable. Lastly the increase in distance between residue 7 of protein1 and residue 45 of protein2 was mainly beyond 1 nm.

3.4 Effect of aggregation on conformation of protein:

We were interested to see that whether, aggregation has any effect on the changes of the conformation of the monomers and to what extent. From the literature it was found that the denaturation temperature of lysozyme was above 337 K. So it was certainly interesting to study whether aggregation will affect the conformation of protein or not. In literature, MD simulations were performed at a range of temperatures from 300 K–500 K to speed up the unfolding rate of

proteins[52]. Valerie Daggett has mentioned in the paper "Increasing Temperature accelerates protein unfolding without changing the pathway of unfolding" that proteins unfold following the same pathway at higher temperatures[53]. A general conclusion appearing from these MD simulations is that distortions in the three-stranded β-sheet domain are the initiation of unfolding of lysozyme[54,55], which is also consistent with experiments[56]. If we have a closer look at our contact map analysis (Figure 5 and Figure S5 of supporting information), we would get a similar conclusion.

To analyse the nature of changes in the conformation of the individual monomers at the two temperatures, we have calculated angle between ß-strand1 (residue no 43-45) and ß-strand2 (residue 51-53) and between ß-strand2 and ß-strand3 (residue no 58-59) from the coarse grained system. Changes were visible for the angle between ß-strand2 and ß-strand3 of protein1 at 300K (Figure S5: a of supporting information) whereas at 340K a visible change was noticed for the angle between ß-strand2 and ß-strand3 of protein2 (Figure S5: b of supporting information) (all others plots are in the supporting information figure S5). To obtain a clear depiction of the change of orientation for both the above mentioned systems, angle distribution was plotted (Figure 7), where it was found that at 300 K anti parallel orientation is prevented but at 340 K, both parallel and antiparallel orientations were present.

3.5 Stability of aggregated system:

In order to verify the system stabilization along the MD simulations, we have analyzed interaction energy between two proteins, between protein and water and radius of gyration of the two proteins together. Figure 8 shows that the interaction energy for protein-protein and protein-water remained almost constant after 1 μs at both temperatures. The non-bonded cut off

was 1.1 nm. Average Coulombic interaction energy and average Lenard-Jones interaction energy between two lysozyme molecules at 10 μs was -32.70±3.3 and -1127.94±7.1 kJ/mol at 300 K and was -22.51±1.1 and -1347.95±19 kJ/mol at 340 K respectively. It was found that at the end of 10 μs at 300 K, average Coulombic interaction energy between protein1 and water was -3370.81±8.7 and between protein2 and water was -3367.69±15 kJ/mol. However, at 340 K average Coulombic interaction energy between protein1 and water was -3061.26±33 and between protein2 and water was -3233.64±17 kJ/mol.

From the Figure 9 it was perceived that at both temperatures after 1 μs the Rg value of dimer remains almost constant. Also, at both temperatures the Rg value of dimer was found to be less than the summation of Rg of two proteins individually. Moreover, in case of 340 K temperature Rg of the aggregated system was less than at 300 K throughout the entire simulation. For last 0.1 μs the average Rg of dimmers were 1.79±0.01 and 1.72±0.01 at 300K and 340 K temperature respectively.

Interaction energy between the helices within in a protein and helices between two proteins was analysed. It was observed that at 340 K interaction energy between two helices within a protein was much more than at 300 K (Figure S7). Even the interaction energy between helices of two proteins at 340 K was more for first 1 μs than at 300 K (Figure S8: a and S8: b, Table 3). If we consider last 1 μs it was found that interaction energy between inter helices was even more at 340K than at 300K (Figure S8: c and S8: d, Table 3).

4. DISCUSSION:

We have monitored inter protein distance which indicate that irrespective of temperature two proteins come together and form aggregated. Structural properties were included to explore the

unfolding and partial unfolding of proteins. Deviations in RMSD signify conformational changes of the initial structure. Also the increase in Rg and SASA of proteins were indicating towards the partially unfolded structure of proteins. For coarse grained system at 340 K large fluctuation in Rg and SASA between 1 to 3 μs and after 3 μs again the changes became almost constant which imply the stability of aggregated system. We found an alteration in the information about the conformational change of proteins by the analysis of protein fraction of alpha carbon contacts, intra protein hydrogen bonding, protein-solvent hydrogen bonding and secondary structure. At both temperatures, along with these conformational changes as the two proteins came closer to each other and aggregated, so it may be said that pathway of aggregation of protein passes through partially folded proteins.

Intra-protein residue contact pairs also signified the structural change of proteins at both temperatures as because with the simulation time hydrophobic contact pairs were also changes. Moreover increase in the number of inters protein contact at 340 K than at 300 K indicated the stability of the aggregates formed. It can be attributed to the higher accessible surface areas of the monomers at 340K.

From the contact map analysis between two proteins obtained from coarse grained simulation we found that as the interfacial residues were different at different temperatures, so it might be said that aggregation of lysozymes was not specific. Even the probability distribution of salt bridge pairs clearly indicated a similar conclusion that aggregation was not specific as the distance between salt bridges pairs were varied in most of the cases.

Probability distribution of angle signified that at 300K anti parallel orientation is prevented but at 340K, both parallel and antiparallel orientations were present which signified the conformational

changes of protein at both temperatures. The total interaction energy between two proteins at 340K temperature was more negative than at 300K but interaction energy between protein and water (for both proteins, Figure 8) was more negative at 300K than at 340K. Lower value of Rg of dimer at 340K than at 300K throughout the simulation indicated the system compactness. Therefore, the results obtained from theses analysis, it might be said that above the denaturation temperature a more stabilised and compact aggregated system was formed. Also it might be concluded that protein aggregation rate simultaneously increases with increasing temperature.

So we obtained similar information about the structural changes of proteins from the analysis of RMSD, Rg, and SASA for both all atom and coarse grained molecular dynamics simulation. Even we get some atomic details from the all atom molecular dynamic simulation by analyzing fraction of alpha carbon contacts, intra protein hydrogen bonding, protein-solvent hydrogen bonding and secondary structure which cannot be performed by coarse grained system. The most important advantage of coarse grained simulation is large time scale. So we can easily perform the analysis of contact map, Probability distribution of angle between beta strands, interaction energy between two proteins, Rg of dimer for 10 µs but from all atom molecular dynamics simulation we cannot do these all analysis for a long time due to high computational coast.

5. CONCLUSION:

From the accumulated results, it could be concluded that above melting temperature as the native conformation of lysozyme gets perturb, the aggregation is promoted to a greater extent than at 300 K. At 300 K, the change observed in the conformation of lysozyme was mainly due to aggregation. Our result also suggests that aggregates are not formed from fully unfolded lysozyme but also initiated from the partially folded lysozyme. Experimentally it is found that

temperature affects the reaction kinetics as the rate constant increases exponentially with temperature for activated complex reactions. So the aggregation of lysozyme rate similarly increases with increasing temperature. Above the denaturation temperature of lysozyme more compact aggregates were formed. Our current report provides the information about the residues involved in dimer formation and hence we can conclude a similar conclusion with the experimental result that aggregation of lysozyme is not specific. The real outcome of this study is proposing a method for finding the temperature dependant aggregation mechanism, pathway and its effects on protein's conformation.

Conflicts of interest:

There are no conflicts to declare.

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FIGURE LEGENDS:

Figure 1: Plot of distance between the center of mass of two proteins (D between COM of two proteins) versus simulation time at temperature 300K and 340K respectively.

Figure 2: Plot of RMSD (a, b), Rg (c, d), SASA (e, f) of two proteins versus simulation time at temperature 300K and 340K respectively.

Figure 3: Plot fraction of alpha carbon contacts (a, b), intra protein hydrogen bonding (c, d), protein-solvent hydrogen bonding (e, f) and secondary structure (g, h) of two proteins versus simulation time at temperature 300K and 340K respectively.

Figure 4: Contact map between two proteins. Inter-protein contacts are shown by black circle.

Figure 5: Contact map of intra-protein backbone contacts.(a) Native protein (b) Protein1 for first 1 μs at 300K (c) Protein1 for first 1 μs at 340K (d) Protein2 for first 1 μs at 300K (e) Protein2 for first 1 μs at 340K (f) Protein1 for last 1 μs at 300K (g) Protein1 for last 1 μs at 340K (h) Protein2 for last 1 μs at 300K (i) Protein2 for last 1 μs at 340K.

Figure 6: plot of probability distribution of distance between salt bridge pairs.

At 300K,   Blue bead= Lys 97 of protein1,   Green bead= Asp 101 of protein2

                 Red bead=Asp 87 of protein1,   Yellow bead=Arg 112 of protein2

At 340K ,   Blue bead= Asp 18 of protein1,   Green bead= Lys 13 of protein2

                 Red bead=Glu 7 of protein1,   Yellow bead=Arg 45 of protein2

Figure 7: plot of probability distribution of angle between ß-strands of protein at temperature (a) 300K and (b) 340K.

Figure 8: plot of interaction energy between two proteins and between protein and water versus simulation time at temperature (a) 300K and (b) 340K respectively.

Figure 9: plot of Rg of dimer versus simulation time.

FIGURES:

At 300K ,1μs

At 300K ,10μs

At 340K ,1μs

At 340K ,10μs