Enhancing Riboflavin-Binding Protein Function through Phosphorylated Flavin Compound Interactions: Predictive Insights for Therapeutic Ligand Design

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ABSTRACT

The objective of this study is to predict and understand the interactions of phosphorylated flavin compounds with RfBP and their potential to enhance RfBP function, with the aim of designing novel and more effective ligands for RfBP with a focus on therapeutic applications. In the absence of Crane RfBP's 3D structure, its protein sequence was retrieved from the NCBI database in FASTA format. SWISS-MODEL employed this sequence as the query for comparative modeling, facilitating 3D structure prediction. The Ramachandran plot gauges protein or experimental structure quality, categorizing amino acids into favorable, allowed, or disallowed regions. Hydrogen atoms incorporation into the protein was facilitated by Autodock software before molecular docking preparation. A total of 10 phosphorylated flavin compounds were selected to predict the binding with crane RfBP. Docking experiment was performed with potential active site on RfBP using AutoDock 4.2.6. compounds such as N-sulfo-flavin mononucleotide and Flavin-N7 protonated-adenine dinucleotide exhibit significantly stronger binding interactions, supported by their notably negative binding energies of -6.46 kcal/mol and -5.64 kcal/mol, respectively. Compared to riboflavin, varied binding affinities and distinct interactions showcase phosphorylated flavins' potential biological versatility. These findings affirm crane RfBP's efficacy in delivering phosphorylated flavins for therapeutic applications.

Keywords: RfBP, Autodock, FASTA, SWISS-MODEL, *In silico*

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1.INTRODUCTION

Riboflavin, a water-soluble vitamin essential for energy production and redox reactions, is crucial for metabolic processes (Köllner et al., 2020). Beyond its traditional roles, Riboflavin-binding protein (RfBP) demonstrates potential in therapeutic targets. It's upregulated in cancers like breast and hepatocellular carcinoma (Liu et al., 2019; Wu et al., 2018) and interacts with proteins implicated in diseases like cardiovascular conditions (Zhang et al., 2019). RfBP mediates riboflavin's transport across membranes, compensating for its limited bioavailability due to poor absorption and rapid excretion (Said et al., 2019; Köllner et al., 2020). RfBP's high-affinity binding, conformational

adaptability, and homology with avian counterparts enhance its significance (Ganesan et al., 2014). This study investigates phosphorylated flavin compounds' interactions with RfBP, potentially paving the way for innovative therapeutic ligand design.

Phosphorylated flavin compounds, which encompass riboflavin derivatives carrying additional phosphate groups, hold the potential to influence various aspects of RfBP's behavior, such as binding specificity, conformational dynamics, and structural stability (Said et al., 2019). For the current study, a selection of ten distinct phosphorylated flavin compounds was chosen to explore their effects on RfBP (Ganesan et al., 2014). By delving into these interactions, this research endeavors to unveil new perspectives on RfBP's functionality and shed light on flavoprotein-associated disorders, including conditions like glutaric acidemia type II (Liu et al., 2019; Wu et al., 2018).

Given RfBP's pivotal role in riboflavin transportation, crucial for fundamental metabolic and redox processes, this investigation carries considerable significance (Köllner et al., 2020). The ultimate objective of this study lies in predicting and comprehending the intricate interactions between phosphorylated flavin compounds and RfBP, with a focused ambition of harnessing this knowledge to craft innovative and potent ligands for RfBP. Such ligands, designed with a therapeutic intent, could usher in novel strategies for manipulating flavin homeostasis and optimizing protein function in various health-related contexts (Zhang et al., 2019). Therefore, the objective of this study is to predict and understand the interactions of phosphorylated flavin compounds with RfBP and their potential to enhance RfBP function, with the aim of designing novel and more effective ligands for RfBP with a focus on therapeutic applications.

2. MATERIALS AND METHODS

2.1 RfBP 3D Model Prediction

Due to the unavailability of the 3D structure of Crane RfBP, the protein sequence was obtained in FASTA format from the NCBI database (https://www.ncbi.nlm.nih.gov/protein/2473547392). The obtained Crane RfBP protein sequence was employed as the query sequence for comparative modeling. The 3D structure prediction was performed using SWISS-MODEL (http://swissmodel.expasy.org).

2.2 RfBP Model Evaluation by Ramachandran Plot

The Ramachandran plot is employed to assess the quality of a modeled protein or an experimental structure. Ramachandran plot statistics offer insights into the distribution of amino acid residues across favorable, allowed, and disallowed regions. To evaluate the protein's quality, the VADAR server (http://vadar.wishartlab.com/) was utilized for Ramachandran plot analysis.

2.3 RfBP Preparation for Molecular Docking

To prepare the target for molecular docking, hydrogen atoms were added to the protein using a Autodock software. Next, the protein was assigned with Kollman or Gasteiger charges to represent the electrostatic properties of the protein. Finally, the prepared protein structure was saved in the PDBQT format.

2.4 Preparation of Phosphorylated Flavin Compounds

A total of following 10 phosphorylated flavin compounds were selected to predict the binding with crane RfBP:

1. N-sulfo-flavin mononucleotide
2. Flavin-N7 protonated-adenine dinucleotide
3. 3-Methyllumiflavin
4. 7,8-Dimethyl-10-formylmethylisoalloxazine
5. Flavin-adenine dinucleotide-N5-isobutyl ketone
6. Isoriboflavin
7. 8-demethyl-8-dimethylamino-flavin-adenine-dinucleotide
8. 8-Hydroxy-5-deazaflavin
9. Flavin adenine dinucleotide
10. Flavin mononucleotide

The chemical structures of the above were retrieved from the PubChem database in the SDF (Table-1) and converted into. PDB format by Open Babel software. The 3D structures of the these compounds were retrieved from PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The ligands were prepared for docking by adding hydrogen atoms, assigning partial charges, and optimizing their geometry. This was done using a molecular modeling software package such as AutoDock Tools. For instance, Gasteiger-Marsili charges could be applied using AutoDock Tools. The prepared ligands were saved in a suitable format such as PDBQT, which is compatible with most molecular docking software packages. For comparative analysis riboflavin was used.

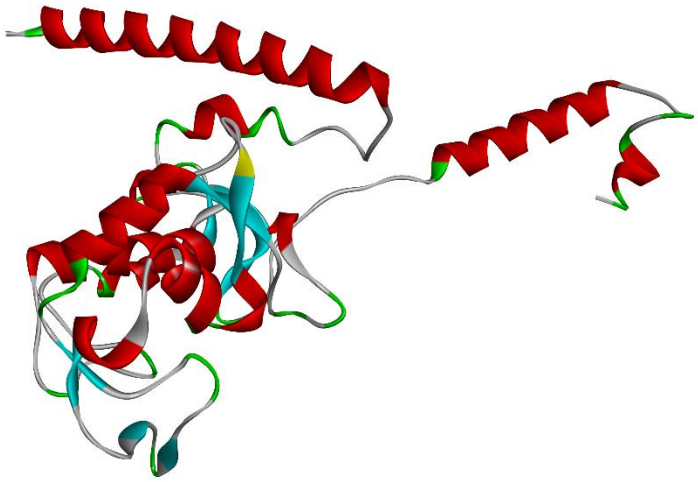


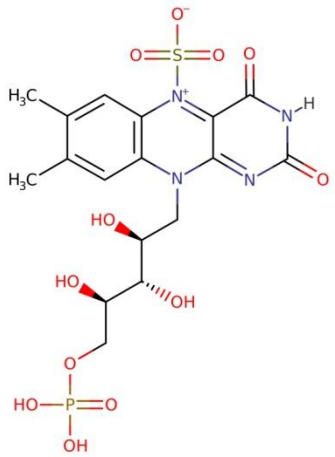
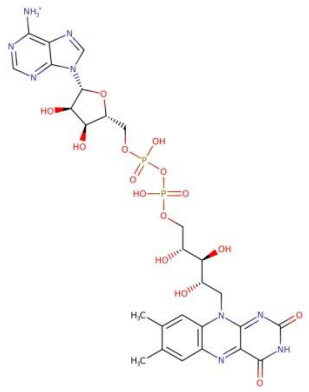
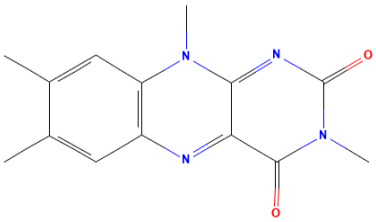
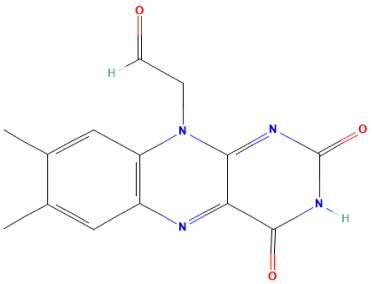
Figure-1. Crane RfBP 3D structure predicted by using SWISS-MODEL web server.

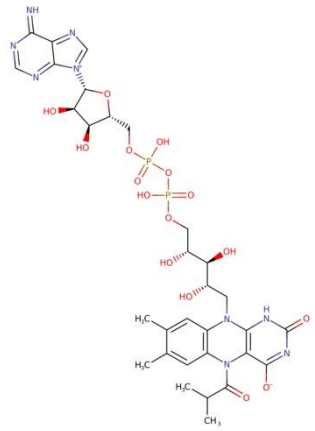
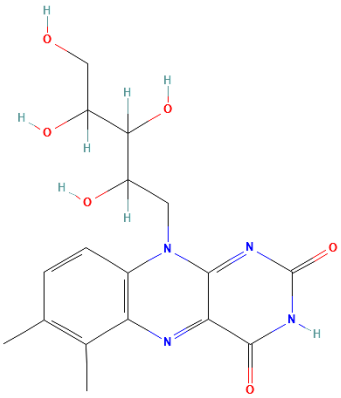
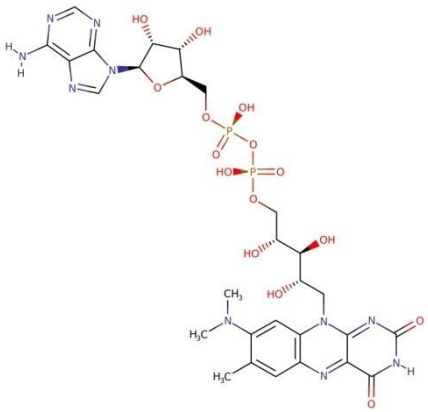
2.5 Molecular Docking

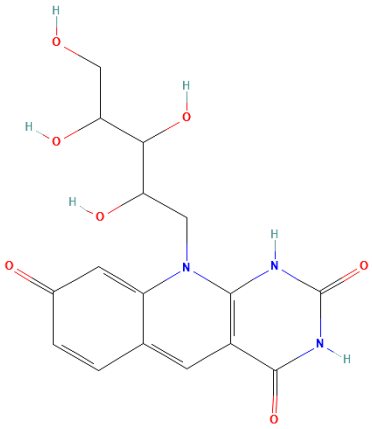
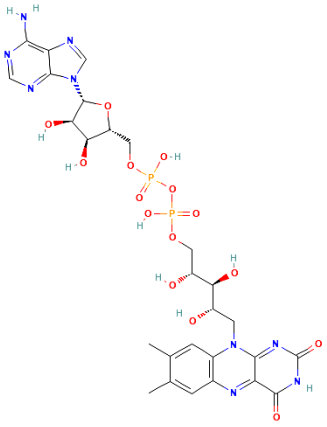
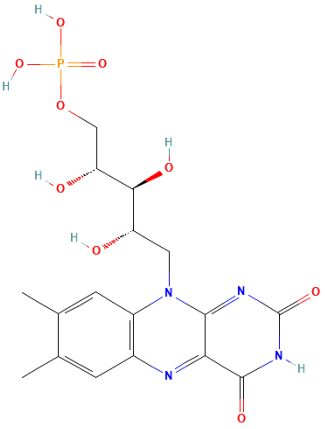
Docking experiment was performed with potential active site on RfBP using AutoDock 4.2.6 (http://autodock.scripps.edu/) to predict the binding modes and affinities of the phosphorylated flavin compounds to the prepared crystal structure of RfBP. During docking at first the explicit hydrogens,

charges, flexible torsions, were assigned by the AD program for both the protein and ligands.

Table-1. Phosphorylated flavin compounds and their 2D structure were obtained from PubChem database

Sl No.	Phosphorylated Flavin Compounds (ID)	2D Structure
1	N-sulfo-flavin mononucleotide (Drug Bank Accession Number DB02164)	
2	Flavin-N7 protonated-adenine dinucleotide (DrugBank Accession Number DB02332)	
3	3-Methyllumiflavin (PubChem CID 87733)	
4	7,8-Dimethyl-10-formyl methyl isoalloxazine/Formyl methyl flavine(PubChem CID 73221)	

5	Flavin-adenine dinucleotide-N5-isobutyl ketone (DrugBank Accession Number DB03531)	
6	Iso riboflavin (PubChem CID165385)	
7	6)8-demethyl-8-dimethylamino-flavin-adenine-dinucleotide (DrugBank Accession Number DB03482)	

8	8-Hydroxy-5-deazaflavin (PubChem CID 122079)	
9	Flavin adenine dinucleotide (PubChem CID_643975)	
10	Flavin mononucleotide (PubChem CID_643976)	

3. RESULTS

3.1 Ramachandran Plot of RfBP

The VADAR server emerged as the most effective tool for model validation, showcasing exceptional results. Typically, favorable resolution values are observed at 95% or higher (Figure-2). Analyzing the Ramachandran plot statistics of the crane RfBP within the VADAR server, an impressive 95.28% of its residues were positioned within the highly favored regions of the plot (Figure-2) indicates the protein validity for docking studies. A quality model is generally anticipated

to exhibit around 90% of its residues within this favorable region.

Delving deeper into the Ramachandran plot statistics from the VADAR server, the Crane RfBP showcased its distribution across various regions: 95.28% in the most favored, 9.7% in additional allowed, 0.0% in generously allowed, and 0.4% in disallowed sections of the plot. Further insights from the Ramachandran plot highlighted specific residue counts: 236 non-glycemic and non-proline residues, 2 end-residues, 8 glycine residues depicted as triangles, and 10 proline residues.

These findings collectively underscore the exceptional quality and favorable structural characteristics of the Crane RfBP model as validated by the VADAR server.

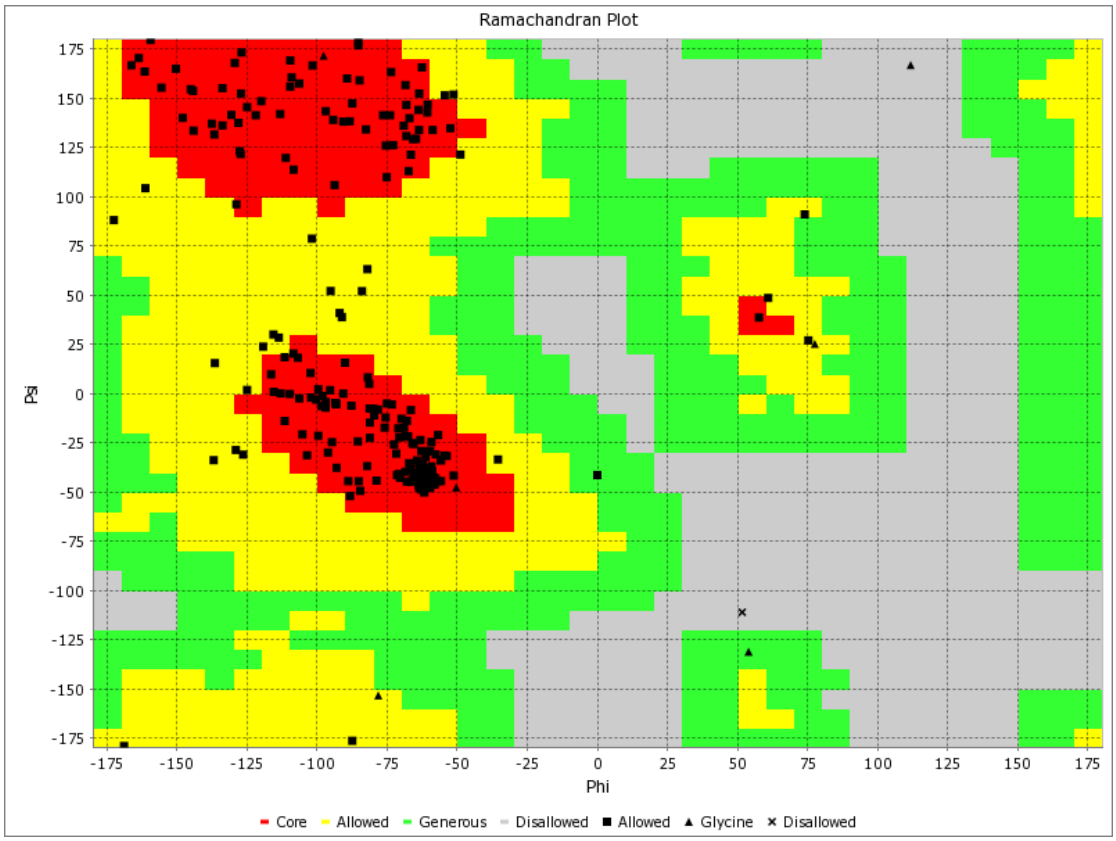


Figure-2. Ramachandran Plot Analysis of Crane RfBP Model Validated by VADAR Server

3.1.2 Molecular Docking Analysis

To predict the binding interactions between crane RfBP and phosphorylated flavin compounds, molecular docking simulations were conducted using Autodock, and the resultant outcomes were analyzed. The table-1 presents the binding energies of different phosphorylated flavin compounds, comparing them to Riboflavin.

Comparing these binding energy values with Riboflavin, it's evident that certain phosphorylated flavin compounds exhibit significantly lower binding energies, indicating a stronger binding affinity. Notably, compounds such as N-sulfo-flavin mononucleotide and Flavin-N7

protonated-adenine dinucleotide exhibit significantly stronger binding interactions, supported by their notably negative binding energies of -6.46 kcal/mol and -5.64 kcal/mol, respectively.

Table. Binding Properties and Interactions of Phosphorylated Flavin Compounds with crane RfBP: Binding Energy, Hydrogen Bonds, and Hydrophobic Interactions

S.No	Phosphorylated Flavin Compounds	Binding Energy Kcal/mol.	No. of H_2 bond interactions	Hydrophobic bond interactive residues	H_2 bond interactive residues
1	N-sulfo-flavin mononucleotide	-6.46	2	Lys71, Met219, Met220, Lys223, Lys247, Lys250, Phe251, Leu254, Lys255, Glu258	Lys71, Lys247
2	Flavin-N7 protonated-adenine dinucleotide	-5.64	5	Gln43, Tyr45, Arg125, Trp126, Ile127, Ser137, Pro139, Lys203, Leu210, Tyr224, Leu225, Leu226, Ser229, Ser230, Glu231	Gln43, Trp126, Lys203,
3	3-Methyllumiflavin	-5.6	3	Glu81, Gln82, Ala84, His85, Pro87, Val88, Ile89, Lys90, Asn92	His85, Ile89, Lys90
4	7,8-Dimethyl-10-formylmethylisoalloxazine	-5.26	3	Tyr45, Arg125, Trp126, Pro139, Lys203, Leu210, Tyr224, Leu225, Leu226, Tyr227, Glu228, Ser229, Glu240	Trp126, Glu228, Glu240
5	Flavin-adenine dinucleotide-N5-isobutyl ketone	-4.15	4	Glu49, Gly50, Asp51, Thr52, His53, Lys54, Leu55, Tyr76, Asn78, Phe79, Gln82, Asn92	Asp51, Leu55, Tyr76, Asn92
6	Isoriboflavin	-3.11	6	Cys42, Gln43, Lys44, Tyr45, Gly46, Cys47, Glu49, Leu55, Lys56, Ile127	Cys42, Glu49, Lys56
7	8-demethyl-8-dimethylamino-flavin-adenine-dinucleotide	-2.95	2	Gln82, His85, Pro87, Val88, Ile89, Lys90, Val91, Tyr95	Gln82, Pro87
8	8-Hydroxy-5-deazaflavin	-2.08	6	Cys42, Gln43, Lys44, Tyr45, Gly46, Cys47, Glu49, Leu55, Lys56, Ile127	Cys42, Glu49, Lys56
9	Flavin adenine dinucleotide	-1.58	4	Gly46, Cys47, Leu48, Glu49, Gly50, Asp51, Thr52, His53, Lys54, Leu55, Tyr76	Cys47, Asp51, Leu55, Tyr76
10	Flavin mononucleotide	-0.97	1	Leu249, Glu252, Lys253, Gly256, Glu257, Glu260	Lys253
11	Riboflavin	-2.29	0	Cys42,Gln4, Lys44,Tyr45, Gly46,Cys47 Glu49,Leu5, Lys56, Ala124,Ile127	-

Additionally, 3-Methyllumiflavin and 7,8-Dimethyl-10-formylmethylisoalloxazine also demonstrate robust binding affinities with energies of -5.6 kcal/mol and -5.26 kcal/mol. In contrast, Flavin adenine dinucleotide and Flavin mononucleotide present comparatively weaker binding interactions, reflected by their less negative binding energies of -1.58 kcal/mol and -0.97 kcal/mol, respectively. These variations in binding energies provide insights into the diverse strengths of interactions, highlighting potential differences in binding capabilities among these compounds and their respective binding partners.

The table-1 highlights the relationship between phosphorylated flavin compounds binding energies and the number of hydrogen bond interactions, drawing comparisons to Riboflavin. Docking of top 4 phosphorylated flavin compounds and their Magnified view in the Riboflavin-Binding Protein pictures are depicted in Figure-3 to 6.

Comparing these binding energy values with Riboflavin, it's evident that certain phosphorylated flavin compounds exhibit significantly lower binding energies, indicating a stronger binding affinity. Notably, compounds such as N-sulfo-flavin mononucleotide and Flavin-N7 protonated-adenine dinucleotide exhibit significantly stronger binding interactions, supported by their notably negative binding energies of -6.46 kcal/mol and -5.64 kcal/mol, respectively. Additionally, 3-Methyllumiflavin and 7,8-Dimethyl-10-formylmethylisoalloxazine also demonstrate robust binding affinities with energies of -5.6 kcal/mol and -5.26 kcal/mol. In contrast, Flavin adenine dinucleotide and Flavin mononucleotide present comparatively weaker binding interactions, reflected by their less negative binding energies of -1.58 kcal/mol and -0.97 kcal/mol, respectively. These variations in binding energies provide insights into the diverse strengths of interactions, highlighting potential differences in binding capabilities among these compounds and their respective binding partners.

The H2 bond interactive residues, along with their respective numbers of hydrogen bonds, hold a pivotal role in orchestrating the binding interactions of phosphorylated flavin compounds (Figures 3-6). These specific amino acid residues, such as Lys71 and Lys247 for N-sulfo-flavin mononucleotide, Gln43, Trp126, and Lys203 for Flavin-N7 protonated-adenine dinucleotide, His85, Ile89, and Lys90 for 3-Methyllumiflavin, among others, contribute to the stabilization of binding through hydrogen bonding. The varying counts of these hydrogen bonds across the compounds emphasize their vital contribution in dictating favorable binding orientations and energies. This intricate interplay between the interactive residues and the compounds underscores their essential role in modulating binding affinity, thus shaping the specificity and strength of the observed interactions within the context of this study.

The hydrophobic interactions involve specific amino acid residues that play a critical role in modulating the binding of these compounds. For instance, N-sulfo-flavin mononucleotide demonstrates a significant number of hydrophobic bond interactive residues, including Lys71, Met219, Met220, Lys223, Lys247, and others, contributing to its strong binding energy of -6.46 kcal/mol (Table-1). Similarly, Flavin-N7 protonated-adenine dinucleotide showcases multiple hydrophobic bond interactive residues like Trp126, Tyr224, Leu225, and Glu231, influencing its favorable binding energy of -5.64 kcal/mol. These hydrophobic interactions, spread across the compounds, highlight their essential role in stabilizing binding interactions, thus underscoring their contribution to the observed binding affinities.

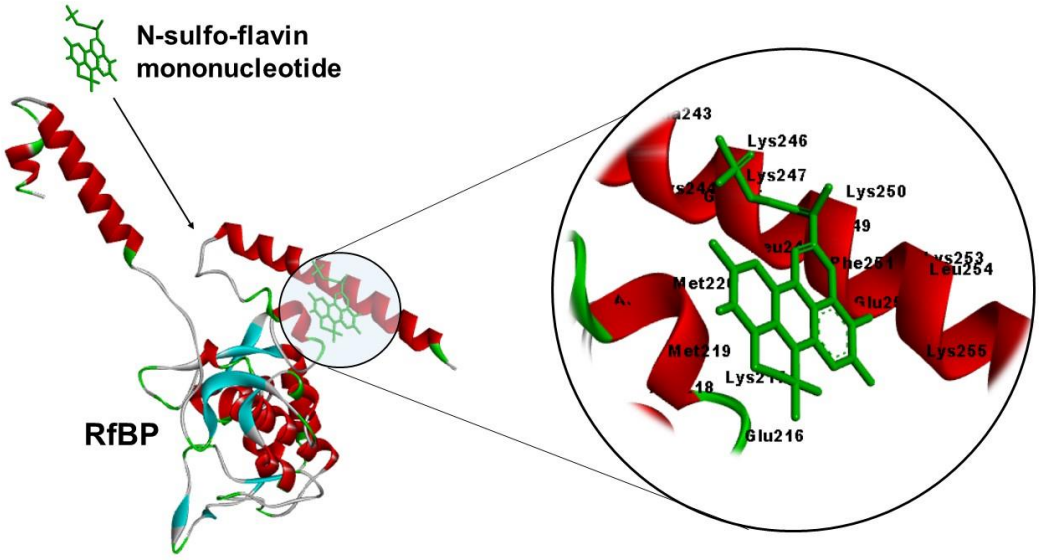


Figure-3. Docking of N-sulfo-flavin mononucleotide into RfBP and Magnified view of N-sulfo-flavin mononucleotide in the Riboflavin-Binding Protein

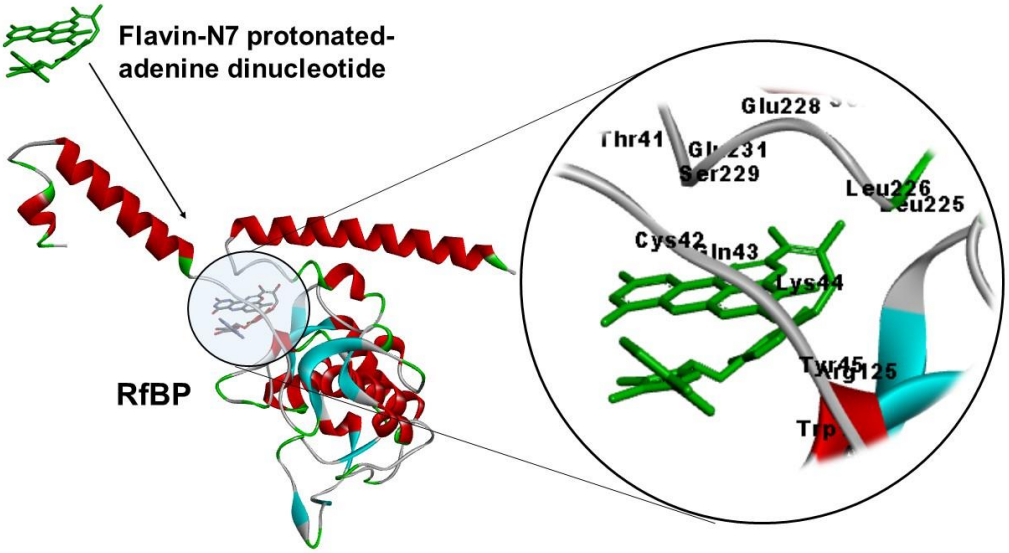


Figure-4. Docking of Flavin-N7 protonated-adenine dinucleotide into RfBP and Magnified view of Flavin-N7 protonated-adenine dinucleotide in the Riboflavin-Binding Protein

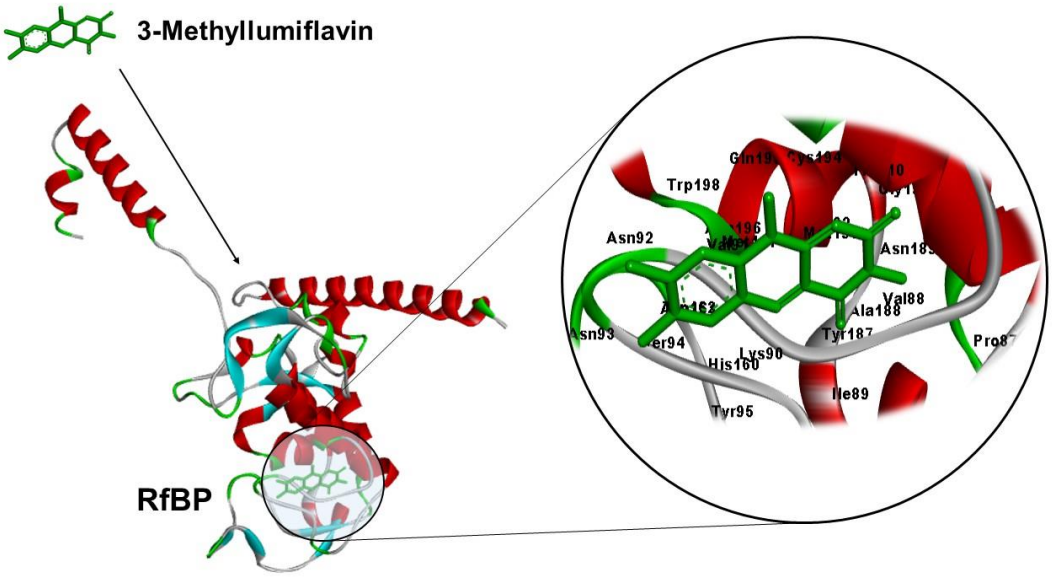


Figure-5. Docking of 3-Methyllumiflavin into RfBP and Magnified view of 3-Methylluminflavin in the Riboflavin-Binding Protein

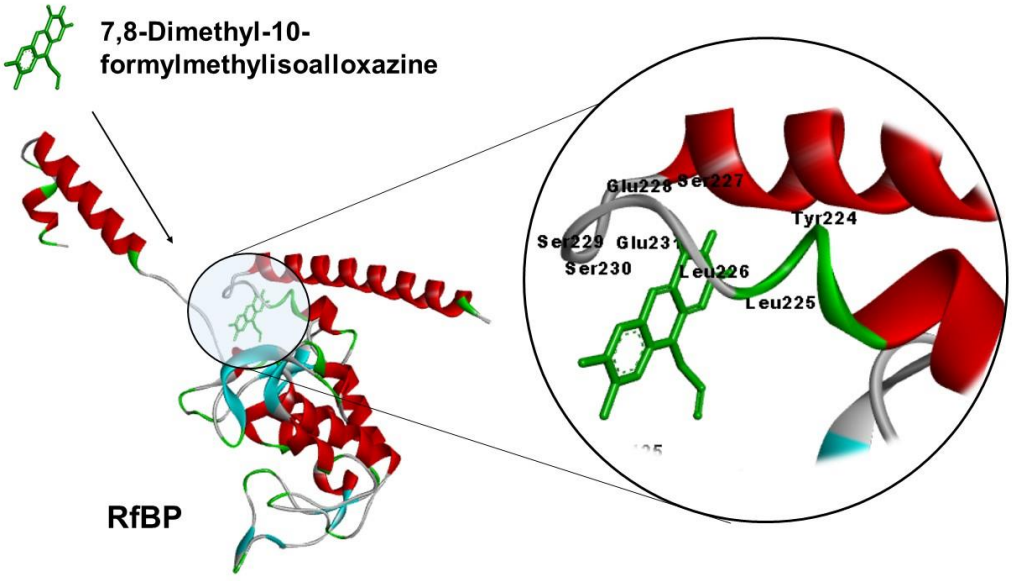


Figure-6. Docking of 7,8-Dimthyl-10-formylmethylisoalloxazine into RfBP and Magnified view of 7,8-Dimthyl-10-formylmethylisoalloxazine in the Riboflavin-Binding Protein

4. DISCUSSION

The binding interaction between crane Riboflavin Binding Protein and phosphorylated flavin compounds were presented in this study. Riboflavin binding protein (RfBP) is a globular monomeric phosphor-glycoprotein that has a high-affinity binding site for riboflavin (vitamin B2). It is involved in the transport and storage of riboflavin in various tissues, especially in the egg white of birds and reptiles (Nabokina et al, 2012).

This understanding holds potential implications for designing targeted interventions in various biological processes where these compounds may play a significant role. For instance, flavoproteins are proteins that contain a nucleic acid derivative of riboflavin, such as FMN or FAD, as a prosthetic group or as a cofactor (Beztsinna, 2016). Flavoproteins are involved in many metabolic pathways, such as oxidative phosphorylation, electron transport chain, fatty acid oxidation, etc., and are essential for cellular energy production and redox homeostasis (Beztsinna, 2016). Therefore, modulating the binding affinity of phosphorylated flavin compounds to flavoproteins may affect their enzymatic activity and function.

Moreover, flavins have been shown to act as a critical liaison between metabolic regulation and photoreceptor function in the retina (Khan et al., 2020). Flavins are involved in the regulation of retinal blood flow, phototransduction, visual cycle, and photoreceptor survival (Khan et al., 2020). Recent research has found a sensitive method of measuring flavins in both diseased and healthy retina, presence of a novel flavin binding protein exclusively expressed in the retina, and the presence of flavin specific transporters in both the inner and outer blood-retina barriers (Khan et al., 2020). Therefore, manipulating the binding affinity of phosphorylated flavin compounds to these proteins and transporters may affect their transport and availability in the retina.

Furthermore, riboflavin has been reported to play a role in plant development and stress responses (Zhang et al., 2014). Riboflavin is the precursor of FMN and FAD, essential cofactors for many metabolic enzymes that catalyze a variety of biochemical reactions (Zhang et al., 2014). Downregulation of leaf flavin content induces early flowering and enhances salt stress tolerance in Arabidopsis (Zhang et al., 2014; Kumar et al, 2020). This suggests that riboflavin biosynthesis and metabolism are linked to plant growth and adaptation (Zhang et al., 2014). Therefore, altering the binding affinity of phosphorylated flavin compounds to riboflavin biosynthetic enzymes or metabolic regulators may affect their expression and activity.

Phosphorylated flavin compounds might have inherent properties or structural modifications that enhance their anticancer activity compared to non-phosphorylated flavins. By associating these flavins with RfBP, their stability, availability, and targeted delivery to cancer cells could be improved, potentially leading to increased efficacy in cancer therapy. Phosphorylated flavins have been suggested to have an impact on cellular energy metabolism. Given that cancer cells often exhibit altered metabolic pathways, targeting these pathways using phosphorylated flavins delivered by RfBP could potentially disrupt cancer cell metabolism and impede tumor growth (Yamamoto et al, 2009; Beztsinna et al, 2016; Gujjeti et al, 2014; Davella et al, 2021).

These results indicate that the identified amino acid residues play a pivotal role in mediating the interaction between the protein and phosphorylated flavin compounds. However, specific

phosphorylated flavins exhibit reduced binding energy and fewer hydrogen bonds with RfBP compared to riboflavin. For instance, flavin adenine dinucleotide's binding energy is -1.58 kcal/mol with four hydrogen bonds, contrasting riboflavin's -2.29 kcal/mol and absence of hydrogen bonds. This suggests weaker affinity and specificity of these compounds to RfBP than riboflavin.

RfBP, a riboflavin carrier pivotal in cellular processes, holds promise for targeted drug delivery by conjugating phosphorylated flavins like derivatives of phosphorylated riboflavin. This strategy may enable precise delivery to tumor cells, exploiting their receptor overexpression. In light of these findings, crane RfBP displays potential in efficiently delivering phosphorylated flavins to target cells, offering therapeutic avenues.

5. CONCLUSION

In summary, the comprehensive examination of phosphorylated flavin compounds offers valuable insights into their binding traits. compounds such as N-sulfo-flavin mononucleotide and Flavin-N7 protonated-adenine dinucleotide exhibit significantly stronger binding interactions, supported by their notably negative binding energies of -6.46 kcal/mol and -5.64 kcal/mol, respectively. Key amino acids like Lys71, Met219, Gln43, and Trp126 play roles in both bond types. Compared to riboflavin, varied binding affinities and distinct interactions showcase phosphorylated flavins' potential biological versatility. These findings affirm crane RfBP's efficacy in delivering phosphorylated flavins for therapeutic applications.

Conflict Of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Acknowledgements

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