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Analyzing Temperature Dependence of Diphtheria Toxin Structure and Stability Using Molecular Dynamics Simulations

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ABSTRACT

The protein denaturation due to the temperature variation and the free energy difference provided useful information on the protein stability. This study was performed to evaluation of the thermodynamic stability of Diphtheria Toxin (DT) and its mutant (E349K). The free energy differences (ΔΔG) were computed between DT and E349K at the different temperature levels using molecular dynamic simulations. Results showed that the thermodynamic stability of DT was decreased at higher temperature (320 K) in comparison with lower temperature (310 K). Results showed that the thermodynamic stability of DT was lower than that of E349K at 320 K. Secondary structures and coils were analyzed by Kabsch–Sander method. The significant increase of the β-sheet, turn and the helix content of mutant observed in comparison with DT at 320 K. The root mean square fluctuation (RMSF) of Ala187 of E349K (RMSF=0.5034) was much more than that of DT (RMSF=0.2746) at 320 K. The disulfide bridge (Cys186–Cys201) was observed in the E349K structure but was not present in the DT structure at 320 K. In the final trajectories reported the radius of gyration (Rg) and the Root Mean Square Deviation (RMSD) of DT were more than those of E349 at 320 K. Accordant to the Rg, results found that the molecular hydrophobicity of DT decreased at 320 K in comparison with E349K. These results demonstrated that the DT stability was affected by the temperature shift due to the protein structural variation.

Keywords: Diphtheria toxin, Free energy difference, Molecular dynamics simulation, Hydrophobicity

INTRODUCTION

The overall stability of the protein structure was considered to be the sum of contributions from electrostatic interactions, hydrogen binding, Vander Waals, and hydrophobic interactions (solvent exclusion). The much attention has been given to ascertaining the role that ionic side chain interactions and solvent ion interactions which play in the stabilization of proteins [1]. Properties of a hydrogen bond network such as an alpha helix can be described as a set of interacting peptide unit dipoles resulting from the electron distribution between the individual bonded atoms [2]. Stabilization of secondary, tertiary and quaternary structures of proteins has been attributed to intramolecular electric fields while long range recognition such as protein-substrate [3,4], protein-protein [5,6], or protein-DNA binding has been attributed to intermolecular electric fields [7]. The exception of unstructured protein domains, the integrity of a protein's structure and function is largely dependent on thermodynamic stability [8]. The functional relationship and stability in mutants is therefore crucial to proteins [9] also in engineering, designing, and evolving, as well as in novel enzymes in the laboratory [10]. Several experimental studies have demonstrated that the secondary structure propensities are essential in the protein structure stability. Recently, there has been significant progress in our understanding of the interactions responsible for helix stability in monomeric peptides and this information has been included in algorithms based on the helix/coil transition theory [11]. The computational modeling techniques such as classical Molecular Dynamics (MD) have a common tool for studies of macromolecular systems at an atomic level [12,13].

Diphtheria toxin is a 535-residue single chain which can be proteolytically cleaved into fragments A (N-terminal; 21 KDa) and B (C-terminal; 37 KDa) [14,15]. Upon reduction under denaturing conditions, 'nicked' toxin may be separated into a 21.1 KDa N-terminal polypeptide (Residues 1–193), which contains the catalytic (C) domain that is in the fragment A, and a 41.2 KDa C-terminal polypeptide (residues 194-535), which carries both the transmembrane (T) and receptor binding (R) domains [14,16,17].

The location of Glu-349 within the have determined crystallography model of DT, which suggested an explanation for its effects and an important role for Glu-349 in the acidic-pH-triggered translocation event [14]. The common procedure consists of substituting one or several residues and determining the corresponding stability changes by measuring the changes in the free energy of unfolding upon denaturation [18-20]. In this work, we have computed the free energy differences (ΔΔG) between diphtheria toxin and its mutant (E349K) under different temperatures (300 K and 310 K; 300 K and 320 K) using molecular dynamic simulation. Also, using the ability of molecular dynamics simulations to explore a wide range of protein conformations, the results were compared with tools in the root mean square fluctuation, the radius of gyration and the root mean square deviation. The secondary structures and coils were analyzed. The tertiary structures were compared using contact maps.

MATERIALS AND METHODS

The diphtheria toxin structure was retrieved from Protein Data Bank (PDB). The selected protein PDB ID: 1F0L was considered. The 1.55 angstrom crystal structure of wild type diphtheria toxin with X-ray diffraction was used [21]. The protonation fixing process was done with the diphtheria toxin types, wild and mutant (E349K) to prepare in the natural pH by using H++ server which was the input for molecular dynamics simulation [22]. The server allows quickly obtaining and estimating of pKa as well as other related characteristics of bio-molecules such as isoelectric points, titration curves, and energies of protonation microstates. It also automates the process of preparing the input files for typical molecular dynamics simulations. Protons are added to the input structure according to the calculated ionization states of the chemical groups at the user specified pH. The output structure is in the PQR (PDB+charges+radii) format. The mutant three-dimensional structure (E349K) of the diphtheria toxin was prepared using the molecular graphics program PYMOL which is a robust, stable and widely used program that we recommend. It is advisable to use Deep View (spdv3.7b2) to preview the file if you know that your structure may be disordered. Deep View will replace any missing side chains we need to add OXT (add c-terminal oxygen) at the C-terminal end. Water molecules in the crystal were reserved in the simulations [23].

The molecular dynamics simulations were done by GROMACS 4.5.4 package by using AMBER99SB force field and periodic boundary conditions [24]. The solute was then embedded in TIP3P water box [25]. Six simulation boxes were defined with dimensions of 9.5 × 9.5 × 9.5 nm. Then the boxes were filled with the appropriate number of water molecules. In order to neutralize the system, the appropriate numbers of Na and Cl ions were added to each box. To eliminate any undesirable contact atoms and also initial kinetic energy in the simulation boxes, the energy was minimized by applying the steepest descent algorithm. Then, each of the defining systems was equilibrating in two stages, including 5 ns, NPT and NVT simulations with pressure fixed at 1 bar. Temperature was determined in the stage simulations as 300 K, 310 K and320 K. To maintain a constant temperature Velocity-rescaling thermostat was used [26]. In order to employ constant pressure during simulations, the Parrinello-rahman barostat [27] was used in each of equilibration steps and molecular dynamic simulations. For each component of the systems PME algorithm [28] was applied to estimate the electrostatic interactions. LINCS algorithm [29] was employed to fix the chemical bonds in the atoms of the protein. Six simulations were carried out at 20 nanosecond including diphtheria toxin and mutant (E349K) at three temperatures 300 K, 310 K and 320 K. The salt concentration was kept at 0.23M. After each simulation, were evaluated various properties like RMSF, radius of gyration and RMSD profiles. Also, were compared the free energy differences (ΔΔG) between DT and its mutant (E349K) at different temperature (300 K and 310 K; 300 K and 320 K). The secondary structures and coils compared by Kabsch–Sander method [30]. All simulations were repeated to test the convergence of the results.

RESULTS

The folded protein itself is described at the normal temperature (300 K). Protein stability is dependent on the difference in free energy (ΔG^{U-F}) between the folded (F) and unfolded (U) states ($\Delta G_{U-F}=G^{U}-G^{F}$) [31]. In order to evaluate the relative free energy contributions of different forces to protein stability, we define unfolding path way in which these interactions are modified as denaturation temperature (310 K and 320 K). The free energy difference (ΔΔG) between DT and E349K is related to $\Delta G_{DT}{}^{U-F}$ and $\Delta G_{E349K}{}^{U-F}$:

$$\Delta\Delta G^{U-F}=\Delta G_{DT}{}^{U-F}-\Delta G_{E349K}{}^{U-F} \qquad (1)$$

The free energy differences, ΔG, were computed using molecular dynamic simulations (Figure 1). Changes in the free energy ΔΔG between DT and E349K were computed at different temperature (300 K and310 K also 300 K and 320 K). The structure of DT in the unfolded state at 320 K and 310 K were compared with the folded state at 300 K. These results showed that the thermodynamic stability of DT (ΔG2=0.01316 kJ/mol) at higher temperature (320 K) was decreased in comparison with the lower temperature (310 K) (ΔG1=0.01224 kJ/mol). The thermodynamic stability of mutant increased at 320 K (ΔG*2=0.00035 kJ/mol) in comparison with 310 K ($\Delta G^{*}1$=0.000040 kJ/mol)), which was attributed to the decreased free energy (Table 1).

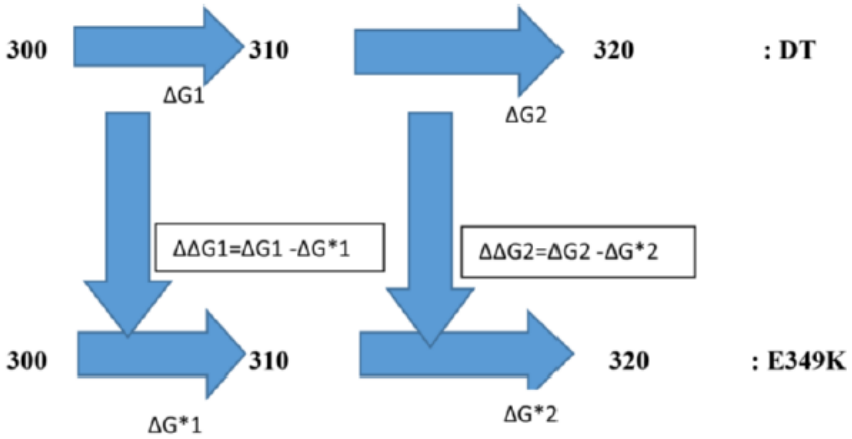

Figure 1: The free energy profile between E349K and DT at 300 K, 310 K and 320 K. ΔG1 is difference of the free energy of DT at 300 K and 310 K; $\Delta G^{*}1$ is difference of the free energy of E349K at 300 K and 310 K; ΔG2 is difference of the free energy of DT at 310 K and 320 K; ΔG*2 is difference of the free energy of E349K at 310 K and 320 K; ΔΔG1 is difference of the free energy between E349K and DT at 300 K and 310 K; ΔΔG2 is difference of the free energy between E349K and DT at 310 K and 320 K

Table 1: The comparison of the free energy differences, between DT and mutant (E349K) in the different temperature levels (300 K, 310 K and 320 K) after 20 ns timescale simulation

	(300 K and 310 K)	(300 K and320 K)
ΔG*1 (E349K) (kJ/mol)	0.000040	----------
ΔG1 (DT) (kJ/mol)	0.01224	----------
ΔG*2 (E349K) (kJ/mol)	----------	0.00035
ΔG2 (DT) (kJ/mol)	----------	0.01316
ΔΔG1 (kJ/mol)	0.01220	----------
ΔΔG2 (kJ/mol)	----------	0.01280

The root mean square fluctuation RMSF is a measure of the average atomic mobility [32] of backbone atoms (N, Cα and C atoms) which were computed during the molecular dynamics (MD) simulations. Results showed that fluctuation of residues of DT and its mutant at 320 K after 20 ns timescale simulation (Figure 2). It has been shown; high fluctuations of residues of the DT structure were in the regions Lys39, Ala187 in the fragment A and in the regions Tyr358, His384 and His520 in the fragment B. However, fluctuation of residue Ala187 of E349K is much more than that of DT at 320 K. Molecular packing is known to be an important aspect of protein stability. Like crystals, globular proteins have high packing densities, which are important for protein native state structure and stability [33].

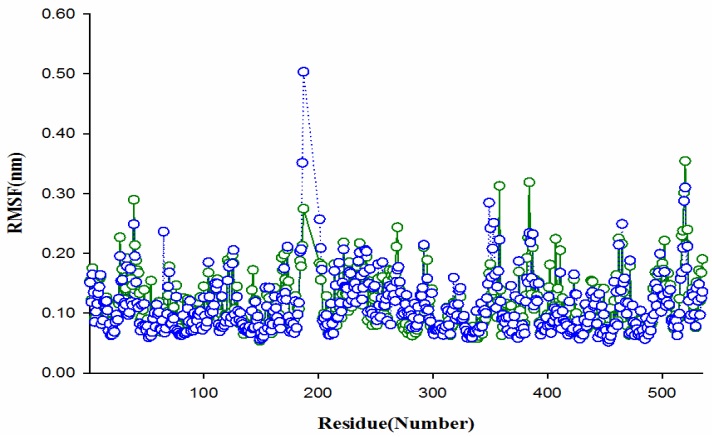

Figure 2: Comparison of the RMSF of DT and E349K at 320 K after 20 ns simulation; the dotted line blue is the E349K at 320 K and the green line is the DT at 320 K

Radius of gyration (Rg) is a parameter that describes the equilibrium conformation of a total system [34]. Rg values of DT and E349K structures at 320 K during simulations were shown in the Figure 3. The gyration radius of the DT structure at 320 K was 2.5162 nm at the beginning of the simulation and it reached to 2.5279 nm at the end of the simulation. The radius of gyration of the E349K structure at 320 K was 2.5150 nm at the initiation of the simulation and reached to 2.5233 nm at the end of the simulation.

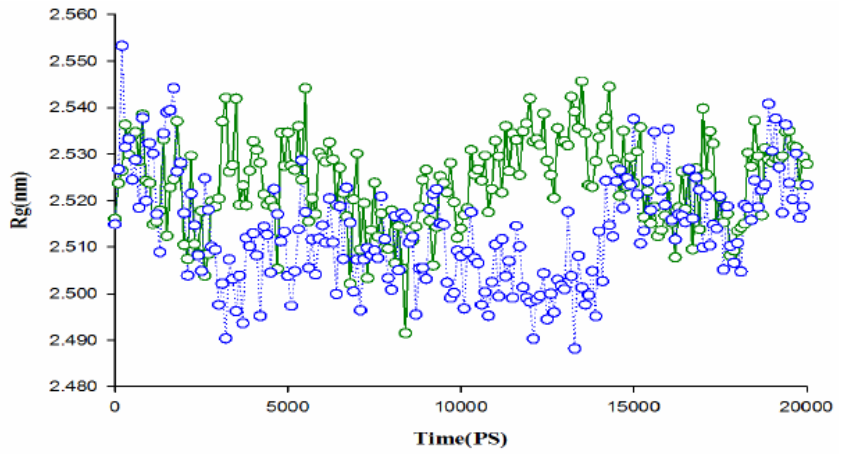

Figure3: Comparison of the Rg of DT and E349K at 320 K after 20 ns timescale simulation; the dotted line blue is the E349K of DT at 320 K, the green line is the DT at 320 K

In this work, we evaluated the stability of the simulated trajectories by monitoring the root RMSD of the calculated structures from the experimental X-ray structure. High deviations from initial native structure can be related to the instability which is imposed by the solvent [35]. The profile of the RMSD for DT and its mutant at 320 K during simulation obtained from molecular dynamics simulation has been given in Figure 4. The RMSD of mutant at 320 K was lower (RMSD=0.1307 nm) in comparison with native of DT (RMSD=0.1421 nm). These results showed that the conformational stability of the E349K structure was more than of the DT structure at 320 K.

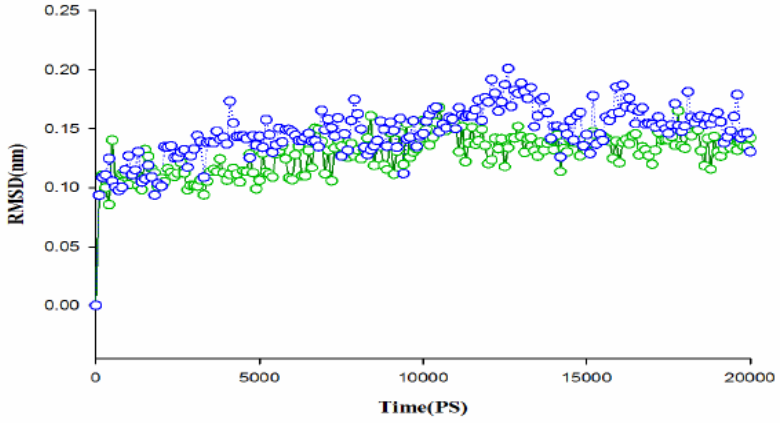

Figure 4: The RMSD of backbone atoms of DT and E349K at 320 K after 20 ns timescale simulation, the dotted line blue is the E349K at 320 K, the green line is the DT at 320 K

To get some insights into the structural stability of DT and its mutant at 320 K, we analyzed relative number of secondary structures and coils by Kabsch–Sander method [30]. As it has been shown in Table 2, turn and β-sheets and 3-10 helix content of the E349K structure increased in comparison with DT at 320 K.

Table 2: The comparison of the secondary structure of the diphtheria toxin types, mutant (E349K) and native, after 20 ns time scale simulation at 320 K

Secondary structure	Helix	Sheet	Turn	Coil	3-10 Helix
E349K (320 K)	28.5%	28.3%	15%	25.6%	2.7%
DT (320 K)	29%	27.1%	14.4%	27.5%	1.9%

DISCUSSION

The free energy difference, ΔG, is referred to as the folding free energy [20]. Adjust with Table 1, Change in free energy (ΔΔG) at 320 K between DT and mutant (0.01280 kJ/mol) was more than that at 310 K with free energy difference 0.01220 kJ/mol. These results showed increased energy level at high temperature in comparison with low temperature in the diphtheria types, mutant (E349K) and native. Replacing the negative charge Glu349 residue with the positive charge Lys residue effected on the electrostatic forces. These results were more valid previous experimental that showed that high temperature caused conformational changes in the diphtheria toxin, and these changes were assumed to reflect denaturation [36]. The change in the β-sheets has an influence on helices and acts as a stability factor. It is unlikely that the folded state of a model β-hairpin peptide resembles a β-sheet in a native protein, with the former sampling a much larger number of conformations of similar energy stabilized by a fluctuating ensemble of transient interactions. Therefore, structural stability of E349K increased in comparison with DT at 320 K.

The disulfide bridge formed between Cys186 and Cys201 is essential for the toxin activity. The reduction of this disulfide, which is necessary for a release of the C domain in the cytosol of the cell, must occur at a late stage of the intoxication process, probably during or after the translocation of C across the membrane of the endosome [37,38]. We observed that the disulfide bridge was available in the mutant at 320 K, whereas it was not present in the DT structure (Figure 5). In addition, it was found that the hydrogen binding between sulfur (S) of Cys201 and hydrogen (H) of Cys186 of DT can impact on the residue fluctuations in this region. *In vivo*, reduction of this disulfide following cleavage of the loop results in the separation of the C domain from the T and R domains (corresponding to the so-called fragment A and fragment B of bacterial toxins with intracellular targets) and to a loss of toxicity [38].

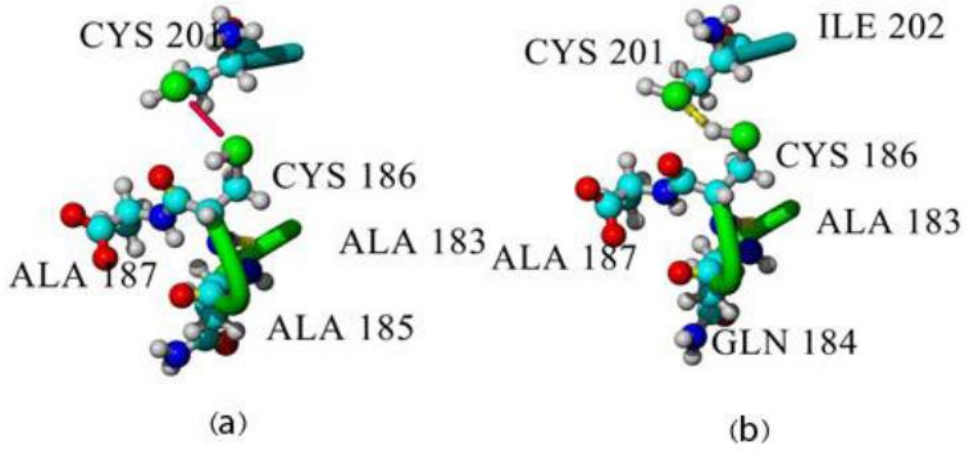

Figure 5: (a) Snapshots of E349K structure in the region 185Ala-202Ile residues at 320 K, (b) Snapshots of DT structure in the region 185Ala-202Ile residues at 320 K

CONCLUSION

Regarding results of free energy differences, we found that the thermodynamic stability of the DT structure at the higher temperature (320 K) decreased in comparison with the lower temperature (310 K). Also our results indicated that the thermodynamic stability of the DT structure decreased in comparison with its mutant (E349K) at 320 K. In addition, we demonstrated that the thermodynamic stability was affected by the temperature shift due to the protein structural variation. The RMSF values indicated that the fluctuation of Ala187 in E349K was considerably more than that in DT at 320 K and this result can be attributed to the hydrogen binding between sulfur of Cys201 and hydrogen of Cyc186. This hydrogen binding did not present in the E349K structure, whereas it was available in the DT structure. Results of the Rg profile showed that the hydrophobicity of DT was less than that of E349K at 320 K. The secondary structures analysis showed increased β-sheet, turn, coil and the helix content of E349K in comparison with DT at 320 K. The RMSD profile showed that the conformational stability of the DT structure declined in comparison with its mutant at 320 K. These results can be explained by the free energy changes due to the folding or unfolding of the proteins. Therefore, temperature as an important factor affected the conformational and the thermodynamic stability of DT. These results can be used in the DT vaccine manufacturing process to ensure the appropriate structural stability of the vaccine regarding the temperature.

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