

Stable Mutant Identification in Bacteriophage T4 Lysozyme Using Message-Passing Neural Network

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Abstract: This study investigates stable mutants and ligand interactions of Bacteriophage T4 lysozyme encoded by PDB ID 2LZM using computational approaches. The objective was to identify mutations that could enhance protein stability or alter functional properties. Using ThermoMPNN, a message-passing neural network (MPNN), a total of 3280 mutants were generated and systematically screened based on their predicted $\Delta\Delta G$ values. The analysis identified the top 10 mutants, with the C54T mutation showing the highest predicted stability enhancement. To understand the impact of these mutations on ligand binding, molecular docking simulations with CBDock Vina were performed, focusing on the interaction between the wild-type protein and β -N, N', N''-triacetylchitotriose. Comparative analysis of docking results for the wild-type and mutant (C54T) proteins suggested that the C54T mutation does not significantly alter the ligand binding properties. These findings offer insights into protein stability and ligand binding, supporting future validation and biotechnological applications.

Keywords: ThermoMPNN analysis; molecular docking; protein-ligand interactions; stability prediction; mutant screening.

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1. Introduction

Proteins are essential biomolecules that fulfil diverse functions ranging from catalysis to structural support and signaling regulation. Understanding the intricate relationship between protein structure and function is pivotal for unravelling the mechanisms underlying cellular processes and diseases, as well as for guiding the design of novel therapeutics. Even a single amino acid mutation at the protein sequence level can alter local atomic changes in its 3D structure, influence diverse aspects of protein behavior such as kinetics of folding, stability,

flexibility, dynamics, and overall activity of the protein [1]. Computational methods have become indispensable tools in this endeavour, offering predictive insights into protein stability, functional residues, and interactions with ligands [2,3].

Here, we focus on Bacteriophage T4 lysozyme (PDB ID: 2LZM) a well-characterized model owing to its potential implications in both biotechnological and biomedical domains. It exhibits significant conformational flexibility, particularly in its hinge-bending domain motion, making it an ideal model for exploring protein dynamics and the effects of different molecular dynamics simulation parameters [4-6]. T4 lysozyme breaks down bacterial cell walls, while N, N', N''-triacetylchitotriose is a substrate and inhibitor of lysozyme, consisting of three N-acetylglucosamine units [7,8]. Its crystal at 1.7 Å resolution provides us with the refined structure of T4 bacteriophage lysozyme, including precise bond lengths and angles, and including 118 solvent molecules, many of which are mostly dispersed on the molecule's surface with clustering near the active site [9]. Computational tools are crucial for identifying stabilizing mutations, but their accuracy is limited by data biases and the greater difficulty of predicting stabilizing changes, even though they aid in protein interaction prediction and drug discovery [10–13]. Thermostability in industrial enzymes is achieved through various engineering strategies, including amino acid substitutions, enhancing hydrogen bonds, salt bridges, hydrophobic interactions, and optimizing secondary structure compatibility to maintain enzyme functionality at high temperature [14-16]. Artificial intelligence, particularly deep learning techniques like 3D convolutional neural networks, has been increasingly employed to predict the impact of mutations on protein stability, addressing longstanding challenges and improving the accuracy and unbiased prediction of both stabilizing and destabilizing mutations [17-19]. These systems outperform modelling protein structures and allow voxel-based analysis, capturing complex biochemical mutation sites, hence advancing prediction quality [20]. Among recent advances, ThermoMPNN, a message-passing neural network, leverages large-scale stability data and transfer learning to predict and screen stable protein mutants with potential functional or structural benefits [21–24]. Using high-throughput experimental datasets, i.e., mega-scale, gives us promising results in predicting mutant stability while addressing current limitations and outlining strategies for future enhancements [25]. Meanwhile, molecular docking simulations provide detailed insights into the binding affinity and conformational arrangements of the wild-type protein with specific ligands, thereby furnishing valuable information on protein-ligand interactions and potential binding sites [26-28]. ThermoMPNN analysis and molecular docking simulations have revolutionized our ability to predict and explore protein structure-function relationships with unprecedented accuracy and efficiency. Valuable protein-ligand dynamics and stability predictions are offered by these techniques, eventually enhancing the identification of potential therapeutic targets [29,30]. By harnessing these computational tools, we can delve deeper into the functional significance of proteins like PDB ID 2LZM, laying the groundwork for potential biotechnological applications and therapeutic interventions in diverse biomedical contexts [31].

The study aims to comprehensively analyze stable mutants of the protein encoded by PDB ID 2LZM and explore its interaction with β -N, N',N''-triacetylchitotriose, a ligand of interest. By combining computational methods with experimental validation, we seek to elucidate the structural and functional implications of specific mutations and their potential applications in biotechnological and biomedical contexts. Overall, this study contributes to our understanding of protein structure-function relationships and lays the groundwork for future research in protein engineering and drug discovery.

2. Materials and Methods

In this study, we employed ThermoMPNN, a message-passing neural network (MPNN) [32], to identify stable mutants within the protein structure encoded by the PDB ID 2LZM. ThermoMPNN utilizes a neural network architecture tailored for molecular property prediction, leveraging message passing between atoms to capture intricate structural and chemical features. Initially, the 3D structure of the protein encoded by the PDB ID 2LZM was retrieved from the Protein Data Bank. The structural quality of PDB ID 2LZM was assessed using PDB validation metrics, including RMSD for bond lengths (0.019 Å) and angles (2.70°), as well as model-to-density fit indicators such as a Fo–Fc correlation of 0.96. The structure shows high overall quality with minimal geometric outliers as per the wwPDB validation report. Subsequently, ThermoMPNN was utilized to predict the stability of various mutant variants generated by introducing single-point mutations at specific residues. The stability predictions were based on thermodynamic properties such as folding free energy or stability scores inferred from the neural network model. This computational approach enabled the systematic screening of potential mutations, aiding in the identification of stable variants with potential functional implications.

In this study, we employed computational techniques to introduce a specific mutation, C54T, in the protein structure encoded by the PDB file "2lzm.pdb". To achieve this, we utilized the Biopython library within the Google Colab environment. Initially, the Biopython package was installed [33], and subsequently, the PDB file was loaded using the PDBParser module. A custom class, MutantSelect, was then defined to facilitate the mutation process. Within this class, the residue at position 54 in chain 'A' was identified and replaced with threonine (THR), as per the mutation requirement. The mutated structure was then saved as a new PDB file, "2lzm_C54T.pdb".

In this study, we employed CB-Dock, a cavity-detection guided blind docking approach [34,35], to investigate the interaction between the wild-type protein structure encoded by PDB ID 2LZM and its mutant counterpart represented by the 2LZM_C54T.pdb file. The docking simulations aimed to explore the binding affinity and conformational preferences of beta-N,N',N''-triacylchitotriose, a ligand of interest, within the binding site of the protein. CB-Dock utilizes a cavity detection algorithm to identify potential binding sites on the protein surface, followed by blind docking simulations to explore ligand binding modes within these cavities. The wild-type and mutant protein structures were subjected to docking simulations separately to compare their respective binding interactions with the ligand. This computational approach facilitated the elucidation of structural and energetic characteristics underlying the ligand-protein interactions, providing insights into the impact of the C54T mutation on ligand binding affinity and specificity.

3. Results and Discussion

3.1. Identification of stable mutants using ThermoMPNN analysis.

In general, the natural form of a protein can be influenced by factors, from within and outside, like changes in its structure due to mutations, pH levels, temperature variations, ions present, in the environment, and many more [36,37]. A total of 3280 mutants were generated using computational methods facilitated by Google Colab and ThermoMPNN. These mutants were systematically screened to identify variants with potentially enhanced stability or altered

functional properties. ThermoMPNN is designed to forecast changes, in energy for proteins. Its accuracy, in predicting the behavior of the homodimeric CcdBl protein raises interest in assessing its efficacy with NA, a tetrameric protein [38,39]. ThermoMPNN analysis enabled the prediction of the $\Delta\Delta G$ (change in free energy of folding) for each mutant, providing insights into the potential impact of single-point mutations on protein stability [32]. ThermoMPNN is a model, for predicting protein stability based on its structure, where it uses learned information from ProteinMPNN to make predictions about the impact of mutations, on protein stability ($\Delta\Delta G$) [40,41]. The top 10 mutants, ranked based on their predicted $\Delta\Delta G$ values, are presented in Table 1. These mutants represent promising candidates for further experimental validation and characterization. Among them, the mutant C54T exhibited the highest predicted stability enhancement, with a $\Delta\Delta G$ value of -0.811 kcal/mol. This mutation involves substituting cysteine (C) at position 54 with threonine (T), potentially leading to improved protein stability or altered structural dynamics. Additionally, mutants such as D70R, V149I, G110C, and G110Y demonstrated significant stability enhancements, indicating their potential utility in protein engineering or therapeutic applications.

The identification of key mutations, such as those at positions 54, 70, 110, and 149, suggests potential functional implications for the corresponding residues in the protein structure. These mutations may affect protein-ligand interactions, catalytic activity, or structural stability, thereby influencing the protein's biological function [42]. For instance, mutations at position 110 (G110C, G110Y, G110L, and G110F) may modulate the local environment around this residue, potentially impacting substrate binding or protein conformational dynamics. Comparative analysis between the wild-type and mutant protein structures provides valuable insights into the structural consequences of specific mutations. Docking simulations and structural modeling techniques can elucidate changes in ligand binding affinity, protein-ligand interactions, and conformational dynamics induced by mutations [43]. Further experimental validation, including biochemical assays and structural characterization, will be essential to validate the functional relevance of the identified mutations and their potential applications in biotechnology and drug discovery. Overall, the comprehensive analysis of mutants generated through computational methods and ThermoMPNN predictions offers valuable insights into protein stability, structure-function relationships, and the potential for rational protein engineering. These findings lay the foundation for future studies aimed at elucidating the functional significance of specific mutations and their applications in various biotechnological and biomedical contexts.

Table 1. Stable mutations identified in T4 lysozyme.

S.No.	Mutation	ddG (kcal/mol)	Position	Wild-type amino acid	Mutant amino acid
1	C54T	-0.811	54	C	T
2	D70R	-0.598	70	D	R
3	V149I	-0.5355	149	V	I
4	G110C	-0.5184	110	G	C
5	G110Y	-0.5009	110	G	Y
6	K16C	-0.4883	16	K	C
7	N140R	-0.4789	140	N	R
8	G110L	-0.4514	110	G	L
9	N68R	-0.4509	68	N	R
10	G110F	-0.4497	110	G	F

3.2. Docking analysis of wild-type protein.

The structural comparison of the wild-type and mutant proteins highlights the mutation-induced conformational changes, as illustrated in Figure 1. The docking analysis of the wild-type protein structure with β -N, N', N''-triacetylchitotriose using CBDock Vina resulted in multiple potential binding poses. The Vina scores ranged from -4.8 to -7.9 kcal/mol, indicating favorable binding affinities between the ligand and the protein (Table 2).

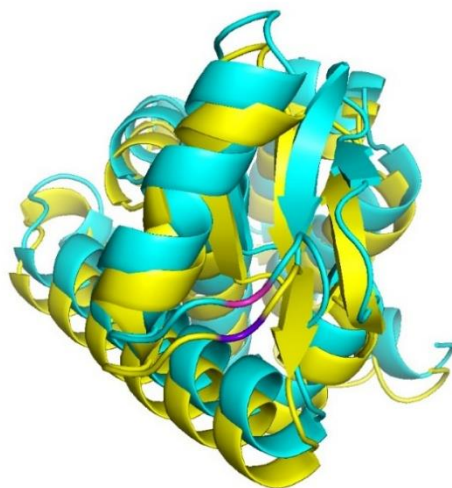


Figure 1. Structural comparison of the wild-type and mutant proteins. The wild-type protein is shown in yellow, while the mutant protein is depicted in cyan. The site of mutation is highlighted in purple in the wild-type and magenta in the mutant structure. This visualization illustrates the spatial positioning and structural alteration induced by the mutation.

Table 2. Docking results of wild-type T4 lysozyme with β -N, N', N''-triacetylchitotriose using AutoDock Vina.

Vina score	Cavity size (\AA^3)	Center coordinates (x, y, z)
-7.9	901	35,16,10
-5.6	46	38,9,-8
-5.4	53	27,14,-4
-5	106	44,7,18
-4.8	60	19,8,0

The top-ranked pose exhibited a Vina score of -7.9 kcal/mol, suggesting a strong binding interaction between the ligand and the protein. This pose corresponded to a cavity with a size of 901 \AA^3 , located at coordinates (x=16, y=10, z=25). Comparatively, the docking analysis of the mutant protein structures also revealed potential binding poses for β -N, N', N''-triacetylchitotriose. The Vina scores for the mutant structures ranged from -5.6 to -7.5 kcal/mol, indicating favorable binding affinities similar to those observed for the wild-type structure. The top-ranked pose for the mutant structures exhibited a Vina score of -7.5 kcal/mol, corresponding to a cavity with a size of 901 \AA^3 , located at coordinates (x=16, y=10, z=25), which was consistent with the top pose of the wild-type structure (Table 3). Figure 2 illustrates the docking poses of wild-type and mutant T4 Lysozyme (C54T) with β -N, N', N''-triacetylchitotriose. Panel A shows the surface representation of the wild-type T4 Lysozyme, revealing the ligand nestled within a prominent cavity, indicating strong binding with a Vina score of -7.9 kcal/mol. Panel B, with the receptor hidden, provides a clearer view of the ligand interactions within the binding pocket, highlighting specific contacts with amino acid residues. Panels C and D display the C54T mutant T4 Lysozyme in surface and hidden receptor representations, respectively. The ligand binding poses in the mutant appear similar to the wild-

type, with a slightly reduced Vina score of -7.5 kcal/mol, indicating that the C54T mutation does not significantly disrupt the binding site's topography or ligand interactions. These visual comparisons support the conclusion that the C54T mutation maintains the binding properties and configuration of the ligand within the pocket, suggesting the mutation enhances stability without compromising functional interactions (Figure 2). Comparative analysis of the docking results between the wild-type and mutant structures revealed similar Vina scores and cavity characteristics for the top-ranked binding poses. Both the wild-type and mutant structures exhibited comparable binding affinities and binding site configurations for β -N, N', N''-triacylchitotriose. This suggests that the C54T mutation may not significantly alter the ligand-binding properties or binding site accessibility of the protein. The consistent docking results between the wild-type and mutant structures indicate that the C54T mutation may not directly influence the protein's interaction with β -N, N', N''-triacylchitotriose. However, further analysis, including molecular dynamics simulations and experimental validation, is warranted to fully understand the functional implications of the mutation and its potential effects on protein-ligand interactions. These findings provide valuable insights into the structural dynamics and ligand binding properties of the protein, laying the foundation for future studies aimed at elucidating its biological function and therapeutic potential [44-46].

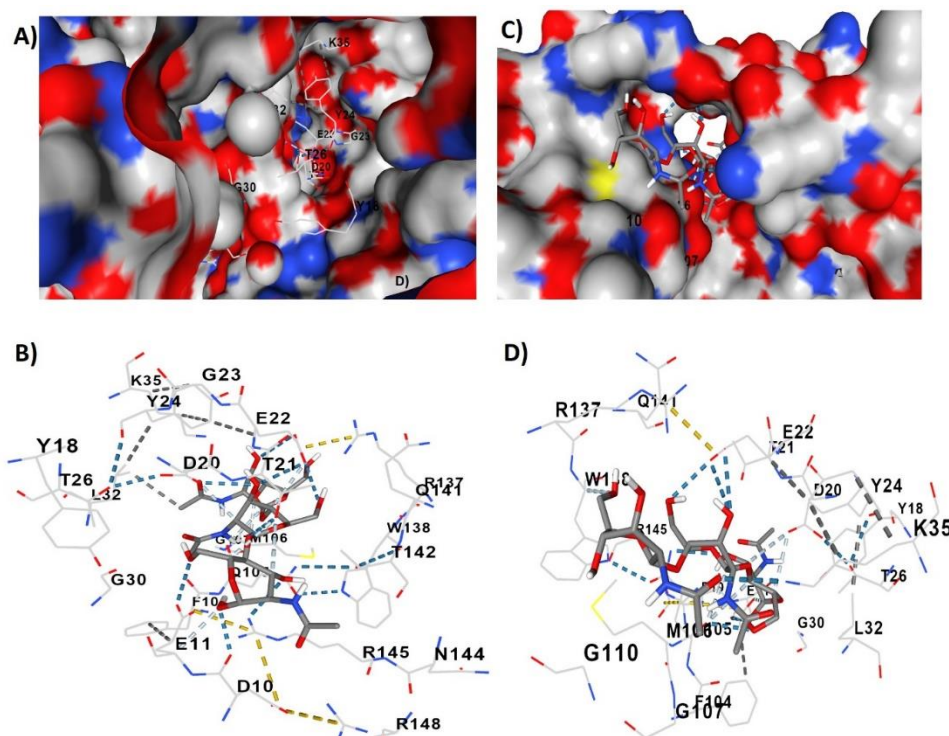


Figure 2. (A) Docking pose image of wild-type T4 Lysozyme with surface representation; (B) Docking pose image of wild-type T4 Lysozyme with hidden receptor representation; (C) Docking pose image of mutant-type T4 Lysozyme with surface representation; (D) Docking pose image of mutant-type T4 Lysozyme with hidden receptor representation.

Table 3. Docking results of mutant-type T4 lysozyme with β -N,N',N''-triacylchitotriose using AutoDock Vina.

Vina score	Cavity size (\AA^3)	Center coordinates (x, y, z)
-7.5	901	35,16,10
-5.9	106	44,7,18
-5.7	53	27,14,-4
-5.6	60	19,8,0
-5.6	46	38,9,-8

3.3. Experimental validation.

In this research, we took advantage of the robust computational tools for docking analysis and mutant stability prediction, but our key limitation was the absence of experimental validation. We may still yield false positives even though they are powerful tools; this is due to their training bias or static structure assumptions. To confirm, site-directed mutagenesis can be used to introduce specific amino acid changes into the T4 lysozyme gene, followed by recombinant protein expression and purification for the predicted stability of mutants like C54T. Also, using differential scanning calorimetry (DSC) or thermal shift assays (TSA), the stability of these proteins can be assessed. Using these methods, we will be able to validate whether the computationally predicted mutant's stability corresponds to actual thermodynamic improvements *in vitro* [47-49].

While this study leverages advanced computational tools to predict protein stability and ligand binding interactions, several limitations must be acknowledged. First, the predictions generated by ThermoMPNN and CB-Dock are inherently dependent on the accuracy of the training data and algorithms, which may introduce biases, particularly in overestimating stabilizing mutations. Consequently, there is a risk of false positives, where predicted stable mutants (e.g., C54T) may not confer actual thermodynamic advantages upon experimental validation. Additionally, molecular docking simulations were performed on static protein structures, which may not fully capture the dynamic conformational flexibility of the protein in a physiological environment. This can limit the reliability of the predicted ligand-protein interaction profiles. Therefore, the findings of this study should be interpreted as preliminary and hypothesis-generating, requiring rigorous *in vitro* and *in vivo* validation to confirm the functional and structural relevance of the identified mutations.

4. Conclusions

In this study, we employed computational methods to identify stable mutants and investigate ligand-protein interactions of the protein encoded by PDB ID 2LZM. Utilizing ThermoMPNN analysis, we generated 3280 mutants and systematically screened them to identify variants with potential stability enhancements or altered functional properties. The analysis facilitated the prediction of $\Delta\Delta G$ values for each mutant, revealing promising candidates for further experimental validation. Among the top 10 mutants identified, C54T showed the highest predicted stability enhancement, suggesting its utility in protein engineering or therapeutic applications. Additionally, mutations at positions 70, 110, and 149 demonstrated significant stability enhancements, indicating their potential relevance in modulating protein-ligand interactions and structural stability. Molecular docking simulations using AutoDock Vina provided insights into the binding affinity and configuration of the wild-type protein with β -N,N',N''-triacetylchitotriose. Comparative analysis with mutant docking results suggested that the C54T mutation did not significantly alter ligand binding properties. These findings offer valuable insights into protein stability, structure-function relationships, and ligand binding properties, laying the foundation for further experimental validation and future studies aimed at elucidating the functional significance of specific mutations and their applications in biotechnological and biomedical contexts.

Author Contributions

Conceptualization, V.K.Y. and S.R.D.; methodology, R.K. and B.S.M.; software, B.K.K.; validation, V.K.Y., S.R.D., and B.S.M.; formal analysis, B.S.M.; investigation, V.K.Y. and S.R.D.; resources, B.K.K.; data curation, B.S.M.; writing—original draft preparation, S.R.D.; writing—review and editing, V.K.Y.; visualization, B.S.M.; supervision, V.K.Y.; project administration, V.K.Y.. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

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Conflicts of Interest

The authors declare no conflict of interest.

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