Enhancing Insulin Thermostability through Strategic Mutations: Integrative Analysis via Thermal Titration Molecular Dynamics Simulations

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ABSTRACT

The thermostability of insulin is essential for its storage and effectiveness, especially in conditions where temperature regulation is difficult. Through Thermal Titration Molecular Dynamics (TTMD) simulations conducted at multiple temperature (300K, 310K, 320K, and 330K) to improve insulin's thermal stability by identifying and targeting flexible residues that lead to structural instability under heat stress. The study identified specific residues in Chain A (Gln5 and Thr8) and Chain B (Gly8, Ser9, and His10) exhibiting significant flexibility and structural instability at physiological conditions (310 K) via Root Mean Square Fluctuation (RMSF), suggesting them prime candidates for mutation. This study produced 6,859 mutations in Chain B and 361 mutations in Chain A of insulin, concentrating on unstable regions. Stability study revealed 80 combinations exhibiting stabilising effects (ΔΔGpred < 0.0 and cpred > 0.95), which were further evaluated for thermal stability using the DeepSTABp server. Two mutants (Mutant 1 and Mutant 2) with the highest melting temperature (Tm) exceeding 50 °C were selected for molecular dynamics (MD) simulations and compared to wild-type insulin. RMSD, RMSF, PCA, and Free Energy Landscape (FEL) analysis demonstrated that Mutant-2 had the most compact energy well and reduced structural flexibility, signifying improved stability. The findings indicate that Mutant-2 (His5 and Lys8 of chain A and Ser8, Thr9, Cys10 of chain B) is a viable candidate for the development of thermostable insulin, potentially enhancing storage and use under fluctuating temperature settings. This integrated methodology, encompassing TTMD, computational mutation analysis, stability prediction, and molecular dynamics, proficiently discovers viable candidates for thermally stable insulin analogues. This study suggests Mutant-2 for further in vitro and experimental investigations to confirm the findings and assess its practical effectiveness.

Keywords: Thermostability, Insulin, Temperature, Insulin analogues

INTRODUCTION

Insulin, a small peptide hormone, is essential for the regulation of blood glucose levels. Insufficient insulin production or function results in diabetes mellitus, a metabolic condition linked to significant health problems and potential fatality if left untreated[1]. Insulin is composed of 51 amino acids arranged into two peptide chains, A and B, connected by two inter-chain disulphide bonds, along with an additional intra-chain disulphide bond in the A-chain[2,3]. This hormone is synthesised in the pancreatic β-cells within the islets of Langerhans, where it is present at micromolar concentrations. At these concentrations, insulin self-associates into dimers, a process likely occurring during expression in the endoplasmic reticulum (ER). Residues participating in dimerisation are situated on the B-chain, specifically at locations B8, B9, B12, B13, B16, and B23–B28[3,4].

However, its inherent sensitivity to temperature poses significant challenges in storage and distribution, particularly in regions lacking reliable refrigeration. Enhancing the thermostability of insulin is therefore a critical objective to improve its accessibility and efficacy worldwide. Protein engineering, especially through site-directed mutagenesis, offers a promising avenue to enhance insulin's thermal stability[5]. By introducing specific amino acid substitutions, researchers can modify the protein's structure to withstand higher temperatures without compromising its biological function.

A previous study presents a computational technique aimed at forecasting alterations in protein melting temperature (ΔTm) due to point mutations, tackling a significant difficulty in protein science with implications for enhancing enzymes for unusual settings[6]. The model utilises standard and temperature-dependent statistical potentials integrated via an artificial neural network, with parameters optimised on a dataset comprising over 1,600 experimentally measured mutations. Thorough evaluation by 5-fold cross-validation exhibited enhanced performance relative to current methodologies, attaining a root mean square deviation (RMSD) of 4.2 °C, which increased to 2.9 °C following outlier elimination.

Another study introduces an innovative technique for enzyme thermostabilizing using computationally assisted protein stapling utilising genetically encoded thioether staples[7]. Incorporating electrophilic amino acids like p-chloroacetamido-phenylalanine (pCaaF) into a myoglobin-based cyclopropanase resulted in improved stability against thermal denaturation (ΔTm′ = +27 °C) and temperature-induced heme loss (ΔT50 = +30 °C), while maintaining catalytic activity and stereoselectivity. Molecular simulations demonstrated that the pCaaF staple limits conformational flexibility, which is associated with increased stability.

A prior study concentrates on improving heat stability in proteins by creating and assessing β-glucosidase mutants through experimental and computational methods. The study offers an extensive investigation of thermal stability parameters, including T50 (temperature at which 50% of activity is preserved), TM (melting temperature), and ΔΔG (change in Gibbs free energy), for 51 mutants by augmenting a previously reported dataset[8]. The efficacy of nine computational stability prediction techniques, such as Rosetta ΔΔG, FoldX, DeepDDG, PoPMuSiC, and SDM, was assessed. Although none were significant predictors of T50, TM, or ΔΔG, these tools demonstrated efficacy in prescreening mutants for their capacity to provide isolatable and soluble proteins. This underscores their significance in preserving folding and soluble production characteristics in protein engineering.

Recent *in silico* analysis have accelerated the development of thermostable insulin variations. Molecular dynamics (MD) simulations have been utilised to assess the effects of single, double, and triple mutations on the stability and unfolding mechanisms of insulin. Thermal unfolding investigations at high temperatures (e.g., 400 K) utilising sophisticated force fields, such as CHARMM36m, have demonstrated the essential function of residues such as AspB10, GluB27, and AspB28 in thermal stability[9]. Alternative methods that integrate computational screening with in vitro validation have effectively found mutants exhibiting elevated melting temperatures (Tm), a crucial sign of thermal stability. These findings highlight the potential of combining computational and experimental approaches to develop insulin analogues with improved thermostability.

In this study, Thermal Titration Molecular Dynamics (TTMD) and molecular applied to identify and change flexible areas in insulin to boost thermostability. Using TTMD at 300 K, 310 K, 320 K and 330 K, specific residues in Chain A and Chain B were identified as highly flexible through Root Mean Square Fluctuation (RMSF) analysis. The multiple mutations were generated in each chains targeting these sites. Stability predictions for the mutated sequences were performed, selecting mutants with stabilizing ΔΔGpred values and high confidence. The stabilized mutants were further screened for thermal

stability, retaining only those with predicted melting temperatures (Tm) above 50 °C. Finally, the best mutants with the highest Tm values were selected for detailed MD simulations alongside wild-type insulin, using RMSD, RMSF, Principal Component Analysis (PCA), and Free Energy Landscape (FEL) analyses to assess and compare structural stability and flexibility.

METHODOLOGY

Thermal Titration Molecular Dynamics (TTMD): Thermal Titration Molecular Dynamics (TTMD) is a progressive modelling method employed to investigate the behaviour and stability of biomolecules, including proteins, under diverse temperature circumstances. It evaluates whether the initial binding configuration is preserved during a sequence of molecular dynamics simulations conducted at increasing elevated temperatures[10]. This study utilised TTMD simulations to pinpoint temperature-sensitive regions of wild-type insulin that get destabilised at increased industrial temperatures. These regions will be prioritised for stability. The protein was simulated via the molecular dynamics (MD) simulation with the GROMACS 2022.4 package[11,12]. The wild-type insulin structure obtained from the Protein Data Bank with PDB ID: 3W7Y was utilised for the TTDM[13,14,15]. The PDB file was converted via the PDB2GMX module. A cubic simulation box was constructed, maintaining a distance of 1.0 nm between the protein and the box edges. Additionally, the system underwent solvation within the solvation box in accordance with the TIP3P model[16]. Subsequently, sodium (Na+) and chloride (Cl-) ions were introduced for the ionisation of the system. The system was subsequently minimised via the steepest descent approach with 50000 steps to eliminate steric conflicts. Moreover, system stability was attained subsequent to the imposition of bond restrictions via the LINCS algorithm[17]. In the equilibration phase, the position of the protein was constrained using the constant number, volume, and temperature (NVT) and isothermal-isobaric (NPT) ensembles. The system's stability was maintained by regulating temperature through velocity scaling method[18] and pressure via Parrinello-Rahman coupling method[19]. An NVT ensemble with a 2 fs timestep was executed, simulating the entire system for 100 ps at various temperatures (300 K, 320 K, 340 K, and 350 K). The same procedure was applied to sustain the system's pressure at NPT conditions: 1 ns at 300K, 320K, 340K, and 350K at 1 atmosphere. During the complete 100 ns production cycle, the structure's coordinates were documented every 10 ps. Root mean square fluctuation (RMSF) and root mean square deviation (RMSD) were employed to evaluate the conformational stability and predictability of the MD results. The post MD analysis was conducted on the visual platform "Analogue," developed by Growdea Technologies[20,21] (https://growdeatech.com/Analogue/).

Mutation: Specific acids in insulin were changed for chains A and B using an internal Python tool. These residues were selected for their considerable flexibility at 310K, as determined by RMSF analysis, and their minimal inter-chain interactions, rendering them appropriate candidates for stabilising mutations. The stability prediction was conducted utilising MaestroWeb[22]. The altered insulin structures were submitted to MaestroWeb for stability assessment. MaestroWeb is a tool designed to anticipate the impact of point mutations on protein stability, available at https://pbwww.services.came.sbg.ac.at/maestro/web/maestro/workflow.

Simulations and predictions were conducted at pH 7 to replicate physiological circumstances. The predicted variation in stability for each mutated sequence was provided as ΔΔGpred (Predicted Variation in Gibbs Free Energy) values in kcal/mol. ΔΔGpred < 0.0 implies a stabilising mutation, indicating that the mutation is predicted to enhance the structural stability of insulin. ΔΔGpred > 0.0 indicates a destabilising effect. The dependability of each ΔΔG prediction is quantified by Confidence Prediction (cpred), a metric that spans from 0.0 to 1.0, where 1.0 denotes high reliability and 0.0 indicates low reliability. This metric provides clarity on the prediction's strength, facilitating the analysis of stabilising or destabilising impacts.
The results from MaestroWeb provided ΔΔGpred values for each mutated sequence, allowing us to assess if the individual mutations enhance the stability of insulin. By focussing on mutations with ΔΔGpred.< 0.0 and high cpred of 0.95, the study prioritise changes that are both stabilising and reliable. This strategy aims to identify mutations that increase insulin's thermostability at physiological pH, so enhancing its resistance to temperature variations while maintaining its biological function.

Additionally, sequences having over 95% confidence prediction (cpred of 0.95) were utilised for screening using the DeepSTABp server to ascertain their thermal stability temperature via melting temperature [°C][23]. DeepSTABp is a predictive instrument that assesses protein stability by determining the melting temperature (Tm) in degrees Celsius, which acts as a crucial indicator of the protein's thermal robustness. A raised melting temperature indicates enhanced stability at high temperatures, implying that the protein structure is more resilient. The sequences exhibiting temperatures exceeding 50 °C were then utilised for molecular dynamics simulation. The sequence exhibiting the highest melting temperature was prioritised due to its superior anticipated stability profile. This selection approach guarantees that only the most thermally resilient versions undergo comprehensive simulation, enabling the study to concentrate on sequences with the highest potential for enhanced thermostability in actual applications.

MD Simulation (Mutated Insulin): The top mutated protein structures were selected for MD simulation at 310 K which showed the most fluctuations for the residues. The MD simulation was performed using the same protocol as mentioned in the section 2.1.

Principal Component Analysis (PCA) and Free Energy Landscape (FEL): The trajectory was preprocessed for principal component analysis by eliminating the periodic boundary condition. The covariance matrix was calculated using the Gmx_covar module of GROMACS. The covariance matrix delineates the correlation among the atomic fluctuations of the protein-ligand combination. The gmx analysis function was utilised to calculate the eigenvalues and eigenvectors of the covariance matrix. The GROMACS tool 'gmx anaproj' was utilised to calculate the principal component (PC) coordinates for every frame. The provision was enacted to exhibit the trajectory on personal computers.

The analysis of the transitional state, represented by the barriers on the Free Energy Landscape (FEL), and the equilibrium state, characterised by the minima on the FEL, might yield essential insights into biological processes such as biomolecule recognition, aggregation, and folding[24]. The energy distribution was estimated utilising Equation (1) to compute the FEL.

$$\Delta G(X) = -kBTln\, P(X) \quad (1)$$

The Boltzmann constant is denoted as kB, Gibbs free energy as G, the reaction coordinate as X, and the system's probability distribution along the reaction coordinate as P(X).

RESULTS AND DISCUSSION

Structure of Insulin

The structure of insulin was retrieved using the PDB ID: 3W7Y as shown in Figure 1. The purpose of this study was to use heat Titration

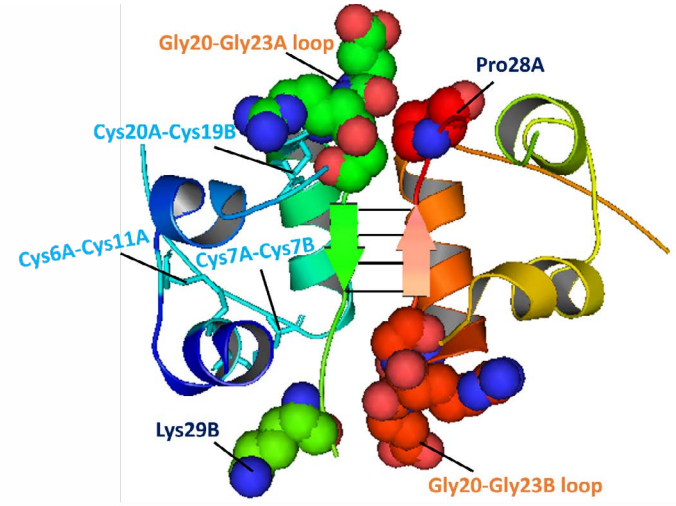

Figure 1. Structure of wild-type human insulin with PDB ID: 3W7Y. The protein structures are depicted in ribbon representation, alongside the interaction surfaces of the Gly20-Gly23 loop. Hydrogen bonds between the β-strands of the insulin monomer are depicted as thin black lines.

Molecular Dynamics (TTMD) simulations to identify which insulin residues are unstable or very flexible under different heat conditions. The goal was to identify insulin residues that can destabilise at high temperatures by studying their structural changes as a function of temperature at the residue level, using metrics such as Root Mean Square Deviation (RMSD) and Root Mean Square Fluctuation (RMSF). Identifying flexible or unstable residues is essential for enhancing insulin's thermostability, allowing for targeted mutations. This strategy systematically identifies the insulin regions most vulnerable to heat stress to develop insulin analogues that are more stable and resilient to storage and use in diverse settings.

A fundamental study conducted by Sanger and Smith clarified the principal insulin sequence[2]. The pancreatic β-cells of the islets of Langerhans generate insulin, a protein with a molecular weight of around 5.8 kDa, which is extremely conserved. Chain A and chain B, the two polypeptides that make up the heterodimer, each contain 21 and 30 amino acids, respectively. Multiple disulphide bridges link the two chains; two of these bridges are located between chains A and B (Cys7A-Cys7B and Cys20A-Cys19B), and one is within chain A (Cys6-Cys11)[25]. All members of the insulin superfamily share these three α-helices (Figure 1), which are held together by these six cysteine residues, in the tertiary structure. Despite advances in biotechnology, insulin is still mostly manufactured as a recombinant protein due to the difficulties posed by the disulphide presence in high-yield chemical synthesis[26].

Hydrogen bonds, which are formed between the B-chain β-strands of neighbouring monomers, stabilise insulin dimers. Hydrogen bonds with distance of approximately 2.9 Å stabilise the T:Rf (Tense- Relaxed form) dimers in wild-type human insulin[27]. Also contributing to dimer formation are van der Waals contacts between monomer Pro28/Lys29 and neighbouring monomer GlyB20-GlyB23 loops (Figure 1). Insulin dimers are able to avoid certain van der Waals interactions when the prolyl and lysyl residues close to the B-chain C-terminus are inverted, which alters the protein backbone's trajectory[28]. A previous showed that the number of hydrogen bonds formed by the insulin lispro dimer between neighbouring β-strands is the same as in wild-type human insulin, but the two closest to the engineered sequence alteration are longer (~3.2 Å).

TTMD OF INSULIN CHAINS

Figure 2 presents a Root Mean Square Deviation (RMSD) plot of insulin at various temperatures (300K, 310K, 320K, and 330K) during a 100 ns molecular dynamics simulation. The study offers insight into the structural stability of insulin at different thermal conditions. The RMSD escalates with time in all simulations, signifying structural changes from the starting configuration as the simulation advances. The RMSD values fluctuate around a certain average at each temperature, indicating that insulin attains a comparatively stable structure, albeit with dynamic variations.

At the minimum temperature (300K), the RMSD remains possibly steady; nonetheless, notable oscillations occur, particularly after 60 ns. The RMSD values range from 0.2 to 0.6 nm, with discontinuous maximum values. At 310K, the overall RMSD was the lowest among the four temperatures, signifying enhanced structural stability. The readings stabilise between 0.3 and 0.5 nm, exhibiting reduced high volatility. RMSD measurements at 320K suggest marginally greater stability than at 300K, exhibiting a narrower fluctuation range, generally between 0.3 and 0.6 nm. The temperature at 330K exhibits the highest fluctuations, with RMSD attaining values of up to 0.8 nm. This implies that elevated temperatures compromise the integrity of insulin's structure, as evidenced by greater deviations.

The results of the root-mean-square (RMSD) analysis suggest to a temperature dependence of insulin's structural stability. Insulin displays relatively consistent behaviour with few outliers at 310K, but its structure becomes more malleable and displays larger fluctuations at 330K, suggesting structural instability. This investigation demonstrates the impact of temperature on the conformational stability of insulin by RMSD assessment. Insulin keeps its structure stable at physiological temperatures (about 310K), but it goes through more drastic structural changes at higher temperatures. Insights gained from these results might direct mutation studies targeted at improving insulin's thermostability, highlighting the significance of thermal stability in insulin design. Insulin could benefit from targeted stabilising mutations or alterations to keep its structure at elevated temperatures, according to the data. This would be particularly beneficial in regions with limited temperature regulation.

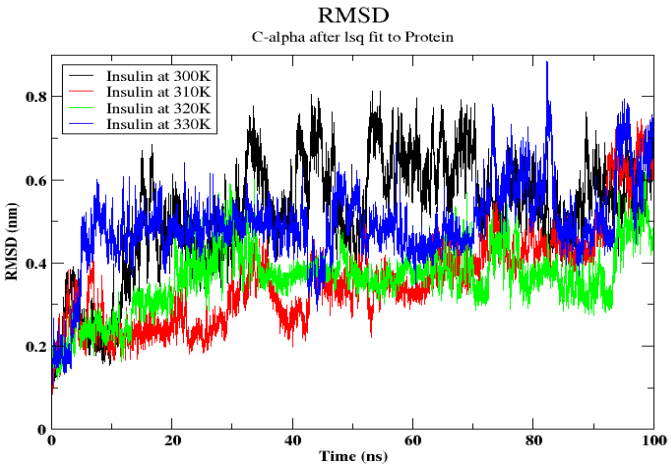

Figure 2. RMSD of the protein Cα atoms of the wild-type insulin analysed after TTMD at 300 K, 310 K, 320 K, and 330 K

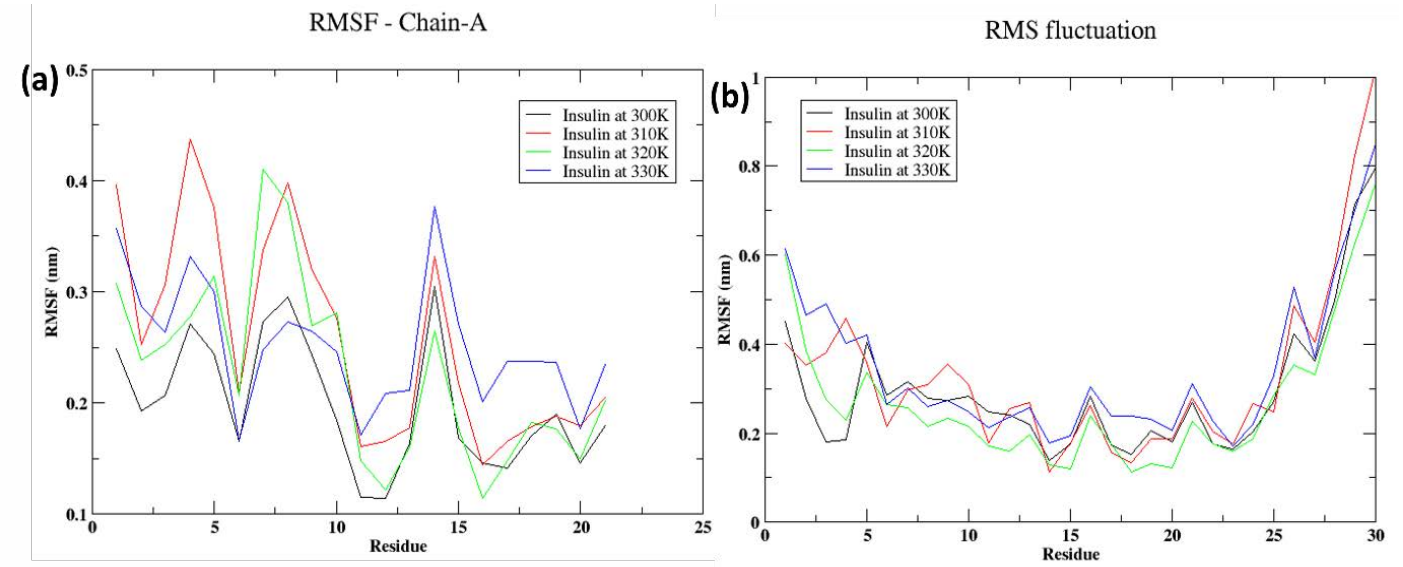

Figure 3

RMSF OF INSULIN CHAINS AT VARIOUS TEMPERATURES

Table 1 summarises the average Root Mean Square Fluctuation (RMSF) values and the count of residues exhibiting RMSF exceeding 0.3 nm for both Chain A and Chain B of insulin at four distinct temperatures: 300 K, 310 K, 320 K, and 330 K. RMSF shown in the Figure 3 quantifies the flexibility of specific amino acid residues across molecular dynamics simulations. Elevated RMSF values signify more flexibility, which may be associated with structural instability.

The average RMSF of Chain A as shown in Figure 3(a) escalates from 0.19 nm at 300 K to 0.25 nm at 310 K, indicating enhanced flexibility with increasing temperature. At 320 K, the average RMSF marginally falls to 0.22 nm. At 330 K, it reverts to 0.25 nm, signifying a steady enhancement in flexibility at elevated temperatures. Only one residue above the 0.3 nm barrier at 300 K. The number of residues >0.3 nm escalates to 8 residues at 310 K, indicating a notable increase in flexible areas. At 320 K, the residues reduce to 4 residues. At 330 K, three residues surpass 0.3 nm, marginally fewer than at 310 K.

Chain B consistently shows higher average RMSF values than Chain A at all temperatures as shown in Figure 3(b). The average RMSF increases from 0.29 nm at 300 K to 0.32 nm at 310 K. It decreases to 0.27 nm at 320 K, then rises to the highest value of 0.34 nm at 330 K. At 300 K, 7 residues have RMSF values exceeding 0.3 nm. This number nearly doubles to 13 residues at 310 K. It decreases to 8 residues at 320 K. At 330 K, the number increases to 14 residues, the highest observed. Both chains exhibit increased flexibility as the temperature rises from 300 K to 310 K and from 320 K to 330 K. A significant reduction in average RMSF and the quantity of extremely flexible residues is observed at 320 K for both chains. This indicates a potential stabilisation impact or a conformational state that diminishes flexibility at this particular temperature.

Elevated RMSF values and an increased number of residues beyond 0.3 nm indicate that certain regions of the protein are becoming more dynamic, which may result in unfolding or functional impairment. Thus, these residues were selected for targeted mutation that could enhance flexibility. A single thermal condition (310K), which showed high instability for both Chain A and Chain B, was selected to optimise the investigation. This temperature approximates physiological settings, rendering it pertinent for investigating insulin stability within the human body, while also exhibiting significant flexibility and instability in specific residues according to the RMSF data. The study focusses on 310K to assess insulin stability in physiologically relevant conditions, where fluctuation in these specific environments may affect functionality and stability. Specific mutations at these residues may enhance the structural integrity of insulin, potentially resulting in a more stable analogue with greater resilience for therapeutic use.

Table 1. Average RMSF of the chain A and B of the wild-type insulin post TTMD

	Chain A		Chain B	
	Average RMSF	Number of residues > 0.3 nm	Average RMSF	Number of residues > 0.3 nm
300 K	0.19	1	0.29	7
310 K	0.25	8	0.32	13
320 K	0.22	4	0.27	8
330 K	0.25	3	0.34	14

RESIDUES SELECTION

The 310 K temperature was selected where both chain A an B of insulin showed high flexibility indicating instability of the structure compared to the others. Further analysis showed that the residues that fluctuated > 0.3 nm for chain A were Gly1, Val3, Glu4, Gln5, Cys7, Thr8, Ser9, Tyr14 and for chain B. were residues Phe1, Val2, Asn3, Gln4, His5, Gly8, Ser9, His10, Tyr26, Thr27, Pro28, Lys29 and Thr30 as shown in Figure 4. As shown in the figure, the terminal residues Gly1 of chain A was not included for the mutations, which may have distinct structural roles. Similarly, the terminal residue Phe1 and Thr30 was not included for mutations.

Further, the interaction between the two chains were observed as shown in Figure 5. It was observed that the chain A showed interaction for the residues Glu4, Val3, Tyr19, Ser12, Ile10, Cys11, Leu13, Tyr14, Glu17, Asn21, Cys20, Leu16, Cys6, Cys7, Ser9 and Chain B showed interaction for the residues Lys29, Pro28, Thr27, Tyr26, Phe25, Phe24, Asn3, Gln4, Val18, Phe1, Arg22, Gly23, Cys19, Ala14, Leu15, Leu11, Leu6, His5 and Cys7. These residues were excluded from mutations due to their potential significance in dimer formation interactions. Thus, the residues Gln5 and Thr8 of chain A and Gly8, Ser9, His10 of

chain B was used for mutation.

The structural integrity of the dimer was protected by avoiding mutations in these interacting residues. Insulin dimer formation relies on specific inter-chain interactions, which are preserved during this selection process, allowing for alterations to be directed to more malleable regions. The emphasis on non-interacting, flexible residues for mutation seeks to improve insulin's thermostability while preserving its functional conformation.

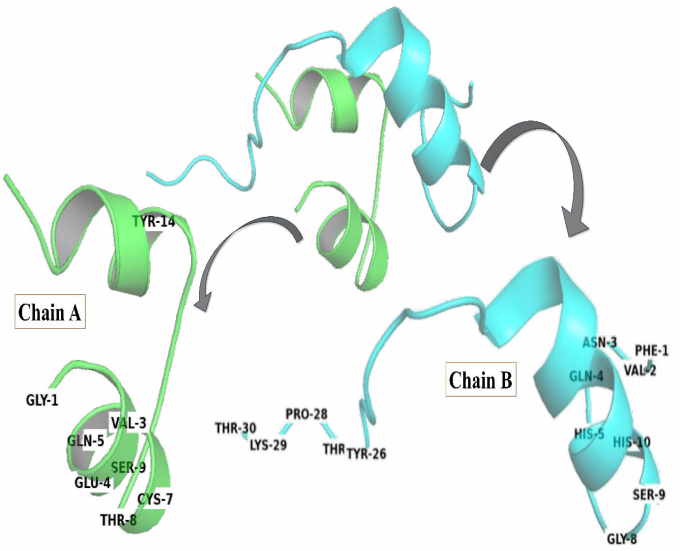

Figure 4. Residues with high fluctuations RMSF > 0.3 nm in chain A and B of insulin at 310 K MD simulation

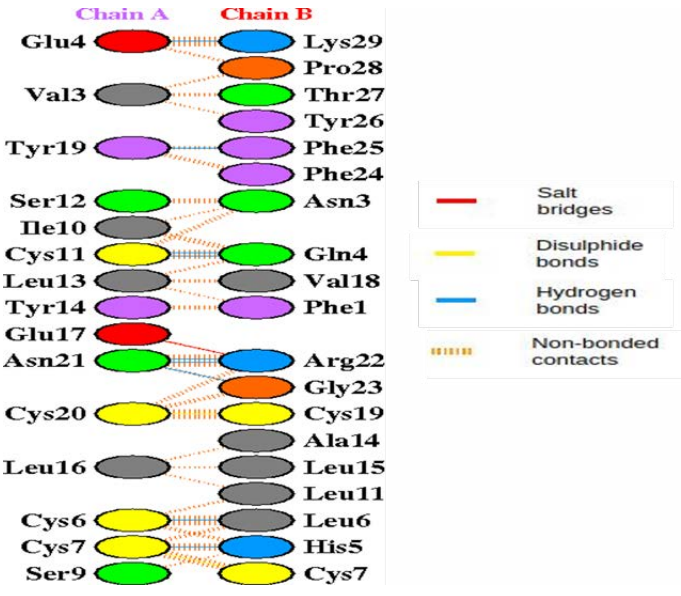

Figure 5. Interaction residues between the chain A and B of the insulin from PDBsum. Blue lines represent the hydrogen bonds, yellow disulphide bonds, red salt bridges and orange non-bonded contacts.

MUTATION AND STABILITY ANALYSIS

A total of 6,859 mutated sequences were generated for chain B by modifying residues at designated places (Gly8, Ser9, His10) recognised as extremely flexible according to RMSF analysis at 310 K. Similarly, 361 mutated sequences were generated for chain A using the sites Gln5 and Thr8. These changes focused on areas with significant structural instability, with the objective of improving insulin's thermostability. The produced mutant sequences were subsequently analysed for stability utilising MaestroWeb, a prediction instrument for evaluating the effects of mutations on protein stability. Among the 6,859 mutated sequences of chain B, 80 exhibited ΔΔGpred values less than 0.0, indicating a stabilising impact, and cpred values exceeding 0.95, signifying high confidence in the predictions. Similarly, only 1 mutated sequence of chain A showed ΔΔGpred < 0.0 and cpred > 0.95 which was selected. This Chain A mutant was then combined with each of the 80 stabilizing Chain B mutants, resulting in 80 unique combination sequences.

Thus, 80 mutated sequences (1 mutated sequence of chain A × 80 mutated sequence of chain B) were made using the combinations from chain A and chain B. The stabilised sequences were further evaluated for thermal stability with the DeepSTABp service, which forecasts the melting temperature (Tm) of proteins. The wild type insulin showed a Tm of 49.04 °C. Only sequences with a predicted melting temperature (Tm) exceeding 50 °C were retained for subsequent investigation. Of them, two mutants exhibited the highest anticipated melting temperatures and were chosen for comprehensive analysis.

The sequences of the wild-type insulin and the two selected mutants are as follows:

GIVEQCCTSICSLYQLENYCNFVNQHLCGSHLVEALYLVC-GERGFFYTPKT (wild-type)

GIVEHCCKSICSLYQLENYCNFVNQHLCSACLVEALYLVC-GERGFFYTPKT (mutant 1)

GIVEHCCKSICSLYQLENYCNFVNQHLCSTCLVEALYLVC-GERGFFYTPKT (mutant 2)

Here, mutant 1 showed mutations His5 and Lys8 of chain A and Ser8, Ala9, Cys10 of chain B where mutant 2 showed similar mutation with Thr9 in place of Ala9. The selected mutants, demonstrating increased thermal stability compared to the wild type indicated by elevated Tm values, were subsequently analysed by molecular dynamics (MD) simulations at 310 K in conjunction with the wild-type insulin sequence. The objective of these simulations was to assess and contrast the structural dynamics, flexibility, and stability of wild-type and mutant insulin under physiologically pertinent conditions. This comparative investigation seeks to ascertain if the alterations enhance thermostability while preserving the overall structural integrity and functional characteristics of insulin.

These findings suggest that the chosen mutations are effective candidates for improving insulin's stability under thermal stress, which could facilitate its application in environments where temperature control is challenging. The high confidence in these predictions further supports their potential for practical implementation in developing thermostable insulin analogs.

MD SIMULATION AT 310 K

RMSD

Figure 6 illustrates the Root Mean Square Deviation (RMSD) graph for wild-type insulin and two mutant variations (Mutant-1 and Mutant-2) during a simulation duration of 100 ns at 310 K. The wild-type insulin has the lowest RMSD values during the simulation, often oscillating between 0.2 and 0.5 nm. This suggests that the wild-type structure is comparatively stable, exhibiting minor changes from the original

shape. Near the conclusion of the 100 ns simulation, the RMSD stays below 0.5 nm, indicating that the wild-type insulin preserves its structural integrity effectively under these conditions. Mutant-1 exhibits the greatest RMSD values of the three, attaining up to 1.2 nm during the simulation. After 40 ns, the RMSD of Mutant-1 oscillates between 0.8 and 1.2 nm, signifying a greater level of structural deviation and flexibility relative to the wild-type. The notable rise in RMSD implies that Mutant-1 may possess a less stable conformation under these simulation parameters, potentially signifying diminished stability relative to the wild-type. Mutant-2 shows intermediate RMSD values, consistently higher than the wild-type but lower than Mutant-1. The RMSD values for Mutant-2 fluctuate between 0.3 and 0.8 nm, stabilising at approximately 0.6 to 0.8 nm during the latter phase of the experiment. This suggests that Mutant-2 exhibits intermediate structural flexibility, demonstrating higher deviation than the wild-type while showing enhanced stability relative to Mutant-1.

The wild-type insulin exhibits superior structural stability, indicated by the lowest RMSD values, implying it preserves its natural conformation more effectively than the mutations. Mutant-1 demonstrates the most pronounced structural departure, signifying it is the least stable under the simulated conditions. Mutant-2 exhibits a moderate stability profile, indicating a potential balance between flexibility and stability, albeit with lower stability than the wild-type.

The elevated RMSD values of Mutant-1 and Mutant-2, especially post 40 ns, indicate that these mutations confer flexibility, potentially impacting thermostability. Given the goal of identifying mutants with enhanced thermostability, Mutant-2 is a superior candidate compared to Mutant-1, as seen by its modest RMSD values and comparatively constant performance in contrast to the extremely variable Mutant-1. The RMSD study reveals that wild-type insulin exhibits the most structural stability under these conditions, whereas Mutant-1 demonstrates the

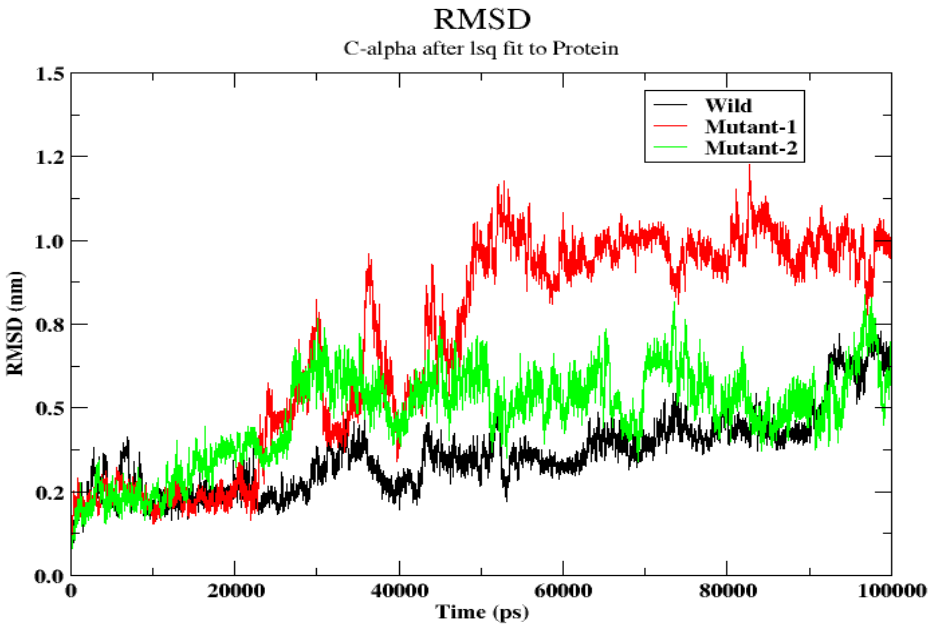

Figure 6. RMSD of the Cα atom of the protein for wild type insulin, Mutant 1 and Mutant 2 during the 100 ns MD simulation

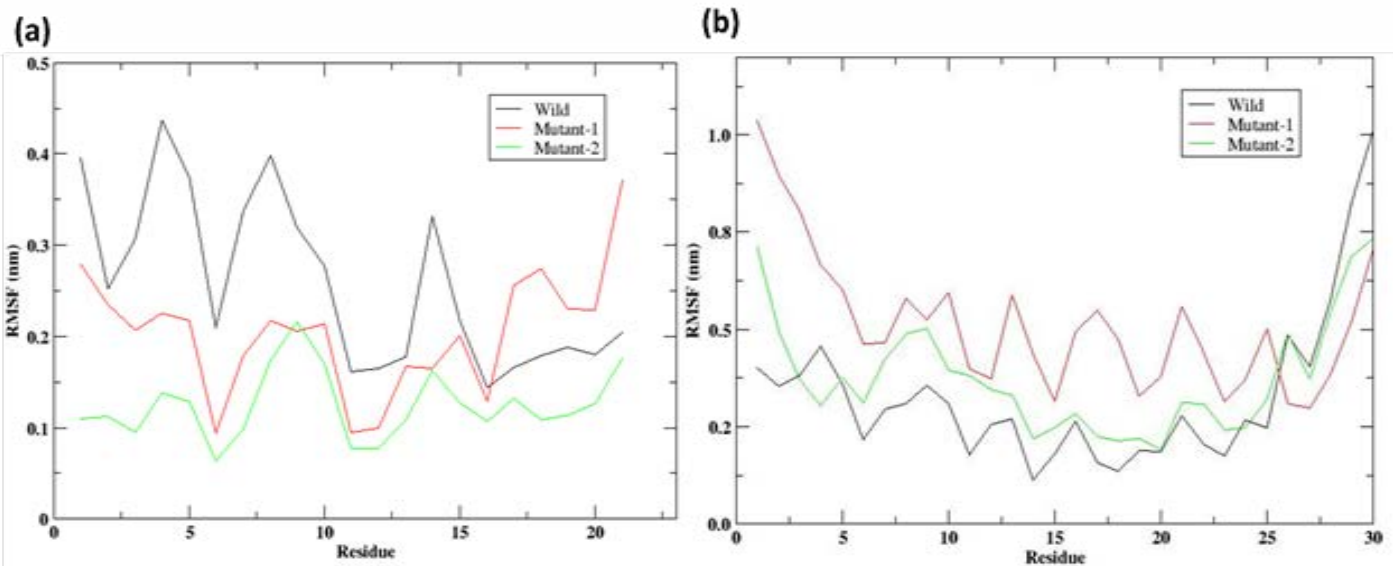

Figure 7. RMSF of the wild type insulin, Mutant 1 and Mutant 2 during the 100 ns MD simulation

lowest stability, exhibiting significant aberrations. Mutant-2 exhibits an intermediate profile, rendering it a viable option for further exploration due to its possible equilibrium between flexibility and stability, which may be beneficial for specific applications.
RMSF

Figure 7 showed the Root Mean Square Fluctuation (RMSF) plots for Chain A (Figure 7(a)) and Chain B (Figure 7(b)) of wild-type insulin and two mutant versions (Mutant-1 and Mutant-2). Mutant-1 has diminished RMSF values relative to the wild-type for the majority of residues in Chain A. It showed peaks at residues 5 and 20 are evident but diminished compared to the wild-type, suggesting that Mutant-1 may exhibit enhanced stability in these regions for chain A. Mutant-2 exhibits the lowest RMSF values among all residues in Chain A, signifying a notable decrease in flexibility relative to both the wild-type and Mutant-1. The diminished fluctuations indicate that Mutant-2 is the most stable mutant for Chain A.

In Chain B, the wild-type insulin exhibits elevated RMSF values for residues adjacent to the termini (e.g., residue 1 and residue 30) as well as for other mid-chain residues. The RMSF values attain 0.8 nm, signifying considerable flexibility in Chain B of the wild-type insulin. Mutant-1 exhibits reduced RMSF values compared to the wild-type over most of Chain B, however remains elevated at residues 1 and 26–30. This indicates a decrease in flexibility; however, the terminal and mid-chain areas continue to exhibit modest elasticity. Mutant-2 exhibits the lowest RMSF values throughout Chain B, especially in mid-chain regions where it displays negligible changes. The terminal residues in Mutant-2 exhibit greater stability compared to both the wild-type and Mutant-1, indicating that Mutant-2 imparts an overall stabilising influence on Chain B.

The average RMSF values for Chain A and Chain B are 0.258 nm and 0.328 nm, respectively as shown in Table 2. These values suggest moderate flexibility in both chains, with Chain B exhibiting slightly higher flexibility than Chain A. In Mutant-1, the average RMSF for Chain A decreases to 0.20 nm, indicating a reduction in flexibility relative to the wild-type, suggesting improved stability in Chain A. However, Chain B shows an increase in RMSF to 0.51 nm, indicating greater flexibility compared to the wild-type. This suggests that while Mutant-1 has stabilized Chain A, Chain B has become more flexible, potentially impacting the overall structural stability. Mutant-2 exhibits the lowest RMSF values for both chains, with 0.125 nm for Chain A and 0.37 nm for Chain B. The reduction in RMSF for Chain A and a slight increase compared to the wild-type in Chain B suggests that Mutant-2 has overall lower flexibility, indicating increased structural stability. The RMSF analysis highlights that Mutant-2 demonstrates the lowest average RMSF values in Chain A and moderately reduced flexibility in Chain B compared to the wild-type. This suggests that Mutant-2 exhibits enhanced structural stability across both chains, particularly in Chain A. In contrast, Mutant-1 shows reduced flexibility in Chain A but increased flexibility in Chain B, which may compromise overall stability. Therefore, Mutant-2 is the most promising candidate for improved thermostability, as it maintains reduced flexibility in both chains, potentially leading to a more resilient insulin structure under thermal stress.

Table 2. Average RMSF of the chain A and B of the wild-type insulin, Mutant 1 and Mutant 2 post MD simulation at 310 K

System	RMSF (Chain A)	RMSF (Chain B)
Wild-type	0.25 nm	0.32 nm
Mutant-1	0.20nm	0.51 nm
Mutant-2	0.12 nm	0.37 nm

The RMSF study indicates that Mutant-2 is the most stable variation, exhibiting markedly less flexibility in both Chain A and Chain B, so positioning it as a robust contender for improved thermostability. Conversely, Mutant-1 exhibits moderate enhancements in stability, whilst the wild-type retains the highest flexibility and likely the lowest stability of the three. These findings correspond with the aim of identifying mutations that enhance the structural stability of insulin, especially under heat stress, by diminishing flexibility in essential regions of the protein.

PCA AND FEL

Principal Component Analysis

Figure 8 illustrates principal component analysis (PCA) plots, showcasing the trajectory data projections for wild-type insulin (black), Mutant-1 (red), and Mutant-2 (green) along two primary eigenvectors. Each plot illustrates the conformational space explored by the proteins during the MD simulation, with each point representing a unique conformation within that space. PCA elucidates the collective dynamics of the protein and facilitates the comparison of the flexibility and stability of each variant. Figure 8(a) illustrates that wild-type insulin exhibits two unique clusters in conformational space, signifying its exploration of numerous conformational states during the simulation. The distribution along both eigenvector 1 and eigenvector 2 indicates that the wild-type insulin demonstrates a wide range of motion, signifying considerable flexibility. This distribution indicates that wild-type insulin exhibits greater conformational diversity, consistent with the RMSF results reflecting increased flexibility.
Figure 8(b) showed that mutant-1 has two principal clusters, but with a broader dispersion along the eigenvectors, particularly along eigenvector 1. This suggests enhanced flexibility or conformation sampling relative to the wild-type, indicating that Mutant-1 may exhibit reduced structural stability compared to the wild-type. The extensive dispersion within the conformational space suggests that Mutant-1 investigates a diverse array of conformations, potentially linked to diminished stability. Figure 8(c) showed that Mutant-2 has a more compact clustering structure, characterised by two well-defined and proximate clusters. The range along both eigenvectors is more constrained than that of the wild-type and Mutant-1, suggesting that Mutant-2 possesses restricted conformational freedom and samples fewer unique conformations. The compactness indicates that Mutant-2 exhibits a more stable conformation throughout the simulation, characterised by reduced flexibility and enhanced structural integrity.

Both wild-type insulin and Mutant-1 demonstrate extensive structural variability, with Mutant-1 exhibiting the greatest range, indicating considerable flexibility and possible instability. Mutant-2 exhibits a more confined conformational space, suggesting it possesses the most constrained mobility among the three and is likely the most structurally stable. In this study, the FEL results indicate that Mutant-2 exhibits a single dominant, deep low-energy basin, signifying a more stable conformation with limited transitions to higher-energy states. This suggests that Mutant-2 has reduced structural flexibility and an overall more rigid framework, which may contribute to its improved thermostability. In contrast, the wild-type insulin demonstrates multiple low-energy basins separated by higher-energy barriers, indicating a greater range of conformational states and enhanced flexibility. Mutant-1, while possessing relatively stable regions, shows a more dispersed low-energy landscape, suggesting an intermediate level of flexibility and stability between the wild-type and Mutant-2.

Comparing transition states and energy barriers, wild-type insulin exhibits multiple transitions between stable states, suggesting greater

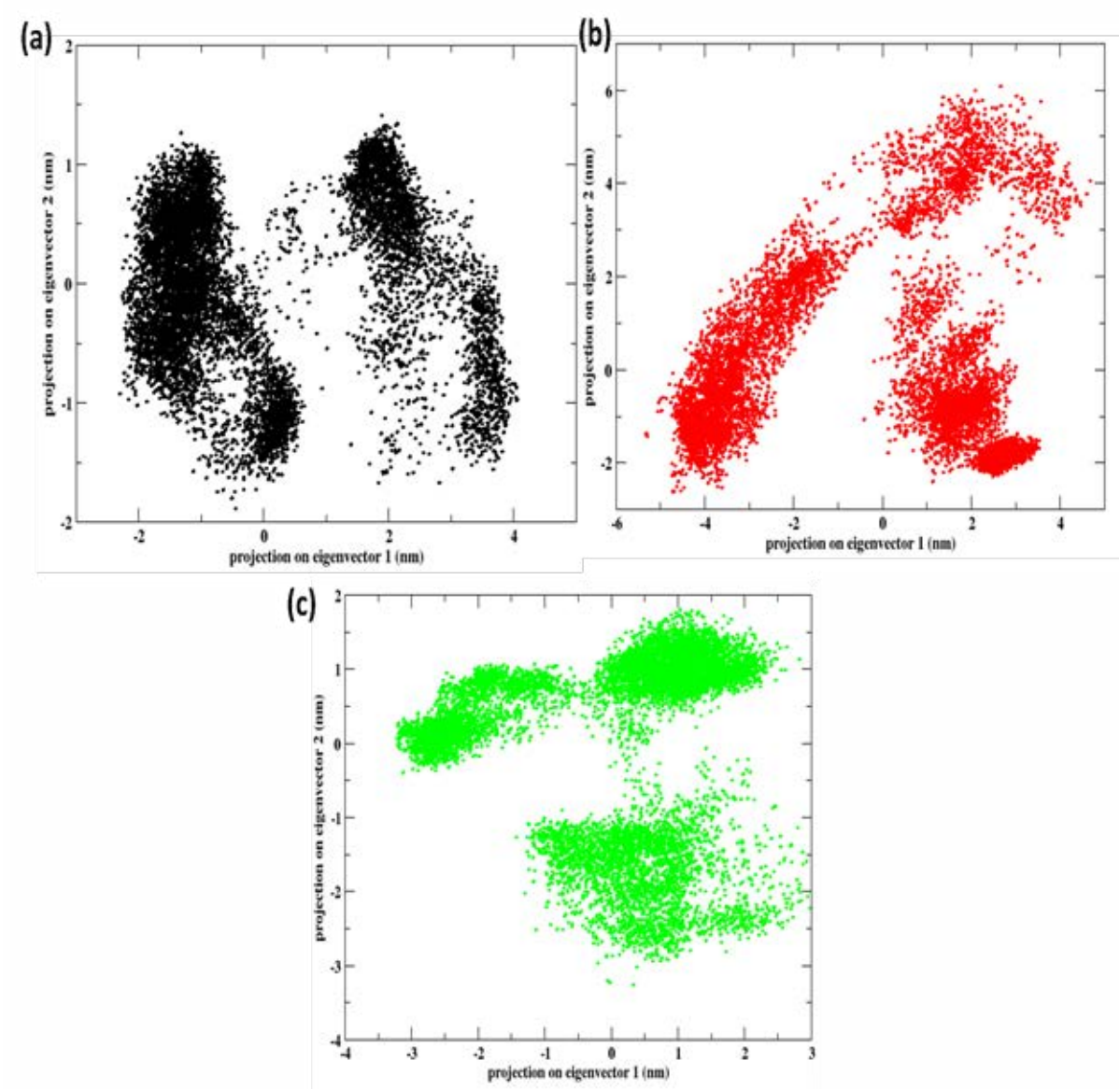

Figure 8. Principal component analysis of the (a) Wild-type insulin (b) Mutant 1 (c) Mutant 2 during the MD simulation at 310 K

conformational adaptability. Mutant-1 has a more widespread distribution of low-energy states, indicating a tendency for increased structural fluctuations. However, Mutant-2 remains in a singular, well-defined energy basin with minimal transitions, reflecting its enhanced structural rigidity and stability.

The PCA analysis corroborates the results from the RMSF and RMSD investigations, demonstrating that Mutant-2 is the most stable variation, exhibiting the least structural flexibility. Mutant-1 exhibits the greatest flexibility, possibly suggesting structural instability. The results indicate that Mutant-2 is a promising candidate for increased thermostability owing to its limited conformational sampling and structural rigidity, which are advantageous for stability under heat stress.

Free Energy Landscape

Figure 9 illustrates the Free Energy Landscape (FEL) contour and three-dimensional surface plots for wild-type insulin and two mutant variants. PC1 and PC2 (Principal Components 1 and 2) denote the two primary eigenvectors or predominant motions, illustrating the distribution of conformational states. The colour scale denotes free energy (ΔG) in kcal/mol, with red signifying low free energy (stable states) and blue representing higher energy (less stable states).

Figure 9(a) illustrates that wild-type insulin possesses two different low-energy basins (red patches) divided by a higher energy region, signifying its exploration of numerous stable conformational states. The dispersion indicates that wild-type insulin can assume several conformations, enhancing its flexibility. Figure 9(b) illustrates a plot depicting deep wells associated with stable conformations, accompanied by abrupt transitions to elevated energy states. This pattern suggests that whereas wild-type insulin possesses stable conformations, it may also attain several high-energy states, indicating structural flexibility. Figure 9(c) demonstrates that Mutant-1 displays a wide distribution in the low-energy zone, characterised by several dispersed basins. This distribution implies that Mutant-1 investigates a broad range of conformational states, possibly signifying diminished structural stability. The energy basins are more dispersed than in the wild-type, indicating an increased level of flexibility. Figure 9(d) illustrates the 3D plot for Mutant-1, which reveals various shallow wells, signifying multiple low-energy states with comparatively diminished stability.

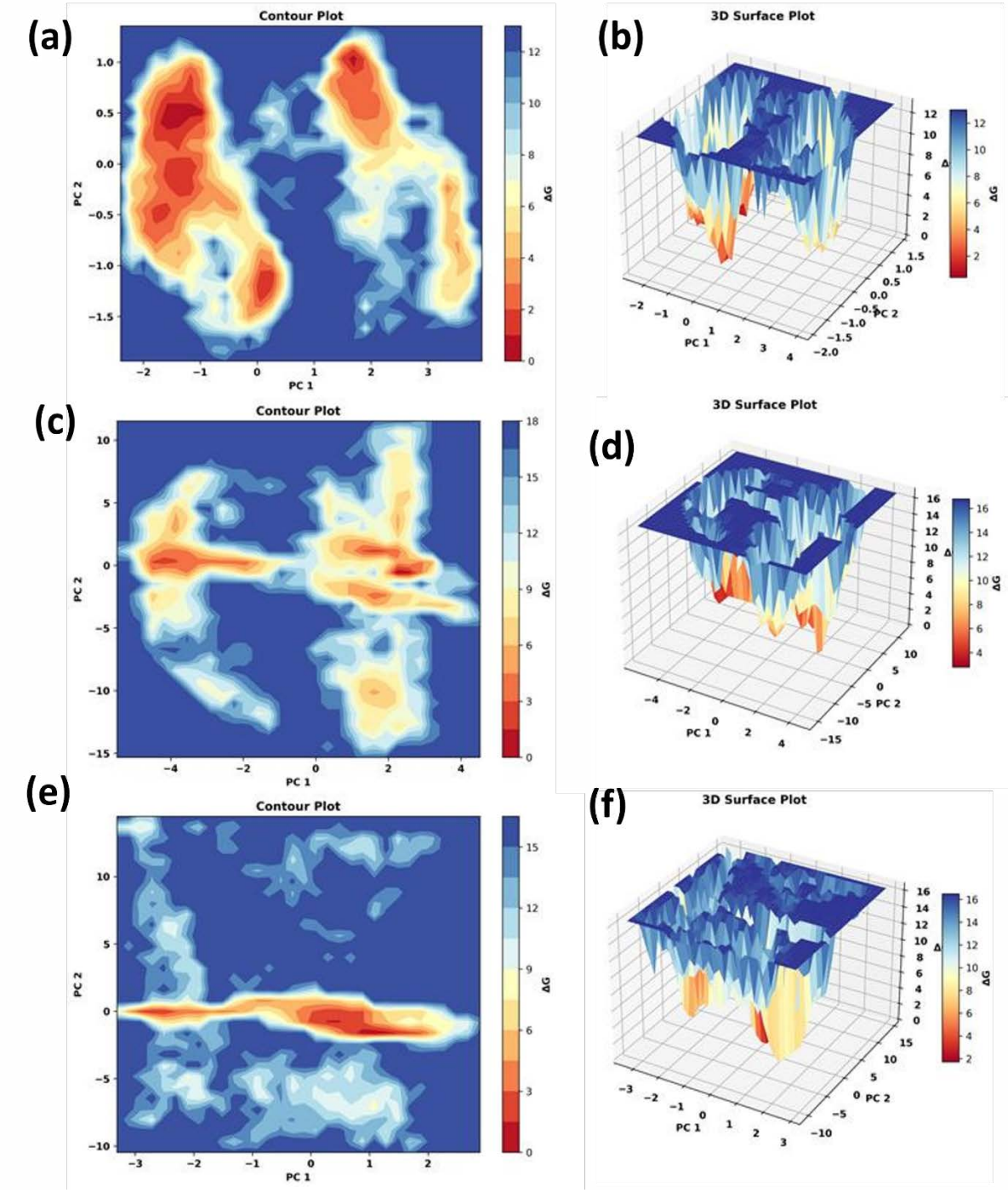

Figure 9. Free energy landscape (FEL) analysis of the (a) Wild-type insulin (b) Mutant 1 (c) Mutant 2 during the MD simulation at 310 K

The expansive, shallow topology indicates that Mutant-1 may experience numerous conformational changes, perhaps resulting in reduced structural stiffness relative to the wild-type. Figure 9(e) illustrates that Mutant-2 has a singular dominant low-energy basin with a more linear and compact distribution, signifying that it explores fewer conformations than the wild-type and Mutant-1. The concentrated energy basin indicates that Mutant-2 preserves a stable conformation, signifying enhanced structural stiffness and stability. Figure 9(f) illustrates the 3D plot for Mutant-2, which reveals a deep, narrow well indicative of a singular predominant low-energy state. The pronounced energy well indicates that Mutant-2 possesses structural stability, with minimal transitions to elevated energy levels, signifying a considerable degree of stability.

The wild-type insulin displays several low-energy basins, indicating its accessibility to diverse structural states. This flexibility enhances its biological activity but may compromise thermostability, as the protein can readily access higher-energy (less stable) states. Mutant-1 possesses several shallow energy basins, signifying a more adaptable structure with diminished stability. The extensive energy landscape indicates that it can assume several conformations, resulting in reduced structural stability relative to the wild-type and Mutant-2. Mutant-2 exhibits a concentrated, profound energy well in both contour and 3D representations, signifying a singular, stable conformation. The constrained energy landscape indicates that Mutant-2 exhibits heightened stability and reduced flexibility, positioning it as a prime candidate for improved thermostability.

The FEL analysis indicates that Mutant-2 is the most stable variation, characterised by a singular dominating low-energy state that limits its structural flexibility. Mutant-1 exhibits diminished stability attributable to its dispersed low-energy states and extensive structural variability. Wild-type insulin exhibits numerous stable conformations, possessing greater flexibility than Mutant-2. Mutant-2 exhibits the greatest promise for enhanced thermostability, as its compact energy landscape restricts structural variations, hence improving tolerance to thermal stress.

DISCUSSION

In this study, Thermal Titration Molecular Dynamics (TTMD) at 300 K, 310 K, 320 K and 330 K was employed to examine the impact of specific mutations on insulin's thermostability, particularly concentrating on residues deemed highly flexible via RMSF analysis. A previous study utilised thermal unfolding molecular dynamics simulations at an elevated temperature of 400 K to assess the effects of diverse point, double, and triple mutations on the structural stability and dynamics of human insulin, employing the CHARMM36m force field and GROMACS software (version 5.1)[9].

This previous study presented ten single mutations, six double mutations, and one triple mutation spanning Chains A and B of insulin. The mutations encompassed residues such as HisA8, ValA10, AspB10, GlnB17, and GluB27, among others, modified to evaluate alterations in stability and structural integrity under elevated temperatures. The previous study evaluated the impact of ten point mutations (HisA8, ValA10, AspB10, GlnB17, AlaB17, GlnB18, AspB25, ThrB26, GluB27, AspB28), six double mutations (GluA13+GluB10, SerA13+GluB27, GluB1+GluB27, SerB2+AspB10, AspB9+GluB27, GluB16+GluB27) and one triple mutation (GluA15+AspA18+AspB3) in the protein sequence on the structure and dynamics of human insulin[9]. This work conducted simulations at 400 K to expedite structural destabilisation and unfold insulin, examining the impact of various mutations on thermal unfolding mechanisms. The investigation elucidated the impact of individual and collective amino acid alterations on the structural integrity of insulin under severe thermal stress.

Conversely, the present study concentrated on improving insulin stability by methodically identifying flexible areas by TTMD at physiological temperature (310 K) and targeting these regions with specific stabilising mutations. This study's methodology aimed to evaluate stability-enhancing mutations via ΔΔGpred values from MaestroWeb and melting temperature (Tm) predictions from DeepSTABp, enabling the selection of the most thermally stable variants prior to conducting comprehensive MD simulations. This study generated 6,859 alterations in Chain B and 361 in Chain A, subsequently screening them for stabilising effects. The best two mutants were selected with the highest anticipated Tm values from this screening and analysed them with wild-type insulin using RMSD, RMSF, PCA, and FEL analyses. Comparative MD analyses, showed that Mutant-2 exhibited reduced flexibility, a compact conformational space, and a single dominant low-energy basin, indicating enhanced stability over the wild-type insulin. Mutant-1 demonstrated moderate stability improvements but was less robust than Mutant-2, while the wild-type insulin displayed the highest flexibility and multiple stable conformations, which may compromise its stability under thermal stress. The results suggest that Mutant-2 is a promising candidate for a thermostable insulin analog due to its enhanced structural integrity and limited conformational flexibility, which are crucial for maintaining stability in variable temperature conditions.

The RMSF analysis provides critical insights into residue-level flexibility, highlighting the regions most susceptible to thermal fluctuations. Notably, at 310K, the wild-type insulin exhibits significant flexibility at residues near the termini and select mid-chain regions, particularly in Chain B. These regions correspond to key structural elements that influence insulin's stability. The introduction of mutations (His5 and Lys8 in Chain A, and Ser8, Thr9, Cys10 in Chain B) was strategically aimed at stabilizing these flexible regions by reducing their conformational fluctuations. Our results indicate that Mutant-2 exhibits lower RMSF values across both chains, particularly in regions where the wild-type showed high flexibility. This suggests that these mutations effectively mitigate structural fluctuations, thereby enhancing thermostability. The correlation between reduced RMSF values and the mutated residues supports the hypothesis that Mutant-2 is more resistant to thermal destabilization. Furthermore, while RMSD trends across temperature variations confirm general stability differences, Mutant-2's comparatively stable trajectory indicates its resilience across the tested temperature range. These findings reinforce that the identified mutations contribute to a more rigid and thermally stable insulin variant. Future experimental validation will further confirm the efficacy of Mutant-2 as a thermostable insulin analog. I will integrate these points into the discussion to strengthen the connection between specific mutations and the observed stability profiles.

One key constraint is the computational cost associated with simulating multiple temperature conditions over extended time scales, which may limit the resolution or duration of simulations compared to experimental techniques. Additionally, TTMD relies on molecular mechanics force fields, which, while well-validated, may not always perfectly capture complex interactions, such as hydrogen bonding dynamics or long-range electrostatic effects, under extreme thermal stress.

CONCLUSION

This study represents a methodical strategy for improving insulin's thermal stability by focussing on mutable flexible residues. This study aimed to increase the thermostability of insulin by systematically identifying flexible and unstable regions in its structure and implementing targeted mutations to boost structural resilience through Thermal Titration Molecular Dynamics (TTMD) simulations. The findings indicate that Mutant-2 is a viable candidate for a thermostable insulin analogue, attributed to its improved structural integrity and restricted conformational flexibility, essential for stability under fluctuating temperature settings. This study illustrates a proficient method for creating thermostable protein variations through the integration of computational simulations, stability forecasts, and molecular dynamics analysis, facilitating subsequent experimental verification of Mutant-2. Further, experimental methodologies may be employed to validate Mutant-2, which could signify a significant advancement in insulin formulation, potentially improving accessibility and efficacy in environments with restricted refrigeration.

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Competing Interest: None

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