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Thermodynamic and Conformational Analysis of GTRNase and Lysozyme Proteins Under Thermal Variations Using Molecular Dynamics Simulations

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Abstract: This study examines the thermodynamic and conformational dynamics of GTRNase and lysozyme proteins under varying temperature conditions using molecular dynamics (MD) simulations. The objective is to evaluate their structural stability, folding behavior, and thermodynamic properties to understand their responses to thermal fluctuations. Protein structures were retrieved from the Protein Data Bank (PDB), refined to remove extraneous molecules, and simulated using GROMACS 2022.2 under NVT and NPT ensembles, spanning temperatures from 270 K to 380 K. The results revealed distinct behaviors for the two proteins. GTRNase exhibited a slight escalation in radius of gyration (Rg) from 1.434 nm at 270 K to 1.445 nm at 380 K, suggesting a marginal conformational expansion. In contrast, lysozyme maintained a consistent Rg of 1.38 nm over the same temperature range, indicating structural compactness. Root mean square deviation (RMSD) data demonstrated increased flexibility in both proteins, with GTRNase escalating from 0.115 nm at 270 K to 0.179 nm at 380 K and lysozyme rising from 0.102 nm to 0.142 nm across the temperature range. Solvent-accessible surface area (SASA) for GTRNase fluctuated between 69 nm² and 73 nm², with the lowest value observed at 300 K and the highest at 370 K. These findings highlight that GTRNase is more susceptible to thermal perturbations than lysozyme, showing greater conformational flexibility and expansion. This research underscores the utility of MD simulations in exploring protein behavior and provides valuable insights for applications in protein engineering and drug design.

Keywords: GTRNase; Lysozyme; MD Simulation; Protein Structure; Protein Folding.

Introduction

The sea turtle, a majestic creature that has roamed the Earth's oceans for millions of years, is an iconic species that has captivated the attention and curiosity of scientists and conservationists alike. The green sea turtle (*Chelonia mydas*) is Indonesia's most commonly found sea turtle species. The species are critically endangered due to their high economic value for eggs and meat. The idea that green sea turtle eggs pose great nutritional value and various health benefits drives consumption [1]. Among the many intriguing aspects of sea turtles, their reproductive biology and the unique composition of their eggs have long been a subject of scientific investigation. It is of paramount academic interest to investigate the constituents and properties of sea turtle eggs [2].

The eggshell of sea turtle eggs comprises three distinct layers: an outer calcareous layer, a middle multi-strata layer, and an inner membrane. Quantitative analysis and area mapping reveal the elemental composition of the eggshell, with calcium (31.18%) being the predominant element in all layers, followed by oxygen (34.08%), phosphorus (0.02%), silicon (0.01%), potassium (0.02%), iron (0.002%), and aluminium (0.002%). The highest percentages of calcium, oxygen, phosphorus, silicon, potassium, iron, and aluminium were found primarily in the middle multi-strata layer of the sea turtle eggshell. A study by Katni et al. reveals the presence of specific nutrients in green turtle eggs, such as high content of Alpha-linolenic acid (ALA) and Linoleic acid (LA) but low content of Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The study found that the energy content of turtle eggs was around 25-29 MJ/kg. The lipid and protein contents in egg yolk were identified as energy sources for embryo development [3-6].

In avian eggs, the primary defence enzyme is commonly known as lysozyme. This enzyme can hydrolyse the β-1,4-glycosidic bond of peptidoglycan. The enzyme's activity can damage the copolymer of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), which are present in bacterial cell walls [7-9]. Interestingly, green turtle eggs contain limited lysozyme but greater RNase activity. This suggests that RNase may act as a self-defence enzyme in the egg white rather than lysozyme. One particular protein found in the eggs of sea turtles, known as GTRNase (Green Turtle Ribonuclease), has recently attracted significant interest due to its potential implications in the embryonic development and survival of green turtle eggs. GTRNase is an enzyme that belongs to the ribonuclease family, which plays crucial roles in the degradation of RNA molecules. The structure of the green turtle egg-white ribonuclease (GTRNase) has been successfully determined using X-ray crystallography. The analysis revealed conserved amino acid residues in the active site, suggesting functional similarity to other ribonucleases. The presence of sulfate ions and glycerol molecules on the molecular surface indicates their potential role in molecular

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packing during crystallisation [10-11]. The characterisation of GTRNase and its enzymatic properties can contribute to developing new therapeutic agents and biotechnological applications. Understanding the structural and dynamic properties of GTRNase is of great importance as it can shed light on its biological functions, evolutionary significance, and potential applications in various fields, including medicine and biotechnology. Overall, the study of GTRNase is important for advancing our understanding of RNase, a superfamily, and its potential applications in various fields [12].

Molecular dynamics (MD) simulation, a computational technique that models the behavior of atoms and molecules over time, provides a powerful tool to investigate the thermodynamic properties and behavior of proteins in a simulated environment. Molecular Dynamics (MD) simulation has made significant advancements, starting with simulating several hundred atoms in biological systems, including any protein in a solution with explicit solvents, proteins interacting with membranes, or large complex macromolecules such as nucleosomes or ribosomes [13-14]. Simulating systems with 50,000-100,000 atoms is routine, and simulations with around 500,000 atoms are typically performed when computational facilities are available. This remarkable progress is largely attributed to utilizing High-Performance Computing (HPC) and developing increasingly simplified basic MD algorithms. The initial model of a system is derived from experimental arrangements or comparative model data. The simulation system can be represented at various levels of detail [15].

In molecular simulations, Newton's Laws of Motion (classical) are employed to calculate acceleration and velocity and update the position of each atom in the system. The motion integration is numerically performed to avoid instability, and for each step of molecular movement, a very short time interval, typically between 1 and 2 fs, is evaluated for atomistic simulations. The short time step determined by a single molecular movement is a major constraint of the simulation method [16-17]. Simulations running for processes within the microsecond range are practically insignificant when considering the time scales of biological processes. Hence, simplified computational methods (coarse-grained methods) are employed in biological simulations, albeit at the expense of the accuracy of ensemble simulation systems [18-19].

Molecular Dynamics (MD) has become an essential computational tool for drug discovery. The main advantage of using MD is its flexible ability to perform structural modifications and account for entropic effects, enabling more accurate thermodynamic and kinetic estimations related to drug target recognition and binding (docking). With the development of better algorithms and hardware architectures, the utilization of MD simulation methods in drug and biomolecular research has significantly increased [20-21]. Currently, unbiased MD simulations allow for the observation of unguided ligand-target binding, assessing how this approach aids in optimizing target affinity and drug residence time towards improved drug efficacy [22-23].

In this study, we aim to explore the thermodynamics of the GTRNase protein found in sea turtle eggs using molecular dynamics simulation. By employing this cutting-edge computational approach, we can obtain insights into GTRNase's structural stability, folding dynamics, and intermolecular interactions. This knowledge will contribute to our understanding of how GTRNase functions and may provide valuable information for developing novel applications or therapies.

Research Methods

This study performed a computational research approach, utilizing molecular dynamics (MD) simulations to analyze the thermodynamic and conformational properties of GTRNase and lysozyme proteins. The research focused on observing the structural responses of these proteins to thermal variations.

Protein Structure Preparation

The protein GTRNase structure from turtle eggs can be obtained in pdb.org under the initial 2zpo, which can be downloaded from[10]: www.pdb.org/pdb?id=pdb_00002zpo. Water molecules (HOH molecules) are removed from the original protein structure as they are unnecessary in the simulation process. The simulation focused on the pure molecule, so the ligand components (SO_4 and GOL) bound to the GTRNase protein are also removed from the protein structure. The cleaned protein structure (PDB code) is then processed for MD Simulation using GROMACS 2022.2 software. By using the `pdb2gmx` tool in GROMACS, the initial protein structure named "GTRNase.gro" and the topology file "topology.top" can be obtained. Both files are required for the GTRNase protein simulation.

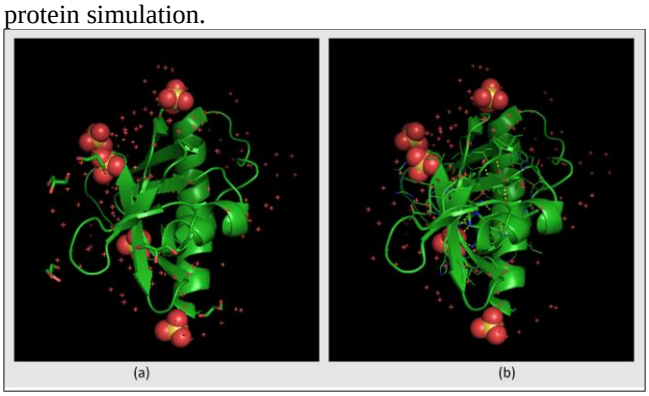

Figure 1. Illustrates the molecular configuration of the GTRNase protein via Pymol. (a) depicts the original protein structure sourced from the Protein Data Bank (PDB). (b) the GTRNase protein is shown in its refined state, with extraneous molecules removed for preparation.

Simulation Procedures

The topology file is obtained by providing input specifying the OPLS-AA/L force field and using the TIP4P water model. It contains fundamental information about the geometry, spatial information, and structure of the substance to be simulated. The simulation involves the GTRNase protein being solvated in water, so 10644 water molecules are added to the system (Figure 2).

Preparation of the Simulation

Before starting a molecular simulation, the stability of the initial simulation system must be ensured. This can be achieved by finding a system conformation with minimal

energy (energy minimization, EM). When the energy reaches its minimum, it ensures that there are no spatial clashes between the water as a solvent and the formed GTRNase protein structure. Calibration is performed once a system with minimal energy (a stable system) is obtained.

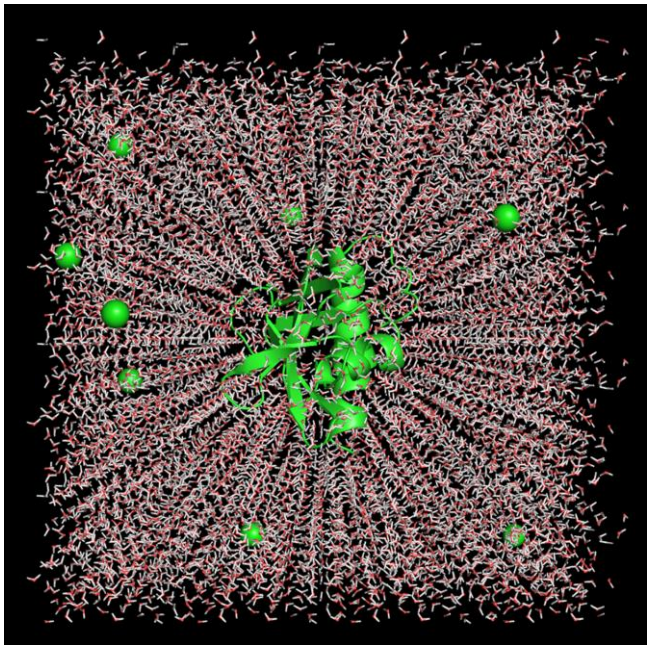

Figure 2. Visualization of the GTRNASE protein in a solvated state with the incorporation of cation and anion species.

A Calibration is often carried out in two phases, namely the NVT ensemble and the NPT ensemble. The first phase calibration is done within the NVT ensemble, where the number of particles, Volume, and Temperature are kept constant. This ensemble is also called "isothermal-isochoric" or "canonical." The duration of such calibration procedures depends on the size and complexity of the system, but in NVT, the system temperature should reach the desired value. If the temperature is not stable, additional time will be required. Typically, a simulation time of 50-100 ps is sufficient. In this study, an NVT calibration is performed for 100 ps.

Pressure calibration is carried out within the NPT ensemble, where the number of particles, pressure, and temperature are all kept constant. This ensemble, also known as an isothermal-isobaric ensemble, closely resembles experimental conditions. In the NPT ensemble, pressure will fluctuate, which is normal in molecular simulations.

From these two calibration stages, the system's density data will be obtained, which should match or be close to the experimental value (in this case, the system density data will be close to the density of water as the main solvent).

Pressure calibration is carried out within the NPT ensemble, where the Number of particles, Pressure, and Temperature are all kept constant on average. This ensemble is also known as an "isothermal-isobaric" ensemble and closely resembles experimental conditions. In the NPT ensemble, pressure will fluctuate, which is normal in molecular simulations. From these two calibration stages, density data of the system will be obtained, which should match experimental values (approximating the density of water as the main solvent).

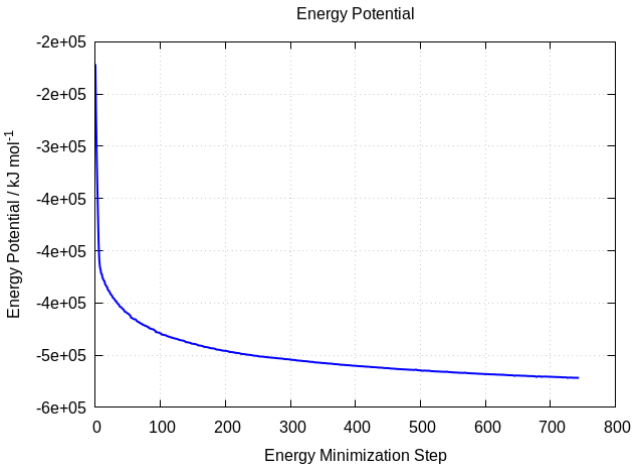

Figure 3. Graph representing the energy potential of GTRNase protein plotted against the simulation step during the energy minimization phase.

Production MD Simulation

After calibrating the simulation system, the Molecular Dynamics (MD) production run is ready to be executed. The entire MD simulation is performed on the solvated structure of GTRNASE in water, along with NaCl salt ions, at temperatures ranging from 270K to 370K for a duration of 2 fs (femtoseconds). Changes in the GTRNASE structure, positions of each atom in the system, and all thermodynamic parameters are recorded at every 5000 simulation steps or approximately 10 ps of real-time. The collected data, including the thermodynamic parameters and atomic position changes, are then analyzed.

Data Analysis

The radius of gyration (Rg) is an important variable in molecular dynamics (MD) simulations of protein structures, offering insights into their compactness. Computed as the root-mean-square distance between protein atoms and the center of mass, it effectively tracks conformational changes during simulations, aiding in the study of protein folding and interactions with ligands[27]. The mathematical expression for Rg:

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

the equation involves the mass of atom I, denoted as m_i, and the position of atom *I* relative to the molecule's center of mass, represented as $\boldsymbol{r_i}$. This formula is very useful in the characterization of polymer solutions and proteins.

Root mean square deviation (RMSD) is a quantitative measure used to assess the structural variability of enzymes during molecular simulations. It provides insights into the extent of conformational changes that occur over time compared to a reference structure. The mathematical expression[16]:

$$RMSD(t_1, t_2) = \left[\frac{1}{M}\sum_{i=1}^{N} m_i \left|\left|\boldsymbol{r}_i(t_1) - \boldsymbol{r}_i(t_2)\right|\right|^2\right]^{\frac{1}{2}}$$

where $M = \sum_{i=1}^{N} m_i$ and $\boldsymbol{r_i}\ (t)$ is the position of atom *i* at time *t*. In this simulation, the structure of GTRNase protein is fitted on the backbone atom of protein (N, Cα, C).

The last method in analyzing the protein structure and conformation is by using the Solvent Accessible Surface

Area (SASA), which refers to the extent of a molecule's surface that is reachable by a solvent. SASA plays a crucial role in assessing the nonpolar component of implicit solvent models, addressing the solvation of an uncharged solute through forming a cavity.

In this research, the atomic coordinate data is visualized using Pymol and VMD, while graphs are produced using the Gnuplot implementation running on a Linux system.

Results and Discussion

In MD simulations, the macromolecular atoms and surrounding solvent molecules undergo relaxation, typically taking tens to hundreds of picoseconds before the system reaches a stable state. This stage of the MD simulation is considered a calibration session. The calibration protocol remains a matter of personal preference, and there are several possibilities depending on the type of simulation being conducted. Some protocols involve complex mechanisms that gradually increase the temperature, while other, more straightforward approaches employ a linear temperature gradient to heat the system to the desired temperature[16].

In biomolecular simulations, specifically protein simulations, two important calibrations are conducted: NPT and NVT. These calibrations aim to achieve equilibrium and ensure the simulated system accurately reflects the desired experimental conditions.

NVT Calibration

The NVT calibration, which corresponds to the canonical ensemble, involves maintaining a constant average number of particles (N), volume (V), and temperature (T) throughout the simulation. The system reaches a stable state where temperature fluctuations occur around a specific average value by directly controlling the temperature during the initialization and data collection phases. This ensures that the protein and solvent molecules interact appropriately, allowing for an accurate representation of the system's thermodynamic behavior[24], [25].

During the NVT calibration of the GTRNASE protein, temperature fluctuations are observed and measured over the course of the simulation. These fluctuations provide valuable insights into the system's dynamic behavior, allowing us to assess the stability and equilibration of the protein in its surrounding solvent environment. These calibrations are crucial in preparing the system for further analysis and exploration of its properties and interactions. By analyzing the fluctuations, we can determine whether the system has reached a thermodynamically stable state and evaluate the accuracy of the simulation.

The temperature fluctuations after the NVT calibration of GTRNASE represent the system's dynamic nature. It showcases the variation in temperature over time, reflecting the thermal energy exchange within the system. This information is crucial for understanding the protein's behavior and its interaction with the surrounding environment.

These temperature fluctuations are a natural characteristic of the simulation process and are expected within a stable system. They demonstrate the dynamic nature of the molecular interactions and the system's ability to adapt to different energy states. The absence of extreme temperature values suggests that the simulation is well-controlled and reflective of realistic conditions. Overall, the temperature fluctuations observed in the simulation are within the expected range and support the system's stability.

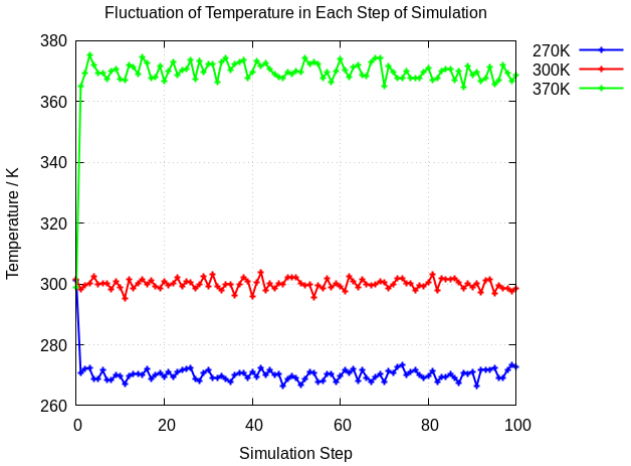

Figure 4. The fluctuations of the blue, red, and green lines during the simulation steps. The blue line represents the average temperature of 270 K, indicating that the system maintains a relatively cool environment. The red line corresponds to a temperature of 300 K, indicating a moderate temperature range. Lastly, the green line depicts a temperature of 370 K, indicating a higher temperature condition.

NPT Calibration

The NPT calibration is a crucial step in molecular dynamics (MD) simulations as it allows for precise control over temperature and pressure. In this calibration, the system is maintained under constant conditions of particle number (N), pressure (P), and temperature (T). Unlike the NVT ensemble, where only the temperature is controlled, the NPT ensemble also considers changes in system volume to maintain a constant pressure. By allowing the unit volume vector to adjust during the simulation, the NPT ensemble accommodates variations in the system's density. This flexibility is particularly important when studying biomolecular systems, as their conformations and interactions can influence the overall density. Thus, the NPT ensemble provides a more realistic representation of the system's behavior under experimental conditions [26], [27].

During the equilibration phase, the NPT ensemble enables the system to reach the desired temperature and pressure, ensuring that it is stable before data collection begins. The system is primed for accurate and meaningful simulations by achieving thermal and pressure equilibrium.

By incorporating the NPT calibration into MD simulations, we can analyze the dynamic behavior of biomolecules in a realistic environment that accounts for both temperature and pressure fluctuations. This calibration step enhances the accuracy and reliability of the simulation results, enabling a more comprehensive understanding of the system under investigation.

In Figure 5, the observed pressure fluctuations exhibit significant extremes, ranging from 400 bar to -400 bar, deviating considerably from the desired pressure of 1 bar. Thus, relying solely on the pressure data for precise characterization in the NPT ensemble is not the best option.

However, after equilibrating the NPT ensemble, the primary focus shifts to another crucial parameter, which is density.

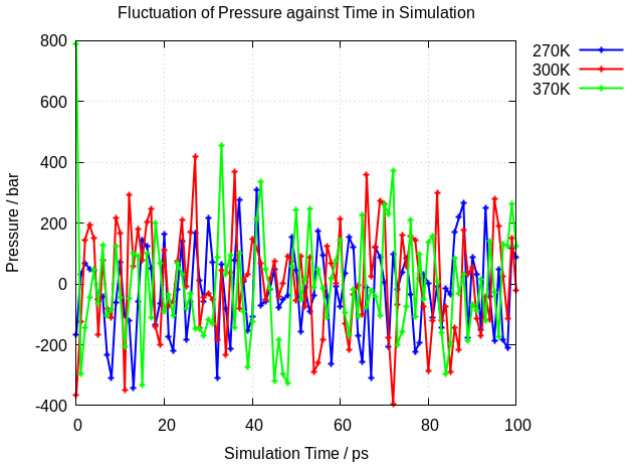

Figure 5. illustrates the pressure fluctuation against simulation time resulting from the NPT calibration of the GTRNase protein. The blue line represents the system at 270 K, the red line represents 300 K, and the green line represents 370 K.

Despite the challenges in pressure control, the density data remains a valuable and reliable metric for assessing the system's behavior. Density reflects the compactness and arrangement of molecules within the system, providing insights into its structural properties. By monitoring the density, one can analyze the system's packing, intermolecular interactions, and overall conformation of the reference structure.

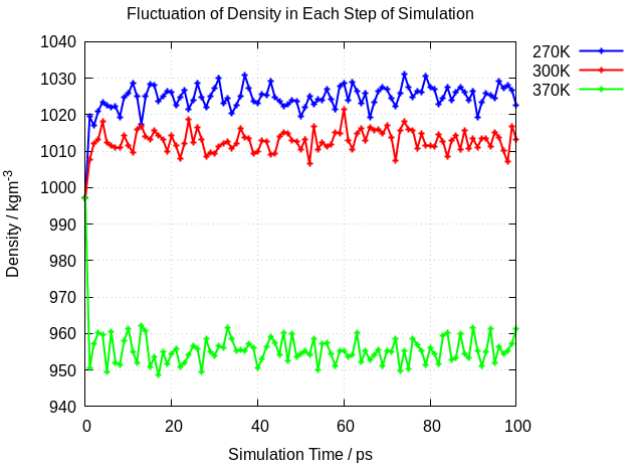

Figure 6. shows the density variations over simulation time resulting from the NPT calibration of the GTRNase protein. The blue line corresponds to a system temperature of 270 K with an average density of 1025 kg/m^3, the red line to 300 K with an average density of 1013 kg/m^3, and the green line to 370 K with an average density of 955 kg/m^3.

Figure 6 presents the density fluctuations of GTRNase protein over time at different temperature levels during NPT calibration, allowing for insights into the protein's thermal response. At lower temperatures, density remains relatively stable, while at higher temperatures, fluctuations are more pronounced, indicating possible conformational changes in the protein structure. The density data (Figure 6) is closer to the experimental value where the density fluctuates within a reasonable range, reflecting that the density data is a reliable parameter in describing the NPT simulation results.

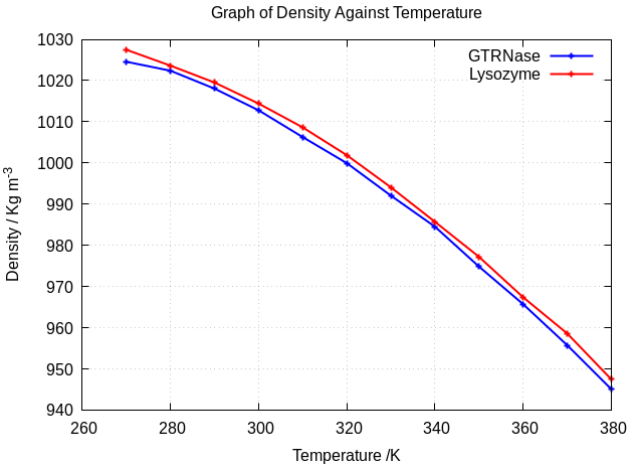

Figure 7. Shows comparison of the density changes between the GTRNase protein and Lysozyme, as temperature decreases from high to low. The blue line represents the density of the GTRNase protein, while the red line represents a well-known enzyme in molecular simulation, lysozyme.

By comparing the density obtained from the simulation of the GTRNase protein with established simulation data of Lysozyme, a process of validation and verification for the simulation model can be conducted. This comparison enables us to assess the accuracy and reliability of the simulation results by observing how closely the density data aligns with the known behavior of lysozyme under similar conditions. Moreover, density plays a crucial role in various thermodynamic calculations, such as computing the molar volume, compressibility, and diffusion coefficients. Density is an essential parameter for understanding phase transitions, solvation behavior, and transport properties within the system.

Radius of Gyration (Rg)

The radius of gyration (Rg) is a fundamental parameter in studying molecular structures, particularly in biophysics, polymer chemistry, and structural biology. It quantifies the distribution of a molecule's mass or atomic positions relative to its center of mass, providing insights into the compactness or spatial extent of the molecule. Beyond proteins, the mean-square radius of gyration also characterizes polymer sizes, encompassing complex structures like hyperbranched polymers and polysiloxanes. This measure's significance extends to its mathematical relationship with segment count and end-to-end distance, impacting the molecule's folding transition from linear to globular forms. Furthermore, through modulation of solubility, it becomes feasible to stabilize intermediate states with fractional dimensions, a factor intrinsically linked to topological properties and molecular interactions, demonstrating the potential for precise control over molecular behavior.

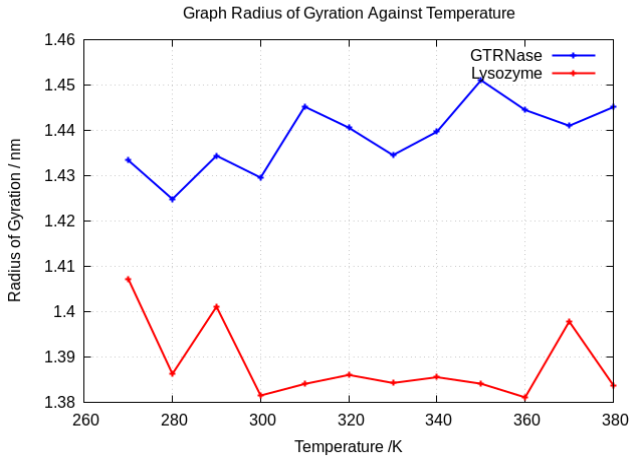

Figure 8. illustrates an increase in Rg with rising temperatures for both the GTRNase protein and Lysozyme.

The Rg data of GTRNase show a slight escalation from 1.434 nm at 270 K to 1.445 nm at 380 K. In contrast, Lysozyme's RMSD data shows stagnation at 1.38 nm from 270 K to 380 K . A slight increase in Rg of GTRNase Protein suggests that the protein's overall size or conformational flexibility is slightly expanding. This expansion could be due to conformational adjustments, an unfolding of certain regions, or an increase in the overall flexibility of the protein's structure. However, in contrast, the Rg of Lysozyme remains relatively constant with increasing temperature, implying that the structural behavior of Lysozyme is notably less affected by temperature variations. This observation suggests a distinctive response of GTRNase and Lysozyme to thermal perturbations, underscoring these proteins' unique conformational dynamics and thermal stability.

Root Mean Square Displacement (RMSD)

RMSD is commonly used in computational studies to evaluate biomolecules' stability, flexibility, and conformational changes over time. By calculating the RMSD, we can determine the average distance between corresponding atoms in the target and reference structures, thereby capturing the overall deviation between each atom [28].

RMSD is a valuable tool for evaluating the stability and dynamics of the enzyme's active site and other important regions in enzyme simulations. It allows us to monitor how the enzyme structure fluctuates, bends, or undergoes structural rearrangements during the simulation. Analyzing the RMSD values allows one to identify critical time points or regions where significant conformational changes occur, providing valuable insights into enzyme function and mechanisms. RMSD analysis also provides important information about the enzyme's structural evolution, its interactions with ligands or substrates, and the conformational transitions it undergoes [29].

The program GROMACS (`gmx rms`) enables the calculation of RMSD for specific atoms within a molecule relative to a reference structure. This is achieved through a least-square fitting process, aligning the molecule's structure with the reference, followed by the computation of the RMSD.

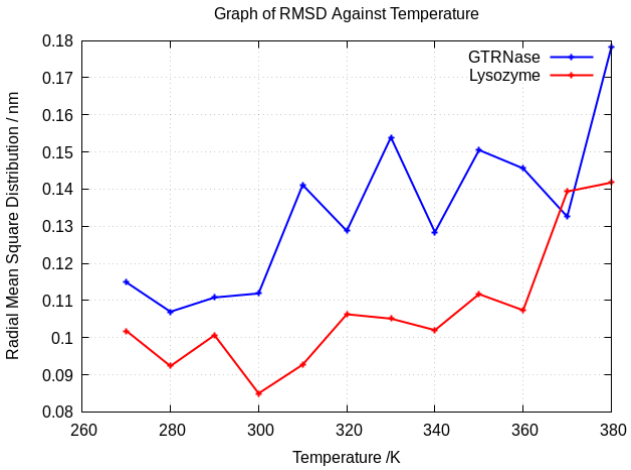

Figure 9. illustrates an increase in RMSD with rising temperatures for both the GTRNase protein and Lysozyme.

The plots display an escalation RMSD data of GTRNase from 0.115 nm at 270 K to 0.179 nm at 380 K, while Lysozyme's RMSD data exhibit a rise from 0.102 nm at 270 K to 0.142 nm at 380 K. A distinctive pattern of RMSD of GTRNase protein in comparison to Lysozyme, wherein GTRNase protein exhibits stability within the temperature range of 270-300 K, contrasting with lysozyme's stability between 320-350 K. Notably, both proteins demonstrate a noteworthy surge in RMSD data at a temperature of 380 K, indicating substantial structural alterations within the protein. The significant RMSD change suggests that the protein's overall structure is undergoing dynamic changes in high temperatures, including potential local unfolding and refolding. This indicates that the protein's conformation is not stable in response to the elevated temperature.

Solvent Accessible Surface Area (SASA)

SASA parameter holds significance in molecular simulations, especially within implicit solvent models where solvation is contextualized within a thermodynamic cycle[30]. Additionally, SASA finds application in investigating surfactant self-assembly[31]. It involves determining the accessible surface area of a molecule, often in relation to water, and is utilized in evaluating surface tension and nonpolar solvation energy within protein simulations[32-34].

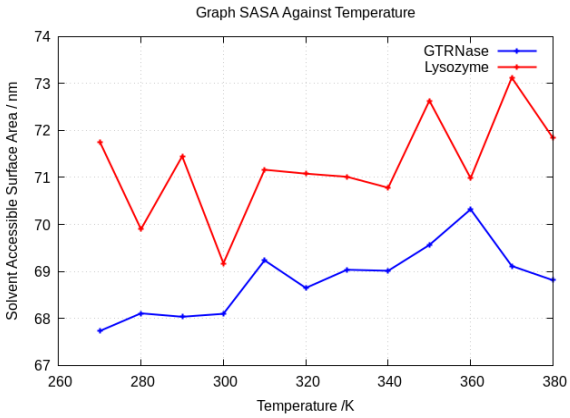

Figure. 10. Graph representing the Solvent Accessible Surface Area plotted against Temperature during the simulation process.

For the GTRNase protein, the SASA values fluctuate between a minimum of 69 nm² and a maximum of 73 nm². Interestingly, the lowest SASA value for GTRNase is observed at 300 K. In contrast, the highest SASA value occurs at 370 K. This suggests a slight but notable increase in solvent accessibility at higher temperatures, although fluctuations are present across the temperature range.

In contrast, the SASA of lysozyme shows a more consistent trend, increasing steadily with temperature. The lowest SASA value for lysozyme is recorded at 67.8 nm² at 270 K. The highest is 70.2 nm² at 370 K. This steady increase implies that lysozyme undergoes gradual surface expansion as temperature rises, which may reflect an increase in molecular flexibility or a response to thermal stress.

Conclusion

This study suggests that the Solvent Accessible Surface Area (SASA) data do not significantly change. Still, there are significant changes in the Root Mean Square Deviation (RMSD) and a slight increase in the protein's Radius of Gyration data during heating, which suggests that the protein is undergoing internal structural changes or fluctuations without experiencing major changes in its overall surface exposure to the solvent. The SASA data provides information about the surface area of the protein that is accessible to solvent molecules. Suppose there is no significant change in SASA during heating. In that case, it indicates that the protein's surface remains exposed to the solvent similarly, suggesting that the protein's overall shape or surface characteristics do not change significantly. The RMSD measures the deviation or difference in atomic positions between two protein structures, in this case, between the heated protein and its initial (reference) structure. A significant change in RMSD during heating indicates that the protein is undergoing structural changes or fluctuations. The significant RMSD change suggests that the protein is experiencing internal conformational adjustments, fluctuations, or even unfolding and refolding certain regions. The Radius of Gyration (Rg) data provides information about the protein's compactness or size. A slight increase in Rg during heating suggests that the protein is undergoing some expansion or structural fluctuations, resulting in a slight increase in its overall size or conformational flexibility. Combining the information from SASA, RMSD, and Rg analyses, the following interpretations can be made: The protein's overall surface exposure to solvent molecules remains relatively constant, as indicated by the stable SASA data. The significant change in RMSD suggests that the protein is undergoing internal structural changes or dynamic fluctuations, possibly related to conformational adjustments or local unfolding and refolding. The slight increase in Rg indicates that the protein is experiencing some degree of expansion or conformational flexibility, which could result from internal structural rearrangements. These observations suggest that the protein is undergoing conformational changes and fluctuations while maintaining its overall surface characteristics. This scenario is often observed in proteins that undergo dynamic motions or conformational changes without undergoing major unfolding or exposing new hydrophobic regions to the solvent. The combination of SASA, RMSD, and Rg analyses provides valuable insights into the interplay between protein dynamics, internal structure, and surface exposure during heating or other biochemical processes.

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