TEMPERATURE EFFECTS ON SPINACH PLASTOCYANIN:
MOLECULAR DYNAMICS SIMULATION STUDY

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In this work, a molecular dynamics approach was used to study the thermal denaturation mechanism of spinach plastocyanin protein. Advantages of related molecular dynamics are also presented. The only significant change in Cα RMSD and Rg plot was seen at 453K. At this temperature, less stability for Met92 with respect to the other residues was found. A more flexible region was found for residues 40-56 at all temperatures.

In our simulation, three steady states, including native, intermediate and denatured states, as well as two transition states ensembles have been found at 453K. Secondary structure analysis have shown that the structure of the first transition state is more similar to that of the native state, while the second transition state is more similar to the denatured state.

Key words: copper protein, transition state, mechanism, molecular dynamics

INTRODUCTION

Denaturation is a process in which macromolecules like proteins lose their tertiary and secondary structure by application of either a chemical denaturant (a strong acid or base, a concentrated inorganic salt, urea) or a physical denaturant (heat and pressure). If proteins in a living cell are denatured, this results in disruption of cell activity and possibly cell death (1). The study of thermal denaturation is easier than that of the other mentioned factors involved in protein unfolding. At higher temperatures, the unfolding process of a protein occurs in a shorter time range, with no mechanism changes (2). So far, there have been several studies on the thermal denaturation of proteins (3-6). Plastocyanin (PC), also

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known as the blue copper (BC) protein, is a small soluble copper protein with a barrel-like structure. Plastocyanin from spinach has been studied by Sandberg and co-workers using Differential Scanning Calorimetry (DSC), absorbance spectroscopy and NMR measurements. One transition temperature (TM) of 344K has been detected by DSC and absorbance spectroscopy in 10mM phosphate buffer at pH=7. Sandberg et al. have also shown that the thermal unfolding of a reduced form of spinach plastocyanin is reversible under anaerobic condition and that the ratio of $\Delta H_{\text{dH}}$ to $\Delta H_{\text{fH}}$, which can be used to determine the occurrence of intermediate states during thermal unfolding, is 0.7; this ratio shows that the denaturation of spinach plastocyanin protein may proceed via intermediate states (7). Here, open questions are related to the analysis of the structural mechanism which underlies the denaturation process. The molecular dynamics technique allows us to simulate the protein under experimental conditions and derive information that is not clear in experimental approaches (8-12). To the best of our knowledge, no molecular dynamics study on thermal denaturation pathways of spinach plastocyanin has been done so far.

The aim of the present work is to investigate the effect of temperature on spinach plastocyanin. Another purpose is to determine the intermediate states of reduced spinach plastocyanin during the thermal denaturation process under anaerobic conditions, using alpha carbon RMSD, radius of gyration and distribution of radius of gyration at different temperatures, including those of below and above the transition temperature.

**MATERIAL AND METHODS**

The initial structure of reduced spinach plastocyanin is taken from the PDB (pdb entry: 1ylb). All calculations were carried out using gromacs4.0.5 package (13) and gromos96 (14-16) force field. Periodic boundary conditions were used to perform molecular dynamics simulation. A $5 \times 5 \times 5nm^3$ cubic simulation box containing 3500 of water molecules was defined by the simple point charges (SPC) model (17). Spinach plastocyanin protein is coordinated at the center of the box. Ions of $[\text{H}_2\text{PO}_4^-]$ and $[\text{HPO}_4^{2-}]$ were added to the simulation box to prepare a phosphate buffer solution with pH=7. To neutralize the system, 57 $\text{Na}^+$ ions were added to the box. A steepest-descent algorithm was performed to minimize the energy of the considered system and to relax water and phosphate ions.

Molecular dynamics simulation was carried out in two stages. In the first stage, a position-restrain simulation was conducted, in which the atoms of the protein molecule were held fixed, whereas water molecules and phosphate ions were free to move around, so that they would reach the equilibrium state. In the second stage, each system was simulated with a time step of 2 fs. LINCS algorithm
was employed to fix all bonds involving hydrogen and SETTLE algorithm in the case of water molecules (18). MD calculations have been done at six different temperatures: 300, 325, 344, 373, 413 and 453K.

Molecular dynamics simulation cannot replicate the protein folding/unfolding process, because this process typically occurs in one second. However, the most of molecular dynamics simulations have been done in nanosecond range of time. Based on literature survey (19-21), it is common to use high temperatures in molecular dynamics simulation to overcome the barrier between two distinct fold and unfold structures in the folding/unfolding process. Daggett has shown that to satisfy experimental evidence in the thermal unfolding process of chymotrypsin, the 94 nanosecond MD simulation in 300 K produced results similar to those provided by the 8 nanosecond MD simulation in 498 K. Also, given that the experimental melting point of spinach plastocyanin protein is 343 K, the above mentioned range of temperature was used in this study to include the protein Tm value and show the apparent changes in structural parameters in the span time of MD simulation used here.

To maintain a constant temperature and pressure for various components during simulations, the Berendsen coupling algorithm was used (22) for each component of the system with relaxation times of 0.1, and 0.5 ps, respectively. As the systems involve many positive and negative charges, the PME algorithm was applied to estimate electrostatic interactions. In this algorithm, each atom interacts with all the atoms from the simulation box and all of their images in an infinite number of identical copies surrounding the main box, which enabled us to obtain a satisfactory level of accuracy for the results of electrostatic interactions (23, 24).

Distribution of radius of gyration was used to determine five states at 453K. Radius of gyration distribution for native, intermediate and denatured structures can be fitted with one Gaussian-like curve. On the other hand, distribution of radius of gyration transition states can be fitted with two Gaussian-like curves. The existence of two Gaussian-like curves in Rg distribution curve simultaneously shows that there is a transition between two states (25).

RESULTS AND DISCUSSION

ROOT MEAN SQUARE DEVIATION (RMSD) ANALYSIS

Figure 1 shows Cα RMSD values in terms of the simulation time. There was no significant change in the values of Cα RMSD for 300, 325, 344, 373, and 413K. For 453K, the only significant change has been found in the region in which Cα RMSD changes from 0.25 to 0.39 between simulation times of 4 ns and 5.5 ns, and also from 0.36 to 0.25 between 12 and 13.5 ns.
The geometry of the copper binding site has an important role in the electron transfer process. Hence, RMSD with respect to position in crystal structure was plotted for ligands of copper ion (i.e., His37, His87, Met92, Cys 87). Figure 2 shows the plot of RMSD in terms of the simulation time. Because of the complexity shown in Fig. 2, statistical analysis, including minimum, maximum and standard deviation, was used to describe the RMSD plot.
Temperature effects on spinach plastocyanin

![Graphs showing temperature effects on spinach plastocyanin.](image_url)
Fig. 2. – RMS deviation of copper ligands.

The statistical analysis of RMSD plots of the copper ligands during the simulation time at six different temperatures is shown in Table 1. The largest mean values of RMSD were seen for His37 ligand at 373K and 453K. In addition, the standard deviations for His37 ligand at 373K and 453K are larger than the related values for other temperatures. Therefore, one can conclude that the mobility of His37 ligand is increased at 373 and 453K. The behavior of the RMSD mean values of His87 ligand is very similar to that of His37 ligand, while the calculated standard deviations of His87 ligand are larger than those related to His37 ligand. Thus, it can be argued that the mobility of His87 ligand is larger than that of the His37 ligand. For the sake of completeness, the related mean values of two other Met92 and Cys84 ligands are considered. Met92 ligand has less stability at 300, 373 and 453K. Cys84 shows an order decrease in stability at temperatures situated above TM.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Min</th>
<th>Max</th>
<th>Max time</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIS37</td>
<td>300</td>
<td>0.026</td>
<td>0.043</td>
<td>3798</td>
<td>0.038</td>
</tr>
<tr>
<td></td>
<td>325</td>
<td>0.02</td>
<td>0.042</td>
<td>336</td>
<td>0.037</td>
</tr>
</tbody>
</table>
Another important factor to study the denaturation process is the radius of gyration. As clearly seen from Fig. 3, the pattern of change in radius of gyration is very similar to Cα RMSD behavior for all temperatures, except 373K. Fig. 3 also shows that protein compactness at 373K is lower than that related to transition temperature (344K). Also, residue-residue contact map of protein at studied temperature was calculated and results were summarized in Fig. 4, which shows the protein structure change in N-terminal and C-terminal region at 498 K.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>HIS87</th>
<th>MET92</th>
<th>CYS84</th>
</tr>
</thead>
<tbody>
<tr>
<td>344</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>373</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>413</td>
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<td></td>
</tr>
<tr>
<td>453</td>
<td></td>
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</tr>
</tbody>
</table>

*RMin, Max and SD refer to minimum, maximum values and standard deviation of RMSDs*
Fig. 3. – Radius of gyration as a function of time for six different temperatures, 300, 325, 344, 373, 413 and 453 K.
INTERMEDIATE IDENTIFICATION

As discussed above, five significant changes in \( C_\alpha \) RMSD and radius of gyration plots were seen at temperature of 453K. These regions are as follow: 0-4ns, 4-5.5ns, 5.5-12ns, 12-13.5ns and 13.5-18ns. Distribution of the radius of gyration was plotted for each of the five time regions at the temperature of 453K (see Fig. 5). One Gaussian-like curve could be fitted for regions of 0-4ns, 5.5-12ns, and 13.5-18ns, which are native state, intermediate, and denatured state, respectively. On the other hand, two Gaussian-like curves were used to fit distribution of radius of gyration for both time regions of 4-5.5ns and 12-13.5ns. This region was named transition state 1 and 2. This result interestingly shows that two ensembles exist simultaneously in the transition state 1 and 2 regions.
ROOT MEAN SQUARE FLUCTUATIONS (RMSF) ANALYSIS

The RMSF analysis was used to explore the flexibility of the residues during simulation. Fig. 6 shows the variation of RMSF values over the residue numbers at 300, 325, 344, 373, and 413K temperatures. RMSF plots have five distinct regions including residues 4-14, 30-40, 40-56, 56-62 and 83-92. Except the N and C terminals, flexibility at residues of 40-56 is greater than that at the other residues. In addition, the effect of temperatures situated above the transition temperature on the flexibility of residues at this region is stronger than that of the temperatures situated below the transition temperature. Residues 4-14, 30-40, 56-62 and 83-92 participate in extended structures, but residues 40-56 make a 3-10 helix. As a result of such observations, the effect of increasing temperature is more obvious on residues that participate in helix structures than on other residues. The flexibility of residues 14-22, 27, 60, 90 and 96 at 325K is higher than that measured at 300K. Moreover, residues of 2-14, 22-38 and 63-94 are more flexible at 344K than at
325K. All residues, except 34 and 38, are more flexible at 373K than at 344K. Finally, the flexibility of all residues is responsible for switching structure from 373 to 413K.

Figure 6. – Root mean squared fluctuation over residues numbers at 300, 325, 344, 373, 413K.

Figure 7 shows the root mean square fluctuation of five detected regions at 453K. As seen explicitly, the pattern of residue flexibility at 453K is more similar to that at other temperatures. For the Transition state 1 structure, residues 6, 21-42, 47-62, 64, 66-84 and 96-100 are more flexible than the native structure. In addition, residues 2-20, 49, 53, 65, 84-96 and 100 in intermediate structure are more flexible than the Transition state 1 structure. For Transition state 2 structure there are three regions, including residues 44, 54-55 and 2, that show a higher flexibility than that of the intermediate structure. Finally, residues of 17-25, 30-33, 36-53 and 58-100 in denatured structure are more flexible than in Transition state 2 structure.

Spinach plastocyanin protein has two negative patches (including residues 42-45 and 59-61, respectively) that participate in electron transfer event through the binding interaction with its partners, including Cytochrome f and photosystem I (26). Our results show that the effect of increased temperatures on the first negative patch is lower than on the second one.
Fig. 7. – Root mean squared fluctuation over residues number s at 453K for native, transition state1, intermediate state, transition state 2 and unfolding structure.

SECONDARY STRUCTURE ANALYSIS

Figure 8 shows three secondary structure types, including extended conformation, coil and turn structures. In this figure, 1, 2, 3, 4 and 5 refer to 300, 325, 344, 373 and 413K structure, respectively. Except for 373K, there are no significant changes in the number of residues that participate in the extended conformation at all temperatures.
Figure 8. – Change of secondary structures with respect to residue number for five structures. Structure index of 1, 2, 3, 4, 5 refer to 300, 325, 344, 373 and 413K structures, respectively.

Figure 9 shows the secondary structure of five ensembles at 453K. In this figure, 1, 2, 3, 4 and 5 refer to the native, first transition state, intermediate, second transition state and denatured mean structure, respectively. Alpha helix structure is not formed in the native structure, but turn and coil structures are formed. Native and the first transition structures are different in some residues that formed turn structures. Accordingly, the structure of the first transition state is more native like. An alpha helix formed by residues 12-45 is seen in the first transition state structure. Similarity between the secondary structure of the native structure and the first transition state is higher than with the intermediate structure. Although extended conformation, turn and coil structures are formed in the intermediate structure, the alpha helix structure is not formed. The structure of the second transition state is more like the denatured structure. On the other hand, less similarity was seen between the second transition state and the intermediate state.
Fig. 9. – Change of secondary structures with respect to residue number for five structures. Structure index of 1, 2, 3, 4, 5 refer to native, transition state 1, intermediate state, transition state 2 and unfolding structures at 453K, respectively.
CARTON REPRESENTATIONS

Carton representations have been used for better representing the difference between structures. Fig. 10 illustrates the average structures of protein at 300, 325, 344, 373 and 413K, with dark color showing the difference between structures. Fig. 11 shows the average structures for five ensembles that were detected in the Gaussian fitting stage; dark color was used to represent the difference between five ensemble structures.

Fig. 10. – Carton representation of the average structures of thermal denaturation simulation of spinach plastocyanin protein at 300, 325, 344, 373 and 413K.
CONCLUSION

In the present work, molecular dynamics simulation of Spinach plastocyanin was carried out within 18ns at six different temperatures. Several analyses, including $C_{\alpha}$ RMSD, RMSD, Rg, RMSF and secondary structure, were used to explain the protein behavior at different temperatures. Five states were detected from $C_{\alpha}$ RMSD analysis at 453K. The RMSD analysis highlighted the fact that Met92 ligand is less resistant to temperature changes. Rg data showed a similar pattern of change as $C_{\alpha}$ RMSD. The intermediate state was checked using
distribution of the radius of gyration at 453K. The RMSF analysis showed different flexible regions for the protein structure. Secondary structure analysis was used to make a more detailed description of the founded structures. This work verified the occurrence of the intermediate state during thermal unfolding using molecular dynamics simulation at 453K.

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