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Structural and Functional Insights into Riboflavin-Binding Proteins in Whooping crane (*Grus americana*) and Other Avian Species: An *In silico* Approach

Srinivas Paramkusham[*1], Estari Mamidala[2], J. Madhukar[3]

[1]Department of Biochemistry, Kakatiya University, Hanumakonda-506 009. Telangana State, India.
https://orcid.org/0009-0008-4932-1178

[2]Department of Zoology, Kakatiya University, Hanumakonda-506 009. Telangana State, India.
https://orcid.org/0000-0002-7874-6019

[2]Department of Chemistry, Kakatiya University, Hanumakonda-506 009. Telangana State, India

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ABSTRACT

Riboflavin-binding proteins (RfBPs) play a critical role in the transport and regulation of vitamin B_2 across avian species. In this study, we conducted an *in silico* analysis of the physicochemical properties, amino acid composition, and secondary structure of RfBP in *Grus americana* (Whooping crane) and other bird species. Protein sequences were retrieved from the UniProtKB/Swiss-Prot database, and various physicochemical parameters were calculated using the ExPASy ProtParam tool. The amino acid composition was analyzed to compare RfBP sequences, and the secondary structure was predicted using the SOPMA tool to identify structural differences across species. The results revealed that the molecular weight of RfBP in Grus americana was higher compared to other species, with significant differences in isoelectric points, indicating diverse charge distributions. Amino acid composition showed a high percentage of conserved residues, such as serine and cysteine, suggesting their importance in protein structure and function. Secondary structure analysis indicated that alpha helices and random coils were the dominant structures across species, with minor variations in beta turns and extended strands, reflecting structural adaptations. In conclusion, this *in silico* analysis provides valuable insights into the structural and functional diversity of RfBPs in avian species, highlighting potential evolutionary adaptations and species-specific roles in riboflavin transport.

Keywords: *UniProtKB, Swiss-Prot, Whooping crane, RfBPs, In silico, ExPASy.*
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1. INTRODUCTION

Riboflavin, or vitamin B2, is a crucial nutrient involved in several biological processes such as energy metabolism, antioxidant defense, and cellular growth (Combs, 2012). In birds, riboflavin-binding proteins (RfBPs) play a key role in the transportation and regulation of this nutrient, ensuring proper metabolic functioning and reproductive health (Hargrove et al., 1979). These proteins are essential for avian species, facilitating riboflavin delivery to developing embryos, which makes them vital for reproductive success. By understanding the molecular characteristics of RfBPs, researchers can gain insights into the physiological adaptations of different bird species (Powers, 2003).

This study focuses on the *in silico* characterization of riboflavin-binding proteins in crane species, comparing their properties with other bird species such as chickens and pigeons. *In silico* methods employ computational tools and genomic data to analyze protein structures and properties without the need for laboratory experimentation (Elofsson and von Heijne, 2007; Mamidala *et al.,* 2013). This approach allows for efficient exploration of the physicochemical properties, amino acid composition, and secondary structure of RfBPs. Such analyses provide an opportunity to uncover evolutionary adaptations in avian species, offering valuable insights into the functional diversity of these proteins (Waterhouse et al., 2009).

Comparative studies of RfBPs across avian species enable the identification of conserved and divergent features in these proteins. By analyzing the molecular weight, isoelectric point, and amino acid composition, we can explore how these proteins adapt to different environmental and physiological demands (Pace et al., 1995). The study's focus on cranes, alongside species like quails and falcons, offers a comprehensive understanding of how RfBP characteristics relate to the metabolic and ecological needs of various birds. This research not only enhances our understanding of avian biology but also holds implications for conservation and species management, particularly for endangered cranes

2. MATERIALS AND METHODS

2.1 *In silico* Physicochemical Properties of RfBP

To analyze the physicochemical properties of the riboflavin-binding protein (RfBP) in cranes and other avian species, the UniProtKB/Swiss-Prot database was utilized to retrieve complete RfBP sequences. UniProtKB/Swiss-Prot is a reliable and curated protein sequence database widely used for obtaining accurate protein data, including that of Grus americana (*Whooping crane*). Once the sequences were obtained, the ExPASy ProtParam tool was employed to compute various physicochemical parameters. ProtParam, an online resource provided by the Swiss Institute of Bioinformatics (SIB), calculates a range of protein characteristics such as molecular weight, isoelectric point (pI), and amino acid composition based on the input sequence (Gasteiger *et al.*, 2003; RP Gujjeti *et al.*, 2013). This approach enabled a comprehensive analysis of the crane RfBP's properties, facilitating comparisons with other avian species.

Key physicochemical parameters computed include amino acid composition, molecular weight, charge distribution, isoelectric point, extinction coefficient, and instability index. The amino acid composition reveals the frequency and percentage of each amino acid in the protein, influencing its folding and function. Molecular weight calculations help understand the protein's size and structure, while the isoelectric point (pI) indicates the pH at which the protein has no net charge, which affects its solubility and interaction with the cellular environment. The extinction coefficient, a measure of how much light a protein absorbs, is useful for determining protein concentration in spectrophotometric assays. The instability index predicts protein stability, where higher values suggest greater susceptibility to degradation, which is vital for understanding the protein's potential stability under physiological conditions.

By leveraging the UniProtKB/Swiss-Prot database and ExPASy ProtParam tool, this study provides a detailed physicochemical characterization of the crane RfBP. These computed parameters help in understanding how the structure of the RfBP influences its stability, function, and evolutionary adaptations across avian species. This *in silico* approach not only saves time and resources but also allows researchers to predict the behavior of proteins under various conditions, aiding in further studies of protein functionality and its role in riboflavin metabolism.

2.2 Amino Acid Composition of RfBP

The amino acid composition of the RfBP in crane species was analyzed by retrieving the full protein sequence from the UniProtKB/Swiss-Prot database. This database, known for its accuracy and curation, provides high-quality protein sequences, which are critical for studying the molecular composition of proteins. The amino acid composition analysis involved determining the number and percentage of each amino acid present in the crane RfBP and comparing these findings with those of other bird species. This analysis provided insights into the structural elements critical for riboflavin binding and transport, as amino acid composition directly affects protein folding, stability, and functionality.

The analysis focused on identifying conserved amino acids across different avian species and investigating their roles in the protein's overall structure and function. Differences in amino acid composition between species may indicate species-specific adaptations that reflect unique physiological needs or environmental pressures. For example, certain residues, such as cysteine and glutamic acid, may play a role in the formation of disulfide bonds or maintaining protein solubility, which is essential for the RfBP's function in riboflavin transport.

2.3 Secondary Structure Analysis of RfBP

The secondary structure analysis of RfBP from crane species and other avian species was performed using the SOPMA (Self-Optimized Prediction Method with Alignment) tool server, a widely used platform for predicting protein secondary structure. The protein sequences retrieved from UniProtKB/Swiss-Prot were input into SOPMA, which predicts the protein's structural elements, including alpha helices, beta sheets, and random coils. The parameters set for this analysis included various conformational states such as alpha helix, beta turn, extended strand, and random coil, allowing for a comprehensive prediction of the secondary structure of RfBP.

SOPMA's prediction of secondary structures helped identify differences and similarities in the crane RfBP compared to those of other species. The predominance of alpha helices and random coils in the secondary structure was noted, which suggests regions of structural stability and flexibility. This comparative analysis of secondary structure across species aids in understanding the evolutionary conservation of these proteins and possible adaptations in RfBP that may be linked to species-specific functional requirements. The findings from this structural analysis contribute to a deeper understanding of how secondary structure elements influence the RfBP's role in riboflavin transport across different bird species

3 RESULTS

3.1 Analysis of *In silico* Physicochemical Properties RfBP

The analysis of the physicochemical characteristics of riboflavin-binding proteins (RfBPs) from different bird species, as shown in Table 1, highlights several variations in protein size, molecular weight, charge, and stability. The number of amino acids in the sequences varies slightly across species, with the *Whooping crane* (Grus americana) having the largest protein

sequence (263 amino acids) and a correspondingly higher molecular weight (30,457.59 Da) compared to other species, which all have around 238-240 amino acids and molecular weights ranging from 27,175.85 to 27,439.20 Da.

Isoelectric point (pI) values differ significantly among species, reflecting the protein's net charge at a given pH. *Dryobates pubescens* (Downy woodpecker) has the highest pI (8.66), indicating a more basic protein, while *Coturnix japonica* (Japanese quail) has the lowest pI (5.36), making it more acidic. This variation in pI likely reflects different environmental or physiological adaptations. In terms of charged residues, the *Whooping crane* RfBP has the highest number of negatively charged residues (36), while Dryobates pubescens has the highest number of positively charged residues (35), which may influence protein folding and function.

The extinction coefficient, which relates to the protein's capacity to absorb light, is highest in the *Whooping crane* (60,025) and lowest in *Coturnix japonica* (46,045). This suggests that the RfBP in the crane has higher light absorption properties. The instability index, an indicator of protein stability, shows that most species have relatively high values, indicating potential instability. Coturnix japonica has the highest instability index (75.55), whereas *Indicator Indicator* (Greater honeyguide bird) has the lowest (59.76), suggesting that this protein may be more stable in the latter species. Overall, these variations reflect differences in protein structure and function, likely tied to the birds' distinct evolutionary paths and environmental niches.

Table-1: Physicochemical Characteristics of Riboflavin Binding Protein Sequences

S.No.	Species Name	No. of amino acids	Molecular weight	PI	-ve charged residues	+Ve charged residues	Extinction coefficient	Instability index
1	*Coturnix japonica* (Japanese quail bird)	238	27237.46	5.36	34	23	46045	75.55
2	*Dryobates pubescens* (Downy woodpecker Bird)	238	27175.85	8.66	27	35	53035	67.42
3	*Indicator Indicator* (Greater honeyguide Bird)	238	27439.20	6.18	32	29	53035	59.76
4	*Malurus melanocephalus* (Red-backed fairywren Bird)	240	27200.86	5.62	32	27	53035	60.11
5	*Falco biarmicus* (Lanner falcon Bird)	238	27275.02	6.94	30	30	54525	65.70
6	*Grus Americana* (*Whooping crane* Bird)	263	30457.59	5.82	36	29	60025	67.38

3.2 Analysis of Amino acid composition of RfBP

The amino acid composition of riboflavin-binding proteins (RfBPs) across different bird species shows both conserved and variable features, which may reflect functional and evolutionary adaptations (Table-2). Serine (Ser) is the most abundant amino acid in all species, ranging from 11.4% in Grus americana (*Whooping crane*) to 13.3% in *Malurus melanocephalus* (Red-backed fairywren). This high serine content suggests a crucial role in the structure or function of RfBPs, possibly contributing to protein flexibility or post-translational modifications like phosphorylation.

Glutamic acid (Glu) also shows significant variation, being particularly high in *Coturnix japonica* (9.2%) and Grus americana (9.1%), indicating a potential role in protein stability through acidic interactions. Interestingly, Lysine (Lys) is highest in *Falco biarmicus* (9.7%) and *Dryobates pubescens* (9.2%), which could suggest a role in protein-protein interactions or binding due to its positive charge, critical for riboflavin transport functions.

Table-2: Amino acid composition of Riboflavin binding protein sequences

Amino acids	Number of Amino acids (in %) of different avian RfBP					
	Coturnix japonica	***Dryobates pubescens***	***Indicator Indicator***	***Malurus melanocephalus***	***Falco biarmicus***	***Grus Americana***
Ala	14(5.9)	14(5.9)	11(4.6)	14(5.8)	14(5.9)	12(4.6)
Arg	9(3.8)	13(5.5)	7(2.9)	6(2.5)	7(2.9)	6(2.3)
Asn	10(4.2)	11(4.6)	12(5)	9(3.8)	10(4.2)	13(4.9)
Asp	12(5)	10(4.2)	11(4.6)	12(5)	11(4.6)	12(4.6)
Cys	19(8)	18(7.6)	19(8)	19(7.9)	19(8)	19(7.2)
Gln	12(5)	10(4.2)	8(3.4)	11(4.6)	9(3.8)	10(3.8)
Glu	22(9.2)	17(7.1)	21(8.8)	20(8.3)	19(8)	24(9.1)
Gly	9(3.8)	11(4.6)	7(2.9)	9(3.8)	8(3.4)	8(3.0)
His	10(4.2)	3(1.3)	7(2.9)	5(2.1)	6(2.5)	10(3.8)
Ile	7(2.9)	8(3.4)	9(3.8)	9(3.8)	8(3.4)	12(4.6)
Leu	15(6.3)	13(5.5)	17(7.1)	16(6.7)	15(6.3)	20(7.6)
Lys	14(5.9)	22(9.2)	22(9.2)	21(8.8)	23(9.7)	23(3.7)
Met	9(3.8)	6(2.5)	9(3.8)	6(2.5)	8(3.4)	10(3.8)
Phe	8(3.4)	9(3.8)	7(2.9)	7(2.9)	8(3.4)	9(3.4)
Pro	8(3.4)	9(3.8)	7(2.9)	8(3.3)	9(3.8)	10(3.8)
Ser	29(12.2)	31(13)	29(12.2)	32(13.3)	29(12.2)	30(11.4)
Thr	11(4.6)	8(3.4)	11(4.6)	12(5)	10(4.2)	11(4.2)
Trp	6(2.5)	7(2.9)	7(2.9)	7(2.9)	7(2.9)	8(3)
Tyr	8(3.4)	9(3.8)	9(3.8)	9(3.8)	10(4.2)	10(3.8)
Val	6(2.5)	9(3.8)	8(3.4)	7(2.9)	8(3.4)	6(2.3)
Pyl	0(0)	0(0)	0(0)	0(0)	0(0)	0
Sec	0(0)	0(0)	0(0)	0(0)	0(0)	0

The amino acid composition of Riboflavin binding proteins (RfBP) across various avian species reveals notable similarities and differences. For instance, **Serine (Ser)** is the most abundant amino acid, with percentages ranging from 29% to 32% across species such as *Malurus melanocephalus* and *Grus Americana*. Additionally, **Glutamic acid (Glu)** also shows significant representation, particularly in *Grus Americana* (24%) and *Coturnix japonica* (22%). Conversely, amino acids like **Valine (Val)** and **Tryptophan (Trp)** are less prevalent, with values around 6% to 8%. This distribution suggests a potential evolutionary adaptation of these species to their respective ecological niches, emphasizing the importance of specific amino acids in the functional roles of RfBP.

Furthermore, the variation in amino acid percentages highlights the evolutionary divergence among these avian species. For example, *Lysine (Lys)* shows a notably higher percentage in *Falco biarmicus* (23%) compared to others, which may indicate a unique physiological requirement or adaptation in this species. In contrast, the absence of **Pyrrolysine (Pyl)** and **Selenocysteine (Sec)** across all species suggests these amino acids are not utilized in the RfBP structure, possibly due to their rarity in avian proteins. Overall, the analysis of the amino acid composition provides insights into the functional diversity and evolutionary adaptations of RfBP in different avian species.

Certain amino acids show more variability across species. Arginine (Arg), for instance, is relatively high in *Dryobates pubescens* (5.5%) but low in Grus americana (2.3%). This variability may reflect differences in protein structure and interactions, as arginine is involved in hydrogen bonding and electrostatic interactions. Additionally, the high cysteine content (7.2-8%) across all species suggests its role in disulfide bond formation, contributing to protein stability and structure. These differences across species indicate both conserved and species-specific adaptations of RfBPs.

3.3 Secondary Structural analysis of Riboflavin binding proteins

The Table-3 provides the values of the secondary structural analysis parameters for each avian species, including *Grus americana*.

The secondary structural analysis of riboflavin-binding proteins (RfBPs) across different bird species reveals notable variations in the distribution of alpha helices, extended strands, beta turns, and random coils. Alpha helices constitute the largest portion of the secondary structure in all species, ranging from 36.67% in *Malurus melanocephalu*s (Red-backed fairywren) to 41.60% in *Falco biarmicus* (Lanner falcon). *Grus americana* (Whooping crane) also exhibits a high proportion of alpha helices (40.68%), suggesting that these helices play a crucial role in maintaining the structural stability and functional integrity of RfBPs across species.

Table-3. Secondary structural analysis of Riboflavin binding proteins

Secondary Strutural analysis parameters	**Secondary structural analysis parameters values of different Avain species**					
	Coturnix japonica	***Dryobates pubescens***	***Indicator Indicator***	***Malurus melanocephalus***	***Falco biarmicus***	***Grus Americana***
Alpha helix	97 (40.76%)	92 (38.66%)	93 (39.08%)	88 (36.67%)	99 (41.60%)	107 (40.68)
3^{10} helix	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Pi helix	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Beta bridge	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Extended Strand	17 (7.4%)	21 (8.82%)	17 (7.14%)	21 (8.75%)	17 (7.14%)	21 (7.98%)
Beta turn	6 (2.52%)	8 (3.36%)	8 (3.16%)	5 (2.08%)	10 (4.20%)	5 (1.90%)
Bend region	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Random coil	118 (49.58%)	117 (49.16%)	120 (50.42%)	126 (52.50%)	112 (47.06%)	130 (49.43%)
Ambiguous state	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Other State	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)

Random coils are the second most abundant structure, comprising 47.06% to 52.50% of the proteins. Malurus melanocephalus has the highest proportion of random coils (52.50%), which might indicate increased flexibility in its RfBP structure. This abundance of random coils may contribute to the protein's ability to undergo conformational changes, potentially influencing riboflavin binding or release.

Extended strands and beta turns are less prevalent but still show some variation. Grus americana and Dryobates pubescens (Downy woodpecker) have slightly higher proportions of extended strands (7.98% and 8.82%, respectively), which may enhance the protein's ability to form stable beta sheets. Beta turns are most common in Falco biarmicus (4.20%), while other species have lower percentages, with Grus americana having only 1.90%. These differences in secondary structure composition may reflect adaptations related to species-specific functional requirements and environmental influences.

The analysis of secondary structural elements such as alpha helix, extended strand, beta turn, and random coil, revealed distinct variations in Grus americana. For instance, the protein structure of Grus americana displayed a higher percentage of alpha helices and extended strands, suggesting a more stable and rigid conformation. Additionally, it exhibited a lower percentage of beta turns and random coils, indicating a more structured protein fold. These differences in secondary structural elements may contribute to functional variations and conformational dynamics in Grus americana, highlighting the importance of further investigations to elucidate the underlying mechanisms.

Comparing the structural analysis parameters of Grus americana with other bird species reveals some unique characteristics. Grus americana exhibits a higher percentage of alpha helices and extended strands, indicating a more stable and rigid secondary structure. Additionally, it has a lower percentage of beta turns and random coils, suggesting a more defined and ordered structure. These differences in secondary structural elements may contribute to functional variations in Grus americana.

3. DISCUSSION

The results from Table-1, which outline the physicochemical properties of riboflavin-binding proteins (RfBPs) across avian species, indicate species-specific variations that likely reflect evolutionary adaptations. The molecular weights of the RfBPs range from 27,175.85 Da in *Dryobates pubescens* (Downy woodpecker) to 30,457.59 Da in *Grus americana* (Whooping crane), suggesting slight structural differences that may influence protein function. Similar patterns were observed by Chou et al., (2008), who found that variations in molecular weight among avian proteins could be linked to metabolic demands. The isoelectric points (pI) also vary, with *Dryobates pubescens* having a more basic pI (8.66) compared to the acidic nature of *Coturnix japonica* (5.36), reflecting differences in protein charge and possibly influencing protein stability and function, aligning with Pace et al. (1995) on the importance of pI in protein interactions.

The amino acid composition, as shown in Table-2, highlights notable conservation of certain residues, such as serine, which makes up 11.4% to 13.3% of the sequences in all species. This aligns with previous findings by Dubchak et al. (1995), who emphasized the significance of serine in protein flexibility and function. The high levels of glutamic acid (Glu) in *Grus americana* (9.1%) and *Coturnix japonica* (9.2%) are consistent with studies showing Glu's role in maintaining protein solubility and charge balance (Rost et al., 1995; Gujjeti et al., 2013). Moreover, variations in arginine and lysine content across species may indicate differing levels of electrostatic interactions within the protein structure, crucial for riboflavin binding, as noted in Powers (2003). These compositional differences support the idea of species-specific functional adaptations in RfBPs.

Table-3, which details the secondary structure, reveals that alpha helices dominate the RfBPs in all species, ranging from 36.67% in *Malurus melanocephalus* to 41.60% in *Falco biarmicus*. This high prevalence of helices is consistent with Madhukar et a., (2012) and Gidhamaari et al., (2012), who found that alpha helices play a key role in maintaining protein stability. The significant proportion of random coils, particularly in *Malurus melanocephalus* (52.50%), indicates structural flexibility, which could facilitate riboflavin binding and release. This mirrors findings by Guruprasad *et al.,* (1990) and Srivastava *et al.,* (2017), A Babu *et al.,* (2022) and Gujjeti et al., (2014) who noted that random coils are often involved in regions critical for protein interaction and function. Comparatively, the beta turns and extended strands are less prominent but may contribute to the overall stability and folding of the protein, further supporting their functional roles in maintaining riboflavin transport across avian species.

4. CONCLUSION

The *in silico* characterization of riboflavin-binding proteins (RfBPs) across avian species reveals significant variations in physicochemical properties, amino acid composition, and secondary structures, indicating species-specific adaptations. The differences in molecular weight, isoelectric points, and charged residues suggest functional variations in protein stability and riboflavin transport. Amino acid composition, particularly the high conservation of serine and glutamic acid, highlights critical roles in protein flexibility and solubility, supporting evolutionary and metabolic needs. Secondary structure analysis shows the dominance of alpha helices and random coils, reflecting structural stability and flexibility, respectively, essential for riboflavin binding. These findings align with previous studies, emphasizing the adaptive significance of RfBPs in avian species. The comparative analysis provides valuable insights into the functional and structural diversity of RfBPs, enhancing our understanding of avian physiology and the evolutionary pressures shaping these proteins. Future studies could further explore how these variations impact species-specific ecological and dietary requirements.

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