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Relative stability of thrombin-hirudin complex is illustrated using molecular dynamics

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ABSTRACT

Thrombin has a vital role in regulating blood coagulation mechanism and its related disorders. Anti-thrombin agents/inhibitors interact mainly at the active site or the exosites I/II of thrombin, leading some structural and conformational changes to form the thrombin-inhibitor complex. Study of such conformational modifications helps to illustrate dynamic molecular interactions between them. Hirudin, a serine protease inhibitor's N- terminal binds effectively to the active site and its C- terminal to exosite-I of thrombin. Here, we elucidate the insights of thrombin-hirudin complex interaction by molecular dynamics (MD) simulation study. The 3-dimensional crystal complex of thrombin-hirudin was neutralized and kept in water cube for 1000 picoseconds (ps) at specific equilibrium. At every frame the movement, interaction and stability of the complex is studied and visualized. Results were analyzed based on change in energy of the system and parameters such as Root Mean Square Deviation (RMSD= 0.45 Å fluctuation), radius of gyration (18.04 Å) and hydrodynamic radius (19.06 Å) etc. It was seen that thrombin-hirudin complex interaction was stable throughout the experiment, similar as in the natural environment. It depicts the stability of drug (hirudin)- target (thrombin) complex and this study would provide valuable insights for thrombin inhibitor design in future.

Keywords: Molecular dynamics, Hirudin, Thrombin

INTRODUCTION

Vascular injury triggers formation of platelet plug, thrombin and fibrin clot through blood coagulation to arrest the bleeding. A minor imbalance in such highly regulated haemostatic system could lead to hemorrhagic disorders or thrombosis [1-3]. Central mechanism and regulation of blood coagulation is controlled by thrombin; it is trypsin-like serine protease. It has high specificity of arginine bond which plays important role in maintaining haemostasis and thrombosis [4,5]. It initiates blood clotting cascade by not only cleaving fibrinogen but also activates platelets, blood coagulation factors such as V, VIII and XIII and anticoagulant enzyme protein C. Blood coagulation factors are essential for the formation of the blood clot [6]. Inhibition of thrombin prevents the generation of both fibrin and platelet thrombi, and hence is the best target for interference [7]. Its positive feedback regulation serves to amplify haemostatic events; but causes life-threatening thrombi in response to aberrations with vascular and cerebrovascular arteries. Research has proved that thrombin's inhibition by diverse potent compounds could provide invaluable drugs to the treatment of disorders related to thrombosis [8]. Many naturally occurring protease inhibitors are largely explored for its anti-haemostatic activities.

From the early age's Hirudin, a potent naturally occurring polypeptide is used for the treatment of various vascular and inflammatory disorders. Hirudin variants are also produced by recombinant DNA technology and are administered to patients undergoing coronary angioplasty, for the treatment of deep venous thrombosis and as a substitute for heparin in patients developing heparin-induced thrombocytopenia [9]. Hirudin was isolated more than 50 years ago from peripharyngeal glands of the medicinal leech *Hirudo medicinalis* [10] . It consists of a polypeptide chain of 65 amino acid residues with three disulphide bridges [11]. It has highly potent antiprotease activity with a strict specificity for thrombin. From the early research work it has been postulated that hirudin non-competitively bind to the thrombin and prevent conversion of fibrinogen to fibrin.

Thrombin being multifunctional, has the ability to interact with structurally distinct substrates and capacity to produce different structural conformations [12-14]. The three dimensional structure shows that thrombin consists of 308 amino acid residues peptide and is divided into two domains; these are namely A-chain or alpha micro-domain and a B-chain or beta macro-domain. The A-chain is composed of 36-49 amino acid residues and B-chain is 259 amino acid residues long [15]. Both A-chain and B-chain are connected by a disulphide bond. Each domain has its own active site A-chain has catalytic site and B-chain has anion- binding exosite. The exosite is independent of the catalytic site and is mainly involved in fibrinogen recognition whereas the catalytic site involves binding of anticoagulant peptides such as hirudin [16].

A crystal complex of thrombin and hirudin shows that the contact area between the two proteins is larger, which accounts for high affinity and selectivity of hirudin for thrombin. The N-terminal domain of hirudin is folded globular unit interactive with the active site of thrombin. Although the first 3 residues of hirudin bind at thrombin catalytic active site but the primary specificity site of thrombin is not occupied by hirudin. While the last 16 residue i.e. the C-terminal of hirudin binds at thrombin anion binding exosite-I which is secondary fibrinogen binding site [17]. Also the intact structure of hirudin molecule is essential for efficient binding of thrombin and hirudin.

Although various publications have documented hirudin– thrombin complex, we propose to study the molecular dynamics of the said complex and explore the binding interaction between these two proteins. Molecular dynamic simulation is one of the most reliable computational tools frequently used in the study of folding, kinetics and various interactions of proteins and biomolecular systems. Experimental and structural outcome from MD simulation allows gathering of insights into the mechanism of protein dynamics [18].

MATERIALS AND METHODS

Molecular dynamics (MD) simulations analyze a compound at all-atom level with respect to the physical movements of atoms and molecules in a system. It is based on Newton's equations of motion for a system of interacting particles, where forces between the particles and potential energy are defined by molecular mechanics semi-empirical force fields. The equation is solved iteratively to simulate the time evolution of the system. Based on the concepts from statistical mechanics, the resulting trajectory can be used to evaluate various time-dependent structural, dynamic, and thermodynamic properties of the system [19]. MD simulations provide powerful tools for the exploration of the conformational energy landscape between biomolecules. It also helps to explore variety of structural and dynamical properties of protein interaction [20].

Initial structure

Crystal structure of Hirudin-thrombin bearing PDB ID: 4HTC with resolution of 2.30Å, was obtained from the Protein Data Bank. Thrombin protein is an alpha- thrombin moiety with subunits L-chain composed of 36 residues and H-chain of 259 residues. Hirudin polypeptide composed of I-chain with 65 amino acid residues.

MD simulation

The molecular dynamics simulations were performed using GROMACS version 4.5 [21]. The simulation procedures of GROMACS include a series of files and programs. There are mainly four processes. (I) In order to avoid improper structure of proteins, we perform energy minimization at the temperature of absolute zero. (II) Then we increase the temperature of system to 300K by giving every atom a prime velocity according to Boltzmann distribution, and the system is balanced for a short period of time. (III) We performed the GROMACS package to make sure that the system almost lies in the minimum points of the energy surface, and then some simulations were executed, such as stretching proteins to investigate the properties of the two proteins. The last frames of each of process (IV) are used as starting points for next simulations.

For proteins standard in GROMACS, amino acid residue topology and parameters based on the OPLS force field were used [22]. The initial structure was immersed in a periodic water box of truncated cube shape (1 nm thickness) and neutralized with one Na+ counterion. During the simulation, grid type neighbor searching was done and long range electrostatics was handled using Particle Mesh Ewald (PME) [23]. Electrostatic energy was also calculated using the PME method [24]. Cutoff distances for the calculation of the Coulomb and Van der Waals interaction were 1.0 and 1.4 nm, respectively. After energy minimization using a steepest decent method, the system was subject to equilibration at 300 K and normal pressure for 100 ps under the conditions of position restraints for heavy atoms and LINCS constraints for all bonds [25]. The system was coupled to the external bath by the Berendsen pressure and temperature coupling [26]. The final MD calculations were performed under the same conditions except that the position restraints were removed. Further the stabilized complex was submitted to maximum of 1000ps simulation steps of steepest descent energy minimization (figure 1).

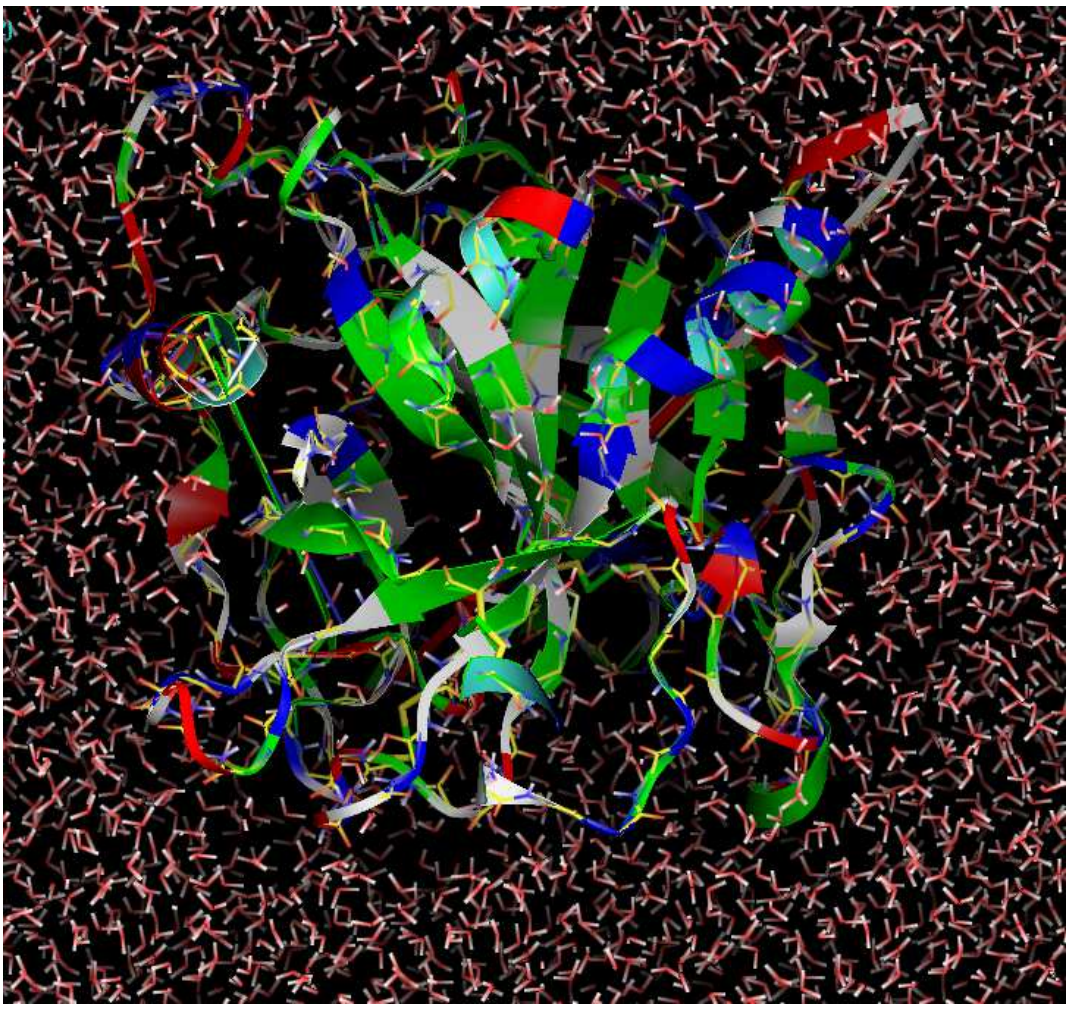


Figure 1: 3D view of thrombin-hirudin complex: It is kept in periodic water box of truncated cube shape (1 nm thickness) and one Na+ ion for 1000 ps

Simulations were carried out on a 10-processor, 195-MHz R10000 Origin 2000 and took 8 days per processor per 1-ns simulation. The results were analyzed using the standard software provided by the GROMACS package. An average structure was refined further using a steepest decent and conjugate gradient energy minimization. GROMACS built in tools were used to compute radius of gyration, hydrodynamic radius, Root Mean Square Deviation (RMSD) and H-bond interaction. All graphs were visualized using Xmgrace (http://www.plasma-gate.weizmann.ac.il/Grace/). The images of the superimposed structures were obtained using Visual Molecular Dynamic (VMD) software [27].

RESULTS AND DISCUSSION

For extensive MD simulations of peptides or protein analysis, it is important to define the properties that give a relative estimate of the stability. We have analyzed the energy gradient throughout the simulation, the root mean square deviation (RMSD) from the crystal structure, the radius of gyration, the hydrodynamic radius and fluctuations in specific residues. These values are plotted as a function of time. In a stable protein these values fluctuate around an equilibrium value; whereas in a protein with changing conformation there will be drift in these values. This simulation study will help to understand more about the interaction and relative stability between the protein chains which is surrounded with water molecules. The dynamic interaction and movements were observed in VMD software. Trajectories of continuous movement and changes in the complex provide details for further analysis.

Dynamic features

1. Energy analysis

Variability in energy levels of the protein complex system was continuously monitored from the initial to the terminal frame of the simulation. The parameters such as number of molecules, temperature, pressure and volume were kept at equilibrium; it is seen that the energy of the system also remains stable throughout the simulation. Total energy, potential energy and kinetic energy were considered for energy analysis of the system; shown in figure 2. A larger sudden change in any of these energies is uncommon for a system in thermal equilibrium with a bath of water molecules.

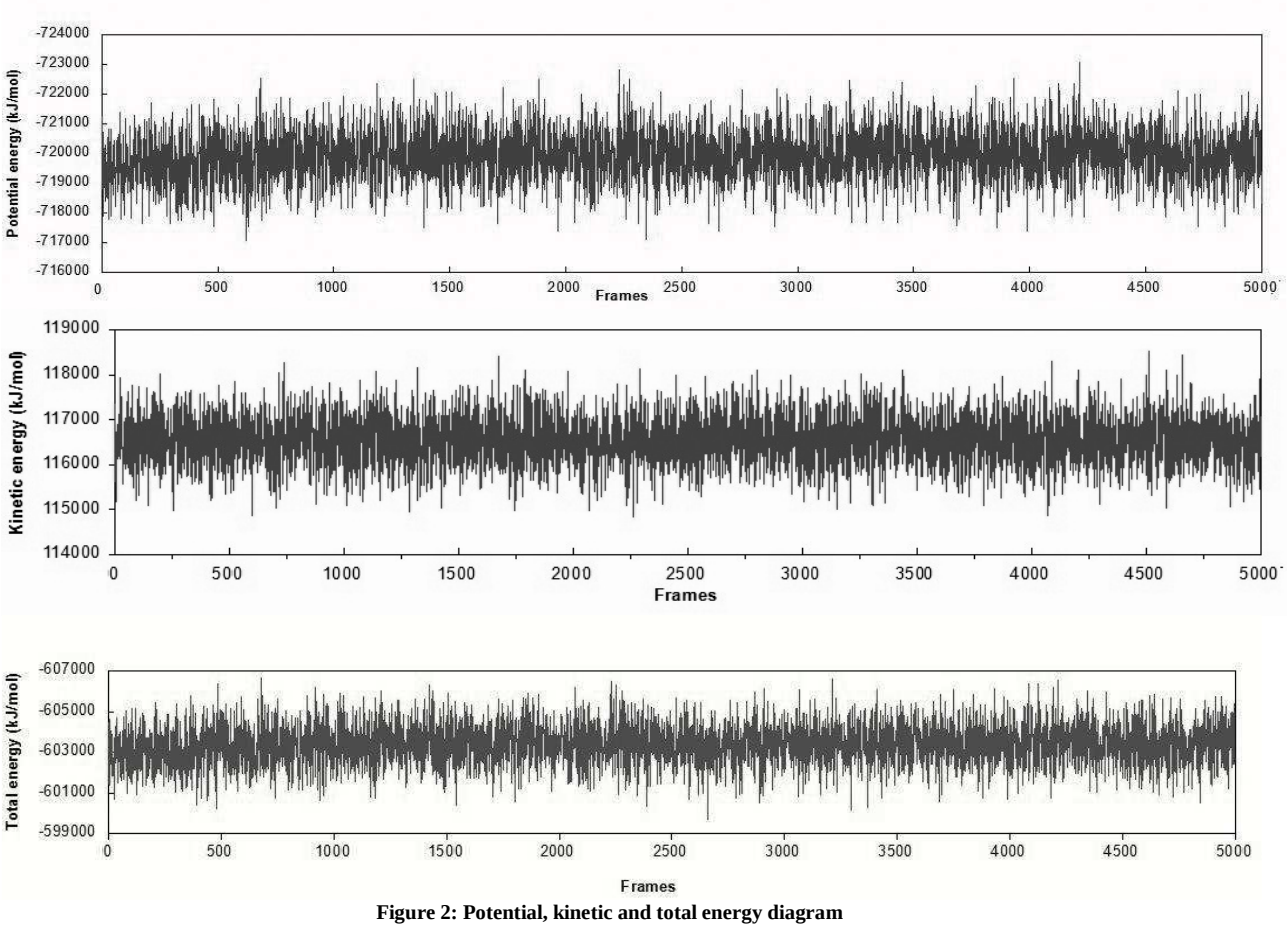


Figure 2: Potential, kinetic and total energy diagram

2. Global structure analysis

Protein structural change as a whole is evaluated based on various parameters such as RMSD, radius of gyration, hydrodynamic radius.

2.1 Radius of gyration

It is the mass weighted scalar length of each atom from its center-of-mass (COM). The radius of gyration is a rough measure for the compactness of a structure. For each peptide the radius of gyration *Rg* was calculated according to Eq. 1 [28]

$$R_g = \left(\frac{\sum_i \mathbf{r}_i^2 m_i}{\sum_i m_i} \right)^{\frac{1}{2}}$$

where m_i is the mass of atom *i* and r_i the position of atom *i* with respect to the center of mass of the peptide.

Radius of gyration *Rg* shown in figure 3, initiates from ~18.06 Å and after 50 frames it starts fluctuating. Initially a steep decrease is seen and from 200 frames, consistency with minimal fluctuation is seen. More or less the *Rg* varies between 17.94 to 18.10 Å.

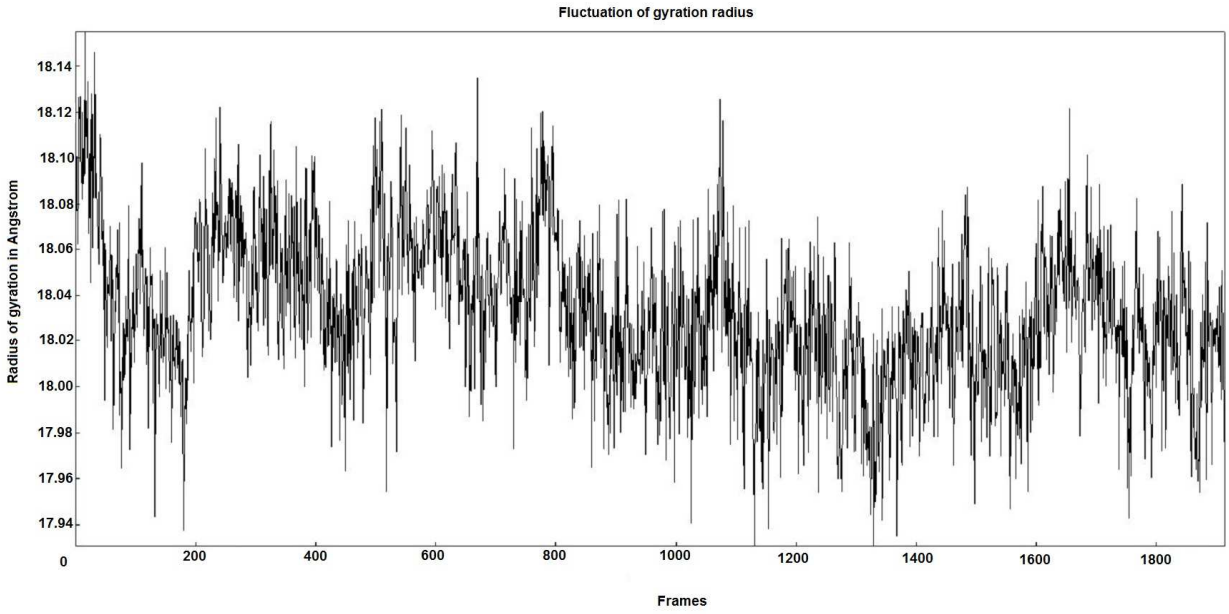


Figure 3: Radius of Gyration

2.2 Hydrodynamic radius

Hydrodynamic radius of a macromolecule or a colloid particle is also called as Stroke radius [29] and is often similar to magnitude of radius of gyration. It is the radius of atom of a polymer in a solvent, measured by assuming that it is a moving body and is resisted by the viscosity of the solvent. Hydrodynamic radius R_{hyd} is calculated using following Eq. 2.

$$\frac{1}{R_{hyd}} \stackrel{\text{def}}{=} \frac{1}{N^2} \left\langle \sum_{i \neq j} \frac{1}{r_{ij}} \right\rangle$$

where r_{ij} is the distance between subparticles i and j, and where the angular brackets $\langle \cdots \rangle$ represent an ensemble average [30]. Figure 4, demonstrates fluctuation in the hydrodynamic radius of the protein complex. No major deviation is seen throughout the simulation frames. Initial count starts with 19.08 Å and at the termination of simulation it end with 19.06 Å. Till 200 frames a steep decrease is seen, after which it increases and remains average throughout. The deviation observed varies between 19.04 to 19.12 Å.

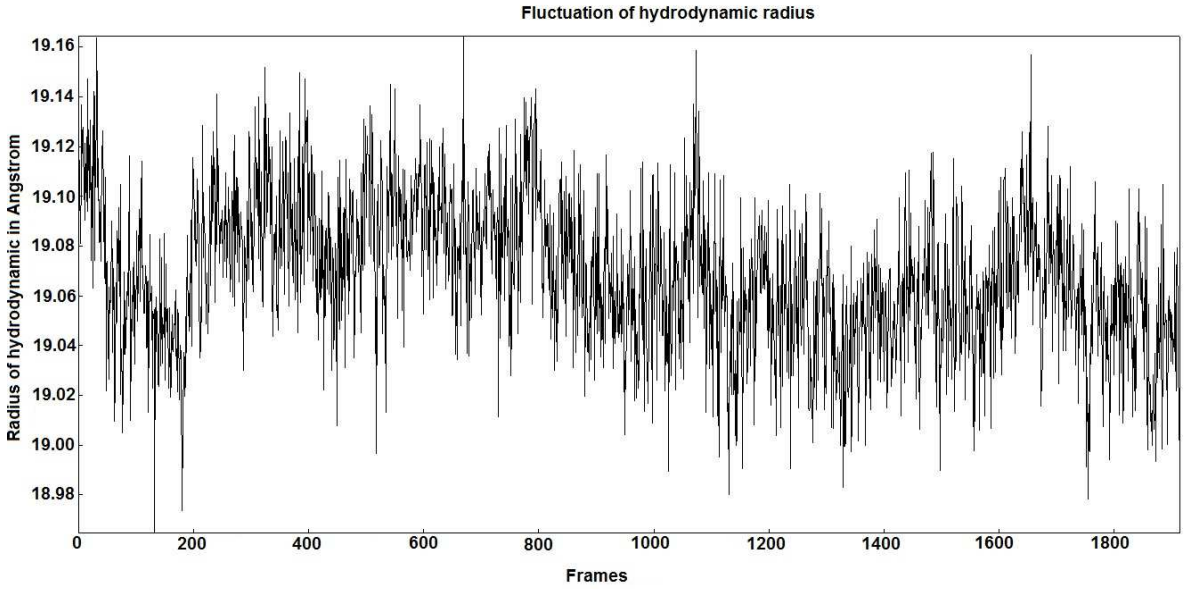


Figure 4: Hydrodynamic radius

2.3 RMSD

The RMSD of two atoms each at the N-terminal and C-terminal was carried out. Usually RMSD is used to measure the scalar distance between atoms of the same type for two structures. In this case, we have measured the average distance between two residues of the superimposed N-terminal and C-terminal (Figure 5). Typically RMSD should not change more than 3 Å within a nanosecond of MD simulation period. Initial reading starts with 1.01 Å with gradual increase to reach 1.3 Å at 400 frame position. After the 400 frame position, a decrease with a steady progression throughout the remaining frames is seen. At the terminal frame the reading is 1.0 Å. Therefore if the variation throughout is considered there is only 0.45 Å fluctuation.

Two residues from the end terminal show not much deviation, that means the N and C terminals remains almost stable all over the simulation period.

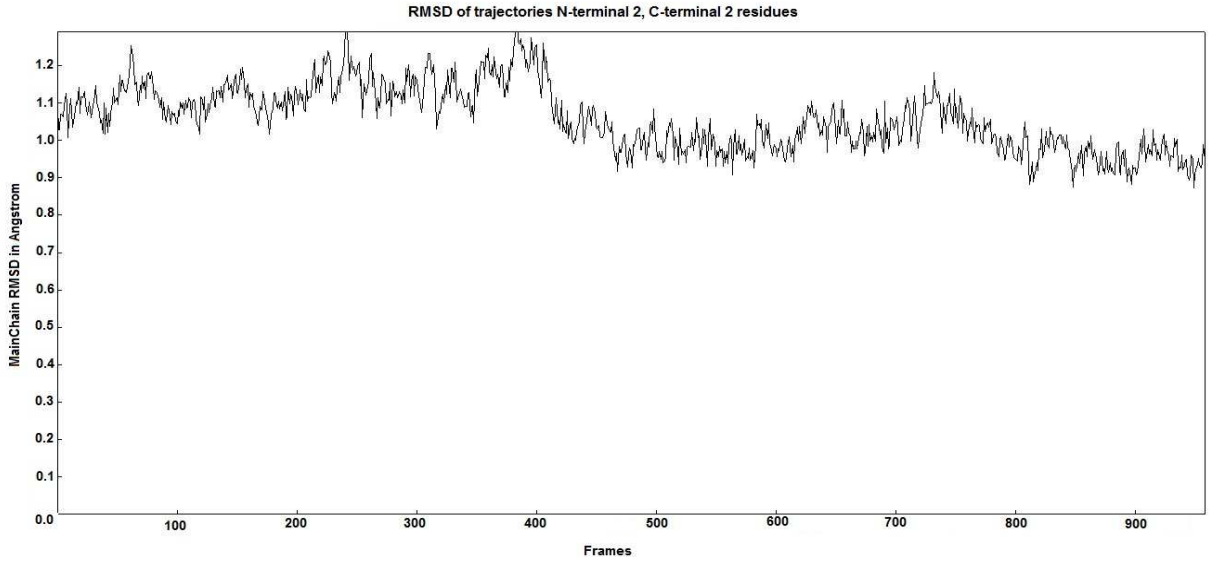


Figure 5: RMSD for 2 residues of superimposed end N and C terminals

2.4 Hydrogen(H) bond analysis

Peptides are linked to each other with the help of H-bond interaction. Any change in the interacting peptides can be assessed based on the number of H-bonds between them. Conformation changes in a peptide complex will be evaluated based on the variations in the number of H-bond interactions. Hence here we have analyzed the thrombin-hirudin conformational changes on the count of H-bond throughout the simulation period.

Figure 6, shows the number of H-bond interaction throughout the entire simulation. Initially number of interactions are 75; however the major fluctuation is seen between 70 to 85 interactions. It means that there are no huge variations in the interacting proteins and no change in the conformation of the protein complex. H-bonds are not reduced and the interaction is quite stable.

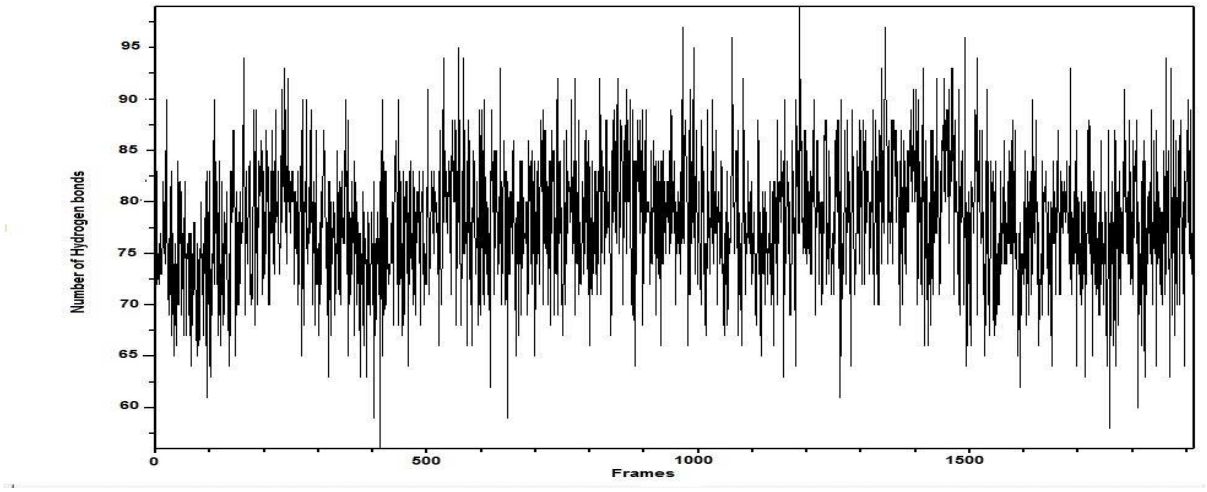
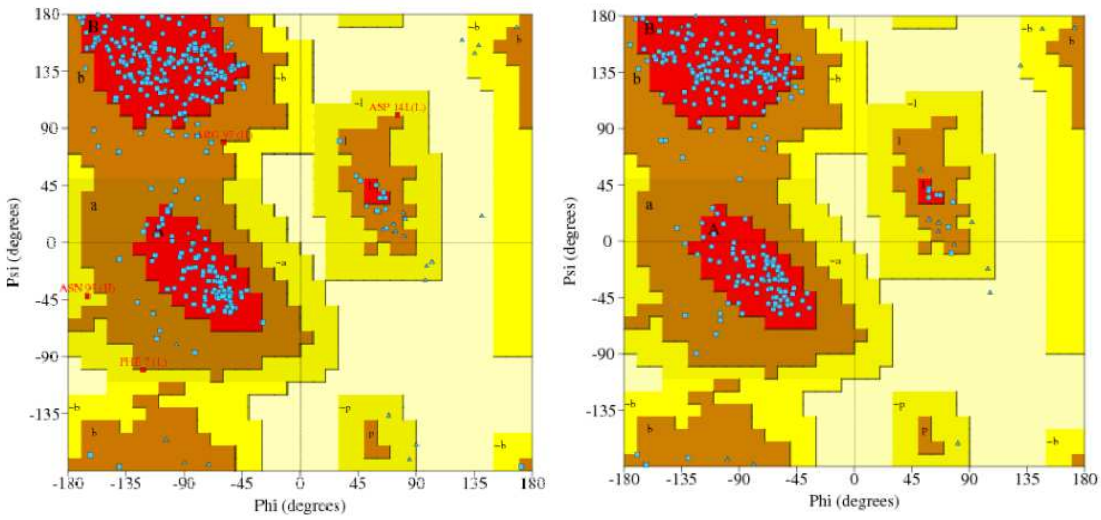


Figure 6: Hydrogen bond count

2.5 Ramachandran plot

To validate structural stability between thrombin-hirudin interaction, the stereochemical property was deduced by drawing the Ramachandran plot. Towards the end of simulation the pdb file of 4HTC is obtained and processed thorough PROCHECK server to retrieve its plot (figure 7). Amino acid residue in favored, allowed and generously allowed regions were observed. Comparative observables were sort by plotting the initial and the terminal stage positions of amino acids in the simulation. The figure 7, A and B illustrates the initial and final respective location of the amino acids. About 84.1% of amino acids fall in the favored region, 15.9% amino acids in 'allowed region' and non in 'disallowed region'.



		No. of residues	%-tage
Most favoured regions	[A,B,L]	201	84.1%*
Additional allowed regions	[a,b,l,p]	38	15.9%
Generously allowed regions	[~a,~b,~l,~p]	0	0.0%
Disallowed regions	[XX]	0	0.0%
Non-glycine and non-proline residues		239	100.0%
End-residues (excl. Gly and Pro)		7	
Glycine residues		22	
Proline residues		16	
Total number of residues		284	

Figure 7: Stereochemical property analysis of the model 4HTC at the end of simulation is drawn from PROCHECK. The plot confirms the stereochemical quality of 4HTC, with 84.1% of amino acids fall in the favored region and 15.9% amino acids in 'allowed region'

CONCLUSION

For this study, MD simulation has been used to investigate flexibility and relative stability between thrombin-hirudin protein complex. Parameters such as energies, RMSD, radius of gyration, hydrodynamic radius etc. helped to study dynamic details of the complex. Simulation study preformed and based on the results from opted parameters, showed that the complex and its interactions are quite stable throughout. These experimental details can be further used as a probe for further docking studies of hirudin-like protein on thrombin and a step towards anti-thrombin drug discovery.

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