Effect of Urea, Arginine, and Ethanol Concentration on Aggregation of 179CVNITV184 Fragment of Sheep Prion Protein

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Supporting Information

ABSTRACT: Understanding protein aggregation is of utmost importance as it is responsible for causing several neurodegenerative diseases and one of the serious impediments in large-scale biopharmaceutical production. The prion protein is responsible for pathological states in fatal transmissible spongiform encephalopathy. The peptid fragment 178−191 of Syrian hamster prion protein is known to be amyloidogenic. Here, we identified the fragment 179CVNITV184 as an aggregation-prone fragment in sheep prion protein. This fragment is conserved sequence among sheep and Syrian hamster prion protein and also falls in the previously identified amyloidogenic sequence. The mechanistic details of the aggregation behavior are analyzed in three different concentrations of urea, arginine, and ethanol. Urea and arginine are found to be aggregation suppressors, but ethanol enhances the protein aggregation through β-sheet formation. We have also analyzed the influence of these osmolyte on water dynamics in the presence of the octamer of this aggregation-prone fragment and correlated this water dynamics with the aggregation behavior of the octamer.

INTRODUCTION

Several neurodegenerative diseases, such as prion disease, Alzheimer's disease, cystic fibrosis, etc., are known to be caused by protein aggregation. Understanding the mechanistic details of protein aggregation is extremely significant not only for biopharmaceutical/biotechnological industries, but also in understanding more than 40 neurodegenerative diseases.

Bovine spongiform encephalopathy (BSE) is the infectious spongiform encephalopathy or prion disease of domestic cattle. The BSE prion is a contagious agent and causes variant Creutzfeldt−Jakob disease (vCJD) in humans after dietary exposure. Aggregates of prion protein (PrP) are the pathological hallmark of prion disease. Here, we demonstrate through simulations that how the aggregating fragment of sheep prion protein gets influenced in the presence of co-solvent and how the co-solvent shifts the compact ensembles of amyloidogenic protein to an extended one, thus affecting the formation of aggregates. These results lead to a considerable insight into aggregation of amyloidogenic proteins.

The most common degradation pathway for protein is protein aggregation. Aggregation leads to a decrease in efficacy of protein drugs and could elicit an immunological response. The prion protein (PrP) is a naturally occurring polypeptide responsible for pathological states in fatal transmissible spongiform conditions, such as Creutzfeldt−Jakob disease and bovine spongiform encephalopathy. Many studies suggest that not whole protein is responsible for determining its aggregation tendency. It seems that protein aggregation is mediated by short “aggregation-prone” peptide segments. These aggregation-prone regions of protein can be detected by utilizing bioinformatics prediction tools based on physiochemical principles (phenomenological models) or molecular simulation approaches. In a previous experimental study, four amyloidogenic peptide fragments of Syrian hamster PrP are identified, viz., 109−122, 113−127, 178−191, and 202−218. Hence, in the present study, we have identified aggregation-prone fragments in the sheep prion protein (Protein Data Bank (PDB) ID: 1UW3) using aggregation servers such as TANGO, AGGRESCAN, and PASTA. We found a common fragment 179CVNITV184 of sheep prion protein as aggregation-prone fragment in the three servers used. For supporting the above-mentioned bioinformatics tools, we have also used Zipper DB, a database that is basically designed for hexapeptide causing aggregation within protein. According to Zipper DB database criteria, hexapeptides forming aggregates must have a Rosetta (scoring function) energy $< -23$ kcal/mol, and composite score (a score combining Rosetta energy, shape complementary, and area of interface) must have the lowest value among all fragments of protein. We found the CVNITV fragment to have a Rosetta energy of $-28.20$ kcal/mol and a composite score of $-46.52$, which is the lowest among all of the fragments of protein.

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sheep prion protein. Surprisingly, this result matches with the experimentally identified aggregation-prone sequence in Syrian hamster prion protein for its aggregation and it is the conserved sequence among these two proteins except one, i.e., valine replaced by isoleucine.

Therefore, to get further insights into the protein aggregation mechanism of this hexapeptide, we performed molecular dynamics (MD) simulation in water and in the presence of osmolytes such as urea, arginine, and ethanol.

Urea has been known to be a strong denaturant for proteins and widely used in protein unfolding or refolding in vitro experiments. Because of the stronger dispersion interaction between urea and protein over urea and water, urea has a stronger denaturing power. Globular native proteins generally fully or partially unfold in the presence of urea and adopt more extended structure. A similar behavior is observed for small unstructured polypeptides. On the other hand, to form β-sheets, protein–protein interaction must be larger than hydrogen bond interaction formed between urea and protein, which slows down the aggregation process in urea. The two different behaviors of urea indicate that it can affect the aggregation in a nonmonotonic way.

Further, an experimental study was performed by Dobson and co-worker’s, where the effect of urea on aggregation of globular protein β-lactoglobulin has been shown. They observed a nonmonotonic behavior of urea with change in concentration for the protein aggregation, where an elaborate balance is achieved between promoting aggregation-prone monomer and inhibiting interpeptide interaction. Hence, it becomes more interesting to investigate whether this nonmonotonic behavior of urea exists for aggregation of this small hexapeptide.

L-Arginine is found to be one of the most commonly used aggregation suppressors of protein and hormones in vitro. In spite of having a series of progress with arginine and its derivatives as an aggregation suppressor, the mechanism of its action is yet to be identified. Arginine shows the best result in preventing the aggregate formation by lysozyme, which is used as a model protein under thermal stress and refolding pathway from previously denatured state in comparison to other 14 amino acids.

Shiraki and co-workers also reported preventive action of arginine toward aggregation with other eight kinds of proteins. Suppressive behavior of arginine toward aggregation was found to be independent of the size/or isoelectric point of proteins. With only minor effect on protein stability, arginine does not facilitate refolding, but suppresses aggregation, while the solubility of aggregation-prone molecule is enhanced. Different mechanisms have been put forward to understand the preventive role of arginine in protein aggregation. Therefore, we have chosen to investigate the aggregation behavior of this hexapeptide in the presence of arginine.

Certain structural changes in protein are also observed in ethanol–water solution, and the degree of aggregation is found to be dependent on the concentration of alcohol used. Ethanol is reported to induce protein aggregation with increase in concentration in experimental results.

In this work, we have selected the hexapeptide of prion sheep protein and performed the MD simulation of octamer of this fragment in water, which forms molecular aggregates. The effects of osmolytes on these fragments were monitored at varying concentrations.

Here, we report the effect of external agents on the aggregation-prone sequence in Syrian hamster prion protein conformation: (a) urea, an osmolyte used to denature globular proteins through presumed perturbations in peptide hydrogen bonding; (b) l-arginine, an osmolyte which has been widely used to suppress protein aggregation; and (c) ethanol, an osmolyte used to induce protein aggregation (depends on the type of protein and the concentration of ethanol). The effect of these agents on the aggregation-prone sequence in Syrian hamster prion protein is studied using standard MD simulation at an isotropic pressure of 1 bar and 300 K temperature. The octamer simulation is expected to show early onset of aggregation as the monomer simulation does not lead to the formation of β-sheets (Figure S1). The primary purpose of this study is to see the structural changes taking place during aggregation and the influence of osmolyte concentrations on aggregation, which could be helpful in giving insights into the mechanistic details of the aggregation of proteins.

## RESULTS AND DISCUSSION

**Prediction of Aggregation-Prone Regions of Protein by Aggregation Prediction Servers.** Several prediction algorithms have been developed depending on the physicochemical and biochemical properties of the amino acids to predict amyloidogenic propensity from the polypeptide sequence. We have used three servers, i.e., TANGO, AGGRESCAN, and PASTA2.0, for prediction of aggregation-prone sequence in sheep prion protein (PDB ID: 1UW3). TANGO, a statistical mechanics algorithm, was developed by Fernandez-Escamilla et al. to predict the β-sheet aggregation of proteins, which is different from amyloid fibril formation tendency, but is highly correlated. Conchillo-Solé et al. developed AGGRESCAN, a natural amino acids aggregation propensity scale derived from in vivo experiments. PASTA designed by Trovato et al. looked for potential β-strand pairs. More details about all of the three servers are given in the Supporting Information. The results obtained from these bioinformatics servers were further validated by protein database tool Zipper DB designed for prediction of aggregation-prone hexapeptides.

We have used these servers on prion sheep protein and found different aggregation-prone sequences of this protein. Only single amino acid sequence, i.e., 179CVNITV_{184} was common among the predicted aggregation-prone sequence of these three servers (Figure 1). This common aggregation sequence obtained was in conformity to the experimental finding on Syrian hamster prion protein.

In Syrian hamster prion protein (PDB ID: 1B10), several fragments have been observed, which form amyloid fibrils, and these fragments include residues 109–122, 113–127, 178–191, and 202–218. Therefore, one of the predicted aggregation-prone sequences from the server matches with the experimental result. The multiple sequence alignment has been done using PRALINE, where valine is replaced by isoleucine, which is another hydrophobic residue in the predicted sequence (Figure 2).

We used this fragment, i.e., CVNITV, for our further analysis.

Standard molecular dynamics simulations were performed on eight fragments of 179CVNITV_{184} of sheep prion protein in the presence of water, urea, l-arginine, and ethanol. System parameters for various simulations of octamer of CVNITV of sheep prion protein are given in Table 1. Figure 3 summarizes...
simulation results in water on the octamer of prion fragment CVNITV.

After simulation, several analyses were performed. First, stability of the protein during the simulation was checked using the DSSP program, which shows the evolution of secondary structure with time, as described by Kabsch and Sander rule. The $\beta$-sheet contents of prion octamer in different osmolytes at various concentrations have been calculated using the DSSP program (Figure 4). It has been observed that the extent of $\beta$-sheet structure increases with the passage of time in water. But a decrease in $\beta$-sheet content is observed at 3 and 8 M urea concentrations. Expectedly at 5 M urea concentration, the $\beta$-sheet content increases. This shows the nonmonotonic behavior of urea in peptide aggregation (Figure 4A). In case of arginine, the $\beta$-sheet content goes on decreasing with increase in concentration (Figure 4B). But when the simulation is performed in ethanol, the extent of $\beta$-sheet structure increases with increase in ethanol concentration (Figure 4C), which indicates that ethanol favors $\beta$-sheet formation with increase in concentration.

**Principal Component Analysis (PCA) and Free-Energy Landscapes (FELs).** Principal component analysis identifies significant motion contributing to the overall dynamics of protein during the entire simulation period. Here, protein dynamics is plotted for octamer of prion protein in pure water and in different osmolytes at different concentrations, i.e., urea (3, 5, 8 M), arginine (0.4, 0.8, 1.2 M), and ethanol (4, 8, 12 M) in a frame called covariance matrix. Any pronounced conformational deviation from the initial structure of protein was captured by first two PCs. FEL analysis of C$\alpha$ atom on first two PCs was computed along the peptide backbone. Projection of two-dimensional free-energy surfaces for oligomers was done onto the first two principal components of C$\alpha$ atoms. To get the diagonalized eigenvectors and eigenvalues, covariance matrix was built on all of the C$\alpha$ atoms using the g_covar command of gromacs utility, which denotes the directions of motion and mean fluctuations, respectively. From the equilibrated portion of trajectories, the essential degree of motion of all of the proteins were obtained to explore the dynamic difference by projecting the motion of C$\alpha$ atoms onto PCs (PC1 and PC2). The cosine content of principal components was also calculated (Table 2) to validate our simulation sampling. The value of cosine content lies between 0 and 1. The value of cosine content closer to 0 is a good indicator of convergence and shows good sampling.

Cosine content can be calculated by the formula given below (eq 1):

$$C_i = \frac{2}{t_{tot}} \left( \int_0^{t_{tot}} \cos \left( \int_0^{t} p_i(t') dt' \right)^2 \left( \int_0^{t_{tot}} p_i(t') dt \right)^{-1} \right)$$

where $t$ is the instantaneous time, $t_{tot}$ is the total simulation time, and $p_i(t)$ is the $i$th principal component at time $t$.

Further two-dimensional plot of free-energy landscape is made by using g_anaeig and g_sham commands in gromacs. To understand the dynamics of oligomer in different osmolytes with different concentrations, the conformational space occupied by oligomer in each osmolyte for the first two principal components (PC1 and PC2) was analyzed. The different conformation of peptides in each osmolyte shows that the extent of sampling varies a lot in each osmolyte (Figure 5). The cosine content of the first PCs was also calculated to check the quality of analysis, as this analysis excludes the random diffusion of atoms interpreted as correlated motion. Conformational changes taking place during the MD simulation were also provided by the cosine content. Cosine contents calculated for oligomer in all osmolytes at all concentrations were found to be closer to zero (Table 2), which confirms actual conformational transitions during the simulation.

**Figure 2.** Multiple sequence alignment of Syrian Hamster prion protein and Sheep prion protein. The rectangular box highlights the common sequence (except isoleucine in 1B10) in the two proteins.
Among the peptides decreases and favors antiparallel concentration, it was observed that the extent of denaturation suppression of aggregation. But at moderate 5 M urea, in loss of secondary structure is predominant, causing urea. At higher urea concentration, the denaturation resulting and peptides become more extended compared to 3 and 5 M simulation in 8 M urea, the oligomers become less compact and minima were observed at 68 and 71 ns. During this period of and 73 ns, respectively, but at 8 M urea concentration, two minima were spotted at 71 and 73 ns. Hence, the PCA analysis showed that the dynamics of peptide during the MD simulation is governed by large-scale collective motions of oligomer in different osmolytes at different concentrations. The structural properties of peptides can be extracted in terms of FELs from our simulation in the case of each osmolyte at different concentrations. The area of FEL is an estimate of the conformational dynamics traversed during simulation. It was observed from FEL that, with increasing concentration of osmolytes (urea and arginine), conformational space occupied by peptides goes on increasing, which indicates relatively more dynamic behavior at higher concentration, but in the case of ethanol, conformational space occupied by peptides decreases with increase in the concentration of ethanol, thus reducing the dynamics of peptides. This might facilitate the formation of more β-sheets in the case of ethanol (Figure 5), as confirmed by the DSSP plot of ethanol (Figure 4C).

Table 1. Conditions for Various MD Simulations of Identified Fragment of Sheep Prion protein

| s. no. | protein PDB:1UW3 | MD time (ns) | no. of water molecules | no. of osmolytes | type | ensemble |
|-------|-----------------|--------------|------------------------|------------------|------|----------|
| 1     | 179CVNITV184    | 100          | 3882                   | 0                | standard MD | NPT      |
| 2     | 179CVNITV184    | 100          | 5295                   | urea (3 M) 356   | standard MD | NPT      |
| 3     | 179CVNITV184    | 100          | 4192                   | urea (5 M) 518   | standard MD | NPT      |
| 4     | 179CVNITV184    | 100          | 3202                   | urea (8 M) 619   | standard MD | NPT      |
| 5     | 179CVNITV184    | 100          | 3778                   | arginine (0.4 M) 34 | standard MD | NPT      |
| 6     | 179CVNITV184    | 100          | 3333                   | arginine (0.8 M) 60 | standard MD | NPT      |
| 7     | 179CVNITV184    | 100          | 2963                   | arginine (1.2 M) 80 | standard MD | NPT      |
| 8     | 179CVNITV184    | 100          | 5633                   | ethanol (4 M) 407 | standard MD | NPT      |
| 9     | 179CVNITV184    | 100          | 4200                   | ethanol (8 M) 895 | standard MD | NPT      |
| 10    | 179CVNITV184    | 100          | 2827                   | ethanol (12 M) 1337 | standard MD | NPT      |

Figure 3. Overview of the contributions of atomistic simulation to study the protein aggregation.

The area of FEL is an estimate of the conformational dynamics traversed during simulation. It was observed from FEL that, with increasing concentration of osmolytes (urea and arginine), conformational space occupied by peptides goes on increasing, which indicates relatively more dynamic behavior at higher concentration, but in the case of ethanol, conformational space occupied by peptides decreases with increase in the concentration of ethanol, thus reducing the dynamics of peptides. This might facilitate the formation of more β-sheets in the case of ethanol (Figure 5), as confirmed by the DSSP plot of ethanol (Figure 4C).

Hence, the PCA analysis showed that the dynamics of peptide during the MD simulation is governed by large-scale collective motions of oligomer in different osmolytes at different concentrations. The structural properties of peptides can be extracted in terms of FELs from our simulation in the case of each osmolyte at different concentrations.

Subsequently, from these FEL, lowest energy conformations were obtained at each osmolyte concentration (Figure 5). In water, a single minimum is spotted with a small region of free-energy surface, and the corresponding structure showed two new antiparallel β-sheets at 57 ns during simulation. The simulations in urea, arginine, and ethanol showed significant conformational changes in the octamer structure. At 3 and 5 M urea concentrations, only a single minima was spotted at 71 and 73 ns, respectively, but at 8 M urea concentration, two minima were observed at 68 and 71 ns. During this period of simulation in 8 M urea, the oligomers become less compact and peptides become more extended compared to 3 and 5 M urea. At higher urea concentration, the denaturation resulting in loss of secondary structure is predominant, causing suppression of aggregation. But at moderate 5 M urea concentration, it was observed that the extent of denaturation among the peptides decreases and favors antiparallel β-sheet formation (Figure 5C). Overall, we observed that increasing urea concentration can suppress the aggregation of CVNITV peptides, but a nonmonotonic behavior is observed at a moderate concentration of urea, i.e., 5 M.

Similarly, in the case of arginine solution, complete inhibition of β-sheet formation was observed compared to oligomers in water (Figure 5A,E,F,G). This is expected as arginine is known to be an antiaggregation osmolyte. At 1.2 M arginine concentration, four minima were spotted in FEL, occupying the larger surface area compared to other arginine concentrations. The corresponding structures to these minima show that peptide gets farther apart from each other during the simulation time.

In the case of ethanol solution, antiparallel β-sheet content of peptide increases with increase in ethanol concentration, and at 12 M ethanol concentration, seven out of eight peptides form the β-sheet structure (Figure 5H–J).

Overall, we concluded that urea and arginine can suppress the aggregation of CVNITV peptides and ethanol favors the formation of β-sheet and cause aggregation of protein. The above results were also confirmed with the calculation of β-sheet content plot obtained from the DSSP program of gromacs (Figure 4), the number of hydrogen bonds formed between all peptide pairs (Figure 6), and radius of gyration, which shows the compactness of peptides during the simulation periods (Figure 7).

Osmolyte Effect on CVNITV Peptide Aggregation.
The effect of osmolytes on the aggregation of CVNITV peptides was evaluated in terms of the number of hydrogen bonds formed between all peptide pairs, radius of gyration for the whole oligomer, and β-sheet structure content obtained from the DSSP program. For each osmolyte at all concentrations, three quantities were recorded with the calculation of β-sheet content plot obtained from the DSSP program of gromacs (Figure 4). The number of hydrogen bonds formed between all peptide pairs (Figure 6), and radius of gyration, which shows the compactness of peptides during the simulation periods (Figure 7).

Osmolyte E
The average number of hydrogen bonds between all pairs of peptides is 1.032 in pure water, and it decreases to 0.582 in urea 3 M. Interestingly, the average number of hydrogen bonds then slowly increases to 0.758 at the urea concentration of 5 M (Figure 6A). With further increase in the concentration of urea, the average number of hydrogen bonds again decreases to 0.541 in 8 M urea. An increase in the number of hydrogen bonds observed at 5 M urea shows the possibility of a slight enhancement for CVNITV aggregation at this concentration.

With the addition of arginine in water, the average number of hydrogen bonds between all peptide pairs sharply decreases from 1.032 to 0.321 from pure water to arginine 0.4 M.
solution. And this number continuously decreases from 0.211 to 0.20 with increase in the concentration of arginine from 0.8 to 1.2 M, respectively (Figure 6B).

In the case of ethanol, at 4 M ethanol concentration, the average number of total hydrogen bonds formed between all peptide pairs is found to be 0.716. With further increase in the
concentration of ethanol, this number increases to 0.89 and 0.97 at 8 and 12 M ethanol concentrations, respectively (Figure 6C). The average number of hydrogen bonds is less than that of pure water, but more $\beta$-sheets are formed in the case of ethanol. Hence, we calculated the average number of interpeptide hydrogen bonds for peptides that are getting converted to $\beta$-sheet (see Table S2). In the case of water, this value is 0.910, while in the case of ethanol, the values are 1.004, 1.051, and 1.131 for 4, 8, and 12 M ethanol, respectively. This suggests that with increase in the concentration of ethanol, the enhancement in aggregation of CVNITV peptides occurs, which is in good agreement with the PCA result discussed earlier.

The radius of gyration has been calculated to ascertain the compactness of the oligomers. Increasing radius of gyration is an indication of swelling in oligomers. The average radius of gyration increases from 2.353 nm in pure water to 2.4 nm in 3 M urea, and this slight increase in the radius of gyration might be because of the destabilization of $\beta$-sheet, as shown in PCA results. At 5 M urea, it decreases to 2.202 nm, which is again because of the small enhancement of the $\beta$-sheet content. When the urea concentration is further increased to 8 M, the radius of gyration sharply increases to 2.59 nm, which suggests that oligomer is swelled and $\beta$-sheet is disfavored. The decrease in the number of hydrogen bonds formed between all pairs of peptide and the increase in the radius of gyration with increase in concentration suggest that swelling of peptides is linked with increase in the extent of urea−backbone interactions. Similarly, the average radii of gyration of oligomer in arginine and ethanol are also calculated (Figure 7B,C). With increase in arginine concentration, the average radius of gyration increases from 2.35 nm in pure water to 2.751 nm in arginine 0.4 M, 2.805 nm in arginine 0.8 M, and 2.875 in arginine 1.2 M (Figure 7B), which indicates continuous extension and suppressed aggregation of CVNITV peptide. In the case of 4 M ethanol, the radius of gyration is 2.05 nm, which is less than that of water, i.e., 2.352 nm, indicating favored $\beta$-sheet formation at this concentration. With further increase in the concentration of ethanol, the radius of gyration further decreases from 2.03 nm at 8 M to 1.44 nm at 12 M ethanol concentration (Figure 7C). This suggests that higher ethanol concentration favors the $\beta$-sheet formation. Thus, we conclude that ethanol acts as protein-stabilizing osmolyte and favors aggregation. These radii of gyration results are further confirmed by our calculated distance of each peptide from center of mass with time (Figure S2). We observed that in water, all of the peptide distances converge with time since $\beta$-sheet content increases with simulation time. At 3 and 8 M urea concentrations, peptide distances from the center of mass increase with time as the unfolding of peptides from helix to coil occurs, while at 5 M urea concentration, only few peptide distances converge with time as these peptides at this urea concentration
concentration transform to \(\beta\)-sheet. In the presence of arginine as co-solvent, all of the peptide distances from the center of mass increases with time as well as concentration. This is because with increase in concentration of arginine, all of the peptides adopt open conformations. Contrary to urea and arginine, since ethanol shows maximum \(\beta\)-sheet aggregation among these simulations, the distances from the center of mass of each peptide converges with time at each concentration (Figure S2).

In summary, the number of hydrogen bonds formed between all peptide pairs; the radius of gyration; the \(\beta\)-sheet content obtained from DSSP; the number of hydrogen bonds formed between protein and protein, protein and water (PW), and protein and osmolyte (PO); and PCA analysis are in excellent agreement. Each quantity explains the nonmonotonic function of urea concentration, arginine as best aggregation suppressor, and ethanol as best aggregation enhancer for this peptide.

Dynamics of Water Molecules in the Presence of Osmolytes. It is known that thin-layer water around protein plays a significant role during the folding of protein. This thin layer forms a hydration shell of a few nanometers around the proteins. Since protein contains both hydrophilic and hydrophobic residues, this hydration shell water causes specific arrangement of these residues; therefore, hydrophobic residues are buried inside the protein core and hydrophilic residues are exposed to water on the surface.\(^{45,50}\) Hence, it was of interest to investigate water dynamics and its role in peptide aggregation in terms of radial distribution function (RDF) and tetrahedral order parameter. The time correlation function for protein–water (\(G_{pw}(t)\)) and protein–osmolyte (\(G_{po}(t)\)) hydrogen bonds formed by the water molecules and osmolytes present in the first hydration layer was also calculated.

We have calculated the radial distribution function of water and osmolytes around protein during the simulation. In pure water simulations, the radial distribution function (RDF) of water plotted in Figure 8A approaches 1 around a radial distance of 2 nm. In urea (3, 5, and 8 M), arginine (0.4, 0.8, and 1.2 M), and ethanol (4, 8, and 12 M) simulations, the RDFs of water and osmolytes with respect to protein are significantly different. We observed substitution of water molecules with urea in the vicinity of the protein to be directly proportional to the urea concentration. At 3 M urea concentration, the water RDF value reaches to 1 at a radial distance of \(\sim 2.3\) nm, while in pure water, the RDF value reaches 1 at a radial distance of 2 nm. As the concentration of urea is further increased, this gradual substitution of water in the vicinity of protein results in approximately similar radial distribution function to protein solvated in pure water (Figure 8B). Also, the distribution of urea around protein continuously increases with increasing concentration and its RDF value reaches 1 only at radial distances of 0.5, 0.6, and 0.35 nm at 3, 5, and 8 M urea concentrations, respectively, which shows replacement of water molecule from protein surface, and thus interaction of urea with protein increases, whereas a non-monotonic behavior is observed at 5 M urea concentration. An increase in the interaction of urea with protein leads to extended conformation of protein and thus inhibits \(\beta\)-sheet formation. First, the hydration peak of water in the presence of urea and in pure water is found to be almost similar, but the first hydration peak of urea has greater altitude than water, which shows larger interaction of urea with protein than water.

In the case of simulation of protein with arginine (0.4, 0.8, 1.2 M), radial distribution of water molecule around protein approaches 1 at a distance of 2.4 nm compared to the case of water, which reaches 1 at a distance of 2 nm (Figure 8C). RDF of arginine reaches 1 only at a radial distance of 0.3 nm and also its RDF value increases with increase in concentration (Figure 8C). Thus, more water molecules are replaced by arginine from the surface of protein compared to urea. The altitude of the first hydration peak of arginine is much higher than that of water, which shows more interaction of arginine...
molecule with protein compared to water. This indicates that accumulation of arginine molecules around protein is relatively higher compared to urea. The differential β-sheet perturbation of protein in arginine and urea solvation as observed in PCA analysis might be because of this unequal distribution of water in urea and arginine solvation.

The RDF value of water in ethanol with respect to protein at 4 and 8 M ethanol concentrations reaches 1 at a radial distance of ~3 nm. The RDF value of water around protein at 12 M ethanol concentration reaches 1 only at a radial distance of 0.68 nm compared to that of water at 2 nm in the case of simulation of protein with water only (Figure 8D). At 12 M ethanol concentration, distribution of water molecule around protein is larger than that at 4 M ethanol concentration (Figure 8D). It should be noted that as the concentration of ethanol molecule increases, the chance of finding water molecule near ethanol molecule gets reduced compared to finding water molecule near protein. As obvious from the RDF of ethanol (Figure 8D), there is no distinct first hydration peak present as in the case of other osmolytes, which shows that no replacement of water molecule is observed by ethanol molecule in the first hydration peak. For this reason the interaction of water with protein in the presence of ethanol is higher compared to other osmolytes. This suggests the possibility that the ethanol molecules tend to form clusters (see Figure S3) and it was further confirmed using g_clustsize of the gromacs utility. For these calculations, the cluster was defined as all molecules of ethanol, which fall within 0.35 nm distance from each other. Such ethanol cluster formation is also reported in previous studies.51

The average number of protein–water and protein–urea hydrogen bonds was calculated, and because of the accumulation of urea around the protein, the number of protein–water hydrogen bonds decreases with increase in the concentration of urea (Figure 9A). The number of protein–arginine hydrogen bonds increases with increase in the concentration of arginine, but compared to urea, protein–arginine hydrogen bond is much less in number (Figure 9B). Contrary to these, in the case of ethanol solvation (4, 8, 12 M), the number of protein–water hydrogen bonds first decreases up to 8 M ethanol concentration and then increases at 12 M ethanol concentration (Figure 9C). This indicates that the hydrogen bonding pattern of protein–water in the case of ethanol is concentration-dependent and shows the signature of observed anomalous behavior.52 We did not observe ethanol–protein hydrogen bond during ethanol solvation. Perhaps this is responsible for enhanced β-sheet formation in this solvation as observed in the β-sheet content obtained from DSSP and also with FEL plot. Figure 9C shows that no hydrogen bond is formed between protein and ethanol, which also supports the formation of cluster result. Thus, ethanol acts as a protein-stabilizing osmolyte.

The presence of high concentration of urea and arginine strongly affects intra- and intermolecular interactions. Increase in the concentration of urea and arginine considerably affects the average number of hydrogen bonds (Table 3), and this change occurs uniformly, i.e., protein–water hydrogen bond decreases with increase in the concentrations of urea and arginine, protein–arginine hydrogen bond increases, and in the case of ethanol, protein–water hydrogen bond decreases up to 8 M and then increases at 12 M ethanol concentration, but no hydrogen bond is observed between protein and ethanol (see Table 3 and Figure 9). Contribution of the average number of hydrogen bonds formed between protein and water in different osmolytes is given in Table S3.

Furthermore, at each concentration of urea and arginine, the number of protein–urea and protein–arginine hydrogen bonds increases with time but no protein–ethanol hydrogen bond is formed in ethanol system during the simulation (Figure 9C), which shows cluster formation by ethanol. Although protein–water hydrogen bond decreases with increase in ethanol concentration, protein–protein hydrogen bond increases with increase in concentration, whereas in other osmolytes, protein–protein hydrogen bond decreases with increase in concentration (Figure 9). These results suggest that the formation of more β-sheets occurs with increase in ethanol concentration, which is in good agreement with the result obtained from DSSP and FEL plot.

It is seen from the above result that presence of osmolytes in protein–water medium alters the regular protein–water hydrogen bond due to the formation of protein–osmolyte hydrogen bond at the surface.

**Table 3. Average Number of Hydrogen Bonds Formed between Protein and Protein, Protein and Osmolytes, and Protein and Water**

| conc. (M) | protein–protein | protein–osmolyte | protein–water |
|----------|-----------------|-----------------|---------------|
| water    | 28.231          | 0.000           | 113.857       |
| urea     | 3 18.620        | 46.971          | 89.447        |
|          | 5 21.420        | 59.173          | 70.602        |
|          | 8 16.309        | 75.182          | 69.580        |
| arginine | 0.4 15.078      | 24.815          | 96.869        |
|          | 0.8 9.509       | 28.540          | 92.568        |
|          | 1.2 9.393       | 34.940          | 91.664        |
| ethanol  | 4 25.234        | 0.000           | 113.270       |
|          | 8 31.938        | 0.000           | 96.689        |
|          | 12 32.990       | 0.000           | 111.548       |
To investigate the relaxation dynamics of these hydrogen bonds formed between protein and water (PW) and protein and osmolytes (PO) present in the first hydration shell, hydrogen bond time correlation function $C(t)$\textsuperscript{53,54} has been used. Two criteria can be used to define the hydrogen bond geometric or energetic. Here, we have used geometric criteria to define the hydrogen bond.\textsuperscript{53–57} $C(t)$ is defined as

$$C(t) = \frac{\langle h(0)h(t) \rangle}{\langle h(0)h(0) \rangle}$$  \hspace{1cm} (2)$$

where $h(t)$ is the hydrogen bond population function, whose value can be either 1 or 0. If a pair of hydrogen bond is formed at particular time, then $h(t) = 1$, otherwise it is 0. The angular bracket indicates the averaged overall hydrogen bond. $C(t)$ is independent of breaking and reformation of hydrogen bond between times 1 and 0. The overall hydrogen bond relaxation time can be extracted from the decay pattern of $C(t)$. Hydrogen bond time correlation functions are best fitted by stretched exponential function of time with stretching exponents ($\alpha$) and (D) ethanol 4 M (red), 8 M (green), 12 M (blue). Table 4. Hydrogen Bond Lifetime ($r'_{HB}$) Stretched Exponential ($n$), Calculated from $C(t)$ for PW and PO Hydrogen Bonds within the First Hydration Layer of Protein

| osmolytes     | protein–water (PW) | protein–osmolytes (PO) |
|---------------|---------------------|------------------------|
| water         | $r'_{HB}$ (ps)      | $n$                    | $r'_{HB}$ (ps) | $n$        |
| urea (3 M)    | 7.96 0.33           |                        | 44.15 0.33    |
| urea (5 M)    | 7.66 0.47           |                        | 25.57 0.33    |
| urea (8 M)    | 7.03 0.48           |                        | 36.59 0.36    |
| arginine (0.4 M) | 4.09 0.38        |                        | 3.89 0.20     |
| arginine (0.8 M) | 3.82 0.43        |                        | 4.95 0.20     |
| arginine (1.2 M) | 3.75 0.37        |                        | 5.13 0.20     |
| ethanol (4 M) | 9.23 0.40           |                        |               |
| ethanol (8 M) | 9.14 0.23           |                        |               |
| ethanol (12 M) | 9.08 0.35           |                        |               |

water molecules present in the first hydration layer of protein in pure water is 7.967 ps, and the $r'_{HB}$ values of water molecules at 3 and 8 M urea concentrations are lower than those of pure water, but they increase at moderate concentrations. The $r'_{HB}$ value of PO at 3 M urea concentration is 44.15 ps, at 5 M urea concentration, it decreases to 25.57 ps, and at 8 M urea concentration, it further increases to 36.59 ps, which shows the nonmonotonic behavior of urea at moderate concentration (Table 4).

In the case of arginine, $r'_{HB}$ of PW continuously decreases with increase in the concentration of arginine and $r'_{HB}$ of protein–arginine increases with increase in the concentration of arginine (Table 4).

In the case of ethanol, since no hydrogen bond is formed between protein and ethanol, $r'_{HB}$ of protein–ethanol is found to be 0 and $r'_{HB}$ of PW in ethanol continuously increases with increase in ethanol concentration, which is in agreement with hydrogen bond results. To get further insights into the above results, interaction energy as a function of time between protein and protein and protein and co-solvent has been calculated in each osmolyte at various concentrations and is plotted in Figure S4. More negative value of interaction energy shows greater interaction. Protein–protein interaction in urea decreases with increase in the concentration of urea, but a slight increase in interaction is observed at 5 M urea (Figure S4). However, protein–urea interaction continuously increases with increase in concentration (Figure S4). In the case of arginine, protein–protein interaction continuously decreases and protein–arginine interaction continuously increases with increasing arginine concentration (Figure S4). In the case of ethanol, protein–protein interaction increases with concentration, but protein–ethanol interaction remains constant at each concentration of ethanol and almost close to 0 (Figure S4), which supports the result that no hydrogen bond formation occurs between protein and ethanol. Data of interaction energy between protein and protein and protein and co-solvent at various concentrations are provided in Table S4.

The reason for the nonmonotonic behavior of urea as reported earlier is the selective accumulation of urea for different amino acids.\textsuperscript{38,59} Radial distribution functions of urea around residues VAL, ILE, and ASN as representative amino acid of hydrophobic and hydrophilic groups are plotted, and it is found that distribution of urea around hydrophobic residues

![Figure 10. Hydrogen bond correlation function of water molecules present in the first hydration layer of protein at various osmolyte concentrations: (A) water (black), (B) urea 3 M (red), 5 M (green), 8 M (blue), (C) arginine 0.4 M (red), 0.8 M (green), 1.2 M (blue), and (D) ethanol 4 M (red), 8 M (green), 12 M (blue). $C(t)$ of osmolytes present in the first hydration layer of protein: (E) urea 3 M (red), 5 M (green), 8 M (blue) and (F) arginine 0.4 M (red), 0.8 M (green) and 1.2 M (blue).]
Concentrations interaction energy of protein−protein interaction energy, respectively. Table 5 shows the result for Lennard-Jones and Coulomb interactions to the overall osmolytes, and protein with water. Total noncovalent the interaction energy of protein with protein, protein with protein, protein with osmolyte, and protein with water. For this, we calculate the nonbonded interaction energy is described by eqs 3−5. The negative value of interaction energy E shows favorable driving force toward unfolding.

\[
E_{\text{PP}} = E_{\text{PP}}^{\text{LJ}} + E_{\text{PP}}^{\text{CB}}
\]

\[
E_{\text{PO}} = E_{\text{PO}}^{\text{LJ}} + E_{\text{PO}}^{\text{CB}}
\]

\[
E_{\text{PW}} = E_{\text{PW}}^{\text{LJ}} + E_{\text{PW}}^{\text{CB}}
\]

where \(E_{\text{PP}}\) is the interaction energy of protein−protein, \(E_{\text{PO}}\) is the interaction energy of protein−osmolyte, and \(E_{\text{PW}}\) is the interaction energy of protein−water. LJ and CB indicate Lennard-Jones and Coulomb interactions to the overall interaction energy, respectively. Table 5 shows the result for the interaction energy of protein with protein, protein with osmolytes, and protein with water. Total noncovalent interaction of the system is also calculated (Table 5). We find that with increase in the concentration of urea, interaction of urea with unfolded conformation of protein continuously increases, as indicated by the negative value of \(E_{\text{PU}}\), but a slight increase in the value of \(E_{\text{PU}}\) is observed at 5 M urea concentration, which indicates the formation of β-sheet. In the case of arginine also, interaction of arginine with protein continuously increases with increase in concentration, as indicated by the negative value of \(E_{\text{PA}}\). The more negative value of \(E_{\text{PA}}\) shows suppression of protein aggregation due to increase in protein−arginine interaction and decrease in protein−protein interaction, as shown by the more positive value of \(E_{\text{PU}}\) in the presence of arginine. In the case of ethanol, protein−ethanol interaction continuously decreases and protein−protein interaction continuously increases with increase in the concentration of ethanol, as shown by increasing value of \(E_{\text{PE}}\) and decreasing value of \(E_{\text{PP}}\) (Table 5). This indicates that ethanol favors protein aggregation and urea and arginine suppress protein aggregation. These results are in good agreement with results obtained from the calculation of the number of hydrogen bonds between protein and protein, protein and osmolyte, and protein and water (Figure 9). Total noncovalent interaction between protein and protein in different osmolytes as a function of simulation time is plotted (Figure S6). Total noncovalent interaction of the system versus osmolytes concentration is also plotted (Figure 11), which shows a variation similar to that shown by hydrogen bonds in each osmolyte.

**Tetrahedral Order Parameter.** Owing to its greater significance, orientational tetrahedral order parameters (\(q\)) are more widely investigated compared to other order parameters. It was first given by Chau and Hardwick and further modified by Errington and Debenedetti.60 Basically, \(q_{\text{tet}}\) is used to define the local structure of water molecule. This refers to a central water molecule surrounded by four water molecules in the first shell, and it can be calculated as
Figure 12. Average tetrahedral order parameter of bulk water molecule as a function of osmolyte concentration.

\[ q_{tet} = 1 - \frac{3}{8} \sum_{j=1}^{\infty} \sum_{k=j+1}^{\infty} \left( \cos(\psi_{j,k}) + \frac{1}{3} \right)^2 \]  

(6)

where \( \psi_{j,k} \) is the angle formed by the central oxygen atom with \( r_{ij} \) and \( r_{ik} \) bond vectors of its four nearest neighbor atoms \( j \) and \( k \). The value of \( q_{tet} \) is 1 for a perfect tetrahedral arrangement and 0 for random arrangement. Average tetrahedral order parameter has been calculated considering only water molecules as nearest neighbors as a function of osmolyte concentration to see the effect of osmolytes. The calculation of tetrahedral order parameters is done by taking mean over all molecules and also over all ensembles following equation

\[ \langle q \rangle = \frac{1}{N} \sum_{i=1}^{N} q_i \]  

(7)

where \( N \) is the total number of molecules.

Tetrahedral order parameters are frequently used in analyzing the water structure, and can be calculated from the angles made by any two of the nearest water molecules to the central water molecule. To investigate the effect of osmolytes on water structure, the average tetrahedral order parameter of the bulk water has been calculated using eqs 6 and 7 as a function of osmolyte concentration. Figure 12 shows the variation of \( q_{tet} \) as a function of osmolyte concentration in different osmolytes.

Assuming the water structure to be tetrahedral, the tetrahedral order parameter provides a good measure of angular arrangements of the nearest neighbors around a central molecule. Any deviation of the tetrahedral order parameter of water in the presence of osmolytes indicates angular distortion in water structure. In such calculation, it is very important to choose nearest neighbors properly. Since in pure water, all of the neighbors are water molecules around central water, but in the binary mixture of osmolyte and water, first four neighbors can be water or osmolyte molecules. In this analysis, we calculated the tetrahedral order parameter by considering the water molecules as four nearest neighbors only (neglecting urea as a neighbor), as a function of osmolyte concentration. From Figure 12A, it has been found that in urea, the value of \( q_{tet} \) decreases as the concentration of urea increases. A similar trend was observed in the case of arginine and ethanol, as shown in Figure 12B,C, with increase in the concentration of osmolyte. A higher value of \( q_{tet} \) is found in the simulation of protein with pure water, and it is 0.588, which continuously decreases with addition of osmolytes with increasing concentration. The average tetrahedral order parameter of pure water is also calculated to be 0.597, which is in good agreement with the value obtained in the simulation of aggregation-causing peptides in pure water (i.e., 0.588), which indicates that water molecules are more ordered around aggregation-causing peptides and its tetrahedral order gets disturbed with the addition of osmolytes. The distribution of tetrahedral order parameter has also been plotted. The distribution \( (P(q)) \) of the tetrahedral order parameter, \( q \), shows the marked variation with increase in the concentration of osmolyte (see Figure S7).61 The distribution \( P(q) \) for pure water (blue line) has a peak at a \( q \) value of 0.588. With increase in the concentration of osmolytes, tetrahedral peaks in the distribution continuously decrease. A similar trend was also observed by Idrissi et al.,62 who concluded that this change is due to breaking in the tetrahedral structure of water molecule by osmolytes. On comparing the effects of urea, arginine, and ethanol on \( q_{tet} \) of water, we observe that the tetrahedral structure of water molecule is more perturbed in the presence of urea (Figure 12A). Since in the presence of ethanol, more \( \beta \)-sheets are formed, \( q_{tet} \) must approach the value of pure water, but because of the formation of cluster by ethanol with increase in concentration, distortion in water structure occurs, and thus the \( q_{tet} \) value decreases, but this decrease is smaller than that in the case of urea.

**CONCLUSIONS**

In this paper, we have reported the atomistic simulation study of the aggregation process of eight CVNITV hexapeptides in aqueous urea, aqueous arginine, and aqueous ethanol solution. To explore the effect of osmolytes, we have used urea, arginine, and ethanol, each with three different concentrations. The number of hydrogen bonds formed between all peptide pairs, radius of gyration, and \( \beta \)-sheet contents are calculated from MD simulation to describe the aggregation behavior.

Increase in the number of urea molecule in solution can suppress the peptide aggregation at 3 and 8 M urea concentrations, while a weak enhancement in secondary structure is observed at 5 M urea concentration. Increase in the number of arginine can suppress the protein aggregation evenly at all concentrations, which can be seen in the analysis of the number of hydrogen bonds, \( \beta \)-sheet content, and radius of gyration. Ethanol is found to be a protein-stabilizing osmolyte in our case and favors \( \beta \)-sheet formation with increase in concentration.

The competition among hydrogen bonds formed between all peptide pairs determines the aggregation process as well as the final structure of oligomer with time. The number of hydrogen bonds formed between protein and water, protein and urea, and protein and protein indicate that urea molecule removes the water molecule from the first solvation shell and gets accumulated around the protein, which leads to the increase in the number of hydrogen bonds between urea and protein with increase in concentration and thus hinders protein aggregation. However, the competition between protein–urea and protein–protein hydrogen bonds leads to a nonmonotonic behavior of CVNITV peptides at moderate urea concentration.
A similar behavior is shown by arginine on the number of hydrogen bonds at all concentrations, which depict the aggregation-suppressing nature of arginine by decreasing the interaction between protein and protein. Ethanol shows an opposite effect, i.e., it enhances the peptide aggregation by favoring the β-sheet formation, and also interaction between peptides increases with increase in the number of hydrogen bonds between peptides.

PCA is in good agreement with the above results, which depicts the aggregation inhibition behavior of urea and arginine and the protein-stabilizing effect of ethanol.

These results could be helpful in understanding the mechanistic details of aggregation of this peptide. Since prion protein aggregation is responsible for several diseases, investigating these aggregation processes is relevant for both control of the disease and developing therapeutics of disease. A complementary extension of the present study could be examining other aggregation-prone peptides of this protein as well as full protein in the presence of different osmolytes and water.

**METHODS**

Identification of aggregation-prone fragment of sheep prion protein has been done using three different aggregation prediction servers, i.e., PASTA, TANGO, and AGGRESCAN, and the results were further validated by using protein database tool Zipper DB designed for prediction of aggregation-prone hexapeptides.

MD Simulation Details. MD simulations of the octamer of identical fragment (179CVNITV184) of sheep prion protein in the presence of single point charge water model and different osmolytes like urea (3, 5, and 8 M concentrations), L-arginine (0.4, 0.8, and 1.2 M concentrations), and ethanol (4, 8, and 12 M concentrations) were performed at 300 K. The force field GROMOS96 53a6 was used during simulations. All simulations were performed using single-precision Gromacs-4.5.4 software. The PDB file for prion protein from RCSB Protein Data Bank was used for MD simulations. A cubical box (size, 6 × 6 × 6 nm³) of osmolytes with each concentration was built and equilibrated using the method of Rocco et al. The GROMOS96 53a6 force field has been used for each osmolyte during box generation. The topology file for urea and ethanol has been generated by Automated Topology Builder using GROMOS 53a6 force field. Initially, the eight fragments of CVNITV were inserted in the simulation box at a distance of 1 nm from each other using the genconf command of gromacs utilities. All of the peptides are allowed to diffuse toward each other under the influence of GROMOS96 53a6 force field. An isotropic force of 1000 kJ/mol nm² was used at all atoms during the steepest descent energy minimization. Thereafter, a two-step simulation was performed, with 100 ps of position-restrained MD using LINCS algorithm, followed by 100 ns of full-standard MD. All of the simulations were performed at 1 bar with a coupling constant of 0.5 ps for pressure and 0.1 ps for temperature, applying the Berendsen weak coupling algorithm in both cases.

The time step for integration algorithm was set at two femtoseconds to integrate Newton’s equation of motion using a leap frog algorithm. PME electrostatics was applied using a Lennard-Jones cutoff of 1.4 nm and a Coulomb cutoff of 0.9 nm with maximum spacing of 0.12 nm for the fast Fourier transform grid to reduce the computational complexity of molecular dynamic simulations. To palliate the system size effects, periodic boundary conditions were applied. All analyses were done ignoring the initial 10 ns trajectory of the simulation for equilibration and using gromacs utilities.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b00875.

Description of the aggregation prediction servers; hydrogen bond calculation; cluster formation by ethanol; radial distribution plot; interaction energy between protein and protein; tetrahedral order parameter distribution; and distance from center of mass of each peptides (PDF)

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**Notes**

The authors declare no competing financial interest.

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