Homology Modeling of Ring Necked Pheasant (*Phasianus colchicus*, Galliformes) Major Hemoglobin Component (HbA)

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Summary: Birds have a specialized system of respiration. Their hemoglobin has been adapted to a vast variety of environmental conditions. To understand their specialized mechanism of respiration we need to understand the three dimensional (3D) structure of its hemoglobin. In this study, we have built a three dimensional homology model of hemoglobin A (HbA) from *Phasianus colchicus* using the 3D structure coordinates of *Meleagris gallopavo* (common turkey) HbA as template. The model was built with the help of MODELLER 9v8 and was evaluated with ProSA and PROCHECK. The present study is about the analysis of effects of intersubunit contacts on oxygen affinity of the hemoglobin. The effect of the following pairs of intersubunit contacts on the oxygen affinity of the hemoglobin have been studied *i.e.* $\alpha^A 99$ with $\beta 101$, $\alpha^A 34$ with $\beta 125$, $\alpha^A 38$ with $\beta 99$ and $\beta 97$, and $\beta 55$ with $\alpha^A 119$. Due to the larger distances between these pairs of residues hydrogen bond is absent between them as has been predicted by the homology model. It has been predicted that the absence of hydrogen bonds between the above mentioned residues might have increased the oxygen affinity of the HbA of *P. colchicus* except the first pair of residues ($\alpha^A 99$ with $\beta 101$) which has been suggested to have lowered the oxygen affinity of the hemoglobin. This study also involves the analysis of the evolutionary relationship of Galliformes with thirteen selected orders of Aves.

Introduction

The transition from anaerobic to aerobic life was a major step in the evolution because it uncovered a rich reservoir of energy. Many folds higher amount of energy is extracted from glucose in the presence of oxygen than in its absence [1]. Vertebrates have evolved two principal mechanisms for supplying their cells with a continuous flow of oxygen. One is the circulatory system and the other is the oxygen carrier molecules. The oxygen carriers in vertebrates is a protein called hemoglobin.

Among vertebrates, birds have acquired the ability to survive under hypoxic conditions either during migration to long distances at very high altitude or during deep see diving [2]. Some work has already been done on the mechanism of respiration of birds [3]. Our study is a continuation of the previous studies. We have made the homology model and have analyzed the three dimensional (3D) structure of HbA of *P. colchicus* (common pheasant). The analysis also includes comparative study of HbA molecules with other avian species HbAs as well as evolutionary status of the Galliformes.

Phasianus colchicus is one of the Galliformes which is a large and diverse group comprising more than 70 genera and more than 250 species also called as gallinaceous birds or game birds [4]. It is commonly found in agriculture farmlands with crops of wheat, corn, oats or in grasslands mixed with small woodlands [5].

Results and Discussion

Homology Model Evaluation

A homology model of the Ring Necked pheasant's HbA (Fig. 1) has been calculated using coordinates of crystal structure of turkey's HbA component [6]. The model has general all alpha topology with no beta strands (Fig. 1) just like all other hemoglobins.
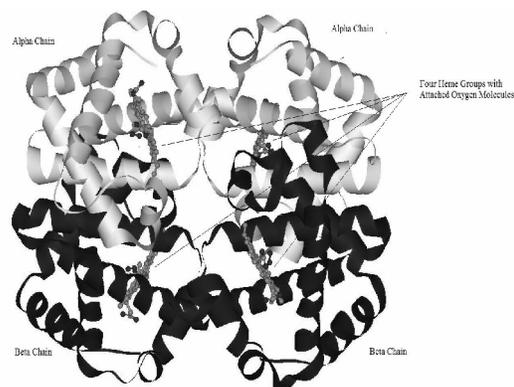

Fig. 1: Schematic representation of the predicted homology model of Pheasant's HbA. Heme is depicted in ball and stick representation whereas globin chains are shown as flat ribbons (Alpha chains: Gray and Beta chains: Black).

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There is a difference of five amino acids in the two hemoglobin's α^A chains, while the beta chains of both the species are identical. There is high enough sequence similarity between the target and template; hence the model will also be highly similar to the template.

Analysis of the model of pheasant shows that 93% residues are in the most favored region, 6.4% in additionally allowed region, and 0.2% in generously allowed region while only 0.4 % residues were found in disallowed region on the Ramachandran plot as evaluated by PROCHECK (Fig. 2). The Fig. 2 shows that only two amino acids *i.e.* Ala18 of chain A and C are in the disallowed region which is due to the presence of two amino acids of the crystal structure of turkey HbA (Fig. 3) because it has been used as a template to create the model. The residues in disallowed region are due to the presence of 0.4% residues in disallowed region in the turkey's HbA's crystal coordinates taken as template. Energy plots of both the chains were below zero just like corresponding template chains' energy plots calculated with the help of ProSA (Fig. 4). The energy values of both the chains are quite similar to the corresponding chains of the template. The low value of the energy of the model and its similarity with the template energy values show the correctness of the model
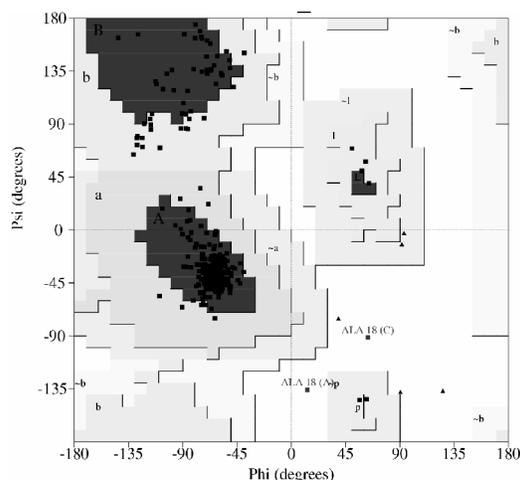
value shows that the model has been built accurately and that the model is almost identical to the template.
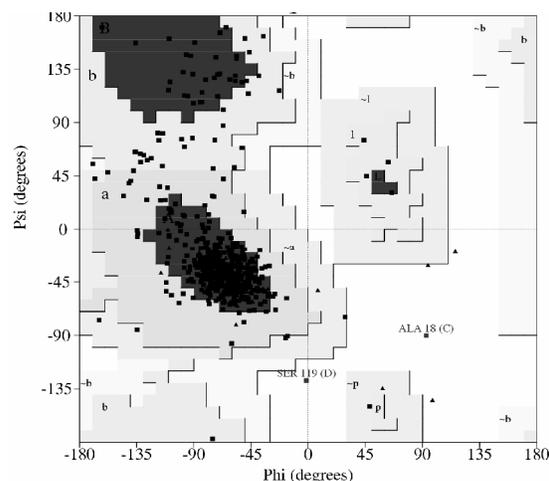

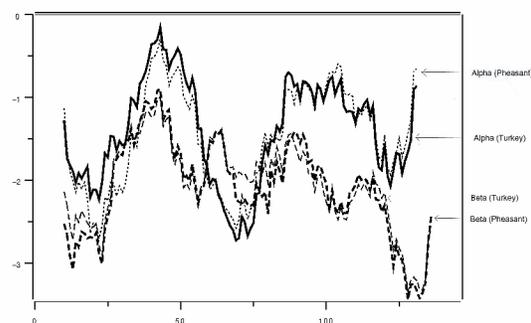Fig. 3:   Ramachandran plot of crystal coordinates of Turkey's HbA.

Fig. 4:   ProSA energy plots of α^A and β chains of both Turkey and Pheasant's HbAs.

Fig. 2:   Ramachandran plot of Pheasant's HbA homology model.

Superposition of Cα-backbone of pheasant's homology model was carried out with the template crystal coordinates with the help of DS Visualizer® (v. 2, Accelrys Software Inc). The backbone superposed well over one another with an rmsd (root mean square deviation) of 0.23 due to high sequence similarity between the two HbAs (Fig. 5). This low
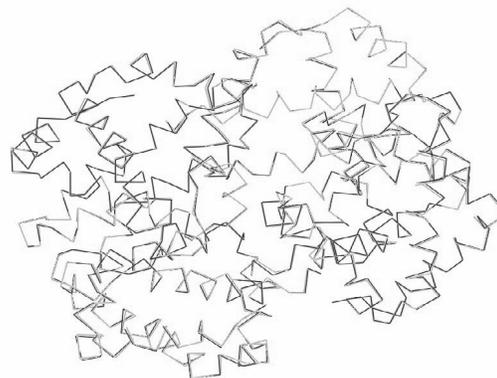

Fig. 5:   Superposition of the Cα backbone of Pheasant's HbA homology model (gray) over Turkey's HbA crystal coordinates (black). Both the structures have been represented in carbon alpha wire form.

Inter-subunit Contacts and Their Effect on Oxygen Affinity

The functional characteristics of hemoglobin are derived from subunit contacts *i.e.* $\alpha^A_1\beta_1$ and $\alpha^A_1\beta_2$ as well as its interaction with effector molecules like Cl, CO2 and organic phosphates [7].

In Tufted duck's HbA, the R (Relaxed) structure is stabilized by a new salt bridge between α^A99Arg and $\beta101$Glu and helps in increasing the oxygen affinity of the hemoglobin [3]. In pheasant's HbA at $\alpha^A_1\beta_1$ contact site α^A99Arg is replaced with α^A99Lys. Due to smaller size of Lys, it is unable to make a salt bridge with $\beta101$Glu as is clear from the distance in between them (Fig. 6). Salt bridge is no possible for this distance. This loss of salt bridge might lower the oxygen affinity of pheasant's HbA as compared to Tufted duck's HbA.
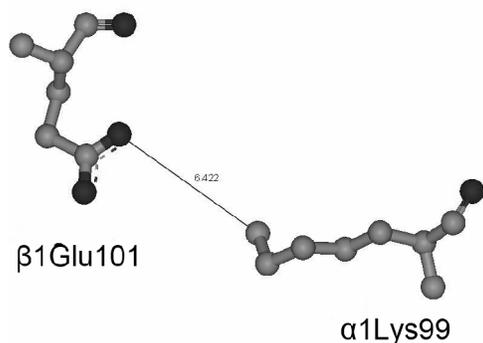

Fig. 6:   The distance between α_1Lys99 and β_1Glu10. The residues have been represented with ball and stick model and the distance has been shown with a black line.

Most birds, including Bar Headed goose, posses Thr at position α^A34 ($\alpha_1\beta_1$ contact site). This residue is involved in forming a hydrogen bond with $\beta125$ stabilizing the T (Tense) structure and hence lowering the oxygen affinity [8]. This hydrogen bond is lost in Tufted duck's HbA because it has been replaced with Ile at α^A34 position [3]. This loss of hydrogen bond stabilizes the R structure and hence increases the oxygen affinity of Tufted duck's HbA. Just like tufted duck's HbA, pheasant's HbA posses Ile at $\alpha34$, which cannot make a hydrogen bond with $\beta125$Glu as is clear from the large distance between them (Fig. 7). Loss of this bond can stabilize the R structure of pheasant's HbA which might raise the oxygen affinity of its hemoglobin.

Anseriformes and some other species posses Gln at position $\alpha38$ [9], which stabilizes the oxy

structure of hemoglobin [10] by making two hydrogen bonds with $\beta99$ and $\beta97$. Tufted duck's HbA posses higher oxygen affinity due to these two hydrogen bonds [3]. In pheasant's HbA, Gln is substituted by Ser at this position, which, due to its smaller size and orientation (larger distance between them), is unable to make hydrogen bonds with $\beta99$Asp and $\beta97$His (Fig. 8). This can lower the oxygen affinity of pheasant's HbA.
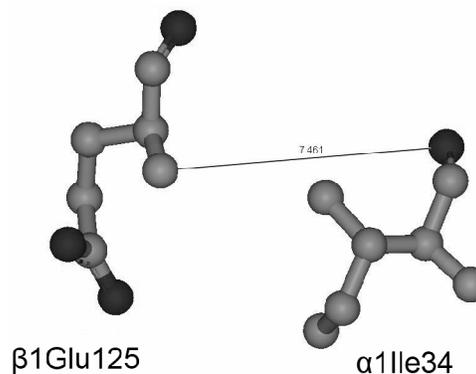

Fig. 7:   The distance between α_1Ile34 and β_1Glu125. The residues have been represented with ball and stick model and the distance has been shown with a black line.

In Human HbA $\beta55$Met is involved in van der Waals interactions with α^APro119, however this type of interaction is not possible in Bar Headed goose and Andean goose because this pair of residues is mutated to smaller residues, *i.e.* $\beta55$Leu and α^A119Ala in Bar Headed goose, and $\beta55$Ser and α^A119Pro in Andean goose [11, 12]. Due to the absence of this contact in Bar Headed goose and Andean goose HbA, T structure becomes unstable increasing the oxygen affinity [3]. In pheasant, Leu is present at position $\beta55$ which due to its smaller size cannot make van der Waals interactions with α^A119Pro due to larger distance (Fig. 9). Loss of these interactions in pheasant's HbA might destabilize the T structure which can possibly increase the oxygen affinity.

Thus it is predicted from the above studies that pheasant's HbA might be able to tolerate hypoxic conditions. However in our previous paper [13], we have analyzed that the HbD component of pheasant's hemoglobin has lower oxygen affinity as compared to high altitude birds. But due to high oxygen affinity of its HbA component it can be predicted that pheasant can survive at higher altitudes.
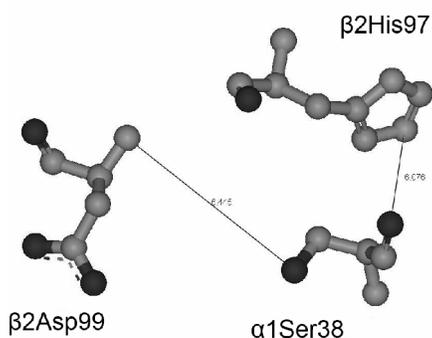
Fig. 8:   The distance between α_1Ser38 and β_2His97, and between α_1Ser38 and β_2Asp99 have been shown in Å. The residues are represented with ball and stick model and the distance has been shown with a black line.
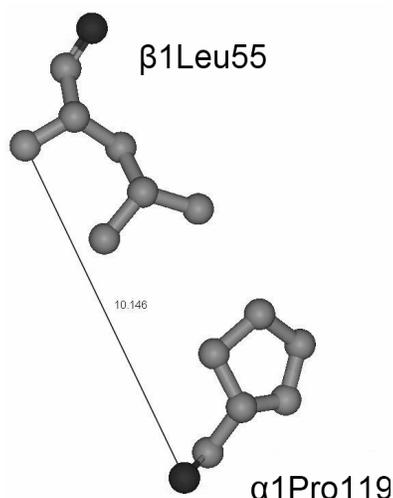

Fig. 9:   The distance between α_1Pro119 and β_1Leu55 has been represented in Å. The residues are represented with ball and stick model and the distance has been shown with a black line.

Phylogenetic Studies

The evolutionary patterns of both α^A and β chains of pheasant were studied using the phylogenetic trees (Fig. 10, Fig. 11). Phylogenetic analysis of both the chains show that all the Galliformes species have been evolved from a single ancestor as it is clear from the single cluster of this order. Order Sphenesciformes seem to be the closest relatives of Galliformes among the orders included in the trees for both the chains. Next closest relatives are Anseriformes and Struthioniformes. The trees for both the chains show that both Passeriformes and

Falconiformes HbAs are more distantly related to the Galliformes.
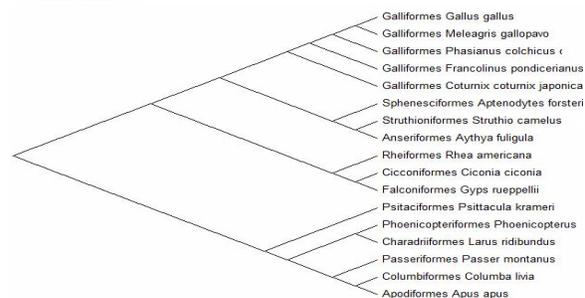

Fig. 10: Phylogenetic lineage of α^A Chain of Avian hemoglobin of species representing thirteen different orders.
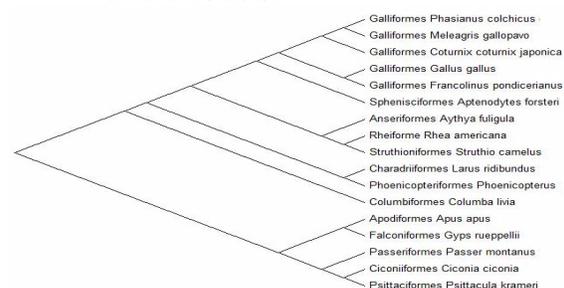

Fig. 11: Phylogenetic lineage of β Chain of Avian hemoglobin from thirteen different orders.

The study of the trees indicates that pheasant is more closely related to Common turkey as compared to chicken. It is also clear from the sequence similarity of their β chains. As their β chains are 100 % identical therefore we infer that these two species might have separated during evolution more recently as compared to chicken.

Methodology

Primary Sequence Analysis

The sequences of α^A and β chains of pheasant's HbA [14] were retrieved from SwissProt Database [15]. To perform similarity searches and template selection BLAST [16, 17] was used. Turkey (*Meleagris gallopavo*) hemoglobin A (PDB ID: 2qmb) was selected as the template because of its highest homology with the target sequence (Fig. 12, 13). The α^A chain of pheasant's HbA shows 96 % identity with α^A chain of turkey's HbA and the β chain of the pheasant is 100 % identical with the β chain of purkey's HbA. The 3D structure coordinates of turkey's HbA were obtained from Brookhaven Protein Databank (PDB) [18]. Alignment was performed with CLUSTAL-X [19].

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                    ********************;********.;***************************.******;***
Pheasant_Alpha_A    VLSAADKNNVKGIFTKIAGHAPEYGAEALERMFITYPSTKTYFPHFDLSHGSAQIKGHGKKVVAALIEAVNHIDDITGTL   80
Turkey_Alpha_A      VLSAADKNNVKGIFTKIAGHAPEYGAETLERMFITYPPTKTYFPHFDLSHGSAQTKGHGKKVVAALIEAANHIDDIAGTL   80
                    1........10.........20.........30.........40.........50.........60.........70.........80


                    **********************************************;************************
Pheasant_Alpha_A    SKLSDLHAHKLRVDPVNFKLLGQCFLVVVAIHHPSALTPEVHASLDKFLCAVGTVLTAKYR   141
Turkey_Alpha_A      SKLSDLHAHKLRVDPVNFKLLGQCFLVVVAIHHPAALTPEVHASLDKFLCAVGTVLTAKYR   141
                    .........90........100.......110.......120.......130.......140.
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Fig. 12: Pairwise sequence alignment of alpha chain of Pheasant with alpha chain of Turkey. Stars above the sequences show identical residues while dot show mismatches.

Model Building and Evaluation

MODELLER 9v8 [20] was used to build the homology models using turkey's HbA as template. Stereochemistry of the models was evaluated by PROCHECK [21]. The energy graphs were calculated with the help of ProSA [22]. The best model (Fig. 13) was selected on the basis of PROCHECK and ProSA results.

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                    ****************************************************************************
Pheasant_HbA_Beta   VHWSAEEKQLITGLWGKVNVADCGAEALARLLIVYPWTQRFFASFGNLSSPTAILGNPMVRAHGKKVLTSFGDAVKNLDN   80
Turkey_HbA_Beta     VHWSAEEKQLITGLWGKVNVADCGAEALARLLIVYPWTQRFFASFGNLSSPTAILGNPMVRAHGKKVLTSFGDAVKNLDN   80
                    1........10.........20.........30.........40.........50.........60.........70.........80


                    ***********************************************************************
Pheasant_HbA_Beta   IKNTFSQLSELHCDKLHVDPENFRLLGDILIIVLAAHFSKDFTPECQAAWQKLVRVVAHALARKYH   146
Turkey_HbA_Beta     IKNTFSQLSELHCDKLHVDPENFRLLGDILIIVLAAHFSKDFTPECQAAWQKLVRVVAHALARKYH   146
                    .........90........100.......110.......120.......130.......140......
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Fig. 13: Pairwise sequence alignment of beta chain of Pheasant with beta chain of Turkey.

To analyze the intersubunit contacts LigPlot [23] was used. The hydrogen bonds between $\alpha^A_1\beta_1$ and $\alpha^A_1\beta_2$ were analyzed with "Protein interfaces, surfaces and assemblies service PISA at European Bioinformatics Institute (http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html), authored by E. Krissinel and K. Henrick [24].

In order to examine any alteration in the Cα backbone of pheasant's HbA, it was superposed onto turkey's HbA molecule with the help of MODELLER 9v8 and rmsd values were calculated. All protein structures and models were visualized and analyzed with the help of DS Visualizer® (v. 2, Accelrys Software Inc).

Phylogenetic Analysis

Program MEGA4 [25, 26] was used for the calculation of tree and phylogenetic analysis. Neighbour joining trees were calculated for α^A and β chains of HbAs of the following species selected from thirteen different orders; Galliformes (*Gallus gallus*, *Meleagris gallopavo*, *Phasianus colchicus*, *Francolinus pondicerianus*, *Coturnix japonica*), Sphenesciformes (*Aptenodytes forsteri*),

Struthioniformes (*Struthio camelus*), Anseriformes (*Aythya fuligula*), Rheiformes (*Rhea americana*), Cicconiformes (*Cicconia ciccnia*), Falconiformes (*Gyps rueppellii*), Psitasciformes (*Psitaccula krameri*), Phoenicopteriformes (*Phoenicopterus rubber*), Charadriiformes (*Larus ridibundus*), Passeriformes (*Passer montanus*), Columbiformes (*Columba livia*) and Apodiformes (*Apus apus*).

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