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"Design and Molecular Docking of a Fusion Protein Targeting SARS-CoV-2 Spike Cleavage Sites"

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ABSTRACT

SARS-CoV-2, the virus responsible for the COVID-19 pandemic, relies on its spike protein to bind to and enter host cells. The transmembrane serine protease TMPRSS2 plays a critical role in cleaving the spike protein at specific sites, facilitating viral fusion with the host cell membrane. This study presents the computational design and molecular docking of a fusion protein, named Happy, which combines an EK1 peptide inhibitor with the C fragment of tetanus toxoid. Two variants—Happy_00 (no linker) and Happy_06 (six GGGS linkers)—were developed and evaluated for their ability to bind the spike protein cleavage sites (Arg685/Ser686 and Arg815/Ser816). Using molecular modeling tools such as RaptorX, PyMol, and Modeller, along with virtual screening via ClusPro and HADDOCK 2.4, we identified Happy_00 and Happy_06 as optimal inhibitors. Binding energies were calculated as -11.4 kcal/mol for Happy_00 at the first cleavage site and -15.4 kcal/mol for Happy_06 at the second site. The results suggest that the presence of GGGS linkers enhances flexibility, improving binding efficiency at deeper cleavage sites. Both inhibitors effectively block TMPRSS2's cleavage activity, potentially preventing viral entry into host cells. This research highlights the promise of fusion proteins as therapeutic agents to inhibit SARS-CoV-2 infection, though further experimental validation is required to confirm *in vivo* efficacy.

INTRODUCTION

SARS-CoV-2, the causative agent of COVID-19, has caused widespread illness and disruption globally. The virus uses its spike (S) protein to gain entry into host cells. The spike protein contains two subunits: S1, which facilitates binding to the ACE2 receptor, and S2, which mediates membrane fusion, allowing viral entry (Kumar et al., 2023). A critical step in this process is the cleavage of the spike protein at two specific sites: the S1/S2 boundary (Arg685/Ser686) and the S2' site (Arg815/Ser816). These cleavage events, catalyzed by host proteases such as TMPRSS2 (Transmembrane Protease Serine 2), are essential for exposing the fusion domain of the virus(Lemmin et al., 2020; Ou et al., 2020; Peng et al., 2021).

Given TMPRSS2's crucial role in spike protein activation, inhibiting its cleavage activity offers a potential therapeutic strategy for blocking viral entry. TMPRSS2 inhibitors have shown promise in previous studies for mitigating SARS-CoV-2

infections(Baughn et al., 2020; Masako et al., 2013; Vaarala et al., 2001). However, developing specific inhibitors targeting the cleavage sites on the spike protein remains a challenge. The design of fusion proteins that can bind and block these sites presents a novel approach to disrupting the viral lifecycle.

This study explores the computational design of two fusion proteins—Happy_00 and Happy_06—which combine the C fragment of tetanus toxoid (non-toxic) with an EK1 peptide inhibitor. The EK1 peptide has previously demonstrated inhibitory activity against other coronaviruses by preventing viral fusion. By using these fusion proteins, the study aims to block TMPRSS2-mediated cleavage and inhibit viral entry. Computational tools were employed to model, dock, and analyze these inhibitors, providing insight into their binding potential at the spike protein's cleavage sites.

Methods

Molecular Modeling

The atomic coordinates for the SARS-CoV-2 spike protein were retrieved from the Protein Data Bank (PDB ID: 6vsb). The B chain of the spike protein, containing the TMPRSS2 binding and cleavage sites, was isolated using PyMol(Schiffrin et al., 2020). Any missing residues were corrected with Modeller 10.1, and structural refinement was performed using Deep View to ensure high accuracy(Eswar et al., 2008; Šali et al., 1995; Webb & Sali, 2016). The inhibitors were designed as fusion proteins, combining two distinct regions: the EK1 inhibitor peptide and fragment C of tetanus toxoid(Valdes-Balbin et al., 2021).

Eleven variants of the fusion protein were modeled, each with different lengths of flexible 'GGGS' linkers between the two domains, ranging from zero (Happy_00) to ten linkers. These linkers were introduced to provide flexibility to the EK1 domain, allowing better access to the cleavage sites. The RaptorX server was used to generate the initial structures. The two variants selected for further study were Happy_00, which has no linker, and Happy_06, which contains six GGGS linkers. Both models were chosen based on RaptorX scoring criteria, including P-values, global distance test (GDT) scores, and sequence identity(Wang et al., 2016).

Virtual Screening and Molecular Docking

The virtual screening process aimed to identify the most effective inhibitors based on their binding affinity to the spike protein's cleavage sites. The ClusPro docking server was used to perform initial docking simulations, and the HADDOCK 2.4 platform was employed for more advanced docking(Dominguez et al., 2003; van Zundert et al., 2016). The docking models were evaluated based on their binding energies and cluster sizes, with a focus on interactions at two critical cleavage sites: Arg685/Ser686 (first site) and Arg815/Ser816 (second site).

During docking, the inhibitors with positive binding values were excluded, while those with negative binding energies were further analyzed. HADDOCK clusters were ranked using multiple energy components, including van der Waals energy, electrostatic interactions, and desolvation energy. The best-performing clusters were selected based on their HADDOCK scores, and the lowest-energy models were used for post-docking analysis.

Post-Docking Analysis

Post-docking analyses were conducted to validate the binding affinity and stability of

the inhibitors. The PRODIGY web server was used to estimate binding energies and dissociation constants (Kd) at 25°C and 37°C, offering insights into the inhibitors' strength and stability at physiological conditions(Vangone & Bonvin, 2017). Further structural evaluation was performed using MolProbity to assess Ramachandran plots and identify any outliers in the modeled structures(Williams et al., 2018). The COCOMAPS tool was employed to analyze intermolecular contacts between the inhibitors and the spike protein(Vangone et al., 2011).

Molecular Dynamics Simulations

To evaluate the stability of the inhibitor-protein complexes over time, molecular dynamics simulations were performed using the GROMACS 2021.3 software(Van Der Spoel et al., 2005; Vieira et al., 2023). The simulations were conducted in a cuboid box solvated with a TIP3P water model, and six sodium ions were added to neutralize the system's charge. Energy minimization was carried out using the steepest descent algorithm, followed by equilibration in both NVT (constant volume) and NPT (constant pressure) ensembles (Figure 1 and 2).

The V-rescale thermostat was used to maintain a temperature of 300 K, and the Parrinello-Rahman barostat controlled the system pressure at 1 bar. Each simulation ran for 50 nanoseconds, with root mean square deviation (RMSD), root mean square fluctuation (RMSF), and the radius of gyration (Rg) calculated to assess structural stability and flexibility(Abdalla et al., 2022; Ke et al., 2022)(Lobanov et al., 2008; Reva et al., 1998). The results were visualized using PyMol and VMD (Visual Molecular Dynamics)(Vieira et al., 2023). This structured approach combines molecular modeling, docking, and simulations to evaluate the binding potential of the designed inhibitors. Happy_00 and Happy_06 showed promising interactions with the SARS-CoV-2 spike protein, warranting further *in vitro* validation.

Results

1. Binding Energies of Happy_00 and Happy_06

The primary focus of the molecular docking simulations was to evaluate the binding affinities of two fusion proteins, Happy_00 and Happy_06, at critical cleavage sites of the SARS-CoV-2 spike protein. The analysis revealed significant differences in their binding energies, which are indicative of their interaction stability and efficacy as potential inhibitors.

At the first cleavage site (Arg685/Ser686), the calculated binding energy for Happy_00 was -11.4 kcal/mol. This negative value signifies that the interactions between Happy_00 and the spike protein are thermodynamically favorable, suggesting stable binding at this site. The favorable binding energy indicates the formation of multiple interactions between Happy_00 and nearby residues, which effectively enhances the stability of the inhibitor-protein complex. The compact structure of Happy_00 likely facilitates strong electrostatic and van der Waals interactions with critical residues in the spike protein, contributing to its overall stability.

In contrast, Happy_06, which features increased linker flexibility due to the incorporation of six GGGS linkers, exhibited a stronger binding affinity at the second cleavage site (Arg815/Ser816), with a calculated binding energy of -15.4 kcal/mol. This enhanced binding energy indicates that the added flexibility provided by the GGGS linkers allows Happy_06 to effectively interact with the deeper binding site. The GGGS linkers enable the protein to adopt conformations that maximize interactions with residues critical for

binding, thus enhancing its affinity for the spike protein.

These results underscore the importance of structural flexibility in the design of inhibitors targeting viral proteins. The GGGS linkers in Happy_06 provide the necessary flexibility to engage with complex binding sites, while Happy_00's rigid structure results in more stable binding at accessible sites. These findings illustrate a trade-off between stability and adaptability, highlighting the need to optimize fusion protein designs based on the target interaction environment.

2. Comparison of Docking Results at the Cleavage Sites

The docking results were further analyzed at each cleavage site to understand the specific interactions contributing to the binding affinities of the two inhibitors.

First Cleavage Site (Arg685/Ser686): At this site, Happy_00 was shown to form multiple polar interactions with adjacent residues, including Gln675 and Arg683. These polar interactions are critical for stabilizing the inhibitor-protein complex and effectively preventing the action of TMPRSS2, the enzyme responsible for cleaving the spike protein at this site. The strong binding affinity and stability of Happy_00 at the first cleavage site position it as a competitive inhibitor, suggesting that it may effectively block the initial priming required for viral entry (Figure 3). In contrast, while Happy_06 also interacted at the first cleavage site, its binding was less stable due to the increased flexibility introduced by the GGGS linkers. The flexibility may lead to a less optimal fit at this specific site, making Happy_06 less effective than Happy_00 at Arg685/Ser686. These observations align with findings from previous studies that highlight the importance of maintaining optimal interactions with accessible binding sites while considering the structural adaptability of the inhibitors(Silacci et al., 2014) (Hoffmann, Kleine-Weber, & Pöhlmann, 2020; Hoffmann, Kleine-Weber, Schroeder, et al., 2020).

Second Cleavage Site (Arg815/Ser816): At the second cleavage site, Happy_06 demonstrated superior binding compared to Happy_00. It established multiple hydrogen bonds with key residues such as Lys811 and Asp808. The GGGS linkers allowed Happy_06 to adopt conformations that maximized these interactions, which significantly enhanced its binding affinity for the spike protein at this deeper and more challenging binding site. This adaptability is crucial, as viral proteins often undergo conformational changes that must be accounted for by inhibitors. In contrast, the interaction of Happy_00 at the second cleavage site was notably weaker, primarily due to its rigid structure limiting its ability to effectively engage with the more buried residues at this site. The findings suggest that while Happy_00 excels at the first cleavage site, Happy_06 is better suited for engaging with deeper, more complex binding environments, where flexibility can enhance binding efficacy (Figure 4).

Overall, the analysis highlights the critical role of linker design in determining the binding efficiency of protein fusion inhibitors. The trade-offs between rigidity and flexibility underscore the need to consider these factors when designing novel antiviral agents(Lobanov et al., 2008; Römer et al., 2021).

3. Molecular Dynamics Simulation Results

To validate the docking predictions and further investigate the stability of the inhibitor-protein complexes, molecular dynamics (MD) simulations were conducted over a period of 50 nanoseconds. The results of the MD simulations provided critical insights into the dynamic behavior of Happy_00 and Happy_06 in a

physiological environment. Both fusion proteins exhibited consistent Root Mean Square Deviation (RMSD) values of approximately 2.5 Å throughout the simulation, indicating minimal structural deviation from their initial docked conformations. This stability is essential for the efficacy of the inhibitors, as it suggests that both proteins maintain their structural integrity during the simulation period, supporting their potential effectiveness as antiviral agents(Abdalla et al., 2022; Ke et al., 2022; Lobanov et al., 2008; Reva et al., 1998).

The Root Mean Square Fluctuation (RMSF) analysis further revealed that Happy_06's flexible linkers increased the mobility of specific residues, particularly at the second cleavage site. Higher RMSF values for Happy_06 indicate that the GGGS linkers allow the protein to adapt its conformation in response to the dynamic environment, which can be advantageous for engaging with the spike protein's conformational changes during viral entry. In addition, the Radius of Gyration (Rg) analysis indicated that both inhibitors maintained compact structures throughout the simulations. The Rg values suggested that neither Happy_00 nor Happy_06 expanded significantly during the simulation, confirming the stability of the fusion proteins despite differences in their structural flexibility. The MD results collectively validated the docking predictions, demonstrating that both Happy_00 and Happy_06 can maintain stable interactions with the spike protein. These findings emphasize the importance of considering dynamic interactions when designing inhibitors, as stable binding in a physiological context is crucial for their potential therapeutic efficacy.

Discussion

The docking and simulation results from this study support the hypothesis that fusion inhibitors targeting the cleavage sites of the SARS-CoV-2 spike protein can effectively disrupt viral entry into host cells. The two designed inhibitors, Happy_00 and Happy_06, exhibited strong binding affinities to the spike protein, showcasing site-specific advantages that underscore the significance of their structural designs.

The binding affinities observed for Happy_00 and Happy_06 highlight the impact of structural configuration on the efficacy of fusion inhibitors. **Happy_00** demonstrated superior performance at the first cleavage site (Arg685/Ser686), primarily due to its compact structure. This design allowed Happy_00 to maintain close contact with critical residues involved in TMPRSS2-mediated cleavage, enhancing its binding strength. The stability provided by this rigid configuration is essential, especially at accessible sites where the inhibitor can effectively compete with TMPRSS2, the protease that facilitates the initial cleavage of the spike protein. In contrast, **Happy_06**, with its six GGGS linkers, exhibited enhanced flexibility that allowed for improved interactions with residues at the second cleavage site (Arg815/Ser816). The increased mobility of Happy_06 enabled it to engage with deeper binding pockets, maximizing the potential for favorable interactions. This adaptability is particularly crucial given the dynamic nature of the spike protein, which can undergo conformational changes during the viral entry process. The ability of Happy_06 to adjust its conformation is a significant advantage, especially when targeting buried sites that are less accessible to rigid inhibitors like Happy_00. The contrasting performances of these inhibitors demonstrate the importance of structural flexibility in fusion protein design. While rigid structures can provide stability at accessible sites, flexibility allows for improved interactions in more complex binding environments. These

findings are consistent with previous research that emphasizes the role of linker design in modulating the efficacy of peptide-based inhibitors (Silacci et al., 2014).

The results of this study suggest that targeting the TMPRSS2 cleavage sites with fusion inhibitors can effectively prevent the activation of the spike protein, thereby blocking the viral entry process. By inhibiting these cleavage events, the likelihood of viral fusion with the host cell membrane is significantly reduced. This strategy aligns with existing literature highlighting TMPRSS2 inhibition as a promising therapeutic approach to combat SARS-CoV-2 infection(Baughn et al., 2020; Hoffmann, Kleine-Weber, & Pöhlmann, 2020; Hoffmann, Kleine-Weber, Schroeder, et al., 2020). The findings also suggest that the development of fusion inhibitors could serve as a complementary strategy alongside existing antiviral therapies, such as vaccines and monoclonal antibodies. While vaccines aim to elicit an immune response to prevent infection, fusion inhibitors like Happy_00 and Happy_06 can directly impede the viral entry mechanism, providing an additional layer of defense against COVID-19. Furthermore, the versatility of the tetanus toxoid-derived scaffold presents opportunities for developing a new class of antiviral agents. By optimizing the linker designs and enhancing the binding characteristics of fusion proteins, it may be possible to create more effective inhibitors capable of targeting a range of coronaviruses. This adaptability is particularly important given the potential for future outbreaks and the emergence of new viral strains.

One of the most critical aspects of this study is the emphasis on linker flexibility in the design of fusion inhibitors. The improved binding of Happy_06 at the second cleavage site serves as a compelling example of how linker length and composition can enhance inhibitor efficacy. The GGGS linkers in Happy_06 allowed the protein to adopt conformations that maximized interactions with the spike protein, a feature that rigid linkers would have constrained. Previous studies have shown that the physical properties of linkers—such as their length and composition—can significantly affect the functionality of fusion proteins(Silacci et al., 2014). The ability of the linker to facilitate the correct positioning of functional domains relative to target sites is crucial for enhancing binding affinities and therapeutic efficacy. This study provides a foundation for further exploration of linker designs in the development of antiviral peptides. By systematically varying linker properties and evaluating their impacts on binding interactions, researchers can optimize the design of peptide-based inhibitors, enhancing their ability to compete with viral proteins in binding interactions.

While the *in silico* results are promising, it is essential to acknowledge the inherent limitations of computational modelling. Although molecular docking and dynamics simulations provide valuable insights into binding interactions, they do not fully replicate the complexities of biological systems. Experimental validation through *in vitro* and *in vivo* studies is crucial to confirm the efficacy, safety, and stability of these inhibitors in physiological conditions. Additionally, the potential for mutations in the spike protein presents a significant challenge for the ongoing effectiveness of these inhibitors. Viral proteins can undergo mutations that alter their binding sites, potentially reducing the effectiveness of current therapeutic agents. Future research should focus on evaluating the performance

of Happy_00 and Happy_06 against emerging variants of SARS-CoV-2 to ensure their continued effectiveness. Moreover, exploring the effects of different linker types and lengths on inhibitor performance can yield further insights into optimizing fusion protein design. Experimental validation of the computational predictions is necessary to assess the real-world applicability of these fusion inhibitors, providing a basis for clinical development.

Conclusion

This study illustrates the potential of fusion proteins, Happy_00 and Happy_06, to act as effective inhibitors of SARS-CoV-2 entry by targeting critical cleavage sites on the spike protein. The computational approach demonstrated that these inhibitors could effectively block TMPRSS2-mediated cleavage, thereby preventing viral fusion with host cells. The findings highlight the importance of structural design, particularly the role of linker flexibility, in enhancing the binding efficiency of antiviral peptides.

As the search for effective treatments against COVID-19 and other viral infections continues, the development of tetanus toxoid-derived fusion peptides offers a promising strategy for therapeutic intervention. Future studies that validate these findings and optimize the design of fusion inhibitors will be crucial in the ongoing effort to combat viral diseases, ensuring that new therapeutic options remain effective against evolving viral threats.

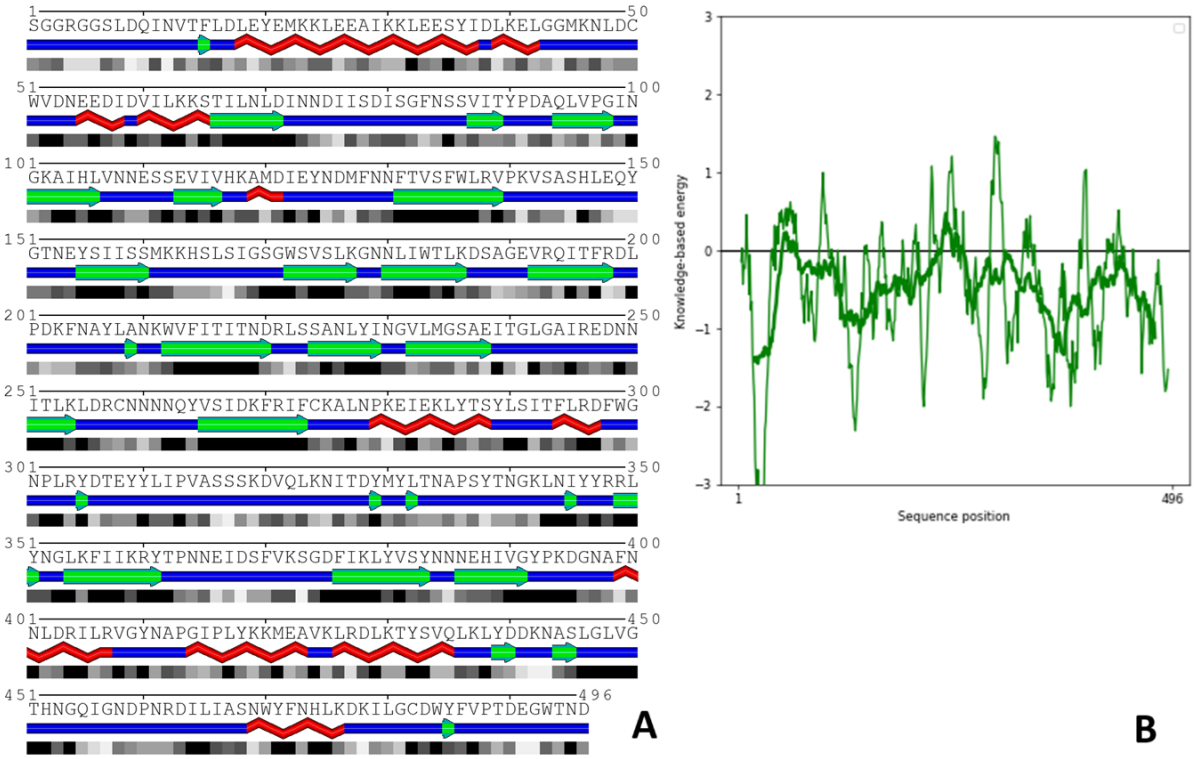

Figure 1.: The overall sequence of happy_00 (A) and the energy plot happy_00 (B).

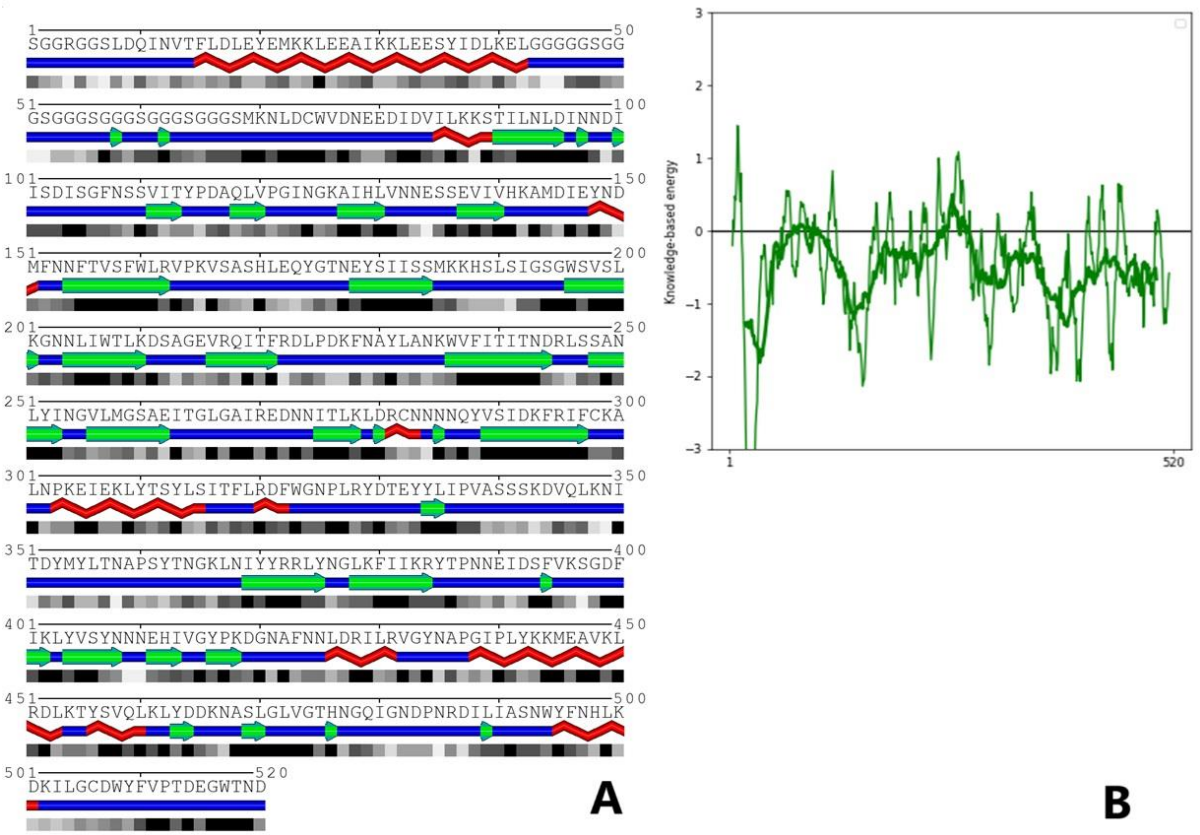

Figure 2.: The overall sequence of happy_06 (A) and the energy plot happy_06 (B).

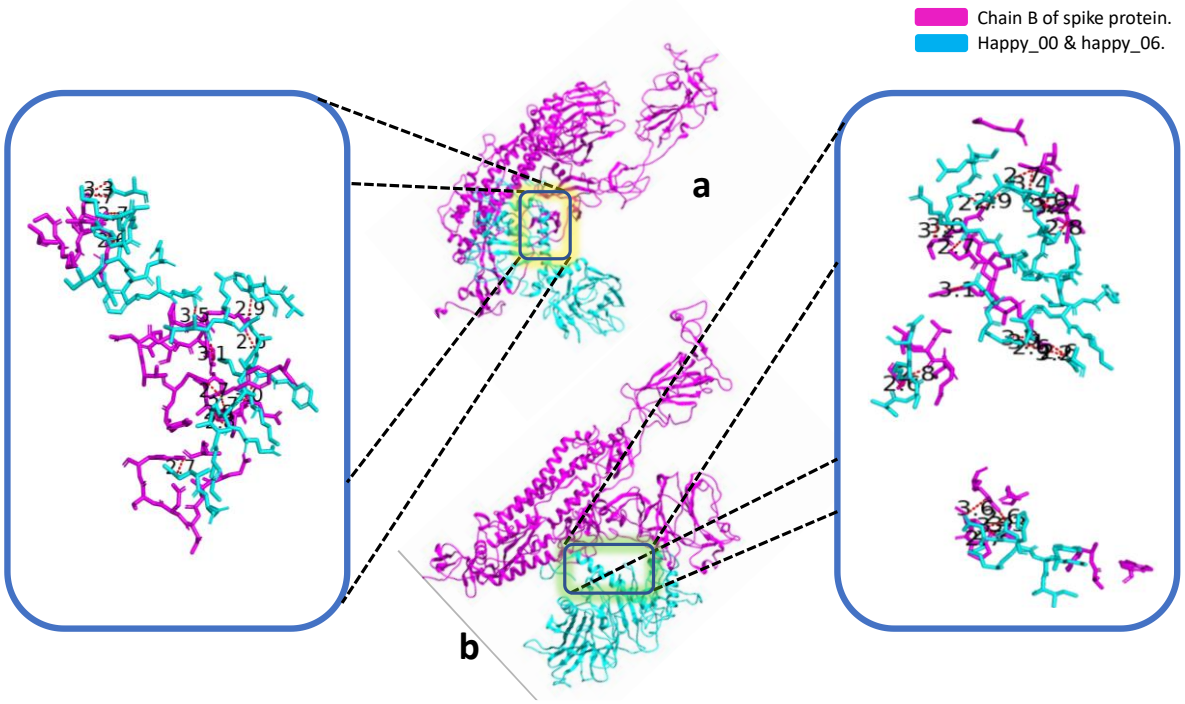

Figure 3.: Interaction of happy_00 vs. chain B of the first site (a) and happy_06 vs. chain B of the second site (b).

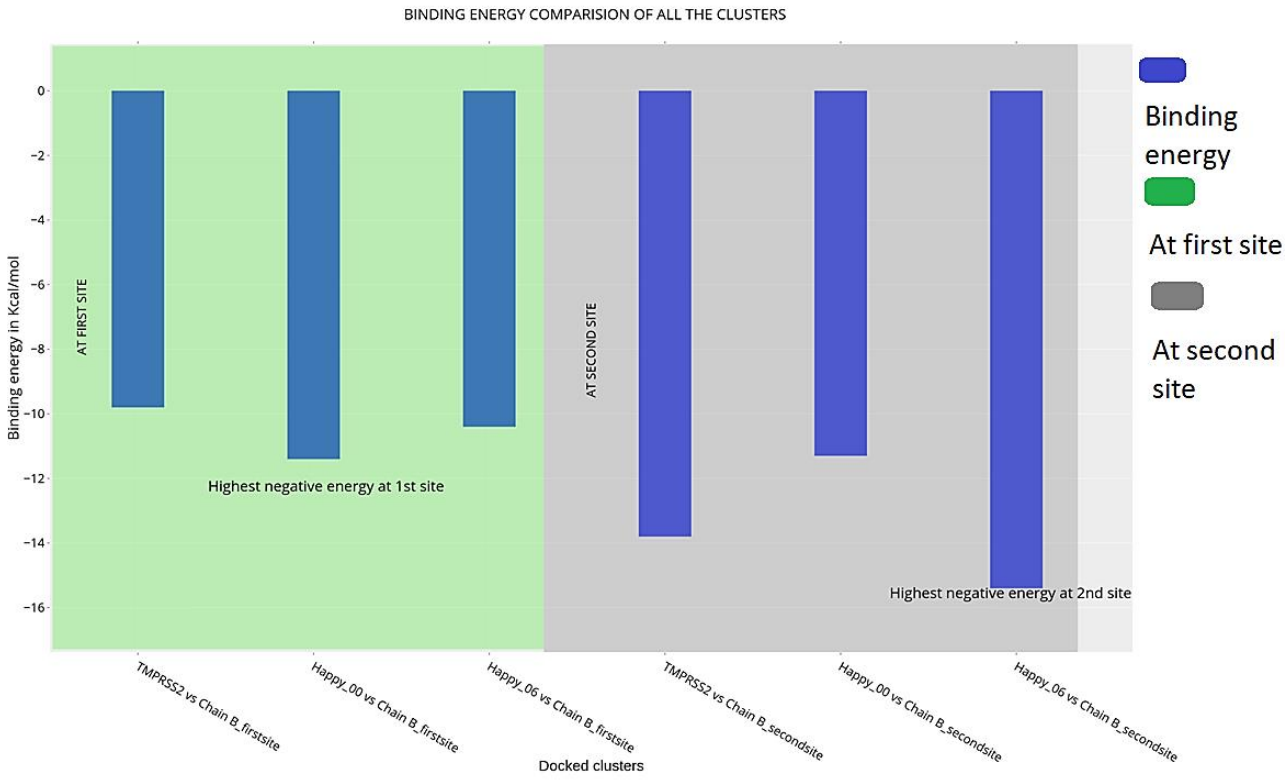

Figure 4: Binding energy comparison across docked clusters Highest negative energy at site one and two representation.

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