

**MOLECULAR MECHANISM OF RESPIRATION IN HIGH ALTITUDE BIRDS:
PRIMARY STRUCTURE AND HOMOLGY MODELING OF HEMOGLOBIN
COMPONENTS FROM COMMON CRANE (*Grus grus*; GRUIFORMES)**



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THESIS

Submitted To

**THE FACULTY OF LIFE & ENVIRONMENTAL SCIENCES
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*In partial fulfillment for the degree
of*

DOCTOR OF PHILOSOPHY

by

ZAHID KHAN

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ZAHID KHAN

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Thesis submitted by

Zahid khan

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SUPERVISOR:

Dr. Ghosia Lutfullah

Professor,

Center of Biotechnology & Microbiology,
University of Peshawar.

Signature: G. Lutfullah

Date: 4.8.2011

CO-SUPERVISOR:

Dr Atiya Abbasi

Professor

HEJ Research Institute of Chemistry,
International Center for Chemistry and Biological sciences
University of Karachi

Signature: _____

Date: _____

EXTERNAL EXERMINAR:

Dr Absar Alum

Assistant Professor Research

Department of Civil, Environmental and Sustainable Engineering

Ira A. Fulton School of Engineering

Arizona State University, Arizona, USA

Signature: Absar Alum

Date: _____

Director:

Dr Bashir Ahmad

Director

Centre of Biotechnology and Microbiology
University of Peshawar

Signature: Bashir Ahmad

Date: _____

In the name of Allah, the Most Gracious, the Most Merciful

Dedicated To My Most Beloved

Parents, Brother, Sisters

and

Centre of Biotechnology & Microbiology,

University of Peshawar

In the name of Allah, the Beneficent, the Merciful



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ABSTRACT

The amino acid sequence of α^A and β -chains from the hemoglobin of Common crane (*Grus grus*) has been determined. Common Crane belongs to the avian order *Gruiformes*, family *Gruidae*. The *gruidae* is a small family with few species most of which are endangered. The common crane is a migratory bird with long migratory route. It migrates to Pakistan during winter from Western Siberia.

Separation of the hemoglobin polypeptide subunits was achieved by RP-HPLC. The globin chains were digested by TCPK treated trypsin. The primary structure of the hemoglobin was established by sequence determination of the tryptic peptides by automatic Edman degradation on a gas-phase sequencer.

The hemoglobin of Common Crane is heterogeneous, having two hemoglobin components, like all many other avian representatives. Position $\alpha 34$ Cys and $\beta 44$ Ala are unique amino acids present in common crane hemoglobin. Position, $\alpha 8$ Ser, $\alpha 55$ Val, $\alpha 64$ Gly, $\alpha 67$ Val and $\beta 13$ Ser, $\beta 43$ Ser, $\beta 55$ Ile and $\beta 119$ Ala are specific to common crane and are present in very few other avian hemoglobins sequenced so far. Homologous comparison with the other avian species showed much conservation in the beta chain as compared to alpha A chain. The automated homology model building was performed using the protein structure modelling package, MODELLER 9v7. The three dimensional models of HbA oxy and deoxy were calculated using crystal structure coordinates of Bar-headed goose [PDB id 1A4F (oxy), 1HV4 (deoxy) hemoglobin. The various exchanges at position critical for structure and function of the hemoglobin are discussed.

LIST OF ABBREVIATIONS

Word	Abbreviation
Adult hemoglobin	HbA
Anseriformes	ANS
Helices B, G and H	BGH
Charadriiformes	CHA
Ciconiiformes	CIC
Columbiformes	CLM
Common crane	CC
Developmental hemoglobin	HbD
Dithiothreitol	DTT
Falconiiformes	FAL
Hemoglobin	Hb
Inositol pentaphosphate	IPP,IP5
N,N,N',N'-tetramethyl-ethylene diamine	TEMED
Oxygen dissociation curve	ODC
Partial pressure of carbon dioxide	pCO ₂
Partial pressure of oxygen	p50
Passeriformes	PAS
Pelecaniiformes	PEL
Phoenicopteriformes	PHO
Relative centrifugal force	RCF
Reserved phase high performance liquid chromatography	RP-HPLC
Rheiformes	RHE
Struthioniformes	STR
Tos-Phe-CH ₂ Cl: (n-tosyl-L-phenylalanyl) chloromethane	TPCK
Trifluoroacetic acid	TFA
Tris(hydroxymethyl) amino methane	Tris

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INTRODUCTION

1.1 GENERAL DESCRIPTION

The word "introduction" is used in two main senses, the philosophical and the literary. The philosophical sense is the one in which the word is used in this book. It is the sense in which the word is used in the title of this book. The literary sense is the one in which the word is used in the title of this book. It is the sense in which the word is used in the title of this book. The philosophical sense is the one in which the word is used in this book. It is the sense in which the word is used in the title of this book. The literary sense is the one in which the word is used in the title of this book. It is the sense in which the word is used in the title of this book.

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1.2 SOME PROBLEMS

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INTRODUCTION

1.1 GENERAL DESCRIPTION

The word respiration is used in two terms generally, the physiological and cellular respiration. The physiological respiration means the transport of oxygen from lungs or skin to different tissues. The cellular respiration means the oxidation of glucose to carbon dioxide and water with the net production of ATPs. Cellular respiration takes place in individual cells while the physiological respiration is the flow and transport of gases and metabolites between the organism and its external environment [1]. In unicellular organisms the exchange of gases with environment takes place by simple diffusion. In contrast, the inner cells of the complex organism like vertebrates etc have greater distance from the external environment and therefore the gas exchange with environment and the inner cells is not possible by simple diffusion. Thus higher organisms need a respiratory system for gases exchange with the environment. In higher organisms the transport of gases is facilitated by a variety of proteins almost all of these proteins are heme proteins.

1.2 HEME PROTEINS

Heme proteins or hemo proteins are metallo-proteins containing a heme prosthetic group. Hemoglobin is part of the large globin family of heme-containing proteins that are involved in the binding and/or transport of oxygen. These proteins are widely distributed in many organisms and have diverse physiological functions including the transport of respiratory gases (oxygen, carbon dioxide). Examples of some globin proteins found in this family are:

1. Myoglobin (used as a reserve supply of oxygen, and facilitates the movement of oxygen within muscles) [2].
2. Neuroglobin (related to oxygen transport in brain) [3].
3. Cytochrome (involved in intracellular oxygen storage or transfer) [4].
4. Leghaemoglobin (provides oxygen to bacteroids, which is essential for symbiotic nitrogen fixation) [5].
5. Erythrocrurin (giant haemoglobin of worms) [6].
6. Flavohaemoglobin (involved in nitric oxide (NO) detoxification) [7].

The heme proteins share a common alpha helical globin structure. With the exception of some deep sea fishes almost all other vertebrates use hemoglobin for the transport of oxygen and carbon dioxide between lungs and tissues. Invertebrates use another protein, erythrocrurin, for oxygen supply [6].

1.3 HEMOGLOBIN

Hemoglobin is a dioxygen transport protein. It is universally present in higher vertebrates as a tetrameric protein, and is found in some bacteria and plants as monomer. Hemoglobin is found in the red blood cells of the body. Each red blood cell (RBC) contains approximately 280 million hemoglobin molecules [8]. The main function of hemoglobin is to transport oxygen from the lungs to the tissues and then transport CO₂ back from the tissues to the lungs. One hemoglobin molecule has the ability to transport up to 4 oxygen molecules. There are two forms of hemoglobin: oxyhemoglobin, which is saturated with oxygen molecules and deoxyhemoglobin, which is desaturated with oxygen molecules [8]. Oxyhemoglobin has a higher affinity for oxygen than deoxyhemoglobin, and deoxyhemoglobin has a higher affinity for CO₂ than oxyhemoglobin. Therefore, oxygen binds to oxyhemoglobin in the lungs and is then

transported through the blood stream until it reaches the tissues. There, the oxygen is released to myoglobin, which then transports it to the mitochondria where it is used for aerobic respiration. In exchange, the deoxyhemoglobin picks up 2 protons and 2 molecules of CO_2 and returns to the lungs, where the CO_2 is released through exhalation [9].

1.3.1 Structure of Hemoglobin

Hemoglobin is a globular protein with a molecular weight around 64.5 KDalton. Hemoglobin is tetrameric molecule consisting of four polypeptide chains [Fig.1.1], two alpha chains each with 141 amino acids and two beta chain each with 146 amino acids [9]. Both alpha and beta chains have different sequences but fold into a very similar structure with only alpha helices and beta sheets. Each polypeptide chain fold into eight helical segments named as A to H. The non-helical segments at the N and C terminals are named as NA (amino terminal of helix A) and HC (corboxy terminal of helix H) respectively [10]. The non-helical segment between two helical segments is named by combining the names of the two helices on both sides of the non helical segment for example; a non-helical segment between helices B and C will be named as BC. The folding of each chain forms a cavity where it holds a prosthetic group, the heme, between E and F helices. A heme group is a macrocycle molecule containing carbon, nitrogen and hydrogen atoms, with a single Fe^{2+} ion at the center. Without the iron atom the heme is called as porphyrin. The iron atom in the heme molecule is bounded by four nitrogen atoms of the porphyrin ring making a nearly flat plane. The heme is covalently bound to a histidine side chain of the helix F making the fifth coordination bond of the iron in heme. The residue making the fifth coordination bond is His87 in the human alpha chain

and 92 in the human beta chain. The sixth coordination position of the heme iron is for the reversible binding of oxygen [11].

A variety of interactions stabilize the hemoglobin tertiary structure including hydrophobic, ionic interaction called salt bridges, hydrogen bonding and Van der Waals interactions. The non-polar amino acid residues are oriented towards the interior of the molecule making a hydrophobic cavity while the hydrophilic amino acids are located on the surface of the molecules making contact with surrounding water molecules [1].

The arrangement of the hemoglobin subunits in the quaternary structure results in four types interactions between the subunits, $\alpha_1\beta_1=\alpha_2\beta_2$, $\alpha_1\beta_2=\alpha_2\beta_1$, $\alpha_1\alpha_2$ and $\beta_1\beta_2$ interactions. There is very limited interaction at the $\alpha_1\alpha_2$ and $\beta_1\beta_2$ interfaces. The interactions at the $\alpha_1\beta_1$ and $\alpha_2\beta_2$ interfaces are mostly constant and do not change during transition from deoxy to oxy structure. While the interaction at the $\alpha_1\beta_2$ and $\alpha_2\beta_1$ interfaces drastically changes during the oxy deoxy transitions. Thus the hemoglobin tetramer is in fact a dimer of $\alpha\beta$ dimers. During the transition from oxy to deoxy structure the $\alpha_1\beta_1$ dimer rotates relative to $\alpha_2\beta_2$ by 15° and a 1 Å relative shift [12]. These changes in interaction, on oxygenation and deoxygenating, result in different types of hemoglobin structures. Monod, Wyman, and Changeux proposed the two energetic state of hemoglobin as T state and R state. The fully oxygenated state is called R (R for relaxed) state while the deoxygenated state is called T state (T for tense) [13]. The two states differ in the number and energy of interaction between the subunits. The two states of hemoglobin also have different affinity for ligands.

The interaction between the subunits in the T state, keeps the hemoglobin constrained and opposes the structural changes resulting from ligand binding (oxygen), therefore the T state hemoglobin has low affinity for oxygen. In the R state these constraints are

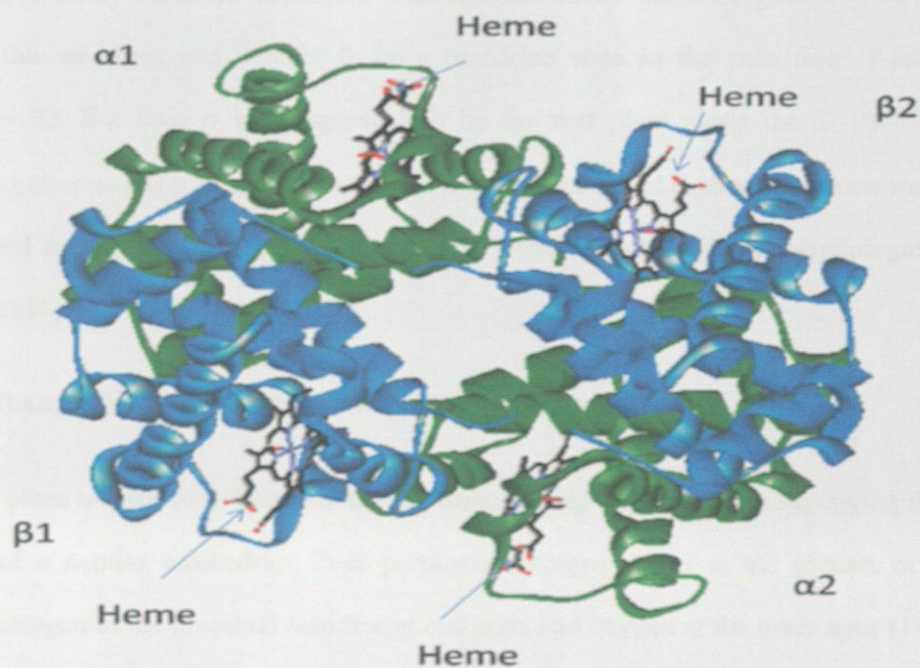


Fig.1.1 Hemoglobin molecule, a tetramer having two alpha and two beta chains: Each chain has a heme group. Image generated by Discovery Studio visualizer using PDB structure coordinates of human deoxy hemoglobin PDB ID: 2DN2.

released and hence the molecule has greater affinity for oxygen. These two states of hemoglobin are in rapid equilibrium. Under controlled conditions the hemoglobin can be crystallized in T or R state [14].

A third quaternary structure called R2 was crystallized by different groups [15-17]. Initially this structure was thought to be a transition state in the path from T-to-R ($T \rightarrow R2 \rightarrow R$). But later it was suggested to be the end point along the $T \rightarrow R \rightarrow R2$ transition pathway and is fully oxygenated [16]. Unlike the T and R structure which were crystallized under high salt condition, R2 was crystallized under almost physiological condition [18].

1.3.2 Changes in the Heme Plane During R-T Transitions

The iron atom is hexa coordinated in the oxyhemoglobin, with six atoms occupying the corners of a regular octahedron. Four porphyrin nitrogen atoms at the corners of a square, nitrogen of the proximal histidine at one apex and oxygen at the other apex [19]. In the oxyhemoglobin the iron atom remain in the heme plane due to the presence of one coordination site on each side of the heme plane, oxygen molecule on one side and the nitrogen atom of Histidine F8 helix on the other side [Fig.1.2]. In the deoxy hemoglobin the oxygen atom on one side of the heme plane is not present and hence the iron atom is penta coordinated. The electrons of the five ligands enter five separate d orbital forming a high spin complex. The electron density of the porphyrin ring and the histidine residue of F8 helix repel each other and push the iron atom out of the heme plane towards the histidine F8. The resulting heme is called dome shaped [Fig.1.3].

In the dome shaped heme the iron porphyrin bonds are longer while the histidine iron bond is shorter than in the planner heme. When oxygen atom binds the iron atom on the

side opposite to the histidine binding site, the iron atom is pulled back into the heme plane. This doming and undoming of the heme during oxygenation and deoxygenation is called as trigger mechanism [20].

1.3.3 Changes in the Alpha Beta Interface Interactions During R, T Transitions

The deoxy-T and oxy-R structure have the same $\alpha 1\beta 1$ and $\alpha 2\beta 2$ interfaces. The two states have different interaction at the $\alpha 1\beta 2$ and $\alpha 2\beta 1$ interfaces between the dimers [21, 22].

During the transition from T to R the $\alpha 1\beta 1$ dimer rotates by $\sim 15^\circ$ relative to the $\alpha 2\beta 2$ dimer. This rotation causes some atoms to shift on the $\alpha 1\beta 2$ and $\alpha 2\beta 1$ interface. Some of the atoms on the interface are shifted by as much as 6 Å. The sliding residues on the $\alpha 1\beta 2$ interface are on C helix and FG corner. The residue His (97) β on the FG corner fits into the quaternary structure interlocking between residues ThrC6(41) α and Pro CD2 (44) α in the deoxy state. On oxygenation the His (97) β shifts and fits between ThrC3(38) α and ThrC6(41) α [23]. These two conformations of the oxy and deoxy hemoglobin state form a ball and socket around which the dimer turns. These contacts, thus act as a switch which permits only two stable quaternary structures, i.e. R or T [24]. A network of intra- and inter subunit linkages is present at the amino and carboxy termini of α - and β -chains in the deoxy-T structure; these linkages break on ligand binding [22].

When an oxygen molecule binds the iron atom in the heme it slightly shifts the F8 helix. This shift is translated to the FG corner at the $\alpha 1\beta 2$ interface which causes a dimer rotation and disrupts the hydrogen bonds salt bridges that stabilizes the T state [25].

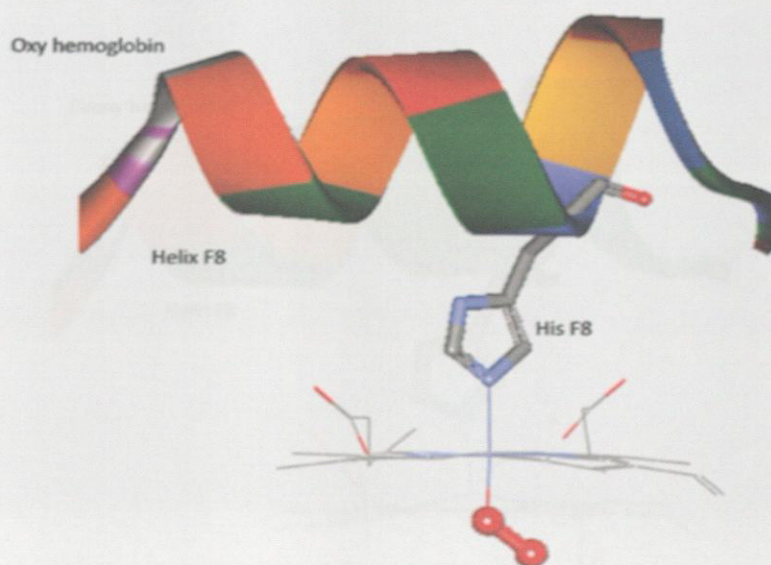


Fig. 1.2 Planner heme structure in the oxygenated hemoglobin: The bonding forces on both sides of the heme plane balance each other and hence the heme is planner shape. The image was generated by discovery studio visualizer.

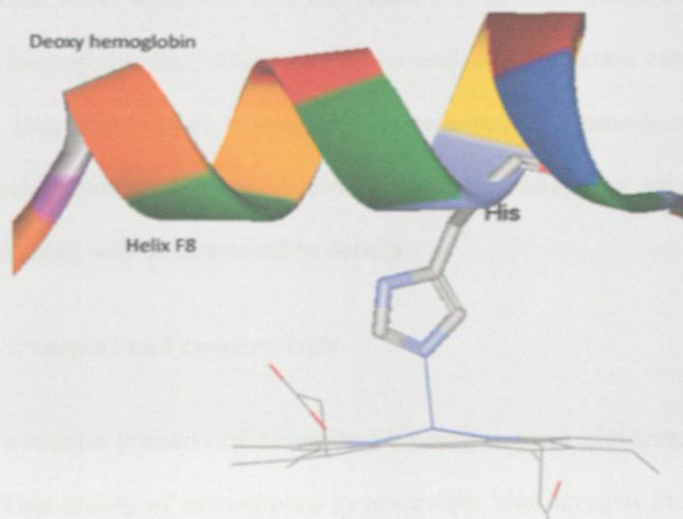


Fig.1.3 Heme plane in deoxyhemoglobin: The heme iron is pulled towards the histidine residue of the F8 helix making the heme adopt a dome shape. The image was generated by discovery studio visualizer.

1.3.4 Functions of Hemoglobin

The main function of hemoglobin is to transport oxygen from lungs to tissues and carbon dioxide from tissue to lungs. This ability of hemoglobin to bind these gases reversibly is regulated by different internal and external factors. Internal factor are within in the hemoglobin molecule itself while the external factor are various chemicals. Beside the transport of gases hemoglobin has other function as well, the important other function is nitrite reductase. This function of hemoglobin is required for generating NO which dilates blood vessels under hypoxic conditions [26]. In the following paragraphs these functions of hemoglobin will be discussed in details.

1.3.4.1 Oxygen transport and cooperativity

Hemoglobin has a unique property of reversibly binding oxygen and transporting from lungs to tissues. This ability of hemoglobin to reversibly bind oxygen in the lungs and unload it in the tissues is regulated by several factors. The biggest regulator of the hemoglobin oxygen affinity is oxygen molecule itself. Hemoglobin has high affinity for oxygen in the lungs, where the oxygen levels are high, and this oxygen affinity further increase after binding of first oxygen molecule to hemoglobin [27]. When oxyhemoglobin binds one oxygen molecule its affinity for oxygen increases until it binds four oxygen molecules. Similarly the deoxyhemoglobin has lower affinity for oxygen and lower affinity further decreases as the hemoglobin loses oxygen molecules i.e. the release of first oxygen molecule from hemoglobin lowers the hemoglobin affinity for the remaining oxygen molecules. Thus the binding of oxygen to hemoglobin facilitates the binding of other oxygen molecules to fully oxygenate hemoglobin while the release of one oxygen molecule from the fully oxygenated hemoglobin facilitates the release of the remaining oxygen molecule to fully unload oxygen at tissues level. This regulation is

known as cooperativity and is essential to the functioning of the hemoglobin because it allows the oxyhemoglobin to carry the maximum amount of oxygen to the tissues and then allows the deoxyhemoglobin to release the maximum amount of oxygen into the tissues [27]. This cooperativity of hemoglobin molecule is due to its unique structural characteristics, and this cooperativity effect is lost if the hemoglobin tetramer is split into dimers [9, 28].

The binding of oxygen to hemoglobin is measured in terms of oxygen equilibrium curve or oxygen dissociation curve (ODC). The oxygen dissociation curve is a graph that shows the percent saturation of hemoglobin at various partial pressures of oxygen. The fractional saturation i.e. the fraction occupying the oxygen-binding site is measured as a function of the partial pressure of oxygen [29]. The shape of the equilibrium curve for hemoglobin is sigmoidal which indicates that the oxygen affinity of hemoglobin increases with oxygen uptake [Fig.1.4]. This sigmoidal shape of the equilibrium curve is because the hemoglobin cooperativity. The importance of cooperative behavior is that it allows hemoglobin to be more efficient in transporting oxygen. For example, in the lungs, the hemoglobin is at a saturation level of 98%. However, when hemoglobin is present in the tissues and releases oxygen, the saturation level drops to 32%; thus, 66% of the potential oxygen-binding sites are involved in the transportation of oxygen. The oxygen affinity is characterized quantitatively as P_{50} i.e. the partial pressure of oxygen at which hemoglobin is half saturated. The P_{50} varies inversely with oxygen affinity [30].

Purified hemoglobin binds much more tightly to the oxygen, making it less useful in oxygen transport. However in the physiological conditions there are many factors which affect the oxygen dissociation curve and make hemoglobin a very efficient oxygen transporter. The effect of these factors can be right or left shift in the oxygen dissociation

curve. These factors are temperature, 2, 3-bisphosphoglycerate (2, 3-BPG) and pH (Bohr Effect).

1.3.4.1.1 Effect of temperature on oxygen dissociation curve

With an increase in temperature the hemoglobin oxygen affinity decreases resulting in a right shift in the oxygen dissociation curve [20]. A right shift in the oxygen dissociation curve means high P_{50} which means high oxygen partial pressure is required to maintain 50% oxygen saturation. This indicates a lower oxygen affinity. Similarly hypothermia causes a leftward shift in the oxygen dissociation curve. The changes due to temperature variations are not very drastic.

1.3.4.1.2 Effect of heterotropic ligands

Various chemical factors influence the oxygen dissociation curve of hemoglobin, which are called heterotropic ligands [12, 31]. These heterotropic ligands are D-2, 3-bisphosphoglycerate (2, 3-BPG), CO_2 , H^+ , Cl^- etc. They all bind preferentially with T state hemoglobin [Fig.1.5] and shift the oxygen dissociation curve to the right [Fig.1.6] giving hemoglobin a physiological advantage. Thus the oxygen affinity of hemoglobin is decreased by binding of these heterotropic ligands [29]. The effects of heterotropic ligands arise because the T structure has a low affinity for oxygen and a high one for protons, chloride, organic phosphate and CO_2 . In the R structure these relative affinities are reversed. The transition between the two structures gives rise to the cooperativity of oxygen binding. All these heterotropic ligands stabilize the T structure and hence lower the oxygen affinity. These ligands form salt bridges within and between the hemoglobin chains. In human hemoglobin 2,3 BPG binds in the cavity between two β chains to Val(1) β , His(2) β , Lys (82) β and His(143) β [32].

Mammals and frogs use 2, 3-BPG but teleost fish use ATP, GTP or even lactate instead of 2, 3-BPG as allosteric effector. In birds Inositolpentaphosphate is used as allosteric effector.

1.3.4.2 Carbon-dioxide transport and Bohr effect

The transport of carbon dioxide is another important function of hemoglobin besides the transport of oxygen. Carbon dioxide is transported from tissues, where it is produced as a byproduct of cellular respiration, to the lungs from where it is exhaled.

Carbon dioxide binds directly with the free amino group of the Val(1) α and β chains of globin to form carbamino compounds [33, 34] according to the following reaction:



Ten to 20 percent of carbon dioxide is transported in this carbamino form bound to all four hemoglobin chains. Carbon dioxide is also an allosteric effector. It favors binding to deoxy hemoglobin and has relatively higher affinity for the β chains [33, 34]. The binding of carbon dioxide to these hemoglobin chains is reversible so that it can easily be released in the lungs.

Hemoglobin also indirectly supports the remaining 70 to 80 percent CO_2 transport blood cells in the form of carbonate. Carbon dioxide release by tissues, combine with water to form bicarbonate and a proton according to the following reaction:



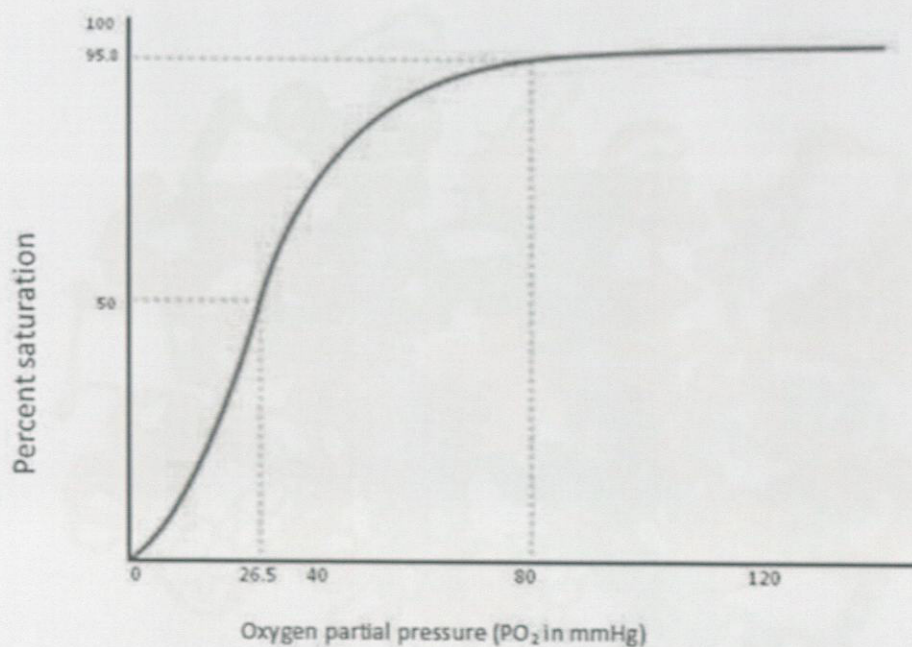


Fig.1.4 The oxygen hemoglobin dissociation curve (ODC): The curve shows that at 26.8 mmHg hemoglobin is 50% saturated. Under basal conditions the P_{O2} of mixed venous blood is 40 mmHg at which point 75% hemoglobin is saturated. Image source, Wikipedia free encyclopedia URL ; http://en.wikipedia.org/wiki/File:Hb_saturation_curve.png.

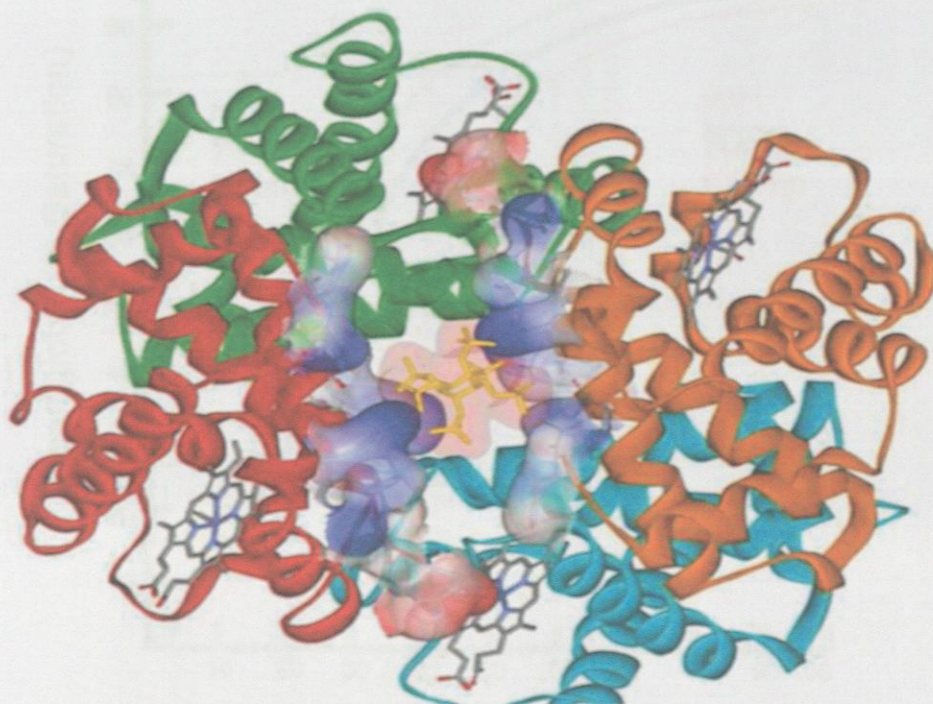


Fig.1.5 Human hemoglobin molecule showing 2, 3-BPG binding pocket: The residues contacting the BPG are shown as Van der Waal surfaces. The image was generated by Discovery Studio Visualizer.

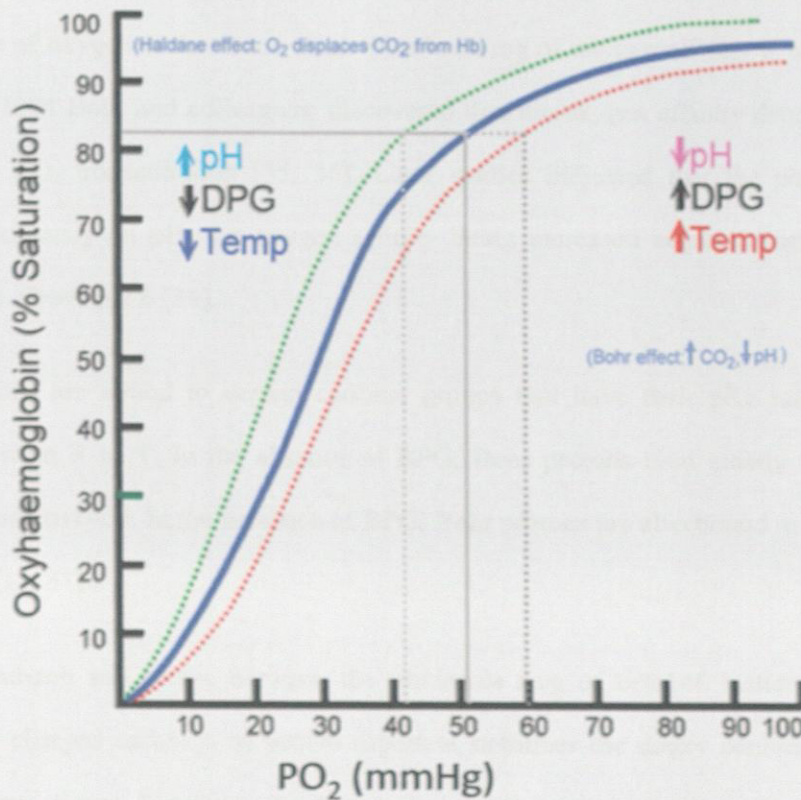


Fig.1.6 Hypothetical oxygen dissociation curve (ODCS): Changes in temperature, pH, and organic phosphates like BPG directly affect the dissociation of oxygen. Image was taken from Wikipedia encyclopedia (http://upload.wikimedia.org/wikipedia/commons/8/8a/Oxyhaemoglobin_dissociation_curve.png).

The released proton is picked up by hemoglobin thus acting as a buffering agent. The binding of proton to hemoglobin lowers the oxygen affinity of hemoglobin and facilitates the release of oxygen at the tissue level. This lowering of oxygen affinity is called Bohr Effect. In 1904 Bohr and colleagues discovered that the oxygen affinity decreases with increasing CO₂ concentration [35, 36]. Later studies indicated that the phenomenon depends primarily on pH; the oxygen affinity being increased at physiological pH in humans i.e. above pH 6 [36].

Bohr protons are bound to certain cationic groups that have their pKa raised in the transition from R to T. In the absence of BPG, Bohr protons bind mainly to Val 1 α , Lys82 β , and His146 β . In the presence of BPG, Bohr protons are also bound to His2 β and His 143 β [12, 31].

An intra-subunit salt bridge between the imidazole ring of beta146 histidine and the negatively charged carboxyl of beta94 aspartate stabilizes the deoxy conformation but not the oxy form. Similarly the terminal amino groups of the alpha-chain and deoxyhemoglobin interact with the carboxy terminal group of the other alpha-chain which increases its affinity for H⁺.

1.4 AVIAN HEMOGLOBIN

The respiratory system of birds is different from mammals but their hemoglobins are very much similar, functionally. Generally birds have two different types of hemoglobin, a major component HbA and a minor component HbD [37, 38]. Some birds have more than two types of hemoglobin while some may have just one hemoglobin (e.g. white stork) component but majority of birds have two hemoglobin components. The two different types

of hemoglobin have same beta chains but different alpha chain. Hemoglobin A (HbA) has alpha A chain while hemoglobin D (HbD) has alpha D chain [37, 38].

In birds the allosteric effector is inositolpentaphosphate instead of 2, 3-BPG. The binding pocket of inositolpentaphosphate is same as 2, 3-BPG except one residue His β 143 which is replaced by Arg β 143.

1.4.1 Different Types of avian Hemoglobins

During the development of birds red blood cells different hemoglobin are synthesized [39, 40]. A number of investigators have worked on chicken hemoglobin to study the nature and properties of avian hemoglobin. Majority of the birds have two types of hemoglobin HbA and HbD, which can be separated by electrophoresis.

During the development stages of birds different types of hemoglobin are synthesized. These hemoglobin types are discussed in the following sections.

1.4.2 Embryonic Hemoglobin

Hemoglobins have often been used as molecular markers of avian embryo development. Because of the ease with which different stages of bird's embryo can be obtained, the embryonic hemoglobins of various birds have been studied. However a detailed study has only been done on chicken.

With the increase in the body mass of the embryo its oxygen demand also increases. This high oxygen demand is met by increasing the hemoglobin concentration, oxygen diffusion capacity, and blood volume and hemoglobin oxygen affinity. Different sets of hemoglobin are synthesized during the developmental stages of the avian embryos. The functional

properties of these embryonic hemoglobins are adjusted so as to meet the needs of the growing embryos [41].

In chicken embryos of up to 6 days incubation, four hemoglobins have been described, two major and two minor. The two major hemoglobins are HbP ($\pi_2\rho_2$) and HbP' ($\pi_2'\rho_2$), and the two minor hemoglobins are HbE ($\alpha_2\epsilon_2$) and HbM ($\alpha^D_2\epsilon_2$). The primary structure of all these hemoglobin chains is known [42].

In mature embryos also, there are two major and two minor hemoglobins. The two major hemoglobins are the same as found in adult chickens, HbA and HbD. The two minor hemoglobins found in mature chicken embryos are HbL and HbH. The globin composition of these hemoglobins is HbA ($\alpha^A_2\beta_2$), HbD ($\alpha^D_2\beta_2$), HbL ($\alpha^D_2\beta^H_2$) and HbH ($\alpha^A_2\beta^H_2$) [43, 44].

The embryonic hemoglobin ρ is most actively expressed on day 6 of the chicken embryo, while the mature embryonic hemoglobin β^H is expressed from day 17 to 22. Carlo Cirotto and Ileana Arangi isolated three other minor hemoglobin components from chicken embryos, these components were named as HbK^E from early embryo and HbK^H and HbK^A from mature embryo [42].

The oxygen affinity of embryonic blood is much higher than in the adult stage. The P_{50} of the whole blood of the 18 day embryo is 21.5 mmHg, that of the 3 day chick is 36.0 mmHg and that of the 30 day chick is 45.0 mmHg. The high oxygen affinity of embryonic blood is a result of the various embryonic hemoglobin components with high oxygen affinity and an increased level of 2, 3-BPG and ATP [45, 46].

1.4.3 Adult Avian Hemoglobins

The hemolysate of the majority of adult bird show two types of hemoglobin components. A major component HbA and a minor component HbD [45]. The major component HbA comprises 60 to 90% of the total Hb. The two components differ in the alpha chains. HbA has alpha subunit while HbD has alpha D subunit. The differences in the alpha subunits of the two hemoglobin components give rise to functional differences. For example in turkey (*Meleagris gallopavo*) HbA has greater oxygen affinity than HbD while in other birds like Rüppell's vulture (*Gyps rueppellii*) HbD has greater oxygen affinity [47]. In some birds species there is only one hemoglobin component in the adult stage. For example pigeon (*Columba livia*), and parakeet (*Ara chloroptera*) and Emperor penguin (*Aptenodytes forsteri*), have only one hemoglobin component in the adult stage [37, 48].

1.4.4 High Altitude Adaptation in Birds

Birds during their flight at high altitude are subjected to hypoxic conditions that challenge their aerobic metabolism. The oxygen levels at high altitude drop significantly to as little as 26% of the sea level oxygen [49]. Birds have shown different level of adaptation to alleviate this hypoxic condition. These adaptations include:

1. More efficient pulmonary gas exchange
2. Change in hemoglobin oxygen affinity
3. Regulation of cardiac output and regional oxygen delivery
4. Cerebral vascular regulation
5. Adaptation at the level of individual tissues [50]

These adaptations make birds the champions of hypoxia tolerance.

1.4.4.1 The respiratory system of birds adapted for efficient gas exchange

To meet the high energy and oxygen demand during flight, birds have evolved a respiratory system that is fundamentally different from the mammalian respiratory system. The respiratory system of birds (lungs/air sacs) is morphologically complex and highly efficient. In addition to the lungs where gas exchange occurs birds have air sacs. The air sacs emanate from the lungs and fill the entire cavity of the bird's body, even penetrating the bones, allowing the air to flow in one direction through the lungs and therefore all the air that passes through the lungs is fresh with high percentage of oxygen. All birds have this one-way flow system. In the bird's respiratory system, air is taken in and pushed down through the fine tubules which interlock with capillaries carrying deoxygenated blood. In the beginning the air comes in contact with fully deoxygenated blood, further down the tubules the air is in equilibrium with the blood in terms of oxygen content. There is no mixing of fresh air and carbon dioxide rich air when the two flow through the air sac system and lungs. Thus, the partial pressure of oxygen in a bird's lungs is the same as the environment, and so birds have more efficient gas-exchange of both oxygen and carbon dioxide than do mammals. Birds living or flying at adapted to high altitude show further anatomical adaptations for example these birds have high muscle capillary-per-fiber number in both highly aerobic pectoral muscles and less aerobic leg muscles thus providing more efficient oxygen delivery to these oxygen demanding muscles [51].

1.4.4.2 Change in hemoglobin oxygen affinity

Hypoxia is one of the most important factors affecting survival at high altitudes. The low partial pressure of oxygen at high altitude can result in a significant decrease in oxygen

saturation of arterial blood and there individuals exposed to high altitude can develop a series sever medical conditions or even death [52].

Birds show a remarkable adaptation to high altitude at molecular level. Species of birds that are well adapted to hypoxia have hemoglobin with high oxygen affinity. The affinity of hemoglobin with oxygen is measured by a term p_{50} which is the pO_2 at which hemoglobin is half-saturated with oxygen [53]. The p_{50} of human blood is approximately 28 mm Hg [54]. Birds adapted to hypoxia have a very low P_{50} which means very high oxygen affinity.

A notable example of the birds' molecular adaptation to high altitude was provided by studying the blood oxygen affinity of two closely related geese species, the bar headed goose and the Greylag goose. The bar headed goose (*Anser indicus*) is a migratory bird and fly through Himalayas at an altitude up to 9,000 m. The blood of Bar headed goose has unusually high oxygen affinity (p_{50} 29.7 mm Hg at pH 7.4 and 37 °C) [12, 31, 55]. The grey lag goose (*Anser anser*) is a very closely related species living in plains and has normal blood oxygen affinity (p_{50} 39.5 mm Hg at pH 7.4 and 37 °C) [12, 31]. The amino acid sequences of the two hemoglobins differ by only four amino acid exchanges. These exchanges are (grey lag goose → Bar headed goose) $\alpha 18\text{Gly} \rightarrow \text{Ser}$, $\alpha 63\text{Ala} \rightarrow \text{Val}$, $\beta 125\text{Glu} \rightarrow \text{Asp}$ and $\alpha 119\text{Pro} \rightarrow \text{A}$. The amino acid substitution 119pro to Ala is unique among birds. This substitution disrupts Van der Waal contact between the $\alpha 119\text{Pro}$ and $\beta 55\text{Leu}$ [12, 31]. Studies on human hemoglobin have shown that this contact stabilizes the T structure therefore in Bar headed goose the disruption of this contact would destabilize the T structure [56] [Fig1.7].

Interestingly the same region is involved in high altitude adaption of the Andean goose hemoglobin (P_{50} 33.9 mmHg at 40°C, pH 7.1) [56, 57]. The Andean goose is a member

birds family Anatidae and is a resident around lakes and marshes in the high Andes, usually well above 3000 m. Similar to the Bar headed goose the Andean goose has a very high oxygen affinity relative to its low land sibling species the Mallard Duck [56]. The substitution responsible for the high oxygen affinity of the Andean goose is $\beta 55\text{Leu} \rightarrow \text{Ser}$. In this case the mutation is on beta chain but it is at the same $\alpha 1\beta 1$ interface position and disrupting the same contact points. This is one of the best examples of convergent evolution. To further validate the hypothesis, Jessen et al [58] and Weber et al [59] introduced the same mutation (Bar headed $\alpha 119\text{Pro} \rightarrow \text{Ala}$ and Andean goose $\beta 55\text{Leu} \rightarrow \text{Ser}$) in human hemoglobin. These studies revealed that the mutant hemoglobin had greater oxygen affinity than normal human hemoglobin [56]. These examples show a strong correlation of hemoglobin affinity and high altitude adaptation.

1.4.4.3 Regulation of cardiac output and regional oxygen delivery

Birds have a very well adapted cardiovascular system. Compared to similar sized mammals, birds have a larger heart with larger stroke volume. The larger size of the heart shows that it can produce more output during hypoxic conditions. During flight the cardiac output of birds increase many fold e.g. the cardiac output of Bar-headed goose during flight increase 6-7 fold. Since the Bar headed goose has greater oxygen affinity for hemoglobin hence more output means more oxygen delivery during flight [50]. The increase in cardiac output is observed only when the paO_2 is below 30 mm Hg [60].

During hypoxia at rest the distribution of blood to various organs is rearranged. For example in Pekin duck during resting hypoxia the blood is shifted away from non vital organs like gastrointestinal tract, spleen and non respiratory muscles and shifted towards organs that are critically dependant on oxygen like brain and heart muscles. In contrast

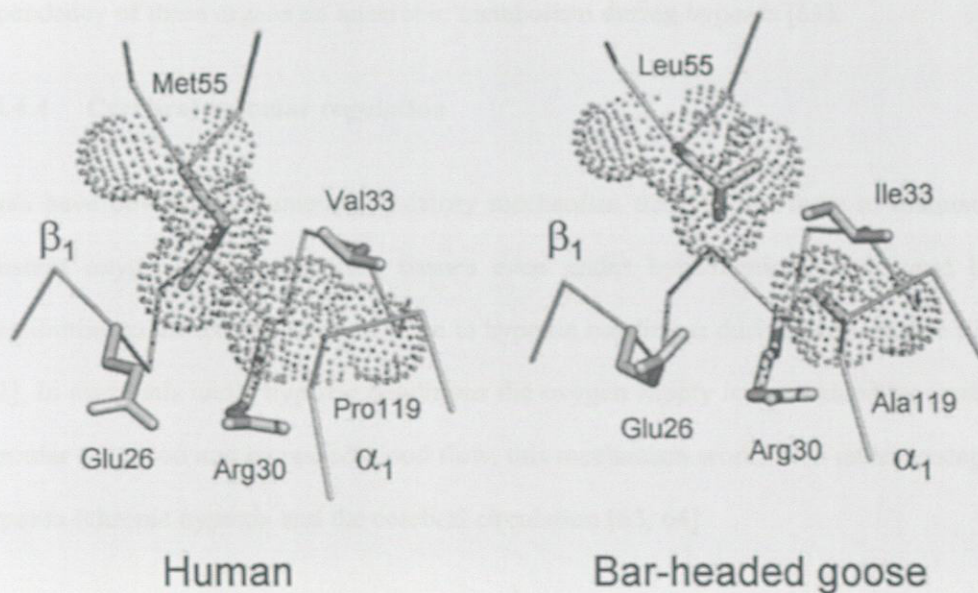


Fig.1.7 Dot representation of Van der Waals radii at an intradimer $\alpha_1\beta_1$ contact in human Hb (left) and Bar-headed geese Hb (right): In Bar headed goose the substitution of pro119 by Ala 119 disrupts the contact between alpha and beta chains. Image taken from Jay et al [56].

the Bar headed goose does not show any significant redistribution of blood flow. The supply of blood to various organs remain almost same in both hypoxic and normoxic conditions [61]. The decreased blood flow to some organs in Pekin duck shows the dependency of these organs on anaerobic metabolism during hypoxia [61].

1.4.4.4 Cerebral vascular regulation

Birds have developed a unique regulatory mechanism that enables them to maintain a constant oxygen supply to brain tissues even under hypocapnic hypoxia and thus contributing to the exceptional tolerance to hypoxic conditions during high altitude flight [62]. In mammals under hypoxic conditions the oxygen supply is maintained by cerebral vascular dilatation and increased blood flow, this mechanism works well under isocapnic hypoxia (chronic hypoxia and the cerebral circulation [63, 64].

However the situation becomes worse under hypocapnic hypoxia. In mammals the cerebral circulation of blood is sensitive to pO_2 and pCO_2 . When the blood pO_2 decreases below 50 mm Hg, the blood flow to brain is increased but at the same time due to hyperventilation caused by hypoxia the pCO_2 also decrease which causes constriction of the vessels and hence lesser blood flow [50]. In humans if the blood pCO_2 is lowered to 10mmHg the blood flow to brains reduces by 50 % [50]. So in hypocapnic hypoxia the two mechanism act opposite to each other and the overall result is reduced oxygen to brain [65]. Therefore mammals are not well adapted to isocapnic hypoxia and high altitudes. In human a decrease in oxygen delivery to brain during ascent to high altitude causes long term neurological impairments [50].

In birds the cerebral circulation is not very sensitive to reduced level of pCO_2 and hence under hypocapnic hypoxia adequate blood flow to brain is maintained [50]. In fact the

blood flow to the brain is markedly increased during severe hypocapnic hypoxia. In addition to reduced sensitivity of cerebral circulation to hypocapnia, birds may also have some other adaptations. For example, birds have extra cranial network of arteries near the eye known as rete ophthalmicum that helps in heat exchange [66]. Bernstein et al have provided evidence that this arteries network can also work as a site of gas exchange. The blood leaving the rete and going to the brain has greater pO_2 and hence supplies more oxygen to brain during hypoxia [66].

1.4.4.5 Adaptation at the level of individual tissues (cellular adaptation)

Other adaptations that benefit oxygen transfer during hypoxia occur at the level of individual tissues. These adaptations include density of capillaries, the number and relative density of mitochondria in cells and the intra cellular concentration of myoglobin in muscles [50]. Birds have higher capillary density in skeletal muscles, heart muscles and brain tissues than mammals [61]. This high capillary density increases the diffusion of oxygen to cells from blood. Another cellular level adaptation is the number of mitochondria per cell. Muscles in birds have greater number of mitochondria per cell than in mammals [67]. In addition to that the activity of oxidative cycle and aerobic oxidation enzymes is also greater. The greater number of mitochondria and more active aerobic oxidation enzyme support the high oxygen demand of birds under hypoxic conditions at high altitude [50]. Another important adaptation is the level of myoglobin in pectoral and heart muscles. Myoglobin has a very high affinity for oxygen (2.5 mm Hg) [68]. In addition to oxygen storage myoglobin also facilitates oxygen transport from cell membrane to mitochondria and as an antioxidant defense [62]. Birds and diving animals have very high levels of myoglobin (50-80 mg /g) in their muscle [69].

1.4.5 Organic Phosphate in Avian Erythrocyte and Their Effect on Hemoglobin Oxygen Affinity

In comparison to mammalian hemoglobin, avian hemoglobin have more positively charged residues at the phosphate binding site and thus have high affinity for organic phosphates [70]. The organic phosphates are normal constituents of red blood cells and modulate the oxygen affinity of hemoglobin. In mammals 2, 3-BPG is used as allosteric effector while in birds inositolpentaphosphate is used [71]. In humans the level of 2, 3-BPG is increased during hypoxic conditions which decreases the oxygen affinity of hemoglobin and this facilitates oxygen release at tissue level [72].

In avian blood three organic phosphates, ATP, IPP and 2, 3-BPG, are present. 2, 3-BPG is the major organic phosphate in the chicken embryo during the last week of incubation which is practically eliminated within 5 days after hatch and during this period ATP become the major organic phosphate [46]. IPP appears after 3rd day of hatching and after 18 days it becomes the major organic phosphate of chicken erythrocytes [45, 46].

In humans generally 2, 3-bisphosphoglycerate (BPG) binds with deoxy-hemoglobin. The affinity of BPG is about 100 times higher than with oxy-hemoglobin. The affinity of IPP for birds' hemoglobin is much higher than human Hemoglobin. At pH 7.2 and 25°C values for the binding constant of about 3×10^5 M for oxyhemoglobin and 4×10^9 M for deoxyhemoglobin are found. For the binding of IPP to human hemoglobin at pH 7.3, 0.1 M NaCl, and 20°C values of 2.5×10^{33} M and 1×10^{63} M for oxy- and deoxyhemoglobin, respectively, have been reported [71, 73, 74]. The much higher affinity of the bird hemoglobin for IPP as compared to human hemoglobin is compatible with the presence of a higher number of positively charged residues located at the phosphate binding site [71].

1.5 PRIMARY STRUCTURE OF AVIAN HEMOGLOBINS

The total number of avian hemoglobin alpha A chain sequenced so far are as follows; *Anseriforms* (15), *Apodiformes* (1), *charadriiformes* (2), *Ciconiformes* (2), *Columbiformes* (2), *Cuculiformes* (2), *Falconiformes* (5), *Galliformes* (5), *Passeriformes* (4), *Pelecaniformes* (1), *Phoenicopteriformes* (1), *Psittaciformes* (2), *Rheiforme* (1), *Sphenisciformes* (2), *Struthioniformes* (1). Total number of avian orders having one or more alpha A chains sequenced is 15 and the total number of species is 46. No sequence of hemoglobin alpha A chain is available from the order Gruiformes.

The total number of hemoglobin beta chains sequenced for different orders of birds is as follows;

Anseriforms (9), *Apodiformes* (1), *Charadriiformes* (2), *Ciconiformes* (2), *Cuculiformes* (2), *Falconiformes* (5), *Galliformes* (5), *Passeriformes* (6), *Pelecaniformes* (1), *Phoenicopteriformes* (1), *Psittaciformes* (2), *Rheiforme* (1), *Sphenisciformes* (2), *Struthioniformes* (1).

The total number of all beta chains available from 14 families is 40. Like the alpha A chains there is no hemoglobin beta globin chain available from any member of the Gruiformes order.

1.6 THE CRANES

Cranes are large, strong flying birds that occur in wetlands and grass lands on all continents of the world except South America and Antarctica [75]. The cranes are among the most ancient and distinctive families of birds on Earth. Their great size and beauty, unique calls, and complex behaviors have for centuries commanded the attention and

respect of people on the five continents where they are found. Species of cranes belong to Aves order Gruiformes and family Gruidae. The family Gruidae consist 15 extant species of cranes. Most of the species are threatened and therefore many countries have adopted laws and rules to protect the population of these cranes [75].

Cranes can be defined as large, long-legged and long necked members of the Gruiformes. They have an elongated and tapering bill, longer than their head. The toes are moderately long and connected at the base by a membrane. The tail is long, usually composed of twelve feathers. Both sexes have similar plumage and are monogamous. They make a loud resonant call. Most species of cranes are vegetarians but they can change their diet according to the season and their own nutrient requirements (opportunistic feeders). They eat a range of items from suitably sized small rodents, fish, amphibians, and insects, to grain, berries and plants [75].

1.6.1 Classification And Phylogeny Of Cranes

Canes belong to the order Gruiformes and family Gruidae. The order Gruiformes is divided into suborder and each suborder is divided into a number of families which consist both extant and recently extinct families [Table.1.1]. The cranes family Gruidae belong to suborder Gruidae which also contains three other extant families namely Aramidae and psophiidae and two extinct families Geranoididae (fossil), Eogruidae (fossil) [75].

The fifteen species of the family Gruidae is subdivided into two sub families Balearicinae and Gruinae [76]. The sub family balearicinae is represented by only two species (The crown cranes) having relatively unspecialized anatomy. The crown cranes cannot withstand low temperature and it is hypothesized that as the earth cooled these

cranes disappeared from the northern continents and survived only in Africa where the tropical condition remained though out the Pleistocene [77].

The subfamily Gruinae is divided into three distinct genera, *Grus* (10 species), *Anthropoides* (2 species) and *Buggeranus* (1 species). These genera are defined on the basis of soft anatomical features. During the past few decades different approaches have been used to describe the evolutionary relationship among cranes species. The first modern phylogenetic analysis of cranes began with Archibald study of unison calls [78]. Subsequent phylogenetic investigations were made using different approaches such as morphometric [79], allozymes [80, 81], DNA hybridization [82], mitochondrial gene sequences [83, 84], morphocladistics [85] and combined mitochondrial-nuclear DNA sequences [86]. The most recent phylogenetic study based on complete mitochondrial genome sequences [77]. All these studies have confirmed the monophyly of the family Gruidae [Fig.1.8].

1.6.2 Cranes of Pakistan

Four types of crane have been sighted in Pakistan. Two species of cranes, the common crane and demoiselle cranes are frequently seen in Pakistan when they migrate through in fall and spring [78]. The other two species, Siberian crane and Sarus crane are very rarely seen in Pakistan. Although extensive network of wetlands is present in Pakistan but the cranes do not use these as a wintering ground and only make a stopover during their migration (*Wild life of pakistan* URL <http://www.wildlifeofpakistan.com/>). The exact routes and corridors used by these cranes are not known. Cranes have often been sighted in different parts of Pakistan in flight or temporary resting [Fig.1.9].

Table.1.1 Classification of 15 crane species of Aves order Guiformes, suborder Gruiformes and family, Gruidae

Subfamily	Genus	Species	
		Common name	Scientific name
BALEARICINAE	<i>Balearica</i>	Black crowned crane	<i>Balearica pavonina</i>
		Grey crowned crane	<i>Balearica regulorum</i>
GRUINAE	<i>Grus</i>	Common crane	<i>Grus grus</i>
		Sandhill crane	<i>Grus canadensis</i>
		Whooping crane	<i>Grus americana</i>
		Sarus crane	<i>Grus antigone</i>
		Brologa	<i>Grus rubicund</i>
		Siberian crane	<i>Grus leucogeranus</i>
		White-naped crane	<i>Grus vipio</i>
		Hooded crane	<i>Grus monacha</i>
		Black-necked crane	<i>Grus nigricolis</i>
		Red-crowned crane	<i>Grus japonensis</i>
	<i>Anthropoides</i>	Blue crane	<i>Anthropoides paradisea</i>
		Demoiselle crane	<i>Anthropoides virgo</i>
	<i>Bucconas</i>	Wattled crane	<i>Bucconas carunculatus</i>

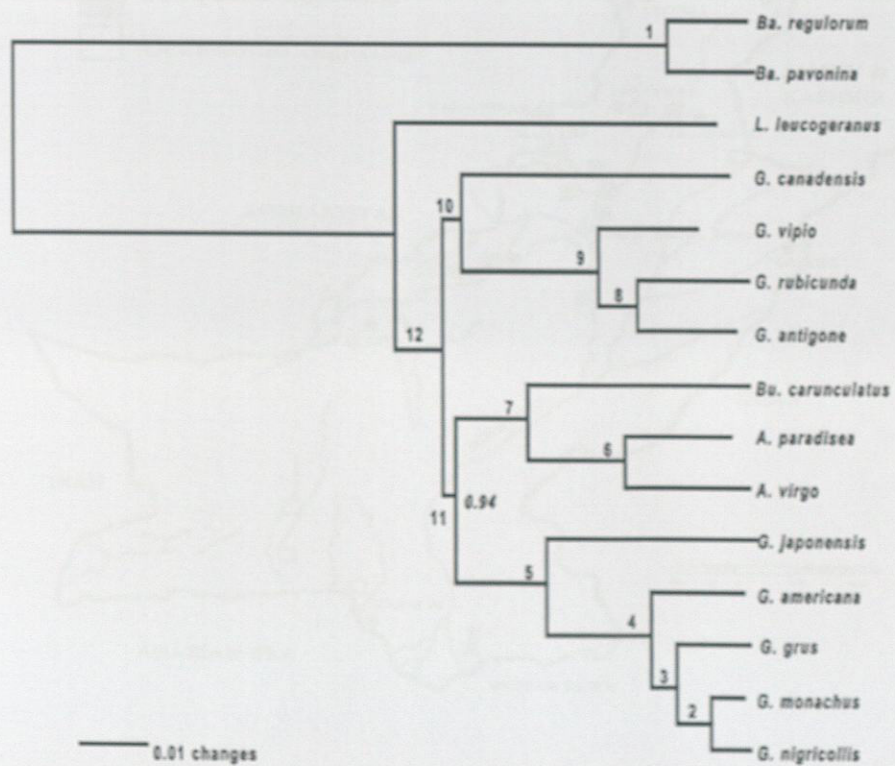


Fig.1.8 A time calibrated tree of all 15 cranes species based on complete mitochondrial DNA sequences: Image source [77].

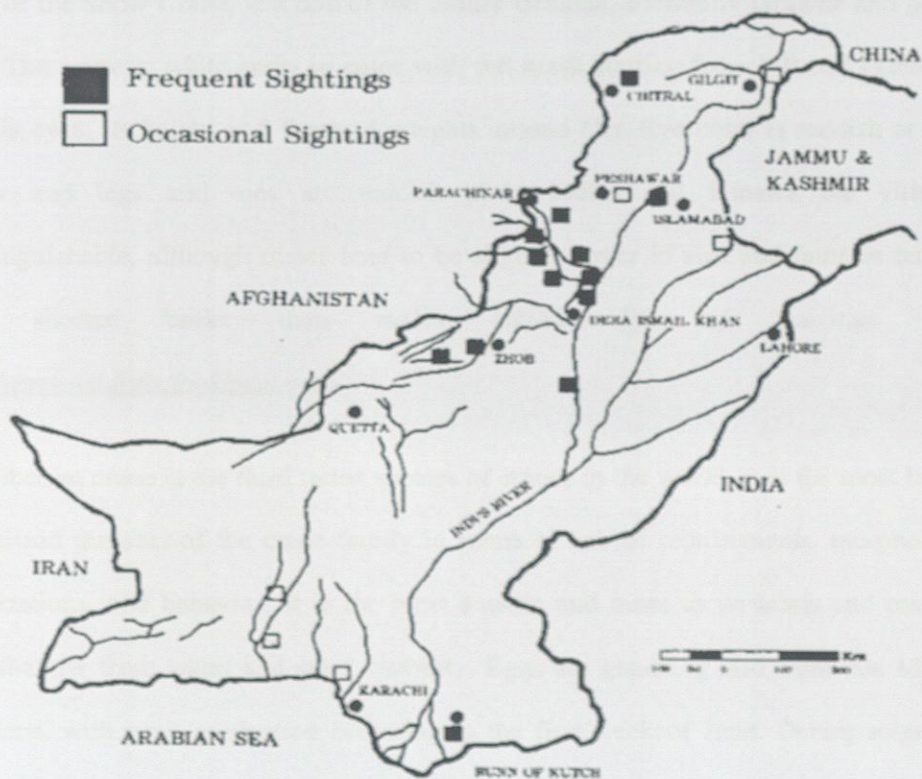


Fig.1.9 Crane sighting records in Pakistan: Image borrowed from [87].

A brief description of the four cranes species seen in Pakistan is presented in the following paragraphs.

1.6.2.1 Siberian crane (*Grus leucogeranus*)

The Siberian Crane, *Grus leucogeranus* [Fig.1.10], also known as the Siberian White Crane or the Snow Crane, is a bird of the family Gruidae, subfamily Gruinae and genus *Grus*. The crane is white snow in color with red mask starting from bill and extending towards eyes. Its height is 5 feet and weights around 6kg. Eye color is reddish or pale yellow and legs and toes are reddish pink. Males and females are virtually indistinguishable, although males tend to be slightly larger in size and females tend to have shorter beaks than males (*Wilde life of pakistan URL <http://www.wildlifeofpakistan.com/>*).

The Siberian crane is the third rarest species of cranes in the world. It is the most highly specialized member of the crane family in terms of habitat requirements, morphology, vocalizations, and behavior. It is the most aquatic and nests in wetlands and marshes with shallow fresh water and good visibility. Eggs are generally laid from late May to mid-June, with peak production occurring in the first week of June. During migration they prefer to rest in wetlands with shallow water 30-60cm and never use drier upland areas. Siberian Cranes consume a wider variety of food items including roots, rhizomes, sprouts of sedges, seeds, horsetails, and berries and cranberries, as well as insects, fish, frogs and other small aquatic animals (*Wilde life of pakistan URL <http://www.wildlifeofpakistan.com/>*).

The total population of Siberian cranes is around 3000 and considered as endangered species. The species is divided into three populations, eastern population, central

population and western population. The majority of species belong to the eastern population and very few belong to central and western population. Due to excessive hunting and shooting in Pakistan, the Siberian cranes have changed their migratory route and are not seen in Pakistan. The Pakistan Crane centre situated in Lakki Marwat is trying to promote education about crane conservation and curtail their shooting so that the Siberian cranes may return to their ancestral migratory route through Pakistan (*International Crane Foundation URL <http://www.savingcranes.org/pakistan-november-3-5-2006.html>*) [Fig.1.11-12].

1.6.2.2 Sarus crane (*Grus antigone*)

Sarus crane [Fig.1.13] is the world's tallest living crane; a large male may stand six feet tall and a wing span of eight feet. The average weight is around 6.3 kg. A notable feature of the Sarus cranes is grey white plumage with red head. Three species of the Sarus crane are recognized with a total population between 15,500 and 20,500 [88] (*International Crane Foundation URL <http://www.savingcranes.org/sarus-crane.html>*). *Grus antigone antigone* (Indian sarus), *Grus antigone sharpii* (Eastern sarus) and *Grus antigone gilli* (Australian sarus). The three subspecies are distinguished mainly by morphological differences. In Pakistan only the Indian Sarus crane is found in a very limited area of Tharparkar District of Sindh province. In recent years the resident Sarus crane was extirpated from Pakistan [88]. With the efforts of the Pakistan crane centre we hope that the once resident Sarus crane will return to Pakistan.

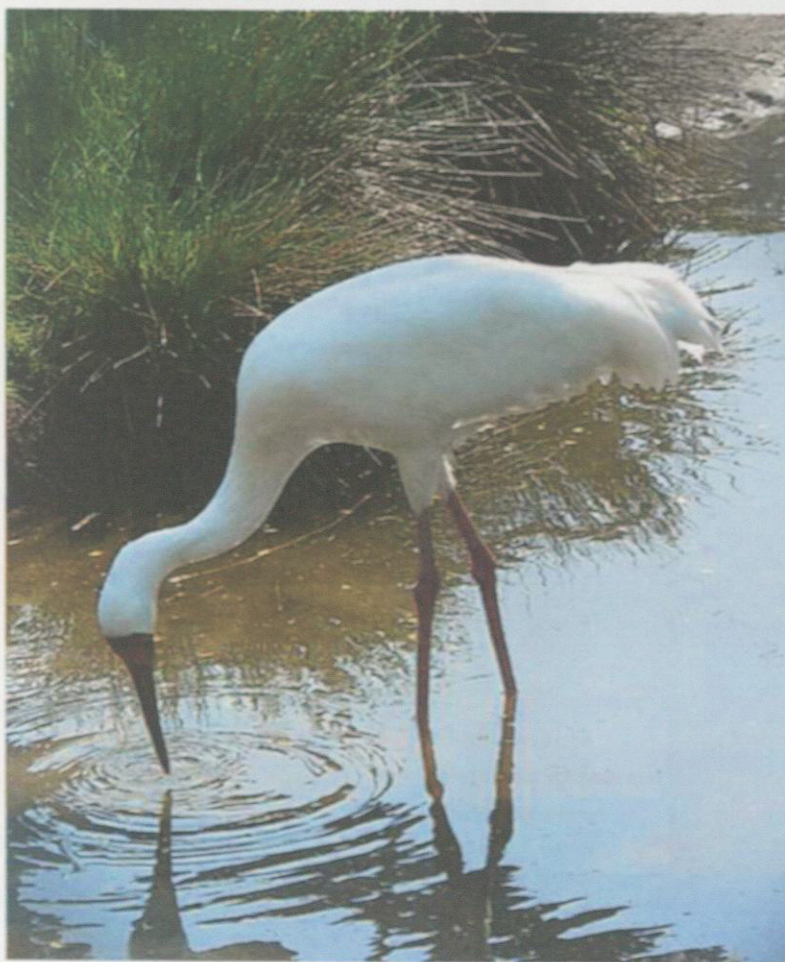


Fig.1.10 Siberian crane: Photo credit Wikipedia Free Encyclopedia

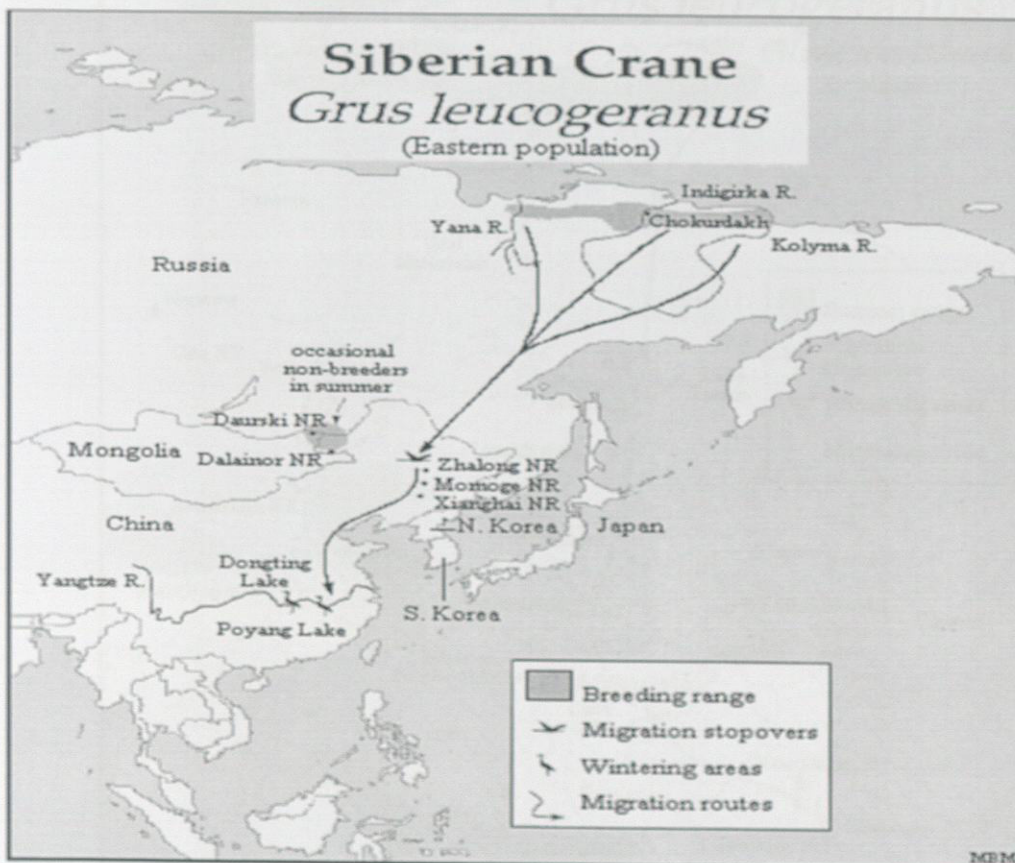


Fig.1.11 Breeding range, migratory route and wintering range of the Eastern population of Siberian crane: The figure was taken from USGS (US Geological Survey 02 Mar 98) website URL (<http://www.npwrc.usgs.gov/resource/birds/cranes/grusleuc.htm>)



Fig.1.12 Breeding range, migratory route and wintering range of the Western and Central population of Siberian crane: The figure was taken from USGS (US Geological Survey 02 Mar 98) website URL <http://www.npwrc.usgs.gov/resource/birds/cranes/grusleuc.htm>)

The current range of the Indian Sarus Crane includes the plains of northern, northwestern, and western India and the western half of Nepal's Tarai lowlands. The population has declined sharply over the last several decades (*International Crane Foundation URL <http://www.savingcranes.org/sarus-crane.html>*). Sarus cranes formerly occurred across the subcontinent from the province of Sindh in Pakistan to Bangladesh.

The Australian Sarus Crane occurs in Northeastern Australia, almost exclusively on the Cape York Peninsula in northern Queensland. Roosting flocks of 2000 individuals have been found on the Artherton tablelands [89]. The eastern Sarus crane is found throughout Indochina, but in the last few decades its population is rapidly declining [Fig.1.14].

1.6.2.3 Demosille crane (*Anthropoides virgo*)

Demosille crane (*Anthropoides virgo*) belongs to the subfamily Gruinae and genus *Anthropoides* [Fig.1.15]. It is the world second most abundant crane. The total population is estimated at 200-204,000. The Demoiselle crane has bluish gray plumage with the face, neck and front of the chest being black. There is no subspecies of the demosille crane. The population is divided into six groups, atlas population, black sea population, Turkey population, Kazakhstan/Central Asia population, Kalmykia population and eastern asian population.

In Pakistan the major population comes from the Kazakhstan/Central Asia population. This population is estimated at 100,000. The breeding grounds are east of the Caspian Sea throughout Kazakhstan. The population migrates through Afghanistan and Pakistan



Fig.1.13 Sarus crane: Photo credit Wikipedia the free encyclopedia website URL ([http://en.wikipedia.Org/wiki/File:Sarus_Crane_\(Grus_antigone\)_at_Sultannpur_I_Picture_151.jpg](http://en.wikipedia.Org/wiki/File:Sarus_Crane_(Grus_antigone)_at_Sultannpur_I_Picture_151.jpg))

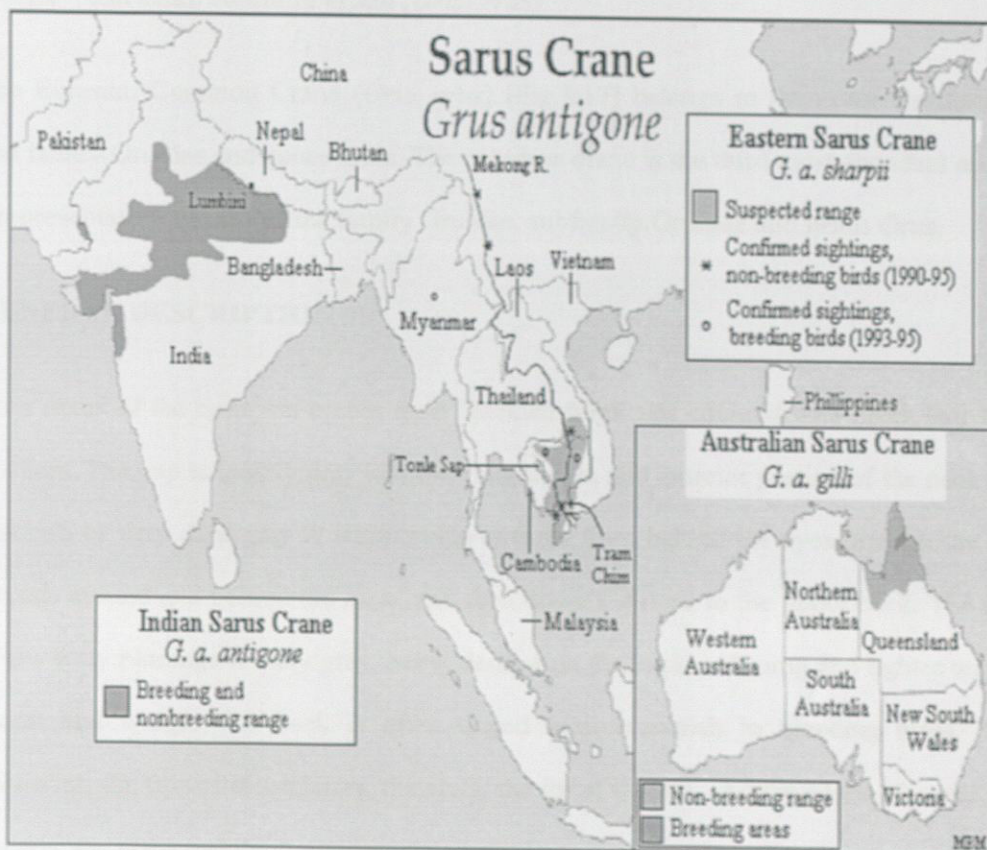


Fig. 1.14 Breeding range, migratory route and wintering range of the Sarus crane:
The figure was taken from USGS (US Geological Survey 02 Mar 98). URL <http://www.npwrc.usgs.gov/resource/birds/cranes/grusanti.htm>

to wintering grounds in the western portions of the Indian subcontinent. The Indian state of Gujarat is the core wintering area [Fig.1.16].

1.6.2.4 Eurasian common crane (*Grus grus*)

The Eurasian Common Crane (*Grus grus*) [Fig.1.17] belongs to the order Gruiformes and family Gruidae and genus *Grus*. The common crane is the third most abundant and is a representative species of the family Gruidae, subfamily Gruinae and genus *Grus*.

GENERAL DESCRIPTION

Both sexes of the common cranes have blackish foreheads covered with black hair like feathers. The nap is largely gray and the chin, throat, and anterior portion of the neck are blackish or very dark gray. A white stripe extends from behind the eyes through the ear coverts around and behind the nape, and then down the neck to the upper back. The rest of the body plumage is slaty gray, being darkest on the back and rump and lighter on the breast and wings. The back is often tinged with brownish in breeding birds. The primaries, the tips of secondaries, the alula, the tip of the tail, and the edges of upper tail coverts are black, while the bases of the rectrices are slaty gray. The inner secondaries may be lighter than the rest of the plumage (*Wilde life of Pakistan URL <http://www.wildlifeofpakistan.com/>*).

The Juveniles common cranes have pale yellowish brown edges on their body feathers, and the crown is covered by feathers; there also are no drooping inner wing feathers. From the first autumn, the new feathers of the head and neck are gray, tipped with cinnamon. The Immature common cranes in their first spring have the crown and forehead partly bare, covered only by some bristle-like feathers. The remaining plumage is like the adult common crane with spotted shabby young feathers. The flight feathers

and most tertials are still juvenile, and are narrower and browner than in adults. Second-year immatures are like adults, but retain some old brown feathers on the body and upperparts, and some flight feathers may still be immature [90].

HABITAT

The Eurasian common crane is fully migratory and used different wintering and breeding grounds. They utilize a very extensive breeding range including a variety of wetlands, marshes, swamps, meadows and bogs. If nearby water is available they may also dry lands. They usually prefer large isolated nesting territories. The nest is a mound of wetland vegetation generally on standing water or near water in an inaccessible and undisturbed bog marsh or wetland.

DISTRIBUTION

The common (Eurasian crane) is the most widely distributed of the fifteen crane species. It is the third most abundant crane species after Sandhill and Demoiselle Crane (*Wilde life of pakistan* URL <http://www.wildlifeofpakistan.com/>). The global population is estimated to be around 270,000 and appears to be increasing. The breeding range of common crane extends across Eurasia from Scandinavia, Western and Central Europe, Ukraine, Belorus and Russia to western and northeastern China, northern Mongolia and eastern Russia. The species' wintering grounds include portions of France, the Iberian Peninsula, North Africa, Sudan, Israel, Jordan, Saudi Arabia, Iran, Iraq, India, southeastern China, and perhaps Indochina. Isolated breeding populations occur in eastern Turkey and the Tibet Plateau. The Eurasian crane has also been recorded as an occasional migrant or wintering bird in Japan, the Korean peninsula, and western North America [91-93].

In Pakistan the major population comes from the Western Siberia Population. During their migration these birds follow a migration corridor southwest toward Afghanistan, and then southeast across Pakistan to wintering grounds in western and central India [91-93]. These birds fly over the Kohe Safed Mountain range at 3800m elevation (*Wilde life of pakistan* URL <http://www.wildlifeofpakistan.com/>) [Fig.1.18].

1.7 OBJECTIVE OF THE STUDY

Hemoglobin is the major oxygen transport protein and shows a high degree of adaptability under different conditions, a feature unique to its primary structure. Thus knowledge of primary structure is of utmost importance both with reference to its function and evolution.

The present study was designed to contribute towards a better understanding of the functional and evolutionary aspects of hemoglobin in birds. This study aims at;

1. Isolation and characterization of common crane hemoglobin (*Grus grus*, Gruiformes) and elucidation of the primary structure of the purified polypeptide chains of hemoglobin.
2. Swquence similarity comparison of the characterized subunits of common crane hemoglobin with other avian species sequenced so far.
3. Prediction of three dimensional homology models of common crane hemoglobin components using protein structure modeling packages modeler and analysis of three dimensional models to explain their structure-function relationship and
4. Phylogenetic analysis of the *Grus grus* hemoglobin to improve our understanding evolutionary relationship among different avian orders on molecular basis.



Fig.1.15 Demoiselle crane (*Anthropoids virgo*): Photo credit Wikipedia free
Encyclopedia (http://en.wikipedia.org/wiki/Demoiselle_Crane)

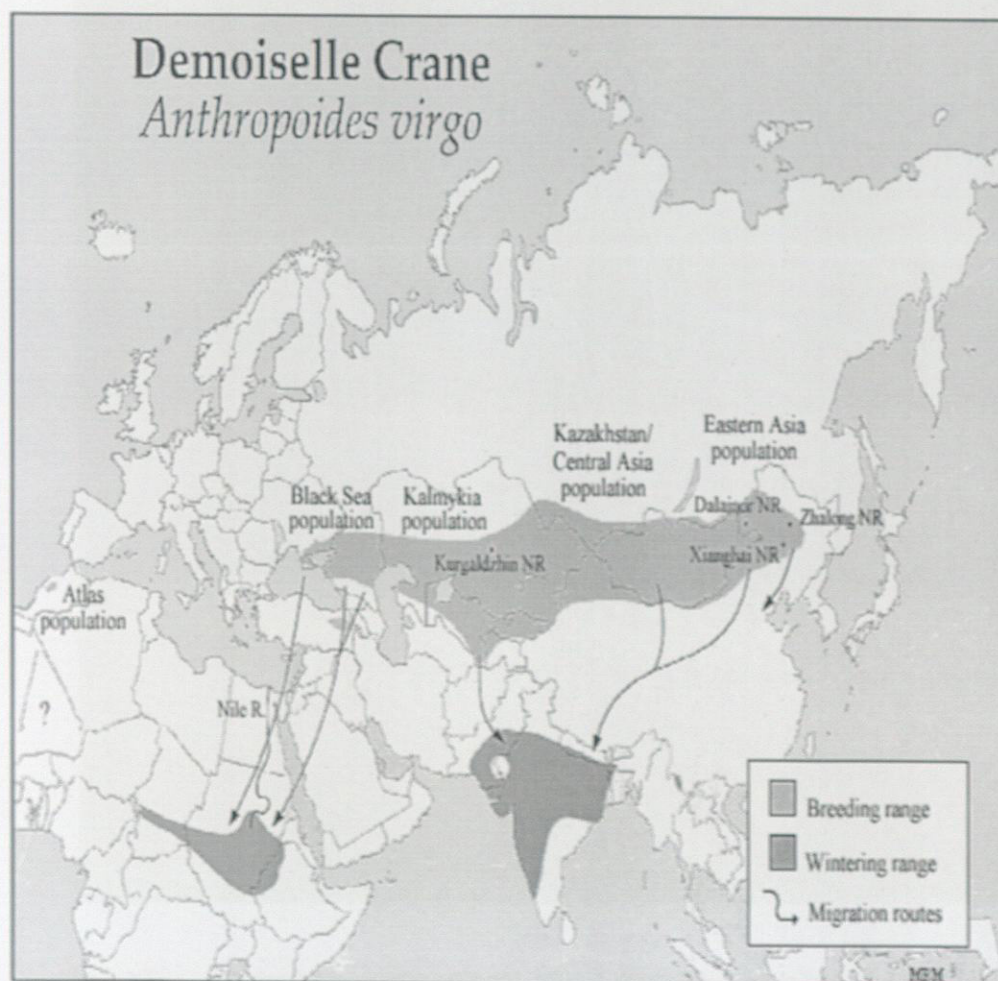


Fig.1.16 Breeding range, migratory route and wintering range of the Demoiselle crane: The figure was taken from USGS (US Geological Survey 02 Mar 98). URL <http://www.npwrc.usgs.gov/resource/birds/cranes/anthvirg.htm>



Fig.1.17 Euroasian common crane (*Grus gus*): Picture taken from Wikipedia free Encyclopedia (http://en.wikipedia.org/wiki/Common_Crane)

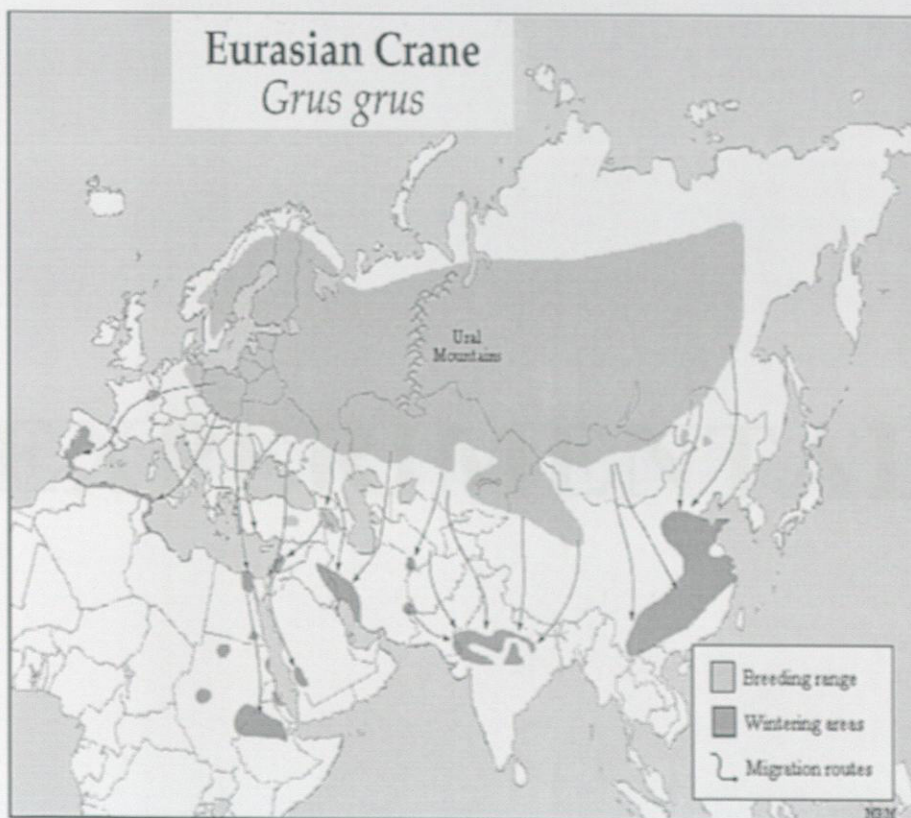


Fig.1.18 Breeding range, migratory route and wintering range of the Eurasian common crane: The figure was taken from USGS (US Geological Survey 02 Mar 98): URL <http://www.npwrc.usgs.gov/resource/birds/cranes/grusgrus.htm>).

1.1. COLLECTION OF FIELD SAMPLES

Crustaceans were collected using a 10-m beam trawl in the Bay of Biscay (43°30'N, 15°30'W). The trawl was towed for 10 min at a speed of 2 km/h. The catch was preserved in 10% formalin solution and stored at 4°C until analysis.

1.2. LABORATORY ANALYSIS

The total lipid content was determined gravimetrically after extraction with chloroform. The extraction was performed using the method of Bligh & Dyer (1959). The extraction was performed using the method of Bligh & Dyer (1959). The extraction was performed using the method of Bligh & Dyer (1959).

EXPERIMENTAL

The method of Bligh & Dyer (1959) was modified to improve the extraction of lipids. A 0.75 g sample of wet weight was weighed and placed in a 100 ml beaker. The sample was then added to 10 ml of chloroform. The extraction was performed using the method of Bligh & Dyer (1959). The extraction was performed using the method of Bligh & Dyer (1959). The extraction was performed using the method of Bligh & Dyer (1959).

2.1 COLLECTION OF BLOOD SAMPLES

Common cranes were purchased from a local market in the Bannu District of NWFP. Blood samples were drawn from the pectoral veins of the non-anesthetized common crane using ethylene diamine tetraacetic acid (EDTA) (1.5 mg/mL) as anticoagulant.

2.2 HEMOGLOBIN ISOLATION

The Drabkin method was used for isolation of the hemoglobin from red blood cells of the common crane [94]. The erythrocytes were separated from the plasma by centrifugation at 845 RPM for 5 minutes at 4°C. The supernatant plasma was discarded and the packed erythrocytes were washed three times with physiological saline (0.9% sodium chloride). The erythrocytes were stored at -4°C till further analysis.

2.3 PREPARATION OF GLOBIN

The method of Rossi-Fanelli Antonini [95] was followed to precipitate the common crane globin. A 0.75 % solution of HCl in acetone was prepared and chilled to -20°C. The packed erythrocytes were lysed by adding two volumes of distilled water. The hemolysate was centrifuged at 845 RPM for 5 min at 4°C to remove cell debris. The pellet was discarded and the supernatant was used for hemoglobin precipitation. The cold acidified solution (stored at -80°C) was taken in a beaker placed in ice bath and the hemolysate was added drop wise. The precipitated globin was separated from the heme by centrifugation at 3000 RPM at 4°C for 15 minutes. The precipitated globin was washed with cold acetone to remove any traces of the acid and then dissolved in water. The final volume was reduced to 2 mL by evaporating on rotary evaporator.

2.4 DIALYSIS

The purified globin was dialyzed for two hours in a dialysis bag using 0.1% acetic acid.

2.5 LYOPHILIZATION

The dialyzed globin was transferred to 100 mL round bottom flask and evaporated to about 5 mL on rotary evaporator. The flask containing globin solution was then immersed in liquid nitrogen with constant swirling so that a thin film of the globin was formed on the lining of the flask. The flask was then immediately connected to the freeze dryer. The lyophilized globin was stored at 4°C until used.

2.6 ELECTROPHORESIS

Electrophoresis of the purified chains were carried out under dissociating conditions using 12% polyacrylamide gel containing 8 M urea [96, 97] and a non-ionic detergent i.e. Triton X-100 [98, 99].

Stock Solutions

The following stock solution were prepared

Solution A (30.8%): 0.4 g bisacrylamide and 15.0 g Acrylamide were dissolved in distilled water and the volume made up to 50 mL. The solution was filtered and stored at 4°C.

Ammonium peroxodisulphate (10%): 0.1g APS was dissolved in distilled water and the volume made up to 1.0 mL

Triton X-100 (20%): 20 mL Triton X-100 was mixed with 80 mL distilled water.

Preparation of gel mixture

Gel was prepared by mixing the following:

Solution-A	10.0 mL
Urea	12 g
Triton X-100(20%)	2.5 mL
Glacial acetic acid	1.25 mL
TEMED	125 μ L
APS (10%)	500 μ L
Final volume	25 mL (with distilled water)

The clear solution was stirred for 1 minute, poured into the glass tubes and left overnight for polymerization.

Sample Preparation

Sample diluting buffer had the following composition:

Urea	4.85 g
Acetic acid (glacial)	1.0 mL
β -mercaptoethanol	1.0 mL
Final volume	10 mL (with distilled water)

Pre-electrophoresis was carried out at 160 V (constant) in 5% acetic acid for one hour. After one hour the electrode buffer was discarded and fresh buffer was added to gel tank. The globin (0.2 mg) was dissolved in sample diluting buffer. Electrophoresis was carried out in 5% acetic acid as electrode buffer. A constant potential of 140 V was applied for

three hours. Gels were stained with a solution of 1% amido black-10B in 7% acetic acid for 1 hour and destained in 7% acetic acid solution overnight.

2.7 SEPARATION OF THE GLOBIN CHAINS BY HPLC

The globin chains were separated on HPLC system (LC-20AT, Shimadzu- Japan), equipped with a diode array detector (SPD-M20A, Shimadzu- Japan) using C4 column (Vydac, Sigma). The lyophilised crude globin was dissolved in 0.1 % acetic acid solution (5mg/mL) and 100 μ L were loaded on column equilibrated with 0.1% Trifluoroacetic acid (TFA). Elution of protein was monitored at 280 nm, 215 nm and 254 nm. The program used for separation of the hemoglobin chains was as follows:

Time	ACN conc (%)
0	20
40	50
50	60
55	50
57	20
60	20

The separated globin chains were lyophilized and stored at 4.0C⁰ for further use.

2.8 LC-MS OF THE PURIFIED CHAINS

The mass spectra of the purified hemoglobin chains were measured on a QSTAR-XL using electrospray ionization technique (Applied Biosystems, USA). Positive ion mode was used to detect the globin chains molecular weight. The cone and capillary voltages were 55V and 3.0 kV respectively. Nitrogen gas was used for drying the solvent from the charged

droplets. The dried hemoglobin chains were dissolved in 200 μ L of 50% acetonitrile (ACN) ACN: H₂O mixture (1:1 v/v) containing 0.1% formic acid and 10 μ L of each globin chain solution were introduced through the electrospray interface with a syringe infusion at a flow rate of 50 μ L/min. The data was acquired using analyst 1.4.1 software over a mass range of 600-2000 amu (atomic mass units). The molecular mass of protein was generated from several multiply charged peaks using Bayesian Protein Reconstruct option in Bioanalyst 1.4 software. Mass calibration was performed using apomyoglobin (16951.3 Da) as external standard.

2.9 PERFORMIC ACID OXIDATION OF THE PURIFIED CHAINS

The purified α and β -chain were oxidized according to the method described by Hirs et al [100]. The oxidizing reagent (performic acid) was prepared by mixing formic acid and hydrogen peroxide in 9:1 ratio. The reagent was kept at room temperature for 2 hours and then cooled at 4°C for one hour. A known amount of the purified sample was dissolved in 500 μ L of the oxidizing reagent. The reaction was carried out for 30 min at 4°C. The oxidation reaction was stopped by diluting with 25mL distilled water. The reaction mixture was lyophilized and stored till further use.

2.10 TRYPTIC DIGESTION OF OXIDIZED GLOBIN CHAINS

A known amount of oxidized globin chains were dissolved in 200 μ L of 50 mM ammonium bicarbonate buffer, pH 9.5. The tosyl phenyl chloromethyl ketone-treated (TPCK) trypsin was added with an enzyme to substrate ration of 1:20. The enzyme was added in two steps.

Initially 50% of the enzyme was added to the substrate and incubated for 90 minutes at 37°. After 90 minutes the pH was adjusted to 8.5 with dilute acetic acid and the remaining 50% of the enzyme was added. The reaction mixture was again incubated for 90 minutes at

37°C. After completion of the reaction the pH of the tryptic digest was adjusted to 4.5 with dilute acetic acid. The digestion mixture was vacuumed dried and stored at 4° till further use.

2.11 fractionation of tryptic peptides by high performance liquid chromatography (hplc)

the tryptic peptides were separated using HPLC system (LC-20AT, Shimadzu- Japan), equipped with a diode array detector (SPD-M20A, Shimadzu- Japan). Samples were injected by a sample injection valve (model 7725i from rheodyne) equipped with 500µl loop. Elution of protein was monitored at 280nm, 215 nm and 254nm.

The column was equilibrated with 0.1% aqueous TFA (solution A) and then 200µl of the tryptic digest was loaded on reverse phase C₁₈ column (Vydac, Biorad-USA). The program used for peptide separation was as follows:

Acetonitrile (solution B): 10-40%B for 40 min, 40-60%B for 10min, 60-60% B for 10 min at a flow rate of 1ml/minute. All the eluting peaks were collected in separate 1mL microfuge tubes and vacuum dried.

2.12 MASS SPECTROMETRY (LC-MS) OF THE FRACTIONATED TRYPTIC PETIDES

The dried tryptic peptides of the globin chains were dissolved in 100µL ACN:H₂O mixture (1:1 v/v) containing 0.1% formic acid and 10µL of each globin chain solution were introduced through the electrospray interface with a syringe infusion at a flow rate of 50µL/min. The data was acquired using Analyst 1.4.1 software over a mass range of 300-3000 amu (atomic mass units).

2.13 SEQUENCING OF THE TRYPTIC PEPTIDES BY PROTEIN SEQUENCER

Amino acid sequencing of the tryptic peptides from hemoglobin alpha and beta chains was carried out by automated gas sequencer (Procise 491, Applied Biosystems, USA) equipped with an on line detector (785A, Perkin Elmer, USA).

2.14 HOMOLOGY MODEL BUILDING

The three dimensional structure predictions for common crane HbA involved the following steps.

- Template selection and sequence alignment
- Homology modeling
- Model evaluation
- Model analysis

2.14.1 Sequence Retrieval and Alignment

The BLAST [101] program against Protein Data Bank (PDB) available at NCBI (National Center for Biotechnology Information) [102] was used to select a template structure for homology modeling of common crane HbA. The sequences of the template models were aligned with the target sequences using the command align 2D of the program modeller.

2.14.2 Homology Model Building

Automated homology model of common crane was constructed by using MODELLER version 9v5 [103]. Modeler builds 3D protein structure by satisfaction of special restraints [104]. The input requirements for modeler were alignment file of the template and target

sequences, the template file in PDB format and the python command file. The coordinates of the crystal structures of Bar-headed goose oxyhemoglobin (PDB=1A4F; determined at resolution 2.0 Å) [105], Bar-headed goose deoxyhemoglobin (PDB code=1HV4; determined at resolution 2.8 Å) [106] and the crystal structure of partially oxygenated human hemoglobin (PDB code=1NIH) [107] were used as templates for building the 3D models of oxy-, deoxy and IPP liganded hemoglobins of common crane respectively.

The MODELLER program was given the task of making twenty models each for oxy-hemoglobin, deoxy-hemoglobin and IHP liganded hemoglobin. The best model for all the oxy, deoxy and liganded hemoglobin was selected through model evaluation.

2.14.3 Model Evaluation

To select the best model generated by the program MODELLER, programs PROCHECK and PROSA were used.

PROCHECK examines the stereochemical quality of the model [108]. It produces a number of postscript files analyzing overall and residue by residue geometry of the model. The checks performed by PROCHECK are covalent geometry, planarity, dihedral angles, chirality, non-bonded interactions, main-chain hydrogen bonds, disulphide bonds, stereochemical parameters and residue-by-residue analysis. The predicted model files of the common crane hemoglobin containing its coordinates were used as input to the PROCHECK program.

PROSA [109] generates energy profile arising from the C β -C β pair interactions and C β surface interactions which identify strained and high energy regions in predicted 3D models. The program is based on energy potentials extracted from databases. A model with

a minimum energy value is considered to be a good model. The input for PROSA software is the atomic coordinates of the model to be evaluated. The output of the PROSA is:

1. The Z-score

The z-score indicates overall model quality. Its value is displayed in a plot that contains the z-scores of all experimentally determined protein chains in PDB database.

2. The plot of residue scores.

This plot shows local model quality by plotting energies as a function of amino acid sequence position i.e. positive values correspond to erroneous parts of the input structure.

2.14.4 Model Analysis

The following software were used for common crane 3D hemoglobin model analysis

1. LIGPLOT: The LIGPLOT software was used for studying the interaction of ligands in HbA with surrounding residues [110]. This software automatically generates schematic 2-D representations of protein-ligand complexes from standard Protein Data Bank file input. LIGPLOT is available free to academic users and was downloaded from anonymous ftp (<ftp.biochem.ucl.ac.uk>).
2. MOLECULAR VISUALIZERS: Two visualizers i.e. the discovery studio visualizer (DS viewer: <http://accelrys.com>) and spdb viewer [111, 112] were used for analyzing the common crane HbA models.

3. 3D model superposition: For comparison the 3D structures of template were superimposed on the common crane HbA models using the Zhang frame of reference [105].

2.15 MULTIPLE SEQUENCE ALIGNMENT AND PHYLOGENETIC ANALYSIS

All alpha and beta sequences available to date were retrieved from swiss-prot/TrEMBL database using the SIB BLAST Network Service [113].

The sequences were manually arranged into different groups according the birds orders and saved in FASTA format. Multiple sequence alignment of the all aves alpha and beta sequences separately was carried out on clustalW embedded in BioEdit with default parameters. The software MEGA4 [114, 115] was used for phylogenetic tree construction.

3.1 ELECTROPHORESIS

The electrophoresis of the crude hemoglobin under denaturing conditions revealed three components identified as α^A , α^D and β [Fig.3.1].

3.2 SEPARATION OF GLOBIN CHAINS BY RP-HPLC

The crude hemoglobin of common crane was separated by HPLC using C4 column [Fig 3.2]. The corresponding peaks from several HPLC runs were pooled to collect enough amounts of globin chains for further processes. The purity of the each collected peak was also confirmed by urea triton gel electrophoresis and each peak showed a single band on the gel [Fig.3.3-4]. The separated peaks were analyzed on LC-MS and α^A , α^D and β chains were identified.

3.3 LC-MS OF PURIFIED GLOBIN CHAINS

The purified globin chains were identified on LC-MS as α^A , α^D and β with masses 15.303 KDa, 15.8790 KDa and 16.304 KDa respectively [Fig. 3.6-8].

3.4 SEPARATION OF TRYPTIC PEPTIDE BY HPLC

The purified globin chains were digested by trypsin and separated by HPLC using C₁₈ column. The HPLC chromatograms of tryptic peptide digestion mixture of chain A and chain beta are shown in [Fig 3.9 and 3.10] respectively. Fractions were collected 1 mL microfuge tubes and dried in a speed vac. Each HPLC fraction after drying was suspended in 20 μ L 0.1% TFA and analyzed on LC-MS [Table 3.1-2]. After LC-MS analysis suitable peptides were selected for direct Edman sequencing.

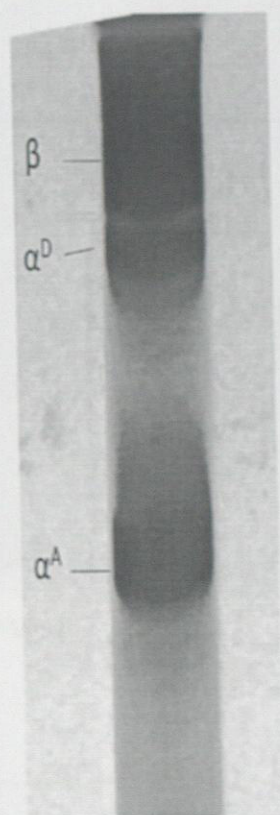


Fig.3.1 Electrophoretic pattern of crude hemoglobin from common crane (*Grus grus*) on polyacrylamide under dissociative conditions

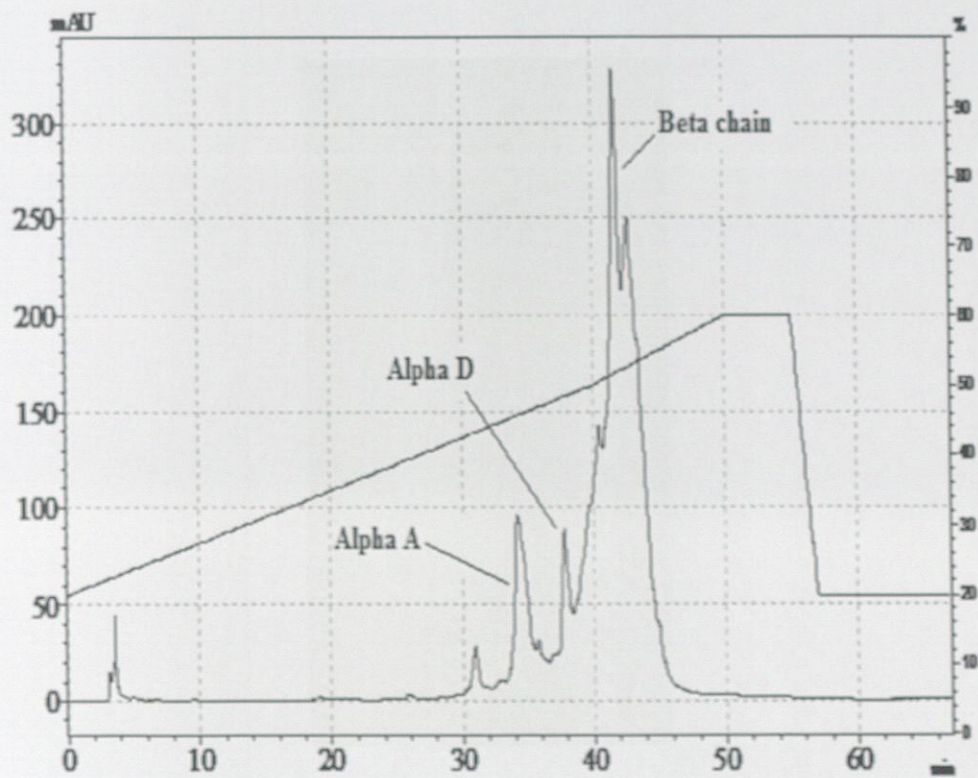


Fig.3.2 Separation profile of common crane (*Grus grus*) globin chains on HPLC C4 column

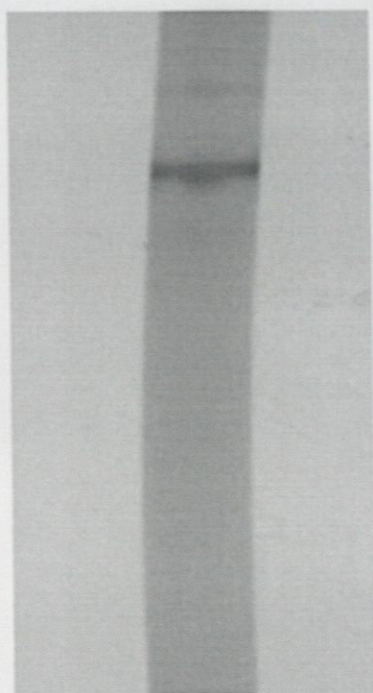


Fig.3.3 Triton urea gel electrophoresis of purified hemoglobin chain B from common crane (*Grus grus*)

Fig.3.4 Triton urea gel electrophoresis of purified hemoglobin chain B plus 20 mM sodium acetate

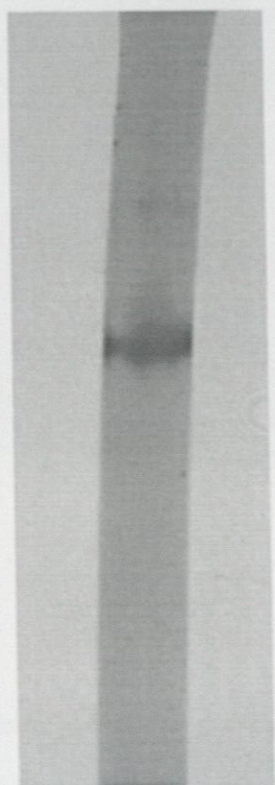


Fig. 3.4 Triton Urea Gel Electrophoresis of purified hemoglobin chain Alpha D from common crane.

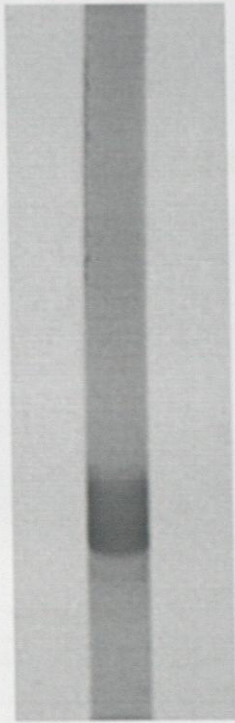


Fig.3.5 Triton urea gel electrophoresis of purified hemoglobin chain alpha A from common crane (*Grus grus*)

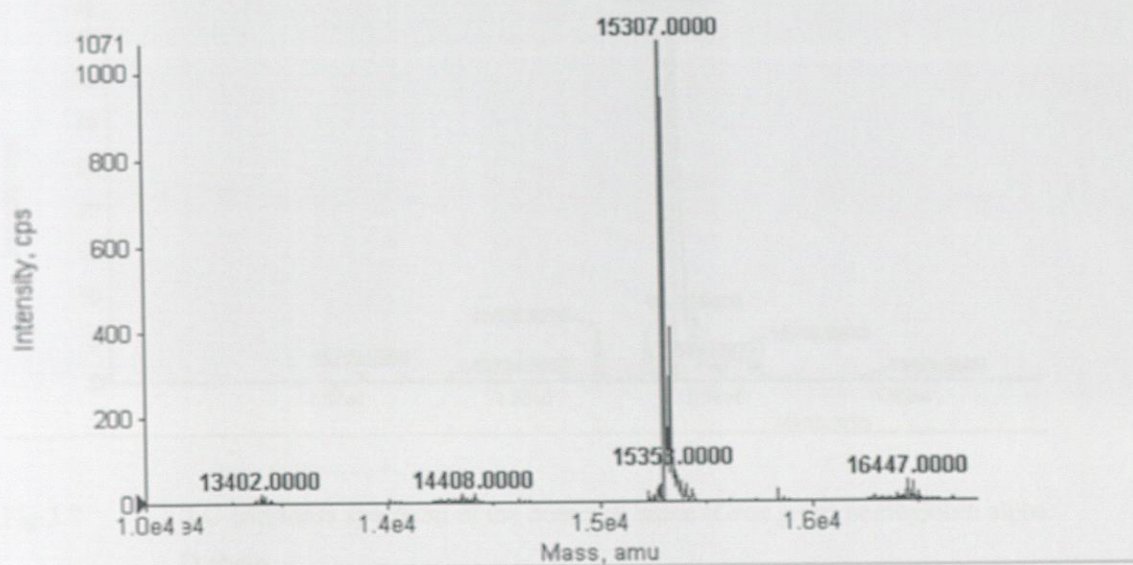


Fig.3.6 LC-MS mass spectrum of the common crane (*Grus grus*) hemoglobin alpha A chain

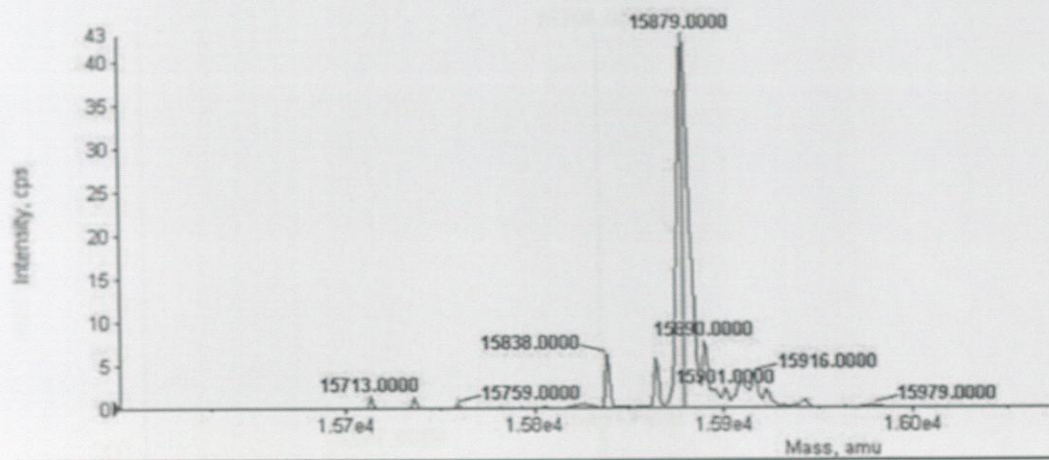


Fig.3.7 LC-MS mass spectrum of the common crane (*Grus grus*) hemoglobin alpha D chain

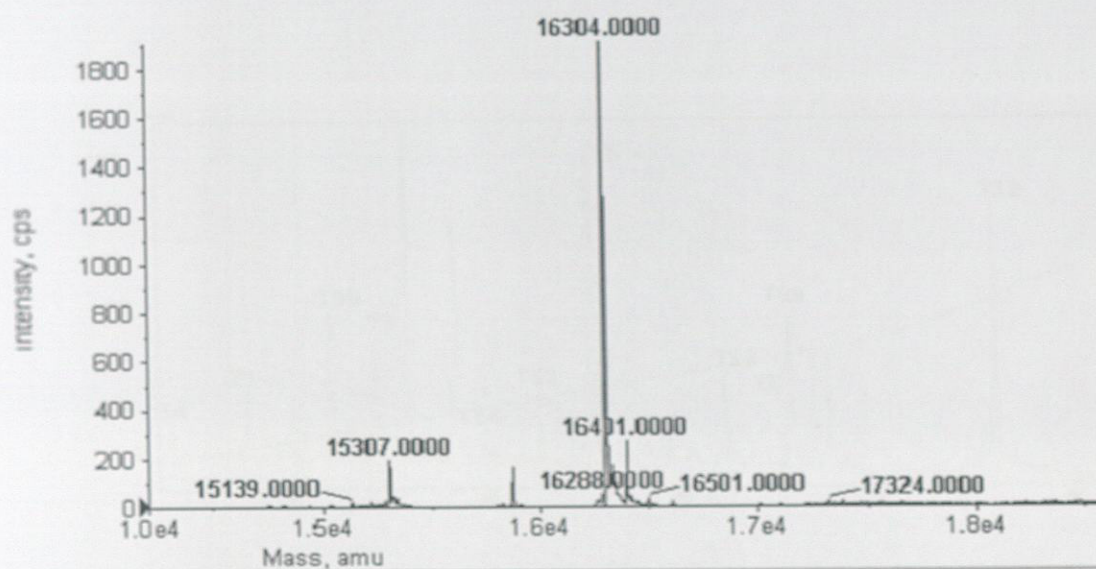


Fig.3.8 LC-MS mass spectrum of the common crane (*Grus grus*) Hemoglobin beta chain

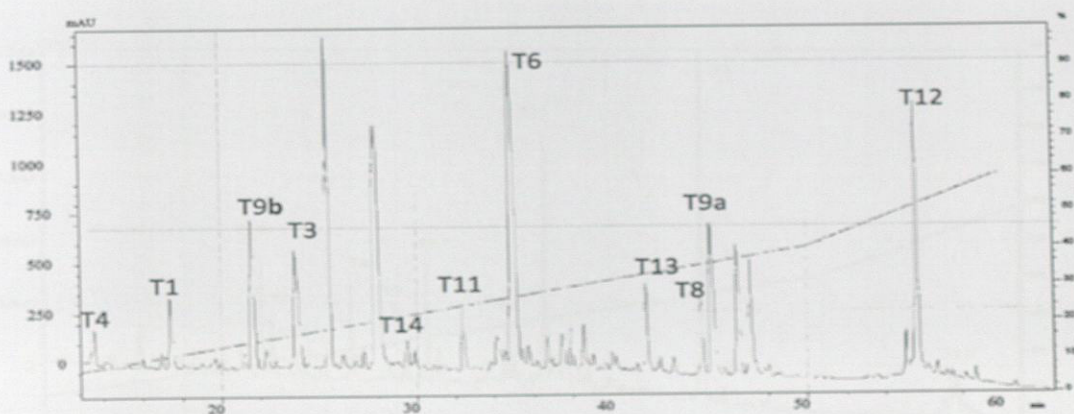


Fig.3.9 Separation profile of common crane (*Grus grus*) alpha A chain tryptic digest on C18 column

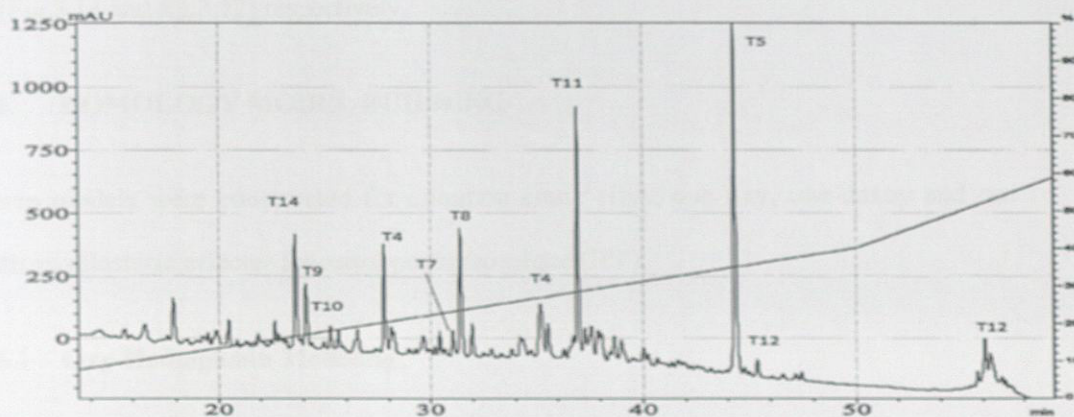


Fig3.10 Separation profile of common crane beta chain tryptic digest on C18 column

3.5 AUTOMATIC SEQUENCE ANALYSIS

Amino acid sequencing of α^A and β -chains was carried out by analysis of enzymatic cleavage products and arranging the peptide by homology with other avian hemoglobin sequences. The sequencing was carried out by automated gas sequencer (Procise 491, Applied Biosystems, USA) equipped with an online detector (785A, Perkin Elmer, USA).

The complete amino acid sequence of the two hemoglobin chains α^A and β -chains is shown in [Fig.3.11 and fig.3.12] respectively.

3.6 HOMOLOGY MODEL BUILDING

Three models were constructed for common crane HbA, one oxy, one dexoy and one with an allosteric effector Ionositol pentaphosphate (IPP).

3.6.1 Oxy-Hemoglobin Modeling

The coordinates of the crystal structure of Bar-headed goose oxyhemoglobin (PDB=1A4F) determined at resolution 2.0Å [105] was used as a template.

The common crane α^A -and β -chains showed 88% sequence identity with the corresponding sequences of the Bar-headed goose HbA. [Fig. 3.13] shows the three dimensional structure of the common crane oxyhemoglobin model as α^A -and β -dimer. The model consists of 287 amino acid residues with two heme molecules.

3.6.2 Deoxy-Hemoglobin Modeling

The 3D model for deoxyhemoglobin was constructed taking the coordinates of the deoxy tetramer hemoglobin of Bar-headed goose as template (PDB code=1HV4) determined at

resolution 2.8 Å [106]. Fig.3.14 shows the three dimensional deoxyhemoglobin model of common crane $\alpha_2\beta_2$ tetramer.

3.6.3 IPP-Liganded Hemoglobin Modelling

The crystal structure of human partially oxygenated hemoglobin (PDB code=1NIH) [107] was used as a template for modeling the 3D structure of common crane Hb with IPP ligand attached. The 3D model of partially oxygenated hemoglobin from common crane HbA is shown in [Fig.3.15].

3.7 HOMOLOGY MODELS EVALUATION

The stereo-chemical and structural evaluation of the homology models of HbA of common crane hemoglobin was carried out with PROCHECK. The stereo chemical evaluation of both oxy and deoxy models shows that all the parameters implicated in PROCHECK have been satisfied.

3.7.1 PROCHECK Of Oxy HbA

The Ramachandran plot of common crane oxy HbA shows a good distribution for the psi/phi angles of the 287 amino acid residues of dimeric form of HbA of common crane, as shown in [Fig.3.16]. 94.9% residues occupy the most favored and 4.7% residues are present in the additionally allowed regions. Only one residue $\alpha 119\text{Ala}$ lies in the generously allowed region of the Ramachandran plot. The same residue is also present in the generously allowed region in the crystal structure of Bar-headed goose (PDB id= 1A4F).

```

      10      20      30      40
1  ....|....| ....|....| ....|....| ....|....|
   VLSGNDKSNV KGVFSKIGGH AEEYGAETLE RMFCTYPQTK 40

      50      60      70      80
41 ....|....| ....|....| ....|....| ....|....|
   TYFPHFDLQH GSAQVKAHGK KVAGALIEAA NHIDDIAGAL 80

      90     100     110     120
81 ....|....| ....|....| ....|....| ....|....|
   SKLSDLHAQK LRVDPVNFKL LGQCFLVVVA IHHPSVLTPE 120

      130     140
121 ....|....| ....|....| .
     VHASLDKFLC AVGNVLTAKY R 141

```

Fig.3.11 Amino acid sequence of the common crane hemoglobin alpha A chain

```

      10      20      30      40
1  ....|....| ....|....| ....|....| ....|....|
   VHWSAEEKQL ITSLWGKVVN ADCGAEALAR LLIVYPWTQR 40

      50      60      70      80
41 ....|....| ....|....| ....|....| ....|....|
   FFSAFGNLSS PTAIIGNPMV RAHGKKVLTS FGEAVKNLDN 80

      90     100     110     120
81 ....|....| ....|....| ....|....| ....|....|
   IKNTFAQLSE LHCDKLHVDP ENFRLLGDIL IIVLAAHFAK 120

      130     140
121 ....|....| ....|....| ....|.
     DFTPDCAAW QKLVRVVAHA LARKYH 146

```

Fig.3.12 Amino acid sequence of the common crane hemoglobin beta chain



Fig.3.13 Homology model of common crane oxy HbA: The globin is shown as solid ribbons while the heme groups are shown as sticks. The image was taken by discovery studio (DS) visualiser.

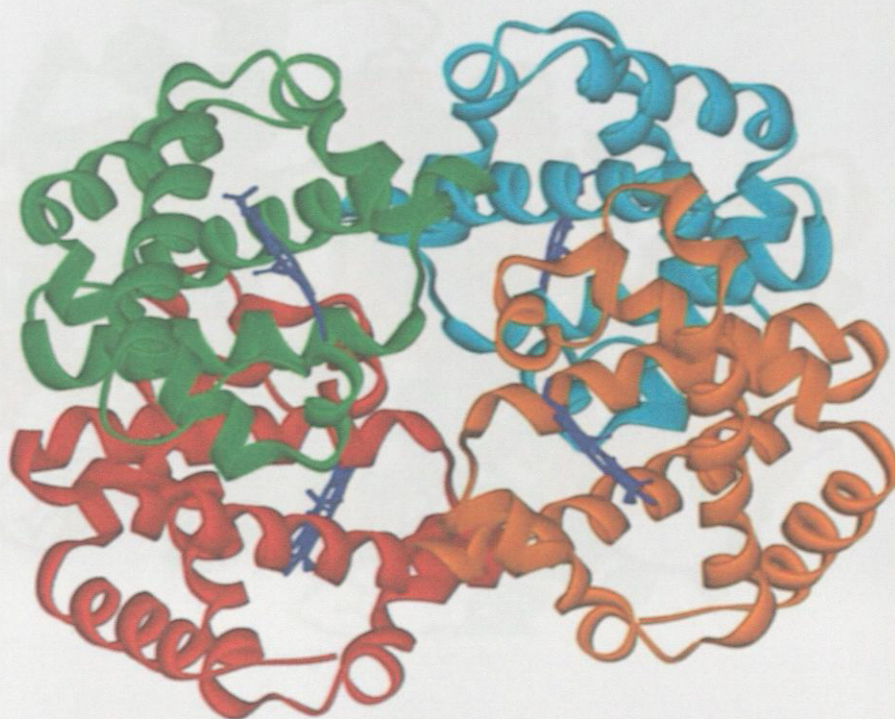


Fig.3.14 Homology model of common crane deoxy HbA tetramer: The globin is shown as solid ribbons while the heme groups are shown as sticks. The image was taken by discovery studio visualiser.

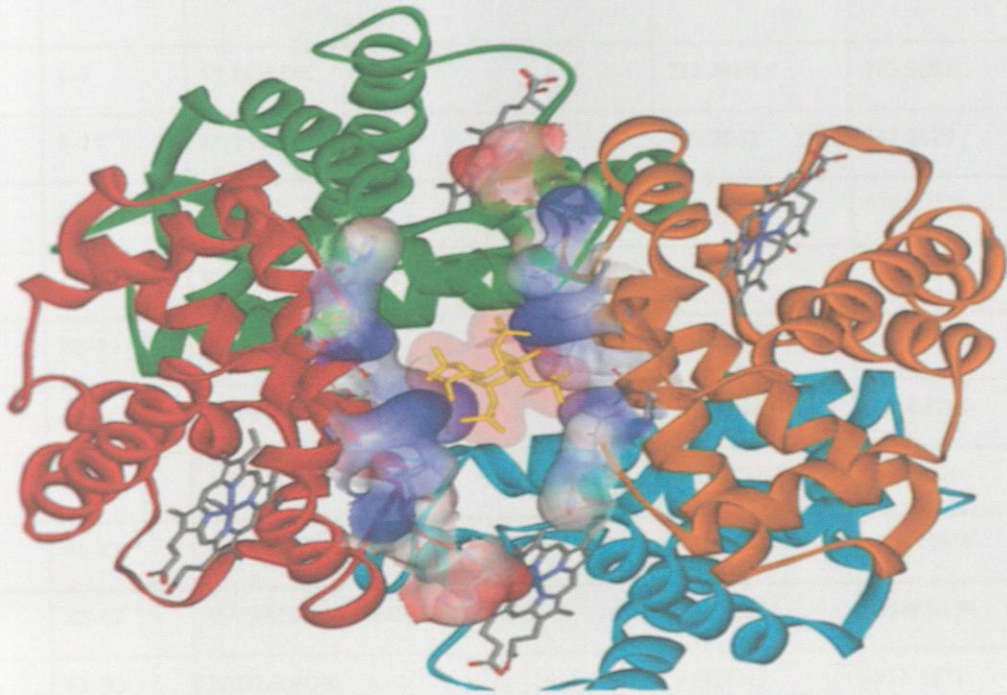


Fig.3.15 Homology model of common crane HbA tetramer with IHP ligand: The globin is shown as solid ribbons while the heme and IHP ligands are shown as sticks. The IHP and its contacting residues are enclosed in Van der Waals semitransparent surfaces. The image was taken by DS viewer.

Table 3.1 ESI-MS of tryptic peptides of α^A -Chain from common crane (*Grus grus*)

Peptide No	Residue No	Sequence	Mass Calculated	Mass Observed
T1	1-7	VLSGNDK	732.3886	732.3253
T2	8-11	SNVK	447.2562	447.2429
T3	12-16	GVFSK	537.3031	537.2929
T4	17-31	IGGHAEYGAETLER	1631.7659	1630.7211
T5	32-40	MFCTYPQTK	1118.5009	1118.5420
T6	41-56	TYFPFIDLQHGSAQVK	1874.9184	1874.8780
T7	57-61	AHGKK	540.3253	540.4120
T8	61-82*	KVAGALIEAANHIDDIAGALSK	2177.1924	2177.1516
T9	62-82	VAGALIEAANHIDDIAGALSK	2049.0974	2049.0974
T10	83-90	LSDLHAQK	911.4945	911.4871
T11	91-99	LRVDPVNFK	1087.6250	1087.2518
T12	100-127*	LLGQCFLVVVAIHHPVLTPEVHASLDK(ox)	3022.6546	3070.591
T13	100-139	LLGQCFLVVVAIHHPVLTPEVHASLDK FLCAVGNVLTAK(ox)	4239.3184	4240.1420
T14	128-139	FLCAVGNVLTAK(Ox)	1283.6816	1283.6511
T15	140-141	YR	338.1823	338.1128

Table 3.2 ESI-MS of tryptic peptides of β -Chain from common crane (*Grus grus*)

Peptide No	Residue No	Sequence	Mass Calculated	Mass Observed
T1	1-8	VHWSAEEK	985.4738	985.4466
T2	9-17	QLITSLWGK	1045.6041	1045.6001
T3	18-30	VNVADCGAEALAR	1288.74 12	1288.7411
T4	31-40	LLIVYPWTQR (ox)	1336.6298	1336.5147
T5	41-61	FFSAFGNLSSPTAIIGNPMVR _(ox)	2242.1325	2258.4701
T6	62-66	AHGKK	540.3253	540.3253
T7	66-76	KVLTSFGGEAVK	1178.6780	1177.6789
T8	67-76	VLTSFGGEAVK	1050.5830	1050.1278
T9	77-82	NLDNIK	716.3937	716.3879
T10	83-95	NTFAQLSELHCDK (ox)	1505.7053	1553.6146
T11	83-104	NTFAQLSELHCDKLHVDPENFR	2661.8453	2661.2177
T12	105-120	LLGDILIIIVLAAHFAK	1707.0567	1707.0826
T13	105-135	LLGDILIIIVLAAHFAKDFTPDCAAWQKLVR	1758.45	1758.9980
T14	136-143	VVAHALAR	835.9401	835.4999
T15	144-146	KYH	447.2350	447.2355

3.7.2 PROCHECK Of Deoxy HbA

The Ramachandran plot of common crane deoxy HbA shows a good distribution for the psi/phi angles of the 570 amino acid residues of tetrameric form of HbA deoxy of common crane, as shown in [Fig.3.17]. 93.7 % residues occupy the most favored and 5.7% residues in the additionally allowed regions. Only 3 residues are present in the generously allowed regions of the Ramachandran plot. The three residues present in the generously allowed region are Leu α 2, Ala β 119 and Arg β 143. The same residues are present in the generously allowed region of the Bar-headed goose deoxy HbA (PDB ID =1HV4) Ramachandran plot.

3.7.3 ProSA Results

3.7.3.1 ProSA Z-score

The Z-score of a protein is defined as the energy separation between the native fold and the average of an ensemble of misfolds in the units of the standard deviation of the ensemble. The Z-score is often used as a way of testing the knowledge-based potentials for their ability to recognize the native fold from other alternatives [116].

The Z-score of the Common crane models is shown in [Fig. 3.18-21]. The Z-score for HbA oxy alpha chain was -5.63 and beta chain was -7.45. The Z-score values for the template alpha and beta chains are -5.29 and -7.31 respectively. Both of these values are within the expected allowed region of the Z-score plot of all the known X-ray structures available.

The Z-scores calculated for common crane deoxy hemoglobin model alpha and beta chains are -5.58 and -7.15 respectively. The Z-score of all the homology models of

common crane are within the range of scores typically found for native proteins of similar size.

3.7.3.2 ProSA plot of residue scores

The residue score plots for all the predicted 3D models of common crane hemoglobin are shown in [Fig.3.22-25]. To remove large amount of fluctuations the plots were smoothed a window size of 40. The residue score plots were superimposed on the corresponding template structures and it was observed that all the predicted models have pair potentials below zero.

3.8 MODEL SUPERIMPOSITION

The 3D models of the common crane oxy and deoxy hemoglobin were superimposed on corresponding crystal structure of human and Bar-headed goose oxy and deoxy hemoglobin respectively [Fig 3.26-27]. The Zhang frame of reference [105] was used for superimposing the 3D models. The Zhang frame of reference contains the following residues α 3 to 13, 31 to 42, 54 to 73, 80 to 84, 97 to 104, 117 to 136 and β 26 to 37, 62 to 68, 106 to 114 and 127 to 139, a total of 117 amino acid residues (76 in the alpha chain, 41 in the beta chain) and 468 atoms. The root mean square deviation (rms) deviations of main-chain atoms among the common crane hemoglobin, Bar-headed goose and human hemoglobin in the oxy and deoxy forms have been shown in Table 3.3.

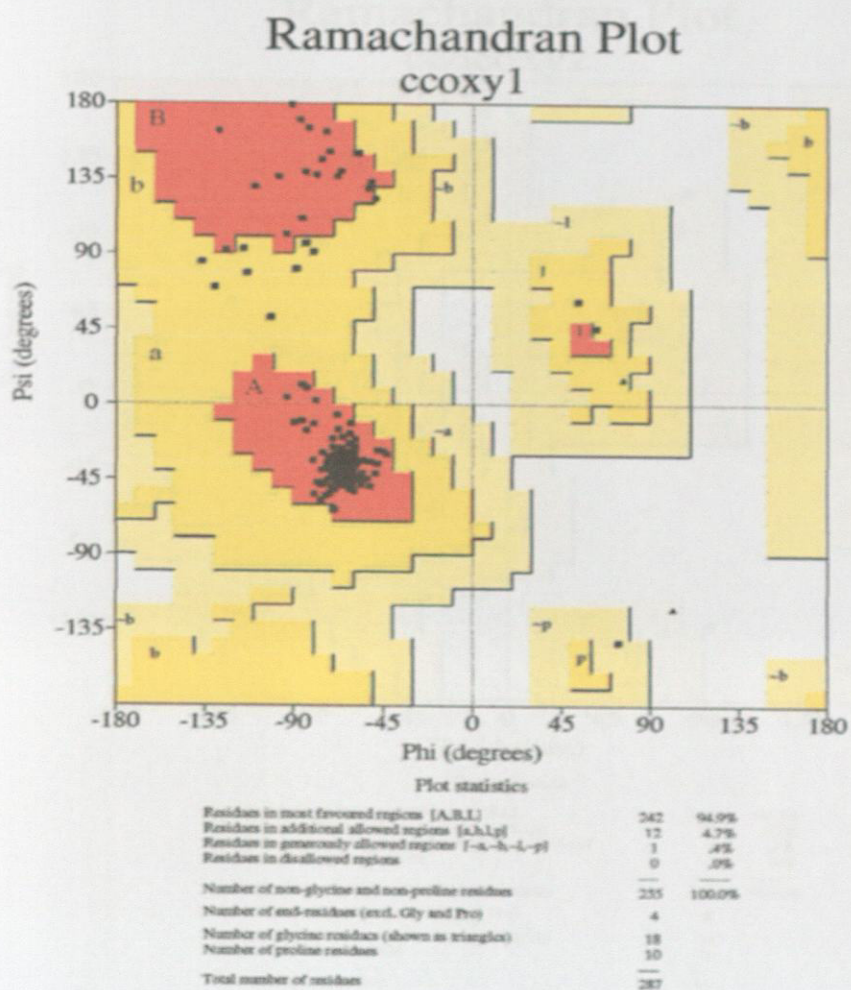


Fig.3.16 Ramachandran plot of the 3D homology model of common crane oxy HbA

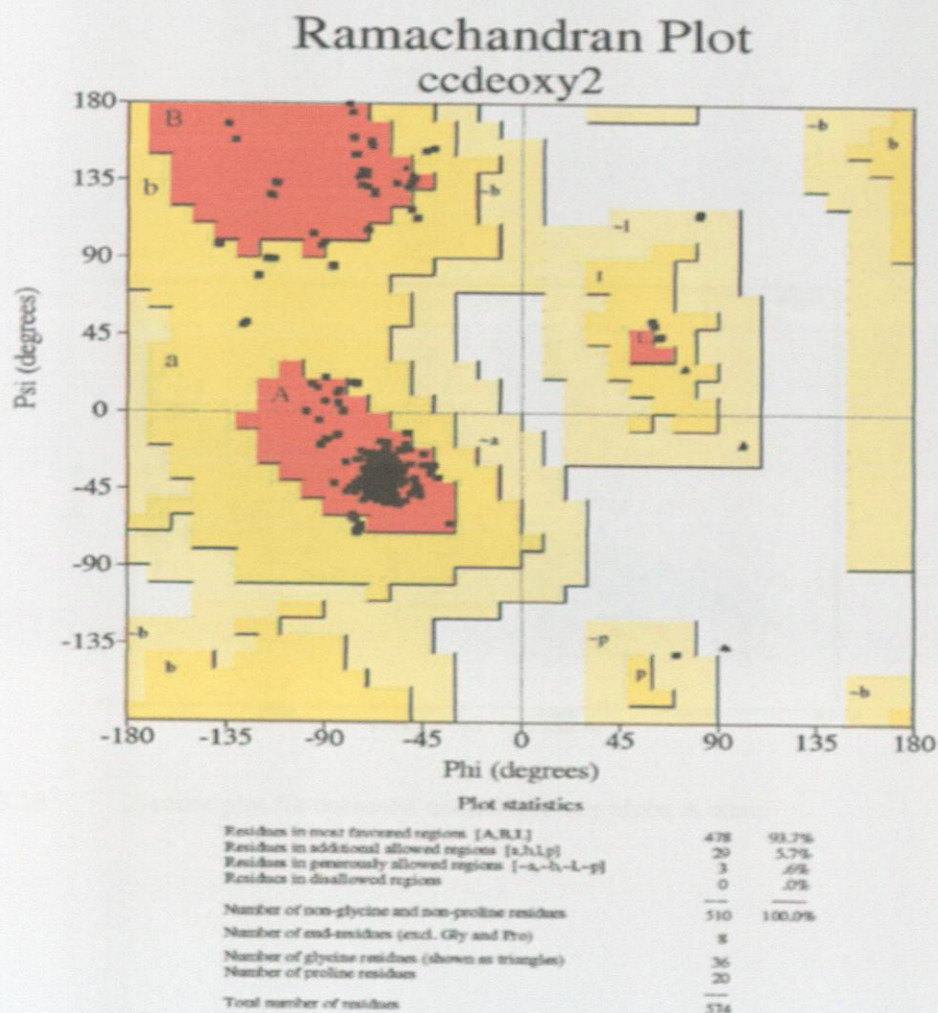


Fig.3.17 Ramachandran plot of the 3D homology model of common crane deoxy HbA tetramer

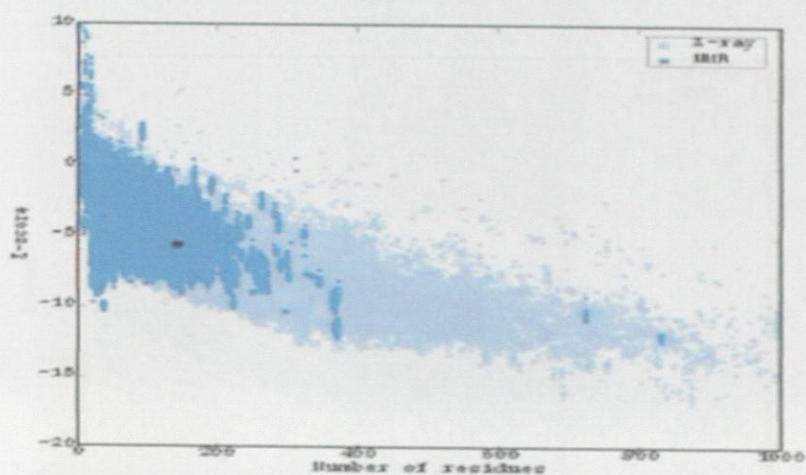


Fig.3.18 Z-score plot for common crane HbA oxy alpha A chain

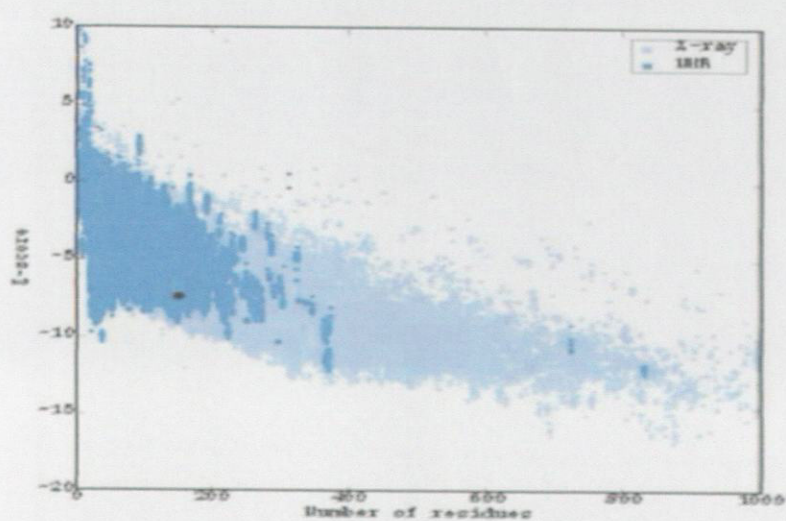


Fig.3.19 Z-score plot for common crane HbA oxy beta chain

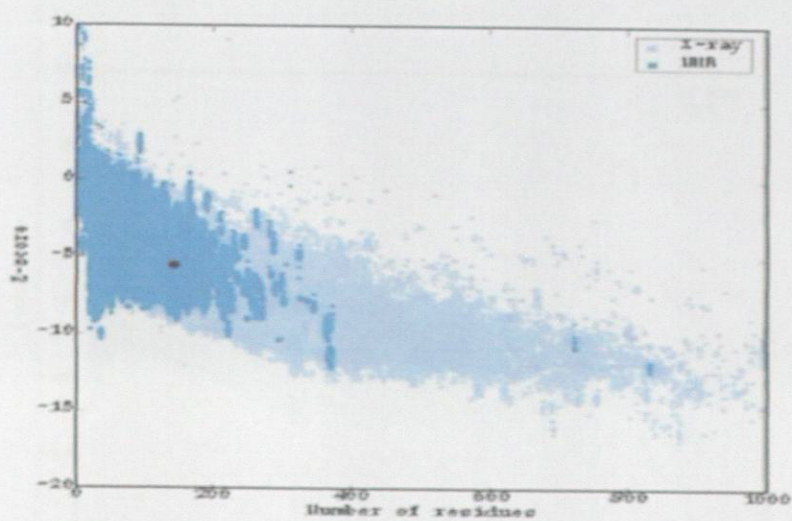


Fig.3.20 Z-score plot for common crane HbA deoxy alpha A chain

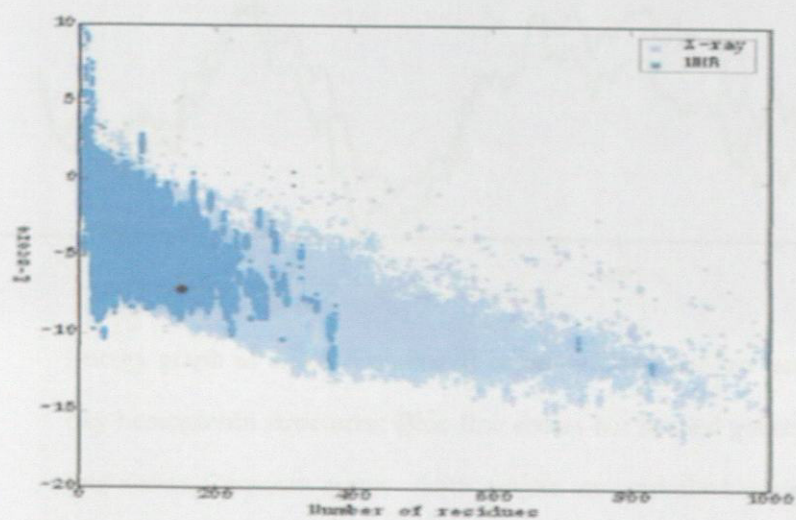


Fig.3.21 Z-score plot for common crane HbA deoxy beta chain

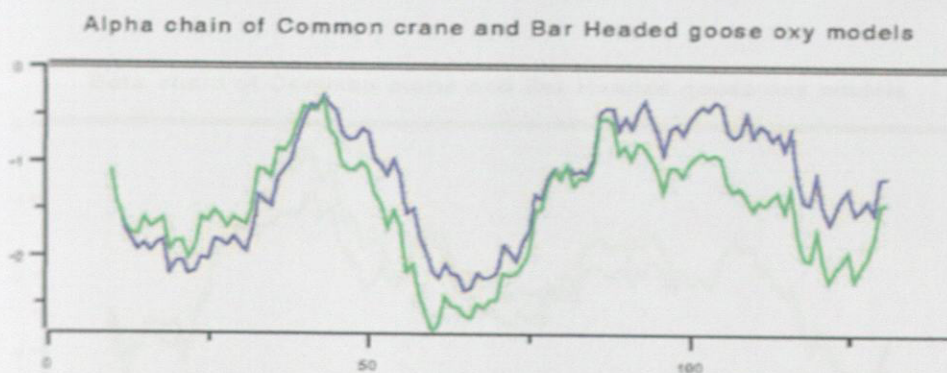


Fig.3.22 Energy graph of alpha A chains of common crane and bar headed Goose oxy hemoglobin structures: Blue line shows bar headed geese and green line shows Common crane. Both graphs are smoothed using a window size of 20 residues.

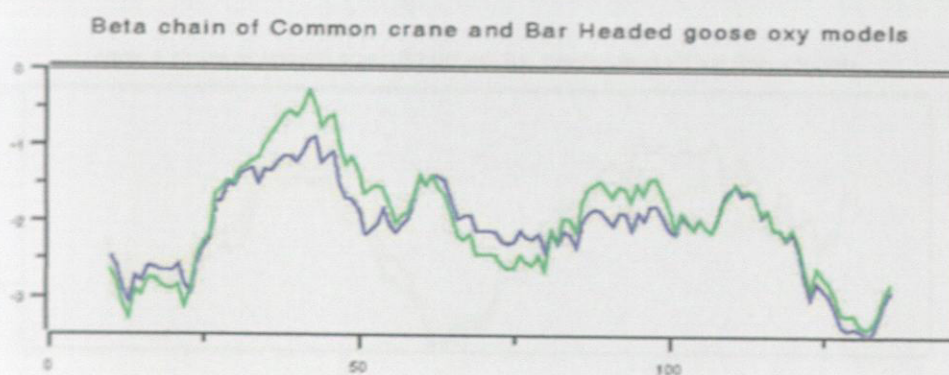


Fig.3.23 Energy graph of beta chains of common crane and bar headed goose oxy hemoglobin structures: Blue line shows bar headed geese and green line shows common crane. Both graphs are smoothed using a window size of 20 residues.

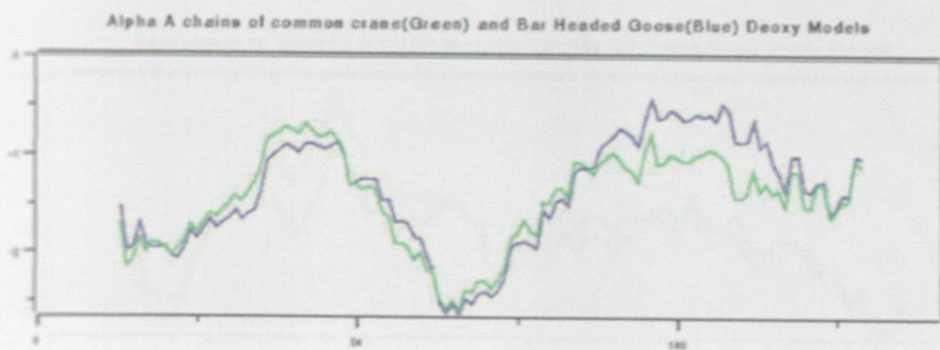


Fig.3.24 Energy graph of alpha A chains of common crane and bar headed goose deoxy hemoglobin structures: Blue line shows bar headed geese and green line shows common crane. Both graphs are smoothed using a window size of 20 residues.

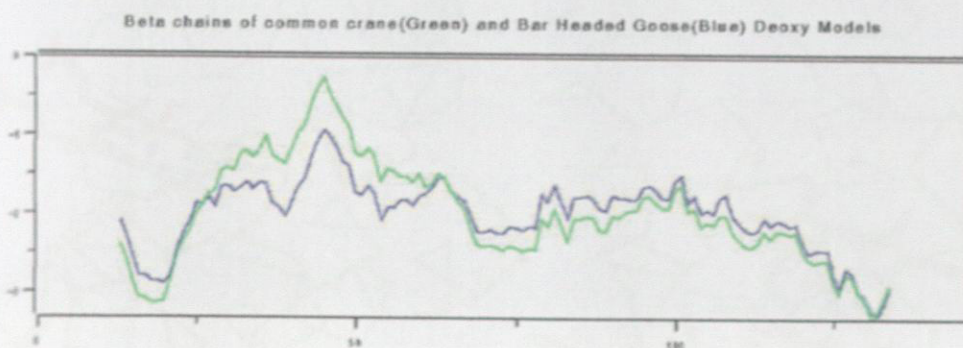


Fig.3.25 Energy graph of beta chains of common crane and bar headed goose deoxy hemoglobin structures: Blue line shows bar headed geese and green line shows common crane. Both graphs are smoothed using a window size of 20 residues.

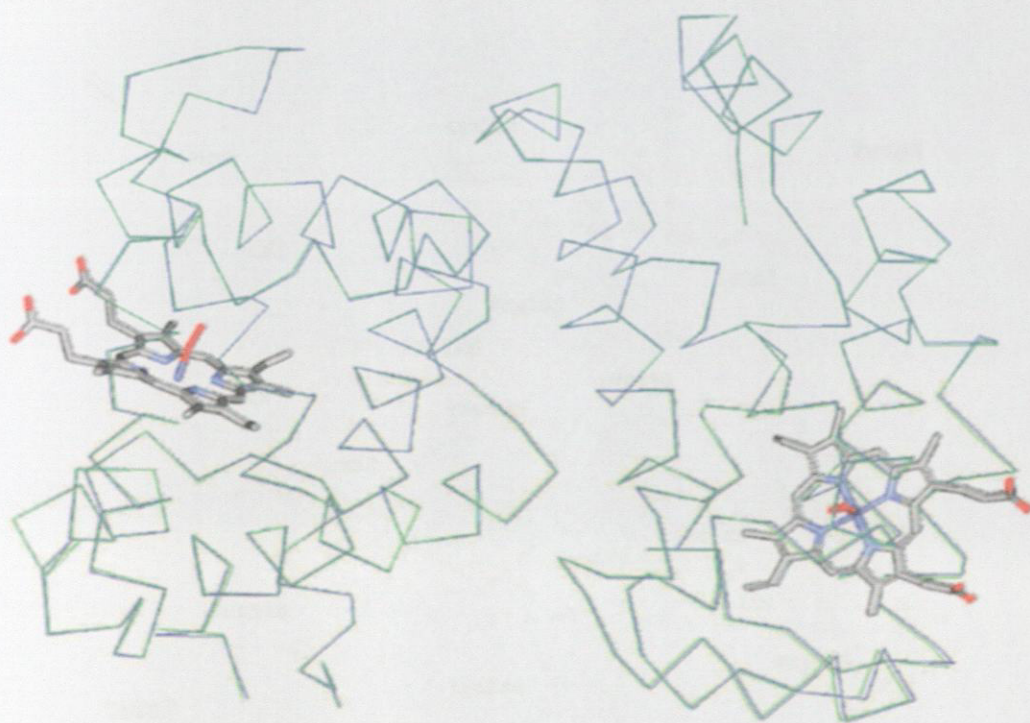


Fig.3.26 The common crane HbA oxy superposed on the crystal structure coordinates of the bar headed goose oxy hemoglobin with Zhang frame of reference: The green color shows common crane HbA and the violet color shows the bar headed goose HbA.

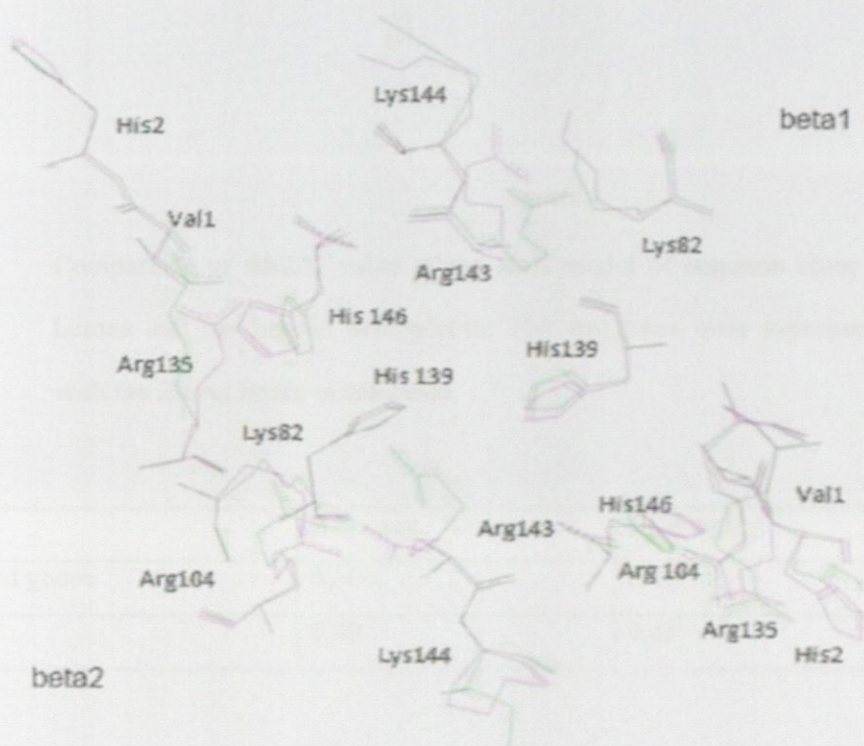


Fig.3.27 IPP binding site of common crane (*Grus grus*) deoxy HbA (violet) and bar headed goose deoxy HbA (green) superimposed with BGH frame.

Table.3.3 Comparison of RMSD value of the HbA model of common crane with human and bar headed hemoglobin: The structures were superimposed with the Zhang frame of reference.

Species	HbA oxy	HbA deoxy
Bar Headed goose	0.16	0.15
Human	0.49	0.65

DISCUSSION

4.1 HEMOGLOBIN COMPONENTS

The common crane has two hemoglobins HbA and HbD as revealed by the triton gel electrophoresis of crude globin. Both HbA and HbD have identical beta chains but different alpha chains [Fig. 3.1]. HbA is a major component having two alpha and two beta chains ($\alpha^A\beta_2$) in its tetrameric form while HbD is a minor component with two alpha D and two beta chains ($\alpha^D\beta_2$) in tetrameric form. HbA 85 to 90 % and HbD are from 10 to 15% composition estimated from gel band intensities. High performance liquid chromatography HPLC of the crude globin showed three major peaks [Fig. 3.2]. After pooling the corresponding fractions from several HPLC runs, they were analyzed by electrospray ionization mass spectrometry. The three peaks were identified as α^A -, α^D - and β -chains. The purity of the separated globin chains was confirmed by polyacrylamide gel electrophoresis in the presence of urea and triton X-100. The gels for individual chains showed clear single bands indicating the purity of the separated hemoglobin chains [Fig. 3.1, 3.3, 3.4 and 3.5]. Electrospray ionization mass spectrometry revealed a molecular mass of 15.3070, 15.8790 and 16.3040 KDa respectively for α^A , α^D and β -chain [Fig. 3.6-3.8]. Thus like many of the avian species the common crane hemoglobin is also heterogeneous. Other avian species known to have heterogeneous hemoglobins are *Anser Indicus* (anseriformes), *Cygnus olor* (anseriformes), *Meleagris gallopavo* (Galliformes), *Phasianus colchicus* (Galliformes), *Anseranas semipalmata* (anseriformes), *Apus apus* (anseriforme), etc. Avian species with single hemoglobin components are; *Columbia livia* (columbiformes), *Rock hopper Penguin* (sphenisciformes) etc.

4.2 SEQUENCE DETERMINATION OF THE ALPHA AND BETA GLOBIN CHAINS

The purified globin chains were oxidized by performic acid. The amino acids cysteine, methionine and tryptophan are oxidized during this process. The oxidized chains were digested with TCPK treated trypsin (Sigma). The tryptic peptides of the globin chains were separated on reverse phase HPLC C₁₈ column (Vydac, Sigma). The separated peptides of the individual chains were analyzed on Electrospray ionization mass spectrometry [Table 3.1-3.2].

The sequences of all peptides obtained from the tryptic digest of the performic acid oxidized polypeptide were determined completely by gas-phase sequencer. The complete primary structure of α^A and β -chains from common crane hemoglobin have been determined. The amino acid sequences of the hemoglobin alpha and beta chains are shown in Fig. 3.11 and 3.12.

4.3 ANALYSIS OF PRIMARY STRUCTURE OF α^A AND β -CHAINS FROM COMMON CRANE

The amino acid sequence of α^A -chain of Common crane hemoglobin shows 15 Ala (A), 3 Arg (R), 5Asn (N), 7Asp (D), 3Cys (C), 5Gln (Q), 6Glu (E), 11Gly (G), 9His (H), 5Ile (I), 15Leu (L), 12Lys (K), 1Met (M), 7Phe (F), 8Pro (P), 8Ser (S), 6Thr (T), 4Tyr (Y), 14Val (V) and no Trp (W). There are some differences shown by the α^A -chain of common crane at positions which are generally conserved in other avian alpha A chain. For example the amino acid α 8Ser, α 34Cys, α 55Val and α 64Gly are present in very few avian species. The α 34Cys is unique amino acids which is not present in any other avian hemoglobin alpha chain sequenced to date.

The theoretical pI of the α^A is 8.46 as calculated by Compute pI/Mw [117, 118] available online on Expasy tools server [102]. The total number of negatively charged residues in common crane α^A is 13 while total number of positively charged residues is 15. The percent composition of all amino acids is given in Table 4.1a.

The β -chain of Common crane contains 18Ala, 6Arg, 10Lys, 7Asn, 7asp, 3Cys and 4Trp. The total number of positively charged residues is 13 and the total number of negatively charged residues is 18. The theoretical pI for beta chain is 8.85 as calculated by Compute pI/Mw. The amino acid percent composition of beta chains is shown in Table 4.1b. There are 90 amino positions which are conserved in all avian hemoglobin beta chains sequenced so far. Similarly there are 69 amino acid positions conserved in all avian alpha A chains sequenced so far. Thus the beta chain seems to be more conserved than α^A chains.

4.4 THE OVERALL STRUCTURES OF HbA HOMOLGY MODEL

Three models were constructed for common crane HbA; one oxy, one dexoy and one with an allosteric effector IPP.

4.4.1 Oxy-Hemoglobin Modeling

The coordinates of the crystal structure of Bar headed goose oxy hemoglobin (PDB=1A4F) determined at resolution 2.0Å [105] was used as a template. The common crane α^A - and β -chains showed 32 and 54% sequence identity with the corresponding sequences of the Bar headed goose HbA. Fig. 3.13 shows the three dimensional structure of the common crane oxy hemoglobin model as α^A - and β dimer. The model consists of 287 amino acid residues with two heme molecules.

4.4.2 Deoxy-Hemoglobin Modeling

The 3D model for deoxy hemoglobin was constructed taking the coordinates of the deoxy tetramer hemoglobin of Bar headed goose as template (PDB code=1HV4) [106].

The overall topology of the 3D model is similar to the Bar headed goose hemoglobin. The model was evaluated by PROCHECK and PROSA and all parameters were found to validate the model. The alpha helical content of the model is identical to the human and Bar headed goose hemoglobin.

4.4.3 The Heme Pocket

The heme pocket of common crane HbA is very similar to the heme pocket of Bar headed goose and Human Hb. In both the models the equivalent residues overlap closely and are well-conserved. Fig. 4.1-4 shows the heme pockets of common crane Hb. There is no mutation in the heme vicinity which could change the oxygen affinity. The hydrophobic residues making the heme binding pocket are Val132, Leu101, Phe98, Leu91, Val62 and Phe43 of alpha chain. The amino acid residues Val98, Val67, Phe42 and Phe41 make up the hydrophobic heme pocket of β chain. The distal His residues which coordinate to the iron atom (Fe) of the heme are conserved in both alpha and beta chain. In alpha chain the proximal His87 is ligated to the iron of the heme. In beta chain the proximal His92 is ligated to the iron of the heme. The distal residues in contact with the ligand i.e. E7His and E11Val of common crane hemoglobin have very similar conformations and environments as has been observed in human hemoglobin. The geometry of the heme groups for the 3D of common crane hemoglobin A, bar-headed goose oxy-hemoglobin A (184) and human oxy-hemoglobin (13) is shown in Table-4.2.

Table 4.1a Amino acid percent composition of common crane alpha A chain

Amino acid three letter code	One letter code	Total number of amino acids	Percent composition
Ala	(A)	15	10.60%
Arg	(R)	3	2.10%
Asn	(N)	5	3.50%
Asp	(D)	7	5.00%
Cys	(C)	3	2.10%
Gln	(Q)	5	3.50%
Glu	(E)	6	4.30%
Gly	(G)	11	7.80%
His	(H)	9	6.40%
Ile	(I)	5	3.50%
Leu	(L)	15	10.60%
Lys	(K)	12	8.50%
Met	(M)	1	0.70%
Phe	(F)	7	5.00%
Pro	(P)	5	3.50%
Ser	(S)	8	5.70%
Thr	(T)	6	4.30%
Trp	(W)	0	0.00%
Tyr	(Y)	4	2.80%
Val	(V)	14	9.90%
Pyl	(O)	0	0.00%
Sec	(U)	0	0.00%

Table 4.1b Amino acid composition of common beta chain

Amino acid three letter code	One letter code	Total number of amino acids	Percent composition
Ala	(A)	18	12.30%
Arg	(R)	6	4.10%
Asn	(N)	7	4.80%
Asp	(D)	7	4.80%
Cys	(C)	3	2.10%
Gln	(Q)	5	3.40%
Glu	(E)	6	4.10%
Gly	(G)	7	4.80%
His	(H)	7	4.80%
Ile	(I)	8	5.50%
Leu	(L)	17	11.60%
Lys	(K)	10	6.80%
Met	(M)	1	0.70%
Phe	(F)	8	5.50%
Pro	(P)	5	3.40%
Ser	(S)	7	4.80%
Thr	(T)	6	4.10%
Trp	(W)	4	2.70%
Tyr	(Y)	2	1.40%
Val	(V)	12	8.20%
Pyl	(O)	0	0.00%
Sec	(U)	0	0.00%

Table 4.2 The geometry of heme groups from common crane homology models of hemoglobin A, bar-headed goose oxy-hemoglobin A, and human oxy-hemoglobin.

Distance	Common crane		Bar headed goose		Human	
	α^A	β	α^A	β	α^A	β
Fe-O distance (Å)	1.933	1.890	1.78	1.81	1.66	1.86
Fe-O-O angle (°)	164.6	155.7	170.2	158.7	152.7	158.7
His F8 N ^e -Fe distance (Å)	2.22	2.22	2.10	2.07	1.94	2.07
His F7 N ^e -Fe distance (Å)	2.74	2.73	2.53	2.57	2.68	3.49
Val E11C ^{r2} Ne-Fe distance (Å)	3.24	3.29	3.22	3.25	3.25	3.36

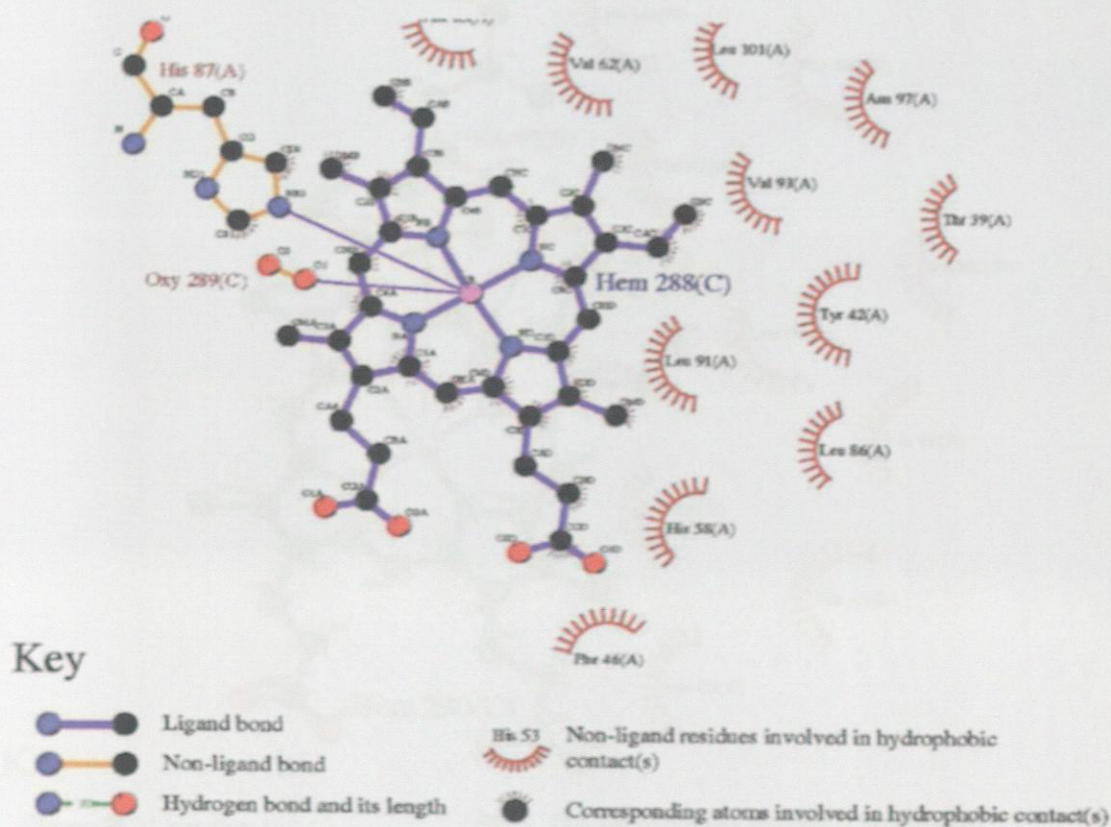


Fig.4.1 The heme binding pocket of common crane oxy HbA model chain A: The figure was generated by Ligplot. The heme binding residues have been flattened by Ligplot to get a 2D picture.

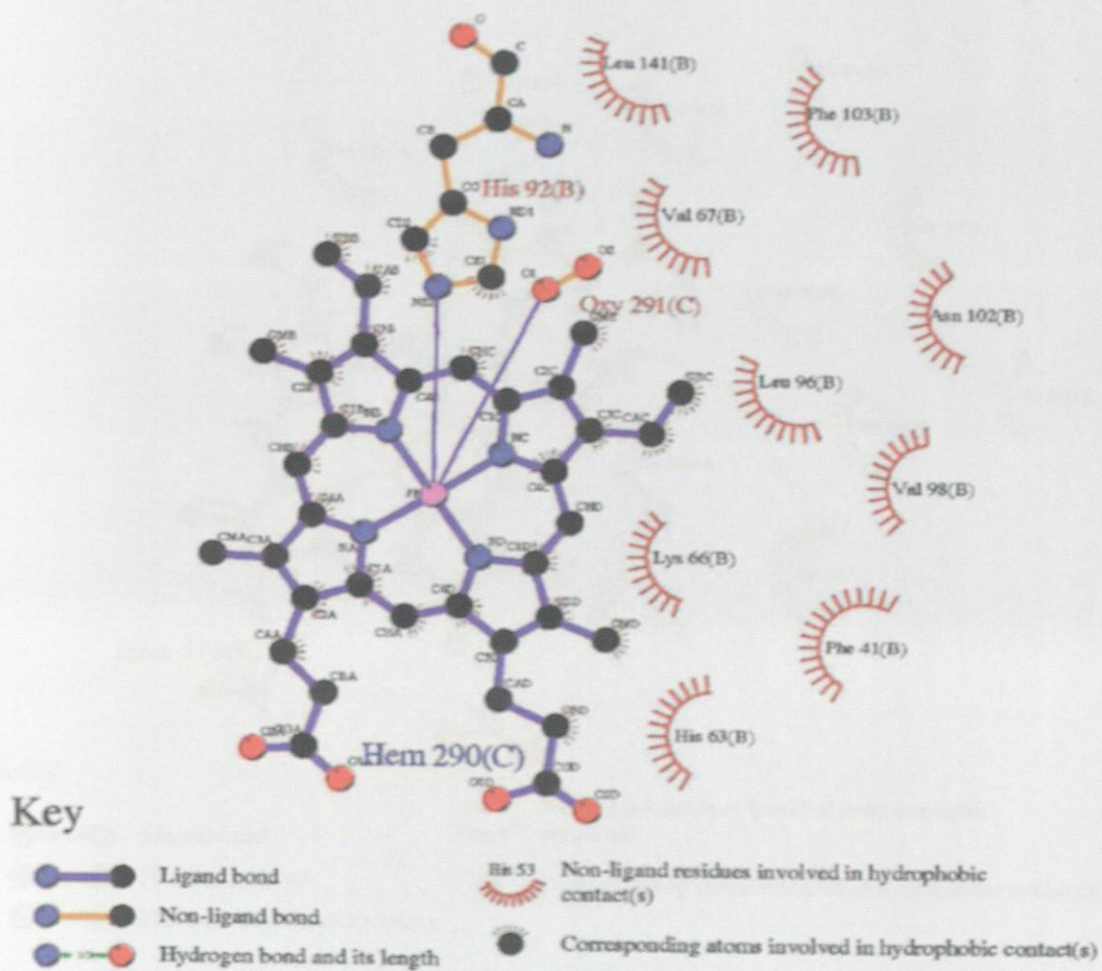


Fig.4.2 The heme binding pocket of common crane oxy HbA model chain beta:
The figure was generated by Ligplot. The heme binding residues have
been flattened by Ligplot to get a 2D picture.

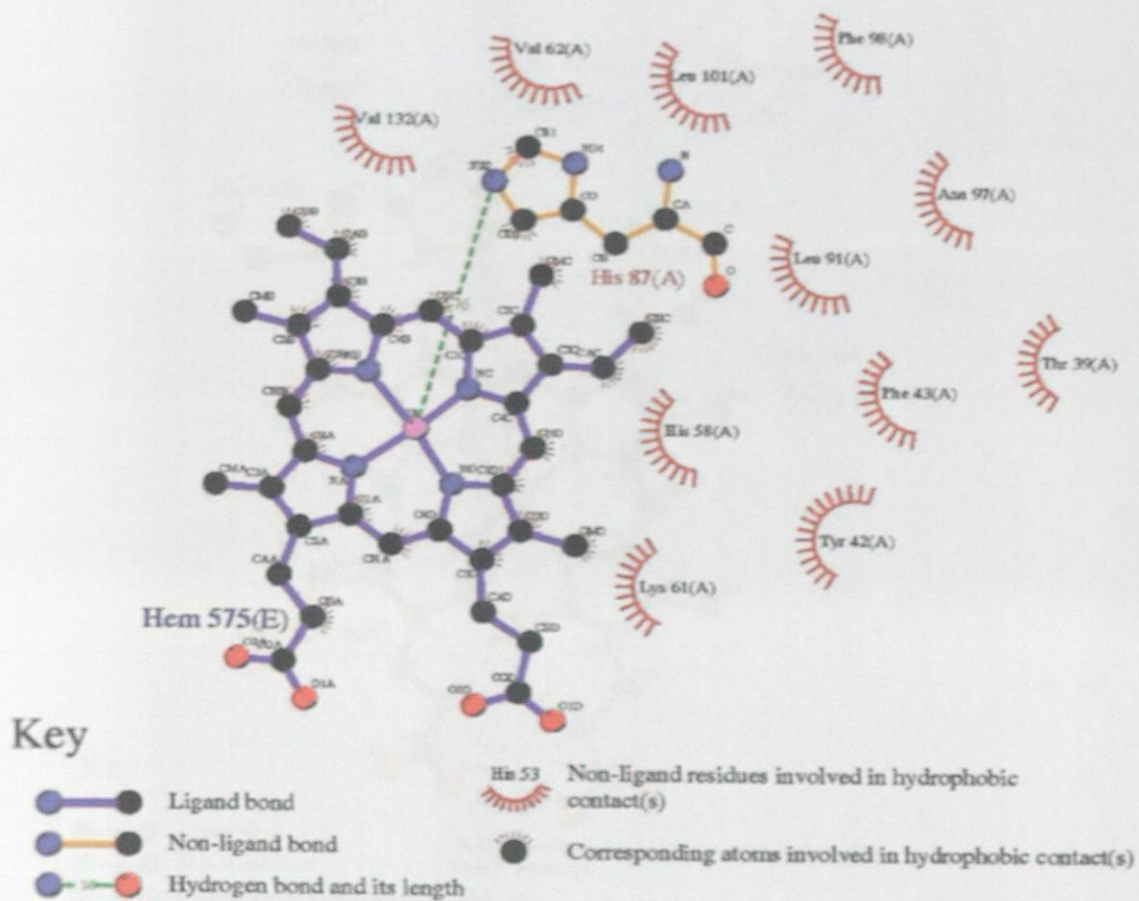


Fig.4.3 The heme binding pocket of common crane deoxy HbA model chain alpha: The figure was generated by Ligplot. The heme binding residues have been flattened by Ligplot to get a 2D picture.

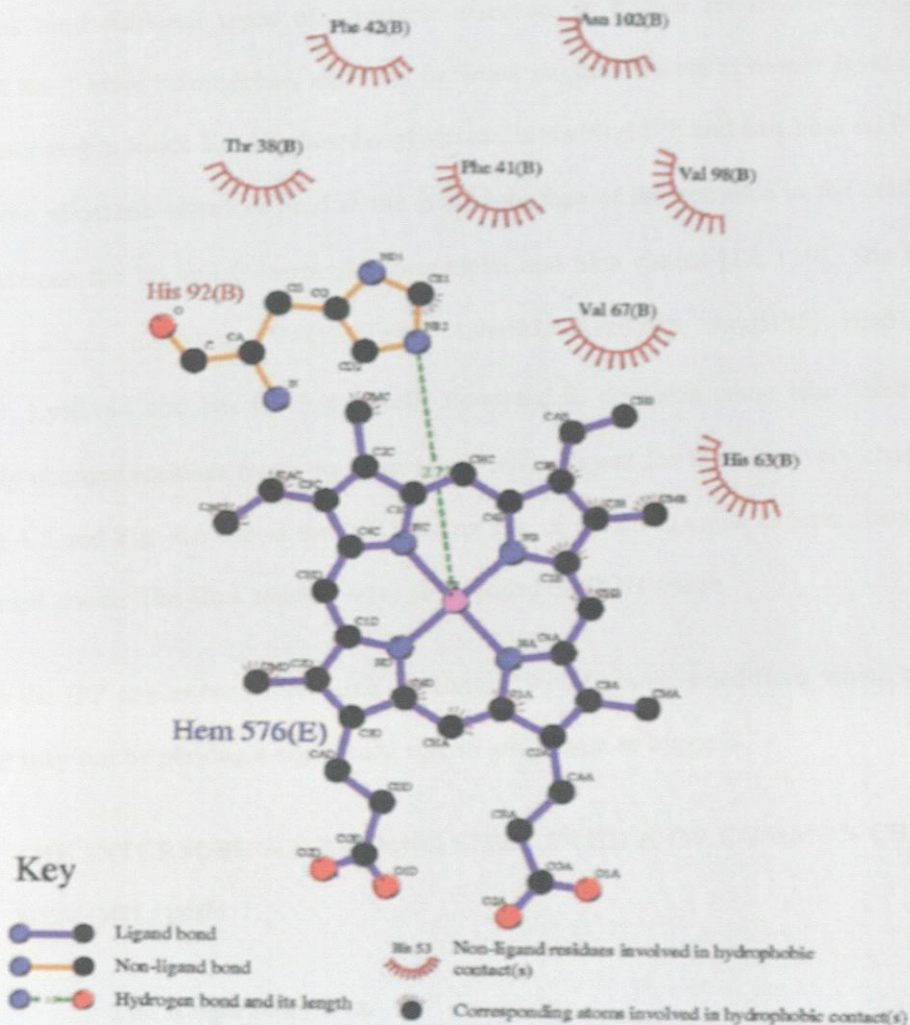


Fig.4.4 The heme binding pocket of common crane deoxy HbA model chain beta: The figure was generated by Ligplot. The heme binding residues have been flattened by Ligplot to get a 2D picture.

4.4.4 The IPP-Binding Site

Vertebrates bind different types of allosteric effectors to further reduce the oxygen affinity of the T state hemoglobin, and thus facilitate oxygen release at tissues level i.e. human hemoglobin binds 2, 3 bisphospho-glycerate, birds bind IPP and fish bind ATP or GTP. These allosteric effectors bind at the $\beta 1\beta 2$ interface at the entrance to the central cavity between the N- and C- termini of the alpha and beta chains [12, 119]. The IPP binding residues including, Val $\beta 1$, His $\beta 2$, Lys $\beta 82$, Arg $\beta 104$, Arg $\beta 135$, His $\beta 139$, Arg $\beta 143$, Lys $\beta 144$ and His $\beta 146$ are well conserved in common crane Hb. All these positively charged residues make an appropriate environment for the negatively charged IPP. Fig 4.5 and Fig. 4.6 shows the IPP binding site of common crane superimposed on Bar headed goose. The HbA models were superposed on BGH frame.

In birds the IPP concentration remains unchanged with hypoxic conditions which mean that IPP may not be playing a significant role in adaptation to hypoxia.

4.5 THE INTERSUBUNIT BINDING SITES IN HB A OF COMMON CRANE HEMOGLOBIN

4.5.1 The Respiratory Box

The region on hemoglobin alpha chain from $\alpha 34$ to $\alpha 38$ accumulates both $\alpha 1\beta 1$ and $\alpha 1\beta 2$ contacts. This region is called as respiratory box [47] due to the presence of these interface contact. There are three $\alpha 1\beta 1$ and two $\alpha 1\beta 2$ contacts in the respiratory box. The amino acid residue in the respiratory box on common crane are; $\alpha 34$ Cys, $\alpha 35$ Thr, $\alpha 36$ Tyr, $\alpha 37$ Pro and $\alpha 38$ Gln. The $\alpha 34$ Cys (C) is unique among all the avian alpha A hemoglobin chains. Table 4.3a and 4.3b show the variation among avian

HbA chains in the respiratory box region. The positions $\alpha 34$ and $\alpha 35$ are highly variable among the avian HbA chains while position $\alpha 37$ Pro is conserved among all avian HbA chains sequenced to date.

4.5.2 The $\alpha 1\beta 1$ Interface

4.5.2.1 The $\alpha 34$ binding site

In common crane alpha A chain the residue $\alpha 34$ Cys (C) is unique among all the primary structures of birds alpha A sequenced so far. In human this position is occupied by Leu (L) while in Graylag goose and Bar headed goose it is Thr (T). In all avian alpha A chains the residue at $\alpha 34$ is Thr (T), Val (V) or Ile (I). Another avian species having a unique amino acid at this position is *Anseranas semipalmata*, *Anseriformes* [120], which has a Gln (Q) at this position. In humans the $\alpha 34$ Leu is at the $\alpha 1\beta 1$ interfaces and makes hydrophobic interactions with $\beta 124$ Pro, $\beta 125$ Pro and $\beta 128$ Ala in the deoxy hemoglobin (T-structure) and with $\beta 128$ Ala in oxy hemoglobin (R-structure) [Fig. 4.7]. In *Gyps rueppellii*, falconiforms, and Bar headed goose this residue is replaced by Thr which results in the loss of hydrophobic contacts at the $\alpha 1\beta 1$ interface in the T state and therefore destabilize the hemoglobin T state [Fig. 4.8]. This in turn stabilizes the R-structure thereby accounting for the higher oxygen affinity in these high altitude flying birds. *Gyps rueppellii* fly at an altitude of approximately 11,300 meters. This bird has three different hemoglobins which function over a wide range of oxygen pressure and thus making it an ideal bird for adaptation to variations in oxygen availability. Substitution of $\alpha 34$ Leu by $\alpha 34$ Cys, in common, results in further weakening of Van der Waals interactions compared to human hemoglobin. The loss of contacts may destabilize the HbA T state and therefore shift the equilibrium towards R state which means more oxygen

affinity [Fig. 4.9]. The replacement of $\alpha 34$ Thr by $\alpha 34$ Cys in common crane creates a gap at the $\alpha 1\beta 1$ interface due to the absence of the bulky methyl group in Cys at beta carbon atom [Fig. 4.10]. The total contact area of the $\alpha 34$ Leu, Thr and Cys for human, Bar headed goose and common crane respectively, was calculated by online server available at (<http://ligin.weizmann.ac.il/cma/>). The contact area as shown in Table 4.4 also explains the loss of contacts due to these substitutions. The contact area decreases with decrease in the length of the side chain of $\alpha 34$ residue with mutation.

4.5.2.2 The $\alpha 119$ and $\beta 55$ binding site

The contacts at the $\alpha 1\beta 1$ interface at position $\alpha 119$ and $\beta 55$ have an important role in the high altitude adaptation. In human hemoglobin the residues $\alpha 119$ and $\beta 55$ are Pro and Met respectively. These residues make Van der Waals contacts at the $\alpha 1\beta 1$ and stabilize the deoxy hemoglobin T state. In avian hemoglobins these contacts are minimized by substituting $\alpha 119$ Pro or $\beta 55$ Met to a smaller residue. The loss of contacts due to substitution of these residues destabilizes the T-structure and shift the T-R equilibrium towards R-state and hence more oxygen affinity [12]. In human oxy hemoglobin the distance between $\alpha 119$ Pro and $\beta 55$ Met is about 4Å thereby enabling Van der Waals interaction at $\alpha 1\beta 1$ interface Fig. 4.11.

the $\alpha 1\beta 1$ interface [Fig 4.12] and thereby results in the high oxygen affinity of the Bar headed goose hemoglobin [106]. The distance between the two residues in Bar headed goose is about 4.6Å, which does not allow the two residues to have Van der Waals interactions.

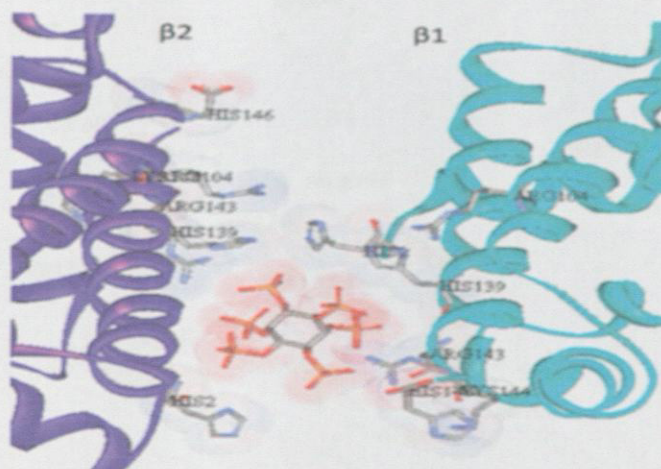


Fig.4.5 The IHP binding pocket of common crane hemoglobin: Five positively charged residues from each beta chain make the IHP binding pocket. The increased positive charge at the IHP binding pocket relative human hemoglobin explains its high affinity for ligand binding. The ionositol hexaphosphate (IHP) ligand and positively charged residues are enclosed in semitransparent Van der Waal surface.

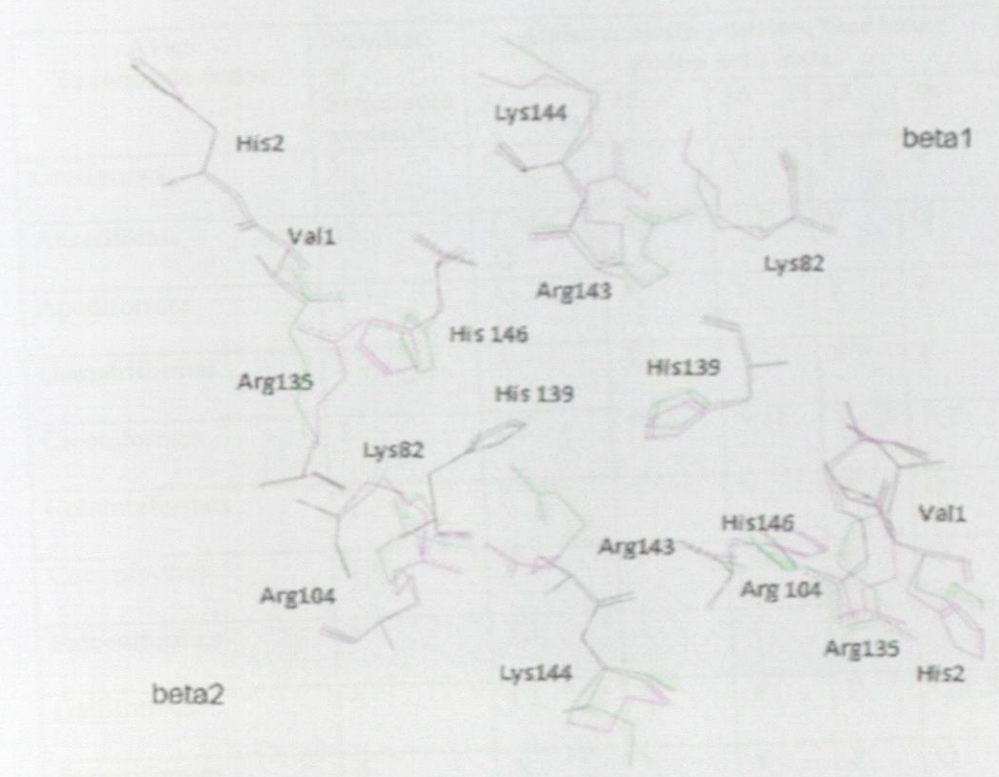


Fig.4.6 The IHP binding pockets of common crane Hb (violet) and bar headed goose Hb (green) beta chains superimposed on BGH frame: The IHP binding positively charged residues are conserved.

Table 4.3a Survey on the respiratory box in alpha A chains of avian hemoglobin

Avies Taxonomic order	Number of Sequences available	Alpha A chain position, One letter amino acid code.				
		34	35	36	37	38
Gruiformes	1	C	T	Y	P	Q
Anseriforms	15	T, V I ,Q	A,T	Y,F	P	Q
Apodiformes	1	A	A	Y	P	P
charadriiformes	2	A	T	Y	P	P
Ciconiformes	2	I,T	T,V	Y,H	P	P,T
Columbiformes	2	I	T	Y	P	Q
Cuculiformes	2	T	V	Y	P	Q
Falconiformes	5	T,I	T	Y	P	P
Galliformes	5	T,I	T	Y	P	Q,P,S
Passeriformes	4	A	T	Y	P	Q
Pelecaniformes	1	T	A	Y	P	Q
Phoenicopteriformes	1	A	A	Y	P	Q
Psittaciformes	2	A,V	T	F,H	P	Q
Rheiforme	1	T	T	Y	P	Q
Sphenisciformes	2	T	T	Y	P	Q
Struthioniformes	1	I	T	Y	P	Q

Table 4.3b Comparison of $\alpha 1\beta 1$ interface residues playing key role in hypoxia adaptation [121]

Birds species	$\alpha 119$	$\beta 55$	$\alpha 34$	$\beta 125$	Max altitude (m)
Common crane	Pro	Ile	Cys	Asp	4600
Bar-headed goose	Ala	Leu	Thr	Asp	9 000
Greylag goose	Pro	Leu	Thr	Glu	Sea level
Andean goose	Pro	Ser	Ile	Asp	6 000
Ruppell's griffon	Pro	Ile	Thr	Asp	11 300
Ruppell's griffon (A0)	Pro	Ile	Ile	Asp	11 300
Black-headed gull	Pro	Asn	Ile	Asp	1000
Canadian goose	Pro	Leu	Val	Asp	Sea level
Mute swan	Pro	Leu	Ile	Asp	Sea level
Chicken	Pro	Leu	Thr	Glu	Sea level
Chicken (D)	Pro	Leu	Thr	Glu	Sea level

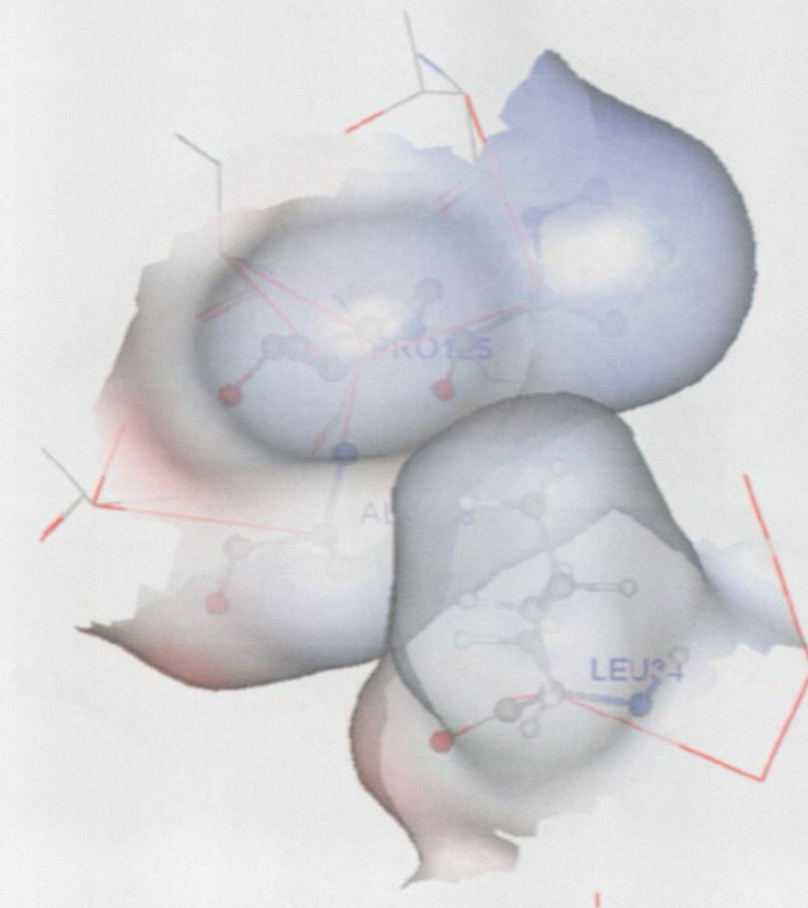


Fig.4.7 The human HbA oxy (PDB code 2DN1) α 34 Thr binding site, (the Van der Waals interactions of α 34Leu(A) with β 124Pro, β 125Pro and β 128Ala in the 3D model of human HbA): The hydrophobic environment on both alpha and beta chains and the bulky methyl groups of alpha34 Leu make possible strong hydrophobic interaction between alpha and beta interface at alpha34 position, therefore shifting hemoglobin to the T-state.

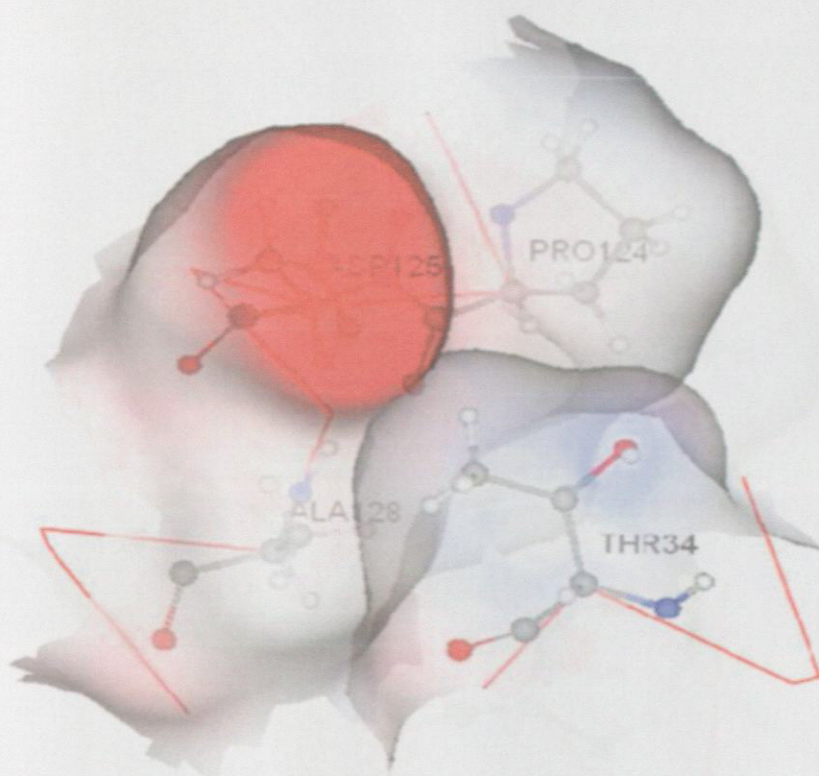


Fig.4.8 The Bar headed HbA oxy α 34 Thr binding site; the Van der Waals interactions of α 34Thr(A) with β 124Pro, β 125Asp and β 128Ala in the 3D model of Bar headed goose HbA): The methyl group present at beta carbon of Thr34 makes hydrophobic contacts, these contact due to methyl group are lost by mutating the Thr34 to Cys34.

Fig.4.8 The Bar headed HbA oxy α 34 Thr binding site; the Van der Waals interactions of α 34Thr(A) with β 124Pro, β 125Asp and β 128Ala in the 3D model of Bar headed goose HbA): The methyl group present at beta carbon of Thr34 makes hydrophobic contacts, these contact due to methyl group are lost by mutating the Thr34 to Cys34.

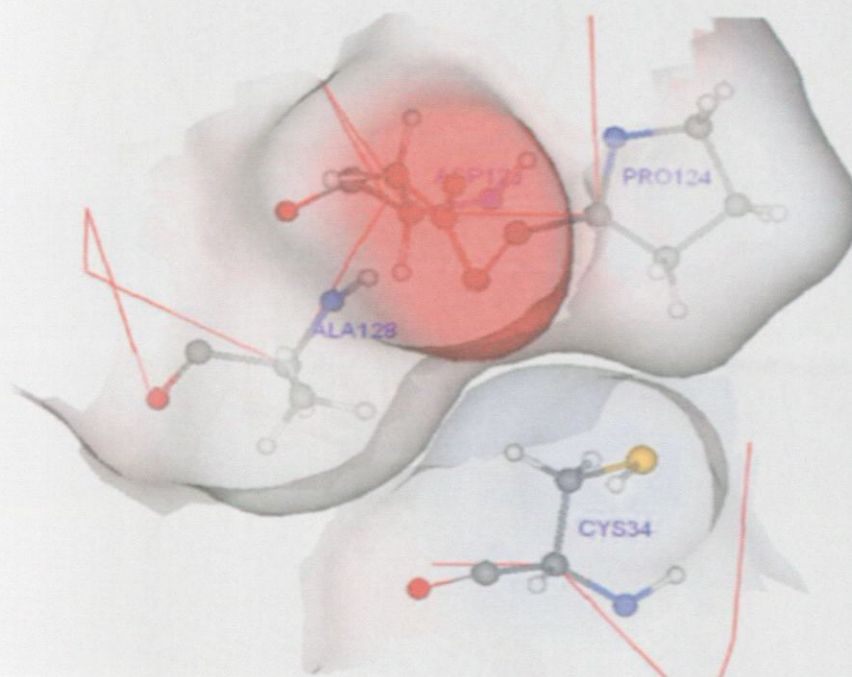


Fig.4.9 The common crane HbA oxy $\alpha 34$ Cys binding site; the Van der Waals interactions of $\alpha 34$ Thr(A) with $\beta 124$ Pro, $\beta 125$ Asp and $\beta 128$ Ala in the 3D model of common crane HbA): A gap visible between the $\alpha_1\beta_1$ interfaces due to absence of methyl group at beta carbon of cysteine which is present in Thr $\alpha 34$ of Bar headed goose.

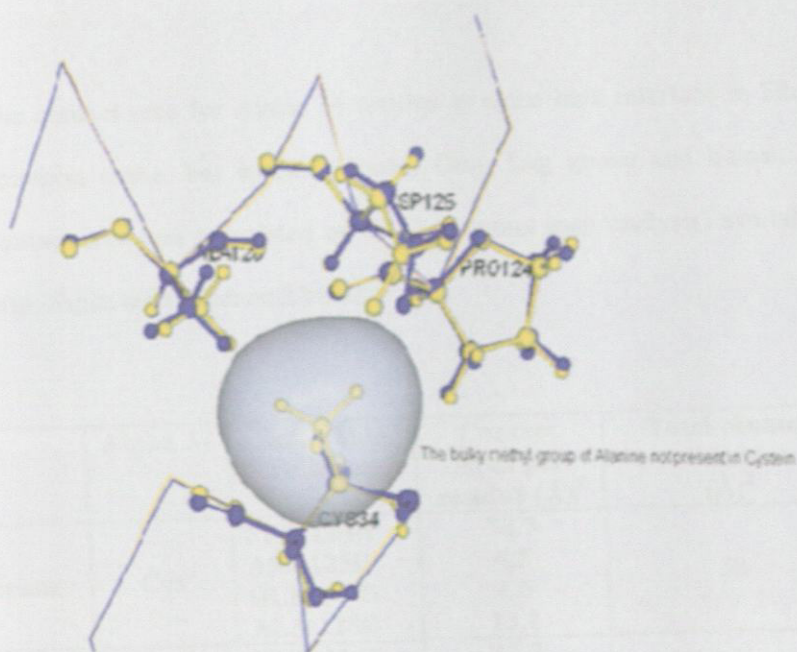


Fig.4.10 The common crane HbA oxy $\alpha 34 \alpha_1 \beta_1$ binding site superimposed on Bar headed goose $\alpha 34 \alpha_1 \beta_1$ binding site; the Van der Waals interactions of $\alpha 34 \text{Thr(A)}$ with $\beta 124 \text{Pro}$, $\beta 125 \text{Asp}$ and $\beta 128 \text{Ala}$ in the 3D model of common crane HbA): Blue color shows common crane HbA and yellow color shows Bar headed goose HbA contact sites. In Bar headed goose the bulky methyl group of Thr34 which makes contacts with beta Pro124, Asp125 and Ala128, is not present in alpha 34 cysteine (Crane). Therefore many of the contacts that are shown by alpha 34Thr of bar headed goose are lost in common crane.

Table 4.4 The contact area for Alpha 34 residue at alpha beta interface in HbA of common crane, bar headed Goose, Gray Lag goose and human. The contact area was calculated on CMA (contact map analysis) available at <http://ligin.weizmann.ac.il/cma/>

Species	Alpha 34	Beta chain residues	Contact Area per residue (Å) ²	Total contact area (Å) ²
Common crane	Cys	PRO 124B	38.7	58
		ASP 125B	4.5	
		GLN 127B	1.6	
		ALA 128B	13.1	
Bar headed goose	Thr	PRO 124B	37.7	59.9
		ASP 125B	8.5	
		ALA 128B	13.7	
Graylag Goose	Thr	PRO 124B	36.5	64.8
		GLU 125B	17.2	
		ALA128B	11.1	
Human	Leu	PRO 124B	29.6	73
		PRO 125B	23.9	
		ALA 128B	18.3	
		LYS 132B	1.2	

In Bar headed goose $\alpha 119$ and $\beta 55$ positions are occupied by Ala and Leu respectively.

The substitution of the $\alpha 119$ and $\beta 55$ residues in Bar headed goose introduces a gap at

In common crane the $\alpha 119$ and $\beta 55$ residues at the $\alpha 1\beta 1$ are Pro and Ile respectively.

The $\alpha 119$ Pro is conserved among all avian hemoglobin alpha A chain except Bar headed goose. The $\beta 55$ Ile is present in few other avian hemoglobin beta chains (Two Falconiformes and two Anseriformes species). *Gyps ruepellii* (Falconiformes) [47] contains exactly the same two residues which the common crane have i.e. $\alpha 119$ Pro and $\beta 55$ Ile.

In common crane the amino acid at $\beta 55$ is Ile as compared to Leu in Bar headed goose and many other avian Hb beta chains. The fig 4.13 shows the $\alpha 119$ and $\beta 55$ contacts. The total contact area is $28(\text{\AA})^2$ which is much smaller as compared to the total contact area ($42(\text{\AA})^2$) in human deoxy HbA for the two residues at $\alpha 119$ and $\beta 55$. In Bar headed goose the total contact area between these two amino acids is $13.9(\text{\AA})^2$.

Other residues which make contact with $\alpha 119$ are Glu26b, Arg30, Ile33, Val34 and Pro51. The total contact area at this position of the $\alpha 1\beta 1$ is inversely related to oxygen affinity of the hemoglobin. Table 4.5 shows the total contact area of common crane, Bar headed goose and human HbA $\alpha 119$ amino acid residue. The smallest contact area is for Bar headed goose which has higher oxygen affinity while the greatest contact area is for human HbA which has lower oxygen affinity relative to Bar headed goose.

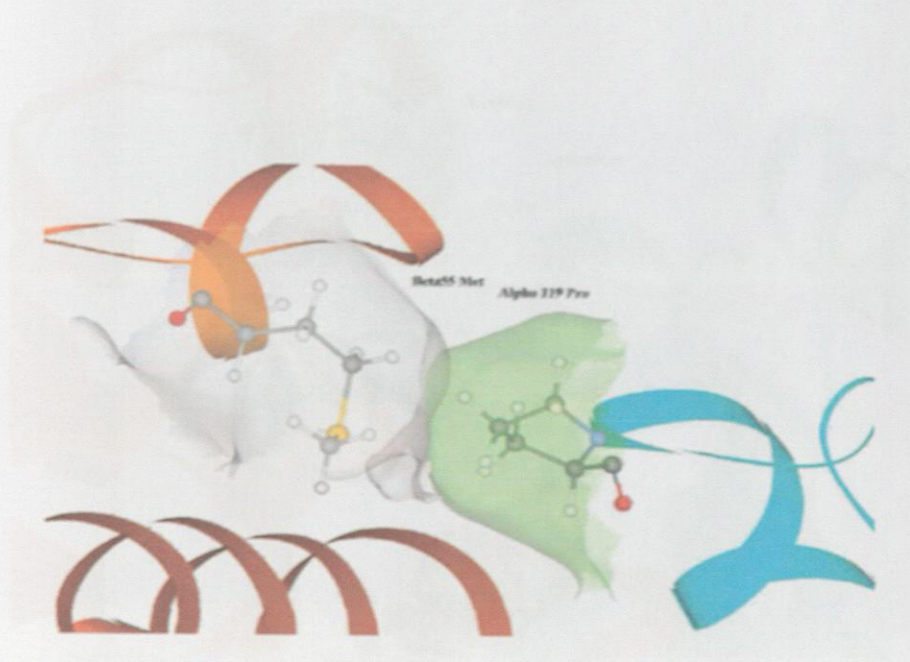


Fig.4.11 Human deoxy α 119Pro and β 55Met contacts at α 1 β 1 interface: The α 119Pro and β 55Met residues are shown as enclosed by semi transparent Van der Waal surfaces.



Fig.4.12 Bar headed goose deoxy $\alpha 119$ Pro and $\beta 55$ Met contacts at $\alpha 1\beta 1$ interface: The $\alpha 119$ Ala and $\beta 55$ Leu residues are shown as enclosed by semi transparent Van der Waal surfaces. A gap is visible between the two residues due to loss of contacts. The minimum distance between the two residues is about 4.65Å which much greater to allow a Van der Waal contact.

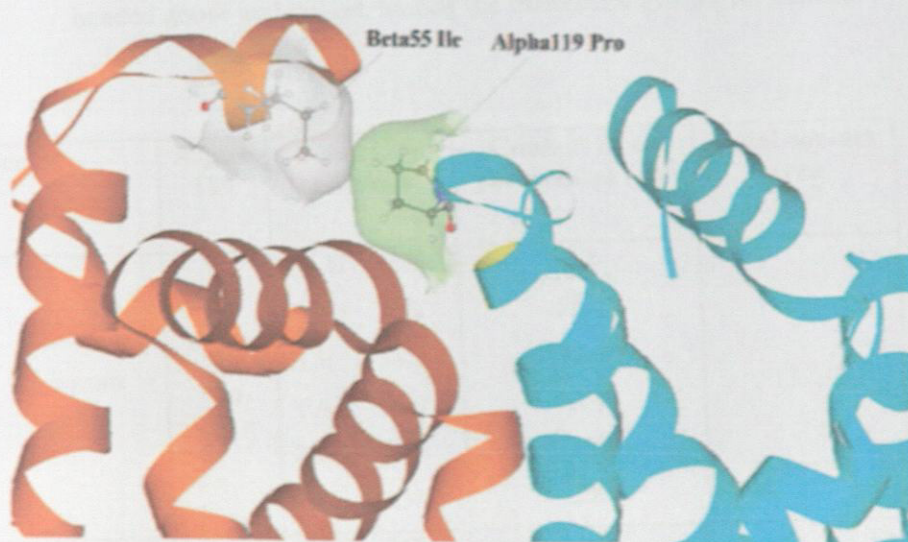


Fig.4.13 Common crane deoxy $\alpha 119$ Pro and $\beta 55$ Ile contacts at $\alpha 1\beta 1$ interface: The $\alpha 119$ Ala and $\beta 55$ Leu residues are shown as enclosed by semi transparent Van der Waal surfaces. The two residues make a very small contact with total contact area of 28 Å as compared to 42 Å in human HbA deoxy.

Table 4.5 The contact area for Alpha 119 residue at alpha beta interface in deoxy HbA of common crane, Bar headed goose and human: The contact area was calculated on CMA (contact map analysis) available at <http://ligin.weizmann.ac.il/cma/>. The contact area is the smallest for bar headed goose and largest, among the three hemoglobin, for human.

Species	Alpha 119	Beta chain residues	Contact Area per residue ($\text{\AA}^2$)	Total contact area ($\text{\AA}^2$)
Common crane	Pro	GLU 26B	2.2	132.1
		ARG 30B	52.7	
		ILE 33B	32.2	
		VAL 34B	9.4	
		PRO 51B	7.6	
		ILE 55B	28	
Bar headed goose	Ala	GLU 26B	1.6	123.8
		ARG 30B	50.4	
		ILE 33B	44.1	
		VAL 34B	11.1	
		PRO 51B	2.7	
		LEU 55B	13.9	
Human	Pro	GLU 26B	1.5	143.7
		ARG 30B	50.4	
		VAL 33B	28.8	
		VAL 34B	11.3	
		PRO 51B	9.4	
		MET 55B	42.3	

4.6 PHYLOGENETIC ANALYSIS BASED HEMOGLOBIN PRIMARY STRUCTURE

Although well studied, the evolutionary relationships among avian groups are contentious. The rapid divergence in bird's early evolutionary history has resulted in various morphologically distinct group e.g. owls, parrots, doves etc. Both molecular and morphological studies support the presence of two nodes at the base of the avian tree. The first node divides birds into two groups paleognathae (which include Ratites and tinamous) and Neognathae (which includes all other birds). The second node divides the Neognathae into two groups Gallanserae (Galliformes and Anseriformes, chickens ducks and allies) and Neoaves (other birds). The Neoaves represent majority of the extant bird species (95 %) and is divided into 26 orders. There are no intermediate forms linking these well defined groups. All these groups appear to have rapidly and independently evolved and therefore show a polytomous phylogeny. Different methods have been used to resolve the relationship among these groups e.g morphology, DNA hybridization, whole mitochondrial genomes, nuclear exon, introns and ribosomal RNAs. All these methods have shown conflicting results.

A most recent attempt was made by Hackett et al [122], using phylogenomics to resolve the relationship among these groups. In this study they used large DNA sequence data set (32kb alignment) from 171 species representing all major Passerine clades. The genomic DNA sequence included coding exons, introns, and UTRs. Their findings strongly supported the division of the avian tree at base into Paleognathae and Neognathae and also the division of Neognathae into Gallanserae and Neoaves.

We made an attempt to find the overall effect of concatenating hemoglobin sequences with cytochrome b sequences on aves phylogenetic trees. Hemoglobin is a ubiquitous

gene and is present in almost all living organism (from bacteria to humans). It has undergone a series of mutations helping the organism to adapt to its specific environment (hypoxia, low temperature etc). The hemoglobin genes therefore carry good information of evolution and adaptation. Concatenating the hemoglobin sequences with known cytochrome b sequences for phylogenetic analysis may improve the phylogenetic tree. We constructed phylogenetic trees using hemoglobin alpha A, hemoglobin beta and cytochrome b protein sequences separately and concatenated. The protein sequences were obtained from Uniprot database. Only those avian species were used in the analysis which had all the three protein sequences (cyt, HBA and HBB) available in the database. Multiple alignments of protein sequences were done with MEGA4 software using the MUSCLE algorithm with default parameters. The phylogenetic trees were constructed with MEGA4 using maximum parsimony (MP) method with bootstrapping value of 1000. Our results show that the phylogenetic tree obtained using all three sequences concatenated resolves the differences among major groups fig 4.14.

The common crane shows affinity with Charadriiformes and thus supports the Hackett et al (2008) phylogenetic analysis. Our study does not support the monophyly of Galloanserae (Galliformes and Anseriformes). Other methods like DNA hybridization [123], cytochrome b [124] and cranial morphology [125] support the monophyly of Galloanserae. The phylogenetic analysis shows Passeriformes as a basal to all living birds. As hemoglobin is one of the many proteins which are ubiquitous in all living organisms therefore we propose that hemoglobin sequences should be included the data set for avian phylogenetic analysis. The current avian taxa, with known hemoglobin primary structure, are very limited. Large scale hemoglobin sequences can be obtained from avian taxa by using rtPCR and DNA sequencing methods.

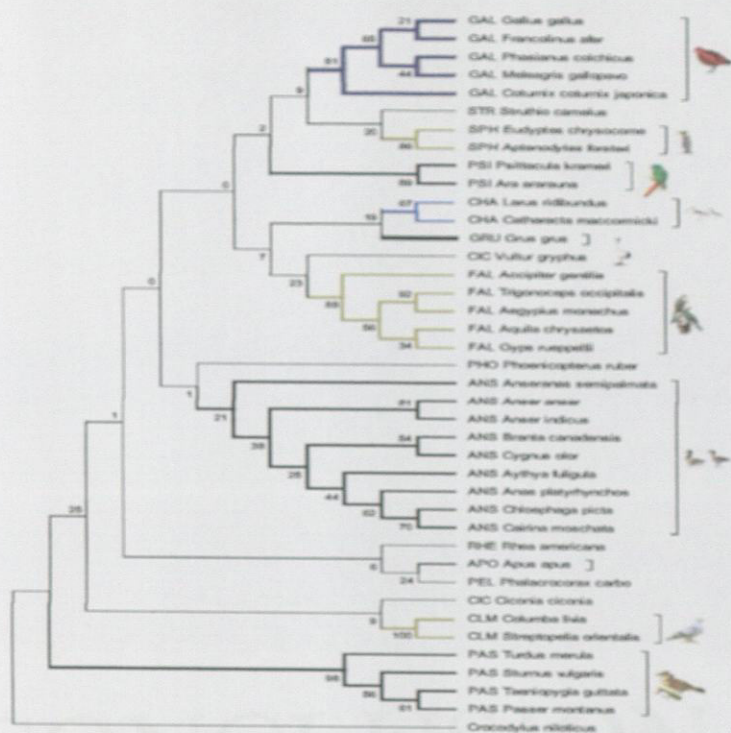


Fig.4.14

Maximum Parsimony analysis of taxa: The evolutionary history was inferred using the Maximum Parsimony method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed [126]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [126]. The MP tree was obtained using the Close-Neighbor-Interchange algorithm (pg. 128 in ref. [127]) with search level 2 [126, 127] in which the initial trees were obtained with the random addition of sequences (10 replicates). The analysis involved 40 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 357 positions in the final dataset. Evolutionary analyses were conducted in MEGA4 [114]. Ordinal codes ANS (Anseriformes), CIC (Ciconiiformes), CLM (Columbiformes), CHA (Charadriiformes), FAL (Falconiiformes), GAL (Galliiformes), GRU (Gruiformes) PEL (Pelecaniformes), PAS (Passeriformes), PHO (Phoenicopteriformes), RHE (Rheiformes).

CONCLUSION

The complete primary structure of common crane (*Grus Grus*) was determined. The alpha A chain shows amino acid substitution at a critical location which we propose is responsible for greater oxygen affinity of hemoglobin and thus enable the common crane to adapt to high altitude hypoxia. These birds fly at an altitude of about 4600m during its migration from Siberia to wintering grounds in India moving across Pakistan. At this level the partial pressure of oxygen drops significantly and therefore the hemoglobin molecule has to have greater oxygen affinity to pick up oxygen at such a low partial pressure. The 3D homology model was constructed by modeler package using the bar headed goose crystal structure coordinates as a template. The structure comparison between Bar-Headed goose and common crane 3D models was done on Swiss PDB viewer. The phylogenetic tree construction and analysis was done on MEGA4 software. Some of the important findings of the research work are given in the following list.

1. The amino acid cysteine $\alpha 34$ is unique among all known hemoglobin sequences. This amino acid occupies a critical position at $\alpha 1\beta 1$ interface.
2. The amino acid $\alpha 34C$ causes the loss of contact at alpha beta interface and therefore destabilizes the T structure shifting the hemoglobin to R structure which has greater affinity for oxygen.
3. Concatenating hemoglobin sequences with cytochrome b sequences give better phylogenetic tree as compared to using hemoglobin or cytochrome b sequences alone.
4. The common crane (order Gruiformes) makes a sister clade with Charadriiforme.
5. The phylogenetic analysis based on concatenated hemoglobin and cytochrome b sequences shows that Passeriformes are basal to all other birds used in the current study.
6. The phylogenetic analysis does not support the monophyly of Galloanserae. Both Galliformes and Anseriformes make separate monophyletic clades.
7. The phylogenetic analysis of birds should include hemoglobin sequences concatenated with other nuclear and mitochondrial genes.

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APPENDIX

Appendix-I

SEQUENCE ALIGNMENT FILE FOR HOMOLGY MODELING of common crane deoxy hemoglobin

>P1;1HV4deoxy

structureX:1HV4deoxy:1 :A:574 :D:Hb:BHG: 1.90: 0.19

VLSAADKTNVKG VFSKISGHAE EYGAETLERMFTA YPQTKTYFPHFDLQH
GSAQIKAHGKKVVAALVEAVNHIDDIAGALSKLSDLHAQKLRVDPVNFKF
LGHCFLVVVAIHHPSALTAEVHASL DKFLCAVGT VLTAKYR/
VHWSAEEKQLITGLWGKVN VADCGAEALARLLIVYPWTQRFFSSFGNLSS
PTAILGNPMVRAHGKKVLT SFGDAVKNLDNIKNTFAQLSELHCDKLHVDP
ENFRLLGDILIIVLA AHFAKEFTPDCQAAWQKLVRVVAHALARKYH/
VLSAADKTNVKG VFSKISGHAE EYGAETLERMFTA YPQTKTYFPHFDLQH
GSAQIKAHGKKVVAALVEAVNHIDDIAGALSKLSDLHAQKLRVDPVNFKF
LGHCFLVVVAIHHPSALTAEVHASL DKFLCAVGT VLTAKYR/
VHWSAEEKQLITGLWGKVN VADCGAEALARLLIVYPWTQRFFSSFGNLSS
PTAILGNPMVRAHGKKVLT SFGDAVKNLDNIKNTFAQLSELHCDKLHVDP
ENFRLLGDILIIVLA AHFAKEFTPDCQAAWQKLVRVVAHALARKYH/....*

>P1;ccdeoxy

sequence:ccdeoxy:1 :A:574 :D:Hb:CC: 2.00:-1.00

VLSGNDKSNVKG VFSKIGGHAE EYGAETLERMFCTYPQTKTYFPHFDLQH
GSAQVKAHGKKVAGALIEA ANHIDDIAGALSKLSDLHAQKLRVDPVNFKL
LGQCFLVVVAIHHPSVLTPEVHASL DKFLCAVG NVLTAKYR/
VHWSAEEKQLITSLWGKVN VADCGAEALARLLIVYPWTQRFFSAFGNLSS
PTAIIGNPMVRAHGKKVLT SFGDAVKNLDNIKNTFAQLSELHCDKLHVDP
ENFRLLGDILIIVLA AHFAKDFTPDCQAAWQKLVRVVAHALARKYH/
VLSGNDKSNVKG VFSKIGGHAE EYGAETLERMFCTYPQTKTYFPHFDLQH
GSAQVKAHGKKVAGALIEA ANHIDDIAGALSKLSDLHAQKLRVDPVNFKL
LGQCFLVVVAIHHPSVLTPEVHASL DKFLCAVG NVLTAKYR/
VHWSAEEKQLITSLWGKVN VADCGAEALARLLIVYPWTQRFFSAFGNLSS
PTAIIGNPMVRAHGKKVLT SFGDAVKNLDNIKNTFAQLSELHCDKLHVDP
ENFRLLGDILIIVLA AHFAKDFTPDCQAAWQKLVRVVAHALARKYH/....*

Appendix-I

SEQUENCE ALIGNMENT FILE FOR HOMOLOGY MODELING of common crane deoxy hemoglobin

>P1;1HV4deoxy

structureX:1HV4deoxy:1 :A:574 :D:HB:BHG: 1.90: 0.19

VLSAADKTNVKG VFSKISGHAE EYGAETLERMFTAYPQTKTYFPHFDLQH
GSAQIKAHGKKVVAALVEAVNHIDDIAGALSKLSDLHAQKLRVDPVNFKF
LGHCFLVVVAIHHPSALTAEVHASLDKFLCAVGT VLTAKYR/
VHWSAEEKQLITGLWGKVN VADCGAEALARLLIVYPWTQRFFSSFGNLSS
PTAILGNPMVRAHGKKVLT SFGDAVKNLDNIKNTFAQLSELHCDKLHVDP
ENFRLLGDILIIVLA AHFAKEFTPDCQAAWQKLVRVVAHALARKYH/
VLSAADKTNVKG VFSKISGHAE EYGAETLERMFTAYPQTKTYFPHFDLQH
GSAQIKAHGKKVVAALVEAVNHIDDIAGALSKLSDLHAQKLRVDPVNFKF
LGHCFLVVVAIHHPSALTAEVHASLDKFLCAVGT VLTAKYR/
VHWSAEEKQLITGLWGKVN VADCGAEALARLLIVYPWTQRFFSSFGNLSS
PTAILGNPMVRAHGKKVLT SFGDAVKNLDNIKNTFAQLSELHCDKLHVDP
ENFRLLGDILIIVLA AHFAKEFTPDCQAAWQKLVRVVAHALARKYH/....*

>P1;ccdeoxy

sequence:ccdeoxy:1 :A:574 :D:Hb:CC: 2.00:-1.00

VLSGNDKSNVKG VFSKIGGHAEEYGAETLERMFCTYPQTKTYFPHFDLQH
GSAQVKAHGKKVAGALIEAANHIDDIAGALSKLSDLHAQKLRVDPVNFKL
LGQCFLVVVAIHHPSVLTPEVHASLDKFLCAVG NVLTAKYR/
VHWSAEEKQLITSLWGKVN VADCGAEALARLLIVYPWTQRFFSAFGNLSS
PTAIIGNPMVRAHGKKVLT SFGDAVKNLDNIKNTFAQLSELHCDKLHVDP
ENFRLLGDILIIVLA AHFAKDFTPDCQAAWQKLVRVVAHALARKYH/
VLSGNDKSNVKG VFSKIGGHAEEYGAETLERMFCTYPQTKTYFPHFDLQH
GSAQVKAHGKKVAGALIEAANHIDDIAGALSKLSDLHAQKLRVDPVNFKL
LGQCFLVVVAIHHPSVLTPEVHASLDKFLCAVG NVLTAKYR/
VHWSAEEKQLITSLWGKVN VADCGAEALARLLIVYPWTQRFFSAFGNLSS
PTAIIGNPMVRAHGKKVLT SFGDAVKNLDNIKNTFAQLSELHCDKLHVDP
ENFRLLGDILIIVLA AHFAKDFTPDCQAAWQKLVRVVAHALARKYH/....*

SEQUENCE ALIGNMENT FILE FOR HOMOLGY MODELING OF COMMON
CRANE OXY HEMOGLOBIN

>P1;1A4Foxy

structureX:1A4Foxy:1 :A:686 :A:HB: BHG: 1.90: 0.19

VLSAADKTNVKGVFSGHAEYGAETLERMFTAYPQTKTYFPHFDLQH
GSAQIKAHGKKVVAALVEAVNHIDDIAGALSKLSDLHAQKLRVDPVNFKE
LGHCFLVVVAIHHPALTAEVHASLDKFLCAVGTVLTAKYR/
VHWSAEEKQLITGLWGKVNVAADCGAEALARLLIVYPWTQRFFSSFGNLSS
PTAILGNPMVRAHGKKVLTSGDAVKNLDNKNTFAQLSELHCDKLHVDP
ENFRLLGDILIVLAAHFAKEFTPDCQAAWQKLVRVVAHALARKYH/....

www

W

www*

>P1;ccoxy

sequence:ccoxy:1 : :574 : :HB:CC: 2.00:-1.00

VLSGNDKSNVKGVFSGHAEYGAETLERMFCTYPQTKTYFPHFDLQH
GSAQVKAHGKKVAGALIEAANHIDDIAGALSKLSDLHAQKLRVDPVNFKE
LGQCFLVVVAIHHPVLTPEVHASLDKFLCAVGNVLTAKYR/
VHWSAEEKQLITSLWGKVNVAADCGAEALARLLIVYPWTQRFFSAFGNLSS
PTAIIIGNPMVRAHGKKVLTSGEAVKNLDNIKNTFAQLSELHCDKLHVDPE
NFRLLGDILIVLAAHFAKDFTPDCQAAWQKLVRVVAHALARKYH/....

www

W

www*

MODELLER COMMAND FILE FOR HOMOLOGY MODEL CONSTRUCTION OF COMMON CRANE DEOXY HEMOGLOBIN

```
# Homology modeling with ligand transfer from the template
from modeller import *      # Load standard Modeller classes
from modeller.automodel import *  # Load the automodel class

log.verbose()  # request verbose output
env = environ() # create a new MODELLER environment to build this model in

# directories for input atom files
env.io.atom_files_directory = ['.', './atom_files']

# Read in HETATM records from template PDBs
env.io.hetatm = True
env.io.water = True

a = automodel(env,
               alnfile = 'alignment.ali', # alignment filename
               knowns = '1HV4deoxy',      # codes of the templates
               sequence = 'ccdeoxy')      # code of the target
a.starting_model= 1          # index of the first model
a.ending_model = 50         # index of the last model
                             # (determines how many models to calculate)
a.make()                    # do the actual homology modeling
```

MODELLER COMMAND FILE FOR HOMOLOGY MODEL CONSTRUCTION OF COMMON CRANE OXY HEMOGLOBIN

```
# Homology modeling with ligand transfer from the template
from modeller import *      # Load standard Modeller classes
from modeller.automodel import *  # Load the automodel class

log.verbose()  # request verbose output
env = environ() # create a new MODELLER environment to build this model in

# directories for input atom files
env.io.atom_files_directory = ['.', './atom_files']

# Read in HETATM records from template PDBs
env.io.hetatm = True
env.io.water = True

a = automodel(env,
               alnfile = 'alignment.ali', # alignment filename
               knowns = '1A4Foxy',        # codes of the templates
               sequence = 'ccoxy')        # code of the target
a.starting_model = 1      # index of the first model
a.ending_model = 50       # index of the last model
                           # (determines how many models to calculate)
a.make()                  # do the actual homology modeling
```


A SHORT VERSION OF HEMOGLOBIN ALPHA A ALIGNMENT

		10	20	30	40	50
						
Common crane	1	VLSGNDKSNVKGVFSKIGGHAEEYGAETLERMFCTYPQTKTYFP	FDLQH			
Gray lag Goose	1	...AA..T.....	TA.....			
Bar Headed Goose	1	...AA..T.....S.....	TA.....			
Chicken	1	M.TAE..KLIQQAWE.AAS.Q..F...A.T..T.....	SP			
Human	1	...PA..T...AAWG.V.A..G.....A.....LSF.T.....	S.			
		60	70	80	90	100
						
Common crane	51	GSAQVKAHGGKKVAGALIEAANHIDDIAGALSKLSDLHAQKLRVDPVNF	FKL			
Gray lag Goose	51	...I.....A..V..V.....	F			
Bar Headed Goose	51	...I.....VA..V..V.....	F			
Chicken	51	..D..RG....L...GN.VKNV.NLSQ.MAE..N...YN.....				
Human	51	G.....D..TN.VA.V..MPN...A.....H.....				
		110	120	130	140	
						
Common crane	101	LGQCFLVVVAIHHPSVLTPEVHASLDKFLCAVGNVLTAKYR				
Gray lag Goose	101	..H.....A.....T.....				
Bar Headed Goose	101	..H.....A..A.....T.....				
Chicken	101	..S..IQ..L.V.MEKDY.....AF....S..SA..AE...				
Human	101	..SH.L..TL.A.L.AEF..A.....AS.ST...S...				

Alignment of common crane HbA sequence with HbA sequences from Human , Gray lag goose, bar headed goose and chicken.

A SHORT VERSION OF HEMOGLOBIN BETA ALIGNMENT

		10	20	30	40	50	
Common crane	1						
Graylag Goose	1	VHWSAEEKQL	ITSLWGKVVV	ADCGAEALAR	LLIVYPWTQR	FFSAFGNLSS	
BarHeaded Goose	1		.G.....			..S.....	
Chicken	1	...T.....	.G.....	.E.....		..AS.....	
Human	1	..LTP...SA	V.A.....	DEV.G...G.	..V.....	..ES..D..T	
		60	70	80	90	100	
Common crane	51						
Graylag Goose	51	PTAIIGNPMV	RAHGKKVLTS	FGEAVKNLDN	IKNTFAQLSE	LHCDKLHVDP	
BarHeaded Goose	51	L.....		.D.....			
Chicken	51	L.....		.D.....			
Human	51	..D.VM...K.	K.....GA	.SDGLAH...	L.G...T...		
		110	120	130	140		
Common crane	101						
Graylag Goose	101	ENFRLLGDIL	IIVLAAHFAK	DFTPDCAAW	QKLVRVVAHA	LARKYH	
BarHeaded Goose	101			E...E.....			
Chicken	101			E...E.....			
Human	101	NV.	VG...H..G.	E...PV...Y	..V.AG..N.	..H...	

Alignment of common crane HbB sequence with HbB sequences from Human , Gray lag goose, bar headed goose and chicken.

COMMON CRANE ALPHA A SEQUENCE ALIGNMENT WITH AVES ALPHA CHAIN

	10	20	30	40	50	60
GRU common crane H	V	L	S	G	N	D
ANS Anser Anser	A	A	T	T	A	I
ANS Brantacanadens	A	A	T	D	V	I
ANS airinamoschata	A	A	T	I	I	I
ANS Cygnus olor	A	A	T	D	I	I
ANS Nomonyx domini	A	A	T	I	D	I
ANS Aythya fuligul	A	A	T	I	I	I
ANS Anas penelope	A	A	T	I	S	I
ANS Anas platyrhyn	A	A	T	I	S	I
ANS Oxyura vittata	A	A	T	D	V	I
ANS Oxyura jamaice	A	A	T	D	V	I
ANS Oxyura austral	A	A	T	D	V	I
ANS Oxyura leucoce	A	A	T	D	V	I
ANS Anser indicus	A	A	T	S	T	I
ANS Chloephaga mel	A	A	A	D	I	I
ANS Anseranas semi	A	A	G	T	G	I
APO Apus apus	A	A	T	A	Q	P
CHA ridibundus	S	T	G	A	A	S
CHA Catharacta mac	S	N	G	A		
CIC Vultur gryphus	S	T	A	D	I	I
CIC Ciconia ciconi	A	R	G	S	A	I
CLM treptopelia or	A	S	A	G	Q	I
CLM Columba livia	A	A	A	Q	G	I
CUC Eudynamys scol	A	A	T	I	A	I
CUC Cuculus canoru	A	A	T	I	T	I
FAL accipiter gen	A	T	N	T	T	I
FAL Gyps rueppelli	A	T	N	T	T	I
FAL Aegypius monac	A	T	T	T	T	I
FAL Trigoniceps oc	A	T	T	T	T	I
FAL Aquila chrysa	A	T	N	T	S	I
GAL Coturnix japon	A	A	T	I	A	I
GAL Gallus gallus	A	A	N	I	T	I
GAL Francolinus po	A	A	N	I	G	I
GAL Phasianus colc	A	A	N	I	T	I
GAL Meleagris gall	A	A	N	I	T	I
PAS Sturnus vulga	A	S	A	A	G	G
PAS Turdus merula	A	A	T	S	A	G
PAS Passer montanu	P	A	A	G	Q	G
PAS Poephila gutt	A	G	A	G	Q	G
PEL Phalacrocorax	A	S	T	A	V	I
PHO Phoenicopterus	S	H	L	G	V	P
PSI Ara ararauna	S	T	I	Q	D	V
PSI Psittacula kra	T	T	S	I	Q	V
RHE Rhea americana	P	T	N	A	D	T
SPH Eudytes crest	A	S	S	S	T	H
SPH Aptenodytes fo	A	S	S	S	T	H
STR Struthio camel	T	T	I	S	S	I

	70	80	90	100	110	120
GRU common crane H	...	...	...	...	...	...
ANS Anser Anser	...	A. V. V.	...	...	...	...
ANS Brantacanadens	...	A. V. V.	...	...	...	...
ANS airinamoschata	...	A. V. V.	...	...	...	...
ANS Cygnus olor	...	A. V. V.	...	...	...	...
ANS Nomonyx domini	...	A. V. V.	S.	...	...	...
ANS Aythya fuligul	...	A. V. V.	...	...	...	...
ANS Anas penelope	...	A. V. V.	...	...	...	...
ANS Anas platyrhyn	...	A. V. V.	...	...	...	...
ANS Oxyura vittata	...	A. V. V.	S.	...	...	...
ANS Oxyura jamaice	...	A. V. V.	S.	...	...	...
ANS Oxyura austral	...	A. V. V.	S.	...	...	...
ANS Oxyura leucoce	...	A. V. V.	S.	...	...	...
ANS Anser indicus	...	VA. V. V.	...	...	...	...
ANS Chloephaga mel	...	A. V. V.	T.	...	...	...
ANS Anseranas semi	...	A. V.	...	...	...	...
APO Apus apus	...	S. V. N.	...	...	...	...
CHA ridibundus	...	A. V.	...	...	...	...
CHA Catharacta mac	...	A. V.	S.	...	...	...
CIC Vultur gryphus	...	V.	AS.	...	...	...
CIC Ciconia ciconi	...	L. V.	...	...	...	...
CLM treptopelia or	...	E. V.	...	...	...	...
CLM Columba livia	...	E. V.	...	...	...	...
CUC Eudynamys scol	...	VA. V. V.	H.	A.	...	...
CUC Cuculus canoru	...	VA. V. V.	H.	A.	...	...
FAL accipiter gen	...	V. V.	...	...	...	...
FAL Gyps rueppelli	...	V. V.	...	...	...	...
FAL Aegyptius monac	...	V. V.	...	...	...	...
FAL Trigoniceps oc	...	V. V.	...	...	...	...
FAL Aquila chrysa	...	V. V.	M.	...	...	...
GAL Coturnix japon	...	A. V.	T.	...	...	...
GAL Gallus gallus	...	VA.	T.	H.	...	...
GAL Francolinus po	...	VA.	T.	H.	...	...
GAL Phasianus colc	...	VA. V.	T. T.	H.	...	...
GAL Meleagris gall	...	VA.	T.	H.	...	...
PAS Sturnus vulga	...	A. V. V.	...	...	...	...
PAS Turdus merula	...	A. V. AV.	...	...	...	...
PAS Passer montanu	...	A. V. V. N.	L.	...	...	...
PAS Poephila gutt	...	A. V. NV.	L.	...	...	...
PEL Phalacrocorax	...	A. V.	...	...	...	...
PHO Phoenicopterus	...	A.	S.	QH.	...	...
PSI Ara ararauna	...	A. V.	T.	...	...	...
PSI Psittacula kra	...	G. S.	TS.	...	...	...
RHE Rhea americana	...	VS. D. N.	Y.	...	...	...
SPH Eudytes crest	...	T. M.	...	...	...	...
SPH Aptenodytes fo	...	A.	...	E.	...	...
STR Struthio camel	...	N. V.	S.	...	...	...

GRU common crane H		VHASLDKFLCAVGNVLTAKYR
ANS Anser Anser	T.....	
ANS Brantacandens	T.....	
ANS airinamoschata	M....A.....	
ANS Cygnus olor	A.....	
ANS Nomonyx domini	M....S.....	
ANS Aythya fuligul	M....AT.....	
ANS Anas penelope	A.....	
ANS Anas platyrhyn	M....A.....	
ANS Oxyura vittata	M....A.....	
ANS Oxyura jamaice	M....A.....	
ANS Oxyura austral	M....A.....	
ANS Oxyura leucoce	M....A.....	
ANS Anser indicus	T.....	
ANS Chloephaga mel	M....A.....	
ANS Anseranas semi	M.....AT.....	
APO Apus apus	AT.....	
CHA ridibundus		
CHA Catharacta mac		
CIC Vultur gryphus		
CIC Ciconia ciconi	T.ST...D...	
CLM treptopelia or	VL...T.....	
CLM Columba livia	VL...T.....	
CUC Eudynamys scol	ST.....	
CUC Cuculus canoru	ST.....	
FAL accipiter gen		
FAL Gyps rueppelli		
FAL Aegyptius monac		
FAL Trigonoceps oc	S.....	
FAL Aquila chrysa		
GAL Coturnix japon	T.....	
GAL Gallus gallus	T.....	
GAL Francolinus po		
GAL Phasianus colc	T.....	
GAL Meleagris gall	T.....	
PAS Sturnus vulga	T.....	
PAS Turdus merula	T.....	
PAS Passer montanu	...P.....T.....	
PAS Poephila gutt	DT.....	
PEL Phalacrocorax	M...AKE.....	
PHO Phoenicopterus	T.....	
PSI Ara ararauna		
PSI Psittacula kra	A.....L.....	
RHE Rhea americana	A.....	
SPH Eudytes crest	S.....	
SPH Aptenodytes fo	I.....S.....	
STR Struthio camel	A.....	

COMMON CRANE BETA SEQUENCE ALIGNED WITH AVES BETA CHAINS

	10	20	30	40	50	60
GRU Common crane H	VHWSAEEKQLITSLW	GKVN	VADCGAEALARLL	LIVYPWTQRFFSA	FGNLS	SPTAIIGNPMV
ANS Anser	G.....	S.....	L.....			
ANS Aythyafuligula	...T....I..G.....	S.....	L.....			
ANS Anser indicus	G.....	S.....	L.....			
ANS Anseranas semi	G.....	E.....	S.....			
ANS Chloephaga mel	...T....G.....	AS.....	S.....			
ANS Anas platyrhyn	...T....G.....	AS.....	L.....			
ANS Brantacandens	...T....G.....	S.....	L.....			
ANS airinamoschata	...T....G.....	AS.....	L.....			
ANS Cygnus olor	...T....G.....	S.....	L.....			
APO Apus apus	.Q.T....G.....	AS.....	A.V.....			
CHA Larus ridibund	G.....	AS.....	N.....			
CHA Catharacta mac	G.....	S.....				
CIC Ciconia ciconi	...T....G.....	DE.....	AS.....	ATAS.....	T.....A.....	
CIC Vultur gryphus	G.....	E.....	AS.....			
CLM Columba livia	I.....	S.....	A.....	S.....	N.....	
CLM Streptopelia o	I.....	AS.....	A.....	S.....	N.....	
FAL Trigoniceps oc	...T....G.....	AS.....				
FAL Aquila chrysa	...T....G.....	AS.....				
FAL Accipiter gent	.Q.A....G.....	AS.....	A.....	VL.....		
FAL Aegypius monac	...T....G.....	AS.....				
FAL Gyps rueppelli	...T....G.....	AS.....				
GAL Gallus gallus	...T....G.....	E.....	AS.....	L.....		
GAL Coturnix cotur	G.....	E.....	AS.....	L.....		
GAL Phasianus colc	G.....	AS.....	L.....			
GAL Francolinus po	...T....G.....	E.....	AS.....	L.....		
GAL Meleagris gall	G.....	AS.....	L.....			
PAS Taeniopygia gu	.Q.T....G.....	E.....G.....	AS.....	LL.....	K.....	
PAS Taeniopygia gu	.N.T....V.T..R..DE.....	VA.....	VS.....	M.....	VL.....	
PAS Turdus merula	.Q.T....G.....	E.....G.....	AS.....	VL.....	K.....	
PAS Sturnus vulgar	.Q.T....G.....	E.....G.....	AS.....	VL.....	K.....	
PAS Passer montanu	.Q.T....G.....	E.....G.....	AS.....	VL.....	K.....	
PAS BTaeniopygia g	.Q.T....G.....	E.....G.....	AS.....	VL.....	K.....	
FEL Phalacrocorax	...T....G.....	E.....	AS.....	A.....	T.....	
PHO Phoenicopterus	G.....	E.....	AS.....	L.....		
PSI Psittacula kra	...T....G.....	TS.....	AS.....	VL.....	N.....	
PSI Ara ararauna	.Q.T....G.....	AS.....	L.....			
RHE Rhea americana	G.....	Q.....	G.....	S.....	S.....	L.....
SPH Eudytes crest	G.....	E.....	AS.....	A.....	VLA.....	
SPH Aptenodytes f	.Q.....SG.....	AS.....	L.....			

	70	80	90	100	110	120
GRU Common crane H	RAHGKKVLT	SFGEAVKNLD	NIKNTFAQL	SELHCDKLH	VDPENFRLL	GDILIVLAAHFAK
ANS Anser	D					
ANS Aythyafuligula	D				V	S
ANS Anser indicus	D					
ANS Anseranas semi						S
ANS Chloephaga mel	D		S			T
ANS Anas platyrhyn	D					T
ANS Brantacandens	D					
ANS airinamoschata	D					T
ANS Cygnus olor	D					
APO Apus apus		S	S			S
CHA Larus ridibund						
CHA Catharacta mac						
CIC Ciconia ciconi	H			K		G
CIC Vultur gryphus			E			
CLM Columba livia	K	D	G		V	I
CLM Streptopelia o	K	D	G	N	K	V
FAL Trigoniceps oc						G
FAL Aquila chrysa						T
FAL Accipiter gent					V	G
FAL Aegyptius monac						G
FAL Gyps rueppelli						G
GAL Gallus gallus	D		S			S
GAL Coturnix cotur	D		S			T
GAL Phasianus colc	D		S			S
GAL Francolinus po	D		S			S
GAL Meleagris gall	D		S			S
PAS Taeniopygia gu	Q		S	S		VV
PAS Taeniopygia gu		R	M	SV	KC	K
PAS Turdus merula	Q		S	G		VV
PAS Sturnus vulgar	Q	D	S	S		VV
PAS Passer montanu	Q		S	S		VV
PAS BTaeniopygia g	Q		S	S		VV
PEL Phalacrocorax			A			
PHO Phoenicopterus						
PSI Psittacula kra						G
PSI Ara ararauna						G
RHE Rhea americana	D					
SPH Eudiptes crest	D	M				
SPH Aptenodytes f	D	M				S
STR Struthio camel	D					T

GRU Common crane H	DFTPD	CQAAWQKLV	RVVAHALARKYH
ANS Anser	E...	E.....	
ANS Aythyafuligula	E...	E.....	
ANS Anser indicus	E.....		
ANS Anseranas semi			
ANS Chloephaga mel			
ANS Anas platyrhyn	...	E.....	
ANS Brantacanadens			
ANS airinamoschata			
ANS Cygnus olor			
APU Apus apus	...	EA.Q..A...	A.....
CHA Larus ridibund	...	S.....	
CHA Catharacta mac	E.....		
CIC Ciconia ciconi			K.....
CIC Vultur gryphus			A.....
CLM Columba livia	...	E.....	
CLM Streptopelia o	...	E.....	
FAL Trigoniceps oc	...	S.....	A.....
FAL Aquila chrysa	...	S.....	A.....
FAL Accipiter gent	...	S.....	A.....
FAL Aegyptius monac	...	S.....	A.....
FAL Gyps rueppelli	...	S.....	A.....
GAL Gallus gallus	...	E.....	
GAL Coturnix cotur	...	E.....	
GAL Phasianus colc	...	E.....	
GAL Francolinus po			
GAL Meleagris gall	...	E.....	
PAS Taeniopygia gu			
PAS Taeniopygia gu	E...	A.....	HE...
PAS Turdus merula			
PAS Sturnus vulgar			
PAS Passer montanu			
PAS BTaeniopygia g			
PEL Phalacrocorax	...	E.....	GA.....
PHO Phoenicopterus	...	E.....	
PSI Psittacula kra			A.....
PSI Ara ararauna	...	E.....	L.....
RHE Rhea americana	...	E.....	
SPH Eudytes crest	...	E.....	E.....
SPH Aptenodytes f			A.....
STR Struthio camel	E...	E.....	

AVES CYT-b ALPHA A AND BETA CHAINS CONCATENATED AND ALIGNED

		10	20	30	40	50	60
Crocodylus nilotic	1						
ANS Anser anser	1	-GSLLGFTLL	VQLASGILIM	MHFLADDSIA	-FMSVAYTSR	EVWYGWLIRS	LHANGASLFF
ANS Aythya fuligul	1	-....AIC.A	T.ILT.L.A	..YT..T...	-S...H.C.	D.Q.....N	F..
ANS Anser indicus	1	F....AVC.A	T.ILT.L.A	..YT..T...	-S...N.C.	N.Q.....N	F..
ANS Anseranas semi	1	-....AIC.A	T.ILT.L.A	..YT..T...	-S...H.C.	D.Q.....N	F..
ANS Chloephaga pic	1	-....AIC.M	T.ILT.L.A	..YT..TT..	-S...H.C.	N.Q.....N	F..
ANS Anas platyrhyn	1	F....AAC.I	A.ILT.L.A	..YT..T...	-S...N.C.	N.Q.....N	F..
ANS Branta canaden	1	F....AIC.A	T.ILT.L.A	..YT..T...	-S...N.C.	N.Q.....N	F..
ANS Cairina moscha	1	-....AIC.A	T.ILT.L.A	..YT..T...	-A...H.C.	D.Q.....N	F..
ANS Cygnus olor	1	F....AIC.V	T.ILT.L.A	..YT..TT..	-S...N.C.	N.Q.....N	F..
APU Apus apus	1	--...AIC.A	T.ILT.L.A	..YT..T...	-A...H.C.	D.Q.....N	F..
CHA Larus ridibund	1	-....IC.A	T.IIT.L.A	..YT..TT..	-S...H.C.	N.Q.....N	F..
CHA Catharacta mac	1	F....IC..	T.ILT.L.A	..YT..TT..	-S...H.C.	N.Q.....N	F..
CIC Ciconia ciconi	1	-....IC..	T.ILT.L.A	..YT..TT..	-T...H.C.	N.Q.....N	F..
CIC Vultur gryphus	1	-....IC.T	M.ILT.L.A	T.YT..TT..	-S...H.C.	D.Q.....N	F..
CLM Columba livia	1	-....IC.M	T.ILT.L.A	..YT..TT..	-S...H.C.	N.Q.....N	F..
CLM Streptopelia o	1	-....IC..	T.ILT.L.A	A.YT..TT..	-S...H.C.	N.Q....R..N	F..
FAL Trigoniceps oc	1	F....IC.M	T.ILT.L.A	A.YT..TT..	-S...H.C.	N.Q.....N	F..
FAL Aquila chrysa	1	-....IC..	T.ILT.L.A	..YT..T...	-S...H.C.	N.Q.....N	F..
FAL Accipiter gent	1	-....IC.M	T.ILT.L.A	..YT..TT..	-S...H.C.	N.Q.....N	F..
FAL Aegypius monac	1	-....IC..	T.ILT.L.A	T.YT..TT..	-S...H.C.	N.Q.....N	F..
FAL Gyps rueppelli	1	-....IC..	T.ILT.L.A	..YT..T...	-S...H.C.	N.Q.....N	F..
GAL Gallus gallus	1	---...IC..	T.ILT.L.FA	..YP..T...	-S...H.C.	N.Q.....N	F..
GAL Coturnix cotur	1	-....AVC.M	T.ILT.L.A	..YT..T...	-S...H.C.	N.Q.....N	F..
GAL Phasianus colc	1	-....AMC.I	T.ILT.L.A	..YT..T...	-S...H.C.	N.Q.....N	F..
GAL Francolinus af	1	-....AVC.I	T.ILT.L.A	..YT..T...	-S...H.C.	N.Q.....N	F..
GAL Meleagris gall	1	-....AMC.I	T.ILT.L.A	..YT..T...	-S...H.C.	N.Q.....N	F..
GRU Grus grus	1	-....AVC.I	T.ILT.L.A	..YT..TT..	-S....C.	N.Q....L.N	F..
PAS Taeniopygia gu	1	-....IC.A	T.ILT.L.A	A.YT..TT..	-S...H.C.	N.Q.....N	F..
PAS Turdus merula	1	-....IC.I	T.IVT.L.A	..YT..T...	-S...HMC.	D.QF.....N	F..
PAS Sturnus vulgar	1	-----	---IT.L.A	T.YT..T...	-N...HMC.	N.QF.....N	M..
PAS Passer montanu	1	-----	---L.A	T.YT..T...	-N...HMC.	N.QF.....N	F..
PEL Phalacrocorax	1	-....IC.I	S.IVT.L.A	..YT..TN..	-S...HMC.	D.QF.....N	F..
PHO Phoenicopterus	1	FWL...IC.M	T.ILT.L.A	..YT..TT..	-S...SH.C.	N.Q.....N	F..
PSI Psittacula kra	1	-----	-----	..YT..T...	-S...N.C.	N.Q.....N	F..
PSI Ara ararauna	1	-----	---LT.L.A	D.YT..T...	-S...NMC.	N.Q.....N	F..
RHE Rhea americana	1	-....IC.I	T.ILT.L.A	..YT..T...	-S...H.C.	N.Q.....N	F..
SPH Eudiptes chrys	1	-....IC.A	T.ILT.L.A	A.YT..TT..	A.S...H.C.	N.Q.....N	F..
SPH Aptenodytes fo	1	-----	T.ILT.L.A	..YT..TT..	-S...H.C.	N.Q.....N	F..
STR Struthio camel	1	-....IC.I	T.ILT.L.A	..YT..TT..	-S...H.C.	N.Q....F..N	F..

		70	80	90	100	110	120
Crocodylus nilotic	59	LCIFLHIGRG	VYYGSYLNN	TWNIGVLLLL	LLMATAFMGY	VLPWGQMSFW	GATVITNIMS
ANS Anser anser	59	I..Y.....	L.....YKE	...T..I...	T.....V..		F.
ANS Aythya fuligul	60	I..Y.....	F.....YKE	...T..I...	T.....V..		F.
ANS Anser indicus	60	I..Y.....	L.....YKE	...T..I...	T.....V..		F.
ANS Anseranas semi	59	I..Y.....	L.....YKE	...T..I...	T.....V..		F.
ANS Chloephaga pic	60	I..Y.....	F.....YKE	...T..I...	A.....V..		F.
ANS Anas platyrhyn	60	I..Y.....	F.....YKE	...T..I...	T.....V..		F.
ANS Branta canadens	59	I..Y.....	L.....YKE	...T..I...	T.....V..		F.
ANS Cairina moscha	60	I..Y.....	F.....YKE	...T..V...	A.....V..		F.
ANS Cygnus olor	58	I..Y.....	L.....YKE	...T..I...	T.....V..		F.
APO Apus apus	59	I..Y.....	F.....YKE	...T..I...	T.....V..		F.
CHA Larus ridibund	60	I..Y.....	F.....YKE	...T..I...	T.....V..		F.
CHA Catharacta mac	59	I..Y.....	F.....YKE	...T..I...	T.....V..		F.
CIC Ciconia ciconi	59	I..Y.....	F.....YKE	...T..I...	T.....V..		F.
CIC Vultur gryphus	59	I..Y.....	L.....YKE	...T..I...	T.....V..		F.
CLM Columba livia	59	I..Y.....	L.....YKE	...T..V...	T.....V..		F.
CLM Streptopelia o	60	I..Y.....	L.....YKE	...T..I...	T.....V..		F.
FAL Trigoniceps oc	59	I..Y.....	L.....YKE	...T..IV...	T.....V..		F.
FAL Aquila chrysaes	59	I..Y.....	L.....YKE	...T..II...	T.....V..		F.
FAL Accipiter gent	59	I..YF.....	L.....YKE	...T..II...	T.....V..		F.
FAL Aegypius monac	59	I..Y.....	L.....YKE	...T..IV...	T.....V..		F.
FAL Gyps rueppelli	57	M..Y.....	L.....YKE	...T..II...	T.....V..		F.
GAL Gallus gallus	59	I.....	L.....YKE	...T..I...	T.....V..		F.
GAL Coturnix cotur	59	I.....	L.....YKE	...T..I...	T.....V..		F.
GAL Phasianus colic	59	I.....	L.....YKE	...T..V...	T.....V..		F.
GAL Francolinus af	59	I.....	L.....YKE	...T..I...	T.....V..		F.
GAL Meleagris gall	59	I.....	L.....YKE	...T..V...	T.....V..		F.
GRU Grus grus	59	I..Y.....	L.....YKE	...T..I...	T.....V..		F.
FAS Taeniopygia gu	59	I..YF.....	I.....KE	...V..I...	T.....V..		L.
FAS Turdus merula	47	I..Y.....	F.....KE	...I.....	T.....V..		F.
FAS Sturnus vulgar	45	M..Y.....	L.....KE	...V..I...	T.....V..		F.
FAS Passer montanu	59	I..Y.....	L.....KE	...I.....	T.....V..		F.
FEL Phalacrocorax	60	I..Y.....	L.....YKE	...T..II...	T.....V..		F.
PHO Phoenixopterus	19	I..YF.....	F.....YKE	...T..I...	T.....V..		G-----
PSI Psittacula kra	39	I..Y...A..	F.....YKE	...T..II...	T.....V..		F.
PSI Ara ararauna	47	I..Y...A..	L.....YKE	...T..II...	T.....G..		F.
RHE Rhea americana	59	I..Y.....	F.....YKE	...T..V...	T.....V..		G.E....F.
SPH Eudiptes chrys	60	I..Y.....	L.....YKE	...T..II...	T.....V..		F.
SPH Aptenodytes fo	50	I..Y.....	F.....YKE	...T..II...	T.....V..		F.
STR Struthio camel	59	I..Y.....	L.....YKE	...T..I...	T.....V..		F.

	130	140	150	160	170	180
Crocodylus nilotic	119	AIPIYVGNTIV	TWIWGGPSVN	SATLTREFTTL	HFLLPFFILLA	AVITHLIFLH ERGSFNPLGL
ANS Anser anser	119	I.Q.L. E.A...F.D NP....FAI	LIAG	ITLV..T...	S..N....I	
ANS Aythya fuligul	120	.L..I.Q.L. E.A...F.D NP....FAI	LIAG	ITLV..T...	S..N....I	
ANS Anser indicus	120	I.Q.L. E.A...F.D NP....FAI	LIAG	ITLV..T...	S..N....I	
ANS Anseranas semi	119	I.Q.L. E.A...F.D NP....FAI	VIAG	ITLI..T...	S..N....I	
ANS Chloephaga pic	120	.L..I.Q.L. E.A...F.D NP....FAI	LIAG	ITLV..T...	S..N....I	
ANS Anas platyrhyn	120	.L..I.Q.L. E.A...F.D NP....FAI	LIAG	ITLV..T...	S..N....I	
ANS Branta canadensis	119	I.Q.L. E.A...F.D NP....FAI	LIAG	ITLV..T...	S..N....I	
ANS Cairina moscha	120	.L..I.Q.L. E.A...F.D NP....FAI	LIAG	ITLV..T...	S..N....I	
ANS Cygnus olor	118	I.Q.L. E.A...F.D NP....FAI	LIAG	ITLV..T...	S..N....I	
APO Apus apus	119	I.Q.L. E.A...F.D NP....FAI	LIAG	ITLV..T...	S..N....I	
CHA Larus ridibund	120	I.Q.L. E.A...F.D NP....FAI	MIAG	LTLI..T...	S..N....I	
CHA Catharacta mac	119	I.Q.L. E.A...F.D NP....FAI	MIAG	LTLI..T...	S..N....I	
CIC Ciconia ciconi	119	I.Q.L. E.A...F.D NP....FAI	AIAG	LTLI..T...	S..N....I	
CIC Vultur gryphus	119	I.Q.L. E.A...F.D NP....FAI	AIAG	LTLI..T...	S..N....I	
CIM Columba livia	119	.V..I.Q.L. E.A...F.D NP....F..	MIAG	LT.I..T...	S..N....I	
CIM Streptopelia o	120	.V..I.Q.L. E.A...F.D NP....F..	MIAG	LT.I..T...	S..N....I	
FAL Tringoides oc	119	.V..I.Q.L. E.A...F.D NP....FAI	VIAG	LTLI..T...	S..N....I	
FAL Aquila chrysaes	119	I.Q.L. E.A...F.D NP....FAI	LIAS	LTLI..T...	S..N....I	
FAL Accipiter gent	119	I.Q.L. E.A...F.D NP....FAI	LIAS	LTLI..T...	S..N....I	
FAL Aegypius monac	119	I.Q.L. E.A...F.D NP....FAI	AIAG	LTLI..T...	S..N....I	
FAL Gyps rueppelli	117	I.Q.L. E.A...F.D NP....FAI	VIAG	LTLI..T...	S..N....I	
GAL Gallus gallus	119	I.H.L. E.A...F.D NP....FAI	AIAG	IT.I..T...	S..N....I	
GAL Coturnix cotur	119	.V..I.Q.L. E.A...F.D NP....FAI	LIAG	IT.I..T...	S..N....I	
GAL Phasianus colic	119	I.Q.L. E.A...F.D NP....FAI	VIAG	IT..M...	S..N....I	
GAL Francolinus af	119	I.Q.L. E.A...F.D NP....FAI	AIAG	IT.I..T...	S..N....I	
GAL Meleagris gall	119	I.Q.L. E.A...F.D NP....FAI	MIAG	IT.I..M...	S..N....I	
GRU Grus grus	119	.V..I.Q.L. E.A...F.D NP....F..	MIMG	LTLI..T...	S..N....I	
PAS Taeniopygia gu	119	I.Q.L. E.A...F.D NP....FAI	VIAG	LTLV..T...	T..N....I	
PAS Turdus merula	107	I.Q.L. E.A...F.D NP....FAI	VIAG	LTLV..T...	T..N....I	
PAS Sturnus vulgar	105	I.Q.L. E.A...F.D NP....FAI	LIVG	LTLV..T...	T..N....I	
PAS Passer montanu	119	I.Q.L. E.A...F.D NP....FAI	LIVG	LTLV..T...	T..N....I	
FEL Phalacrocorax	111	-----	-----	-----	-----	-----
PHO Phoenicopterus	79	I.Q.L. E.A...F.D NP....FAI	VITG	LTLI..T...	T..N....I	
PSI Psittacula kra	99	I.Q.L. E.A...F.D NP....FAI	MITS	L..I..T...	S..N....I	
PSI Ara ararauna	107	I.H.L. E.A...F.D NP....F..	MIGS	LILV..A...	S..N....I	
RHE Rhea americana	119	I.Q.L. E.A...F.D NP....FAI	LIAG	ITLI..T...	T..N....I	
SPH Eudytes chrys	120	I.Q.L. E.A...F.D NP....F..	MIAG	LTLI..T...	S..N....I	
SPH Aptenodytes fo	110	I.Q.L. E.T...F.D NP....FAI	MIAG	LTLI..T...	S..N.HW.I	
STR Struthio camel	119	I.Q.L. E.A...F.D NP....FAI	VIAG	ITLV..T...	S..N....I	

		190	200	210	220	230	240
Crocodylus nilotic	179	TSNADKIPFH	PYFSAKDAMG	MAMAAVLLTT	LTFYFPNLLG	DPENFTPADP	MKTPDHIKPE
ANS Anser anser	179	V.DC.....	...I..IL.	LTIMITP.MA	.ALFS.....	N.	LV..P.....
ANS Aythya fuligul	180	V.DC.....	...F..IL.	FIIMLTP.MA	.ALFS.....	N.	LV..P.....
ANS Anser indicus	180	V.DC.....	...I..SL.	LTIMITP.MA	.ALFS.....	N.	LV..P.....
ANS Anseranas semi	179	M.DC.....	...L..IL.	FTIMLSP.MA	.ALFS.....	N.	LV..P.....
ANS Chloephaga pic	180	V.DC.....	...F..VL.	F.LMLTP.VA	.ALFS.....	N.	LV..P.....
ANS Anas platyrhyn	180	V.DC.....	...F..IL.	FIIMLTP.MA	.ALFS.....	N.	LV..P.....
ANS Branta canaden	179	V.DC.....	...L..IL.	LTIMITP.MA	.ALFS...M.	N.	LV..P.....
ANS Cairina moscha	180	V.DC.....	...L..VL.	FIIMLTP.MA	.ALFS.....	N.	LV..P.....
ANS Cygnus olor	178	V.DC.....	...L..IL.	LIIMLTP.MA	.ALFS.....	N.	LV..P.....
APO Apus apus	179	V..C.....	...T..LL.	FIIMLTP.L.	.ALFS.....	N.	LV..P.....
CHA Larus ridibund	180	Q..C.....	...L..IL.	FIIMFLP...	.ALFS.....	N.	LV..P.....
CHA Catharacta mac	179	V..C.....	...L..IL.	FIIMFLP.A.	.ALFS.....	N.	LV..P.....
CIC Ciconia ciconi	179	V..C.....	...L..IL.	LTLLLLP...	.ALFS.....	N.	LV..P.....
CIC Vultur gryphus	179	V.SC.....X	...TL..IL.	FVIMLLP...	.ALFS.X...	N.	LV..P.....
CLM Columba livia	179	...C.....	...L..IL.	FMVMLLP.M.	.ALFS.....	N.	LV..P.....
CLM Streptopelia o	180	S..C.....	...L..IL.	FMIMFLP.M.	.ALF.....	N.	LV..P.....
FAL Trigoniceps oc	179	I..C.....	...F..IL.	FMIMLLP...	.ALFS.....	N.	LV..P.....
FAL Aquila chrysa	179	I..C.....	...L..IL.	FVIMLLP...	.ALFS.....	N.	LV..P.....
FAL Accipiter gent	179	I..C.....	...L..XL.	FLIMLLP...	.ALFS.....	N.	LV..P.....
FAL Aegyptius monac	179	V..C.....	...F..IL.	FMIMLLP...	.ALFS.....	N.	LV..P.....
FAL Gyps rueppelli	177	I..C.....	...L..IL.	FVIMLLP..A	.ALFS.....	N.	LV..P.....
GAL Gallus gallus	179	S.DS.....	...Y.F..IL.	LTIMLTPFL.	.ALFS.....	N.	LV..P.....
GAL Coturnix cotur	179	S.DS.....	...Y.I..IL.	LTIMLTPFL.	.ALFS..F..	N.	LV..P.....
GAL Phasianus colc	179	S..S.....	...Y.L..IL.	L.LMLTPFL.	.ALFS.....	N.	LV..P.....
GAL Francolinus af	179	S.DS.....	...Y.I..IL.	LTIMLTPFL.	.ALFS.....	N.	LV..P.....
GAL Meleagris gall	179	S.....	...Y.I..IL.	LTIMLTP.L.	..LFS.....	N.	LV..P.....
GRU Grus grus	179	V..C.....	...L..IL.	FMIMLLP.M.	.ALFS.....	N.	LV..P.....
PAS Taeniopygia gu	179	P.DC.....	...YTT..IL.	F.LMLSI.AS	.ALFS.....	N.	LV..P.....
PAS Turdus merula	167	PADC.....	...Y.T..IL.	F.LMLI..VS	.ALFS..A..	N.	LV..P.....
PAS Sturnus vulgar	165	PADC.....	...Y.T..IL.	F.LMLI..VS	MALFS.....	N.	LV..P.....
PAS Passer montanu	179	P.DC.....	...YTV..IL.	FVIMLS..VS	.ALFS.....	N.	LV..P.....
FEL Phalacrocorax	111	-----	-----	-----	-----	-----	-----
PHO Phoenicopterus	139	V..C.....	...L..IL.	FMIMLLP.M.	.ALFS.....	N.	LV..P.....
PSI Psittacula kra	159	P..C.....	...L..LL.	F.LMLLS...	.ALFS.....	N.	LV..P.....
PSI Ara ararauna	167	P..C.....	...L..LL.	FTIMLF...A	.GLFS.....	N.	LV..P.....
RHE Rhea americana	179	V.HS.....	...L...L.	F.LMFIP.L.	.A.FS.....	N.	LV..P.....
SPH Eudytes chrys	180	VA.S.....	...Y.T..IL.	FILLLLP..A	.ALFS.....	N.	LV..P.....
SPH Aptenodytes fo	170	VA.S.....	...Y.T..IL.	F.LMLLP...	..LFS.....	...L...N.	LV.HHTM...
STR Struthio camel	179	I.HC.....	...L..IL.	FTIMFIP.LS	.A.FS.....	N.	LA..P.....

		250	260	270	280	290	300
Crocodylus nilotic	239	WYFLFAYTIL	RCIPNKLTGV	LAMFASILVL	LLLPTLHTSK	RQSMSLRPLS	QLLFWALIAD
ANS Anser anser	239	A..	.S.....G..	..LA..V.I.	F.I.F..K..	QRA.TF....	V.V..
ANS Aythya fuligul	240	A..	.S.....G..	..LA..V.I.	F.I.F..K..	QRT.TF....	T.V.N
ANS Anser indicus	240	A..	.S.....G..	..LA..V.I.	F.I.F..K..	QRA.TF....	V.V..
ANS Anseranas semi	239	A..	.S.....G..	..LA..V.I.	F.S.L..K..	QRT.TF....	T.V.N
ANS Chloephaga pic	240	A..	.S.....G..	..LV..V.I.	F.I.F..K..	QRA.TF....	V.N
ANS Anas platyrhyn	240	A..	.S.....G..	..LA..V.I.	F.V.F..K..	QRT.TF....	T.V.N
ANS Branta canad	239	A..	.S.....G..	..LA..V.I.	F.I.F..K..	QRT.TF....	I.V..
ANS Cairina moscha	240	A..	.S.....G..	..LA..V.I.	F.V.F..K..	QRT.TF....	V.N
ANS Cygnus olor	238	A..	.S.....G..	..LA..V.I.	F.I.F..K..	QRA.TF....	M.V..
APO Apus apus	239	A..	.S.....G..	..LA..V.I.	F.C.F..K..	QRT.TF....	I..V.V.N
CHA Larus ridibund	240	A..	.S.....G..	..YA..V..	F.S.L..K..	QRT.TF....	I.VTN
CHA Catharacta mac	239	A..	.S.....G..	..LA..V..	F.I.L..K..	QRT.TF....	I..T.V.N
CIC Ciconia ciconi	239	A..	.S.....G..	..LA..V.I.	F.C.L..K..	QRT.AF....	T.T.N
CIC Vultur gryphus	239	A..	.S.....G..	..LA..V..	F.M.F..K..	QRT.TF....	T.V.N
CLM Columba livia	239	A..	.S.....G..	..LA..V.I.	F.T.L..K..	QRT.IF....	I.VTN
CLM Streptopelia o	240	A..	.S.....G..	..LA..V..	F.T.L..K..	QRT.IF....	T.VTN
FAL Trigoniceps oc	239	A..	.S.....G..	..LA..V.I.	F.I.F..K..	QRT.TF....	Y....N
FAL Aquila chrysa	239	A..	.S.....G..	..LA..V.I.	F.I.F..K..	QRT.TF....	T...N
FAL Accipiter gent	239	A..	.S.....G..	..LA..V.I.	F.I.F..K..	QRT.TF..F.	I..T...N
FAL Aegypius monac	239	A..	.S.....G..	..LA..V.I.	F.I.F..K..	QRT.TF....	Y.T...N
FAL Gyps rueppelli	237	A..	.S.....G..	..LA..V.I.	F.I.F..K..	QRT.TF....	T...L.V.N
GAL Gallus gallus	239	A..	.S.....G..	..LA..V.I.	F.I.F..K..	QRT.TF....	T...L.V.N
GAL Coturnix cotur	239	A..	.S.....G..	..LA..V.I.	...F..K..	QRT.TF..F.	A...L.V.N
GAL Phasianus colc	239	A..	.S.....G..	..LA..V.I.	...F..K..	QRT.TF....	T...L.V.N
GAL Francolinus af	239	A..	.S.....G..	..LA..V.I.	...F..K..	QRT.TF....	T...L.V.N
GAL Meleagris gall	239	A..	.S.....G..	..LA..V.I.	...F..K..	QRA.TF....	T...L.V.N
GRU Grus grus	239	A..	.S.....G..	..LA..V.I.	F.A.L..K..	QRT.TF....	T.T.N
PAS Taeniopygia gu	239	A..	.S.....G..	..LA.....	F...L....	LR..TF....	I...S.V.N
PAS Turdus merula	227	A..	.S.....G..	..LA..V..	F.T.L..K..	QR..TF....	I...V.N
PAS Sturnus vulgar	225	A..	.S.....G..	..LA..V..	F.M.L....	LR..TF..I.	V...T.V.N
PAS Passer montanu	239	A..	.S.....G..	..LA.....	F.T.L....	FR..TF....	I...V.N
PEL Falacrocorax	111	A..	.S.....G..	..LA..V..	F.S.L..K..	QRTLTF....	T.V.N
PNO Phoenicopterus	199	A..	.S.....G..	..LA..V.I.	F.S.L..K..	QRT.AF..A.	T.P.-
PSI Psittacula kra	219	A..	.S.....G..	..LA..V.I.	F.S.L..K..	QGA.TF..M.	T.A.N
PSI Ara ararauna	227	G.A..	.S.....G..	..LA..V.I.	F.I.F..K..	QR..TF....	I...L.V.N
RHE Rhea americana	239	A..	.S.....G..	..LA..V.I.	F.I.F..K..	QRT.TF....	T.V.N
SPH Eudytes chrys	240	A..	.S.....G..	..LA..V.I.	F.I.L..K..	QRT.AF....	V.N
SPH Aptenodytes fo	230	V.A..	.S.....G..	..LA..V.I.	F.I.L..K..	QR..TF....	F.V.N
STR Struthio camel	239	A..	.S.....G..	..LA..V.I.	F.I.L..K..		

	310	320	330	340	350	360
Crocodylus nilotic	299	FFILTWIGGQ	PVQDPYILIG	QVASEMYFFT	ILILLPLAGM	IENLLIKPTV
ANS Anser anser	299	LLV...V.S.	..EH.F.I..	.L..IA..AI	L.F...A.SA	L..KMLN-F..
ANS Aythya fuligul	300	LLV...V.S.	..EH.F.I..	.L..IT..TI	L.F.F.AVSA	L..KMLN-C..
ANS Anser indicus	300	LLV...V.S.	..EH.F.I..	.L..IA..TI	L.F...A.SA	L..KMLN-C..
ANS Anseranas semi	299	L....V.S.	..EH.F.I..	.L..LS..TI	L.F.F.II.A	L..KMLN-Y..
ANS Chloephaga pic	300	LLV...V.S.	..EH.F.I..	.I..IT..TI	L.F.F.AVSA	L..KMLN-Y..
ANS Anas platyrhyn	300	LLV...V.S.	..EH.F.I..	.L..IT..TI	L.F.F.AVSA	L..KMLN-C..
ANS Branta canadensis	299	LL....V.S.	..EH.F.I..	.L..IA..TI	L.F.F.IVSA	L..KMLN-C..
ANS Cairina moscha	300	LLV...V.S.	..EH.F.I..	.L..IT..TI	.F.F.AVSA	L..KMLN-Y..
ANS Cygnus olor	298	LL....V.S.	..EH.F.I..	.L..IA..TI	L.F.F.I.SA	L..KMLN-C..
APO Apus apus	299	LL....V.S.	..EH.F.I..	.L..LT..TI	L...F..I.A	L..KMLN-Y..
CHA Larus ridibund	300	L....V.S.	..EH.F.I..	.L..LA..TI	L.L.F.AI.A	L..KMLN-Y..
CHA Catharacta mac	299	LL....V.S.	..EH.F.I..	.L..LT..TI	L.L.F.AI.T	L..KMLN-Y..
CIC Ciconia ciconi	299	LL....V.S.	..EH.F.I..	.L..LT....	L...F..I.A	L..K.LN-Y..
CIC Vultur gryphus	299	LL....V.S.	..EH.F.I..	.L..LT..TI	L...F.TL.A	L..KVLS---
CLM Columba livia	299	LL....V.S.	..EH.F.I..	.L..LT..TI	L.V.F.ATAA	L..K.LN-Y..
CLM Streptopelia o	300	LL....V.S.	..EH.F.I..	.L..LT..TI	L...F.AI.A	L..K.LN-Y..
FAL Trigoniceps oc	299	LL.....S.	..EH.F.I..	.L..IT..TI	L.V.F..T.A	L..KVLS---
FAL Aquila chrysaes	299	LL.....S.	..EH.F.I..	.L..LT..S.	L...F..I.A	L..KVLS---
FAL Accipiter gent	299	LL.....S.	..EH.F.I..	.L..LT..SI	L.A.F..T.A	L..KVLS---
FAL Aegyptius monac	299	LL.....S.	..EH.F.I..	.L..IT..TI	L.V.F..T.A	L..KVLS---
FAL Gyps rueppelli	297	LL.....S.	..EH.F.I..	.L..IT..TI	L.V.F..T.A	L..KVLS---
GAL Gallus gallus	299	LL.....S.	..EH.F.I..	.M..LS..TI	L...F.TI.T	L..KMLN-Y..
GAL Coturnix cotur	299	LL.....S.	..EH.F.I..	.M..LS..TI	L...F.MI..	L..KMLN-H..
GAL Phasianus colic	299	LL....V.S.	..EH.F.I..	.M..LS..TI	L...F.AI.T	L..KMLN-F..
GAL Francolinus af	299	LL....V.S.	..EH.F.I..	.T...S..SI	L...F.VI.T	L..KMLN-H..
GAL Meleagris gall	299	LL....V.S.	..EH.F.I..	.M..LS..TI	L...F..I.A	L..KMLN-L..
GRU Grus grus	299	LL....V.S.	..EH.F.I..	.L..LT..TI	L...F.II.A	L..KMLN-Y..
PAS Taeniopygia gu	299	VL....V.S.	..EH.F.I..	.L..LS..TI	...F...AL	L..KML-L..
PAS Turdus merula	287	LL....V.S.	..EH.F.I..	.L..LS..TI	.V.F...AV	L..KML---
PAS Sturnus vulgar	285	LL....V.S.	..EH.F.I..	.L..LS..TI	.V.F...AV	L..KML-L..
PAS Passer montanu	299	VLV...V.S.	..EH.F.I..	.L..LS..TI	.V.F...AI	L..K.L-L..
PEL Falacrocorax	111	-----	-----	-----	-----	-----
PHO Phoenicopterus	259	L....V.S.	..EH.F.I..	.L..LT..T.	L...F.II.A	L..KMLN-Y..
PSI Psittacula kra	277	-----	-----	-----	-----	-----
PSI Ara ararauna	287	L.....S.	..EH.F.I..	.L..LT..TI	.V...IISF	L..KILN---
RHE Rhea americana	299	LL.....S.	..EH.F.I..	.M...S..LI	L.V.F.AI.A	L..KML-Y..
SPH Eudiptes chrys	300	LT.....S.	..EH.F.I..	.L..LT..TI	L...F..I.T	L..KMLN-H..
SPH Aptenodytes fo	290	LI....V.S.	..EH.F.I..	.L..LT..T.	L...F..I.A	L..KMLN---
STR Struthio camel	299	LL.....S.	..EH.F.I..	T..LI	L.V.F.AIAA	L..KM--Y..

	370	380	390	400	410	420
<i>Crocodylus nilotic</i>	359	AVWSKVAGHL	EEYGAEALER	MFCAYPQTKI	YFPHFDSLHG	SAQIRAHGKK
<i>ANS Anser anser</i>	358	G.F..IG..A	T...	..T.....T	Q...	...K....A..V...
<i>ANS Aythya fuligul</i>	359	GIF..IG..A	T...	..IT.....T	Q...	...K....A..V...
<i>ANS Anser indicus</i>	359	G.F..IS..A	T...	..T.....T	Q...	...K....V..V...
<i>ANS Anseranas semi</i>	358	T.FG..IG..A	T.Q.	..QTF....T	QP.	...K....A..V..A.
<i>ANS Chloephaga pic</i>	359	G.F..IG..A	DD....T...	..I.....T	H...	...K....A..V...
<i>ANS Anas platyrhyn</i>	359	G.F..IG..A	T...	..I.....T	Q...	...K....A..V...
<i>ANS Branta canadensis</i>	358	G.F..IG..A	D....T...	..V.....T	Q...	...K....A..V...
<i>ANS Cairina moscha</i>	359	G.F..IG..A	T...	..I.....T	Q...	...K....A..V...
<i>ANS Cygnus olor</i>	357	G.F..IG..A	DD....T...	..I.....T	Q...	...K....A..V...
<i>APO Apus apus</i>	358	G.FA..IG.QA	..AL.G...A.	..A...P..T	P...	...VK....AS..V..A.
<i>CHA Larus ridibund</i>	359	G.FG..IG..A	T...	..AT.....T	Q...	...VK....A..V..A.
<i>CHA Catharacta mac</i>	352	G.FG..IG..A	T...	..AT.....T	Q...	...VK....A..V..A.
<i>CIC Ciconia ciconi</i>	358	G.FG..ISA.A	DD....T...	..TVH.TQ.T	HR.	...K....AG..L...
<i>CIC Vultur gryphus</i>	354	G.FA..IG..A	.D....T...	..IT.....T	Q...	...KG....VG..I..A.
<i>CLM Columba livia</i>	358	..FA..IG.QA	GDL.G....	L.IT.....T	Q...	...KG....AE..V..A.
<i>CLM Streptopelia o</i>	359	..AFG..IG.QA	GDL.GQ....	..IT.....T	Q...	...KG....AE..V..A.
<i>FAL Trigoniceps oc</i>	354	T.FT..IT..A	.D....T...	..IT..P..T	H...	...K....VG..I....
<i>FAL Aquila chrysaes</i>	354	N.FT..IS..A	.D....T...	..TT..P..T	H...	...K....VG..I....
<i>FAL Accipiter gent</i>	354	N.FT..IG..A	T...	..TT..P..T	H...	...K....VG..I....
<i>FAL Aegypius monac</i>	354	T.FT..IT..A	.D....T...	..IT..P..T	H...	...K....VG..I....
<i>FAL Gyps rueppelli</i>	352	N.FT..IT..A	.D....T...	..TT..P..T	H...	...K....VG..I....
<i>GAL Gallus gallus</i>	358	GIFT..I...A	T...	..TT..P..T	H...	...K....VG..I....
<i>GAL Coturnix cotur</i>	358	GIFA..I...A	D...	..TT.....T	V...	...KG....V...I..A.
<i>GAL Phasianus colic</i>	358	GIFT..I...A	T...	..IT..S..T	Q...	...KG....A..V..A.
<i>GAL Francolinus af</i>	358	GIFG..ISS.A	.D....T...	..IT..S..T	Q...	...KG....V...I..A.
<i>GAL Meleagris gall</i>	358	GIFT..I...A	T...	..IT..P..T	Q...	...KG....V...I..A.
<i>GRU Grus grus</i>	358	G.F..IG..A	T...	..T.....T	Q...	...VK....AG..I..A.
<i>FAS Taeniopygia gu</i>	358	..FG..IG.QA	D....D...	..AT.....T	GK.	...VKG....A..V..A.
<i>FAS Turdus merula</i>	344	SAF..IG.QA	D....T...	..AT.....T	GK.	...VKG....A..V..A.
<i>FAS Sturnus vulgar</i>	344	..FG..IG.QA	..F...T...	..AT.....T	GK.	...VKG....A..V..A.
<i>FAS Passer montanu</i>	358	G.FA..IG.QA	D...	..AT.....T	GK.	...VKG....A..V..A.
<i>FEL Phalacrocorax</i>	123	G.FA..G.SA	.A....T...	..T.....T	H...	...K....A..V..A.
<i>PHO Phoenicopterus</i>	318	GLFG..G...	...C..T.A.	..A.....T	QP.	...VK....AG..A..A.
<i>PSI Psittacula kra</i>	289	SIF..IG.QA	DD.....	..VT.....T	V.P.	...VK....AGG.S..A.
<i>PSI Ara ararauna</i>	345	GIF..IG.QA	.D....T...	..ATF....T	V.P.	...VK....A..V..A.
<i>RHE Rhea americana</i>	357	N.FA..IG..A	DA....T...	..TT.....T	H...	...KT....VS..ID..A.
<i>SPH Eudiptes chrys</i>	359	G.F..ISS.A	T...	..TT.....T	H...	...VK....AT..M..A.
<i>SPH Aptenodytes fo</i>	348	SIF..ISS.A	T...	..TT.....T	H...	...VK....A...I..A.
<i>STR Struthio camel</i>	357	GIF..ISS.A	T...	..IT.....T	H...	...K....AN..I....

	430	440	450	460	470	480
Crocodylus nilotic	419	HIDDLPGALC	RLSELHAHSL	RVDPVNFKFL	AQCVLVVVAI	HHPGSLTPEV HASLDKFLCA
ANS Anser anser	418	IA...S	K..D...QK.		GH.F.....	...SA.....
ANS Aythya fuligul	419	IA...S	K..D...QK.	R..	GH.F...L..	...SA.....M..
ANS Anser indicus	419	IA...S	K..D...QK.		GH.F.....	...SA...A..
ANS Anseranas semi	418	IA...S	K..D...QK.		GH.F...L..	...SL.....M..
ANS Chloephaga pic	419	IT...S	K..D...QK.		GH.F.....	...AA.....M..
ANS Anas platyrhyn	419	IA...S	K..D...QK.		GH.F.....	...AA.....M..
ANS Branta canadens	418	IA...S	K..D...QK.		GH.F.....	...SA.....M..
ANS Cairina moscha	419	IA...S	K..D...QK.		GH.F.....	...AA.....M..
ANS Cygnus olor	417	IA...S	K..D...QK.		GH.F.....	...SA.....M..
APO Apus apus	418	N...IA...S	K..D...QK.	L	GH.F.....	...SV.....
CHA Larus ridibund	419	IA...S	K..D...QK.	L	G..F.....	...SV.....
CHA Catharacta mac	412	IS...S	K..D...QK.	L	G..F.....	...SV.....
CIC Ciconia ciconi	418	IA...S	K..D...QK.	L	G..F...V..	...SL.....T
CIC Vultur gryphus	414	IAAS.S	K..D...QK.	L	G..F.....	...SV.....
CLM Columba livia	418	IA...S	K..D...QK.	L	GH.F...V..	...F.SL.....VL.
CLM Streptopelia o	419	IA...S	K..D...QK.	L	GH.F...A..	...F.SL.....VL.
FAL Trigoniceps oc	414	IA...S	K..D...QK.	L	G..F.....	...SV.....
FAL Aquila chrysa	414	MA...S	K..D...QK.	L	G..F.....	...SV.....
FAL Accipiter gent	414	IA...S	K..D...QK.	L	G..F.....	...SV.....
FAL Aegypius monac	414	IA...S	K..D...QK.	L	G..F.....	...SV.....
FAL Gyps rueppelli	412	IA...S	K..D...QK.	L	G..F.....	...SV.....
GAL Gallus gallus	418	IA.T.S	K..D...K.	L	G..F.....	...AA.....
GAL Coturnix cotur	418	IA.T.S	K..D...QK.	L	G..F.....	...AA.....
GAL Phasianus colc	418	IT.T.S	K..D...K.	L	G..F.....	...SA.....
GAL Francolinus af	418	IA.T.S	K..D...K.	L	G..F.....	...SA.....
GAL Meleagris gall	418	IA.T.S	K..D...K.	L	G..F.....	...AA.....
GRU Grus grus	418	IA...S	K..D...QK.	L	G..F.....	...SV.....
FAS Taeniopygia gu	418	NV...A...S	K..D...QK.	L	G..F...T..	RN.SL.....
FAS Turdus merula	404	AV...IA...S	K..D...QK.	L	G..F.T..T.	N.SL.....
FAS Sturnus vulgar	404	.V...IA...S	K..D...QK.	L	G..F...S..	N.AL.....
FAS Passer montanu	418	N...A...S	K..D...QK.	L	G..F...T..	GN.AL.....P.
FEL Phalacrocorax	183	IA...S	K..D...QK.	L	GH.F.....	...TL.....M..
FHO Phoenicopterus	378	IAS...S	K..D...QK.	L	H.F...M..	...SL.....
PSI Psittacula kra	349	IATS.S	K..D...QK.	L	G..F.....	N.SA...A..
PSI Ara ararauna	405	IAT...S	K..D...QK.	L	G..F.....	N.SA.....
RHE Rhea americana	417	N...IY...S	K..D...QK.	L	G..F.....	...SL.....
SPH Eudiptes chrys	419	IA...S	K..D...QK.	L	G..F...M..	...SA.....
SPH Aptenodytes fo	408	IA...S	K..D...EK.	L	G..FM.....	...SA...I..
STR Struthio camel	417	IS...S	K..D...QK.	L	G..F.....	...SA.....

	490	500	510	520	530	540
<i>Crocodylus nilotic</i>	479	VSSVLTSKYR ASFPDPHEKQL	IGDLWHKVDV	AHCGGEALSR	MLIVYPWKRR	YFENFGDISN
ANS Anser anser	478	.GT...A... VHWSAE....	.TG..G..N..D..A...A..L.....TQ.	F.SS..NL.S		
ANS Aythya fuligul	479	.GAT..A... VHWTAE...I	.TG..G..N..D..A...A..L.....TQ.	F.SS..NL.S		
ANS Anser indicus	479	.GT...A... VHWSAE....	.TG..G..N..D..A...A..L.....TQ.	F.SS..NL.S		
ANS Anseranas semi	478	.AT...A... VHWSAE....	.TG..G..N..D..A...A..L.....TE.	F.SS..NL.S		
ANS Chloephaga pic	479	.GA...A... VHWTAE....	.TG..G..N..D..A...A..L.....TQ.	F.AS..NL.S		
ANS Anas platyrhyn	479	.GA...A... VHWTAE....	.TG..G..N..D..A...A..L.....TQ.	F.AS..NL.S		
ANS Branta canadensis	478	.GT...A... VHWTAE....	.TG..G..N..D..A...A..L.....TQ.	F.SS..NL.S		
ANS Cairina moscha	479	.GA...A... VHWTAE....	.TG..G..N..D..A...A..L.....TQ.	F.AS..NL.S		
ANS Cygnus olor	477	.GA...A... VHWTAE....	.TG..G..N..D..A...A..L.....TQ.	F.SS..NL.S		
APO Apus apus	478	.AT...A... VQWTAE....	.TG..G..N..D..A...A..L.....TQ.	F.SS..NL.S		
CHA Larus ridibund	479	.GN...A... VHWSAE....	.TG..G..N..D..A...A..L.....TQ.	F.AS..NL.S		
CHA Catharacta mac	472	.GN...A... VHWSAE....	.TG..G..N..D..A...A..L.....TQ.	F.SS..NL.S		
CIC Ciconia ciconi	478	.T...D... VHWTAE....	.TG..G..N..D..A...A..L.....TQ.	F.AS..NLAT		
CIC Vultur gryphus	474	.GN...A... VHWSAE....	.TG..G..N..E..A...A..L.....TQ.	F.AS..NL.S		
CLM Columba livia	478	.GT...A... VHWSAE....	.TSI.G..N..D..A...A..L.....TQ.	F.SS..NL.S		
CLM Streptopelia o	479	.GT...A... VHWSAE....	.TSI.G..N..D..A...A..L.....TQ.	F.AS..NL.S		
FAL Trigoniceps oc	474	.GN..SA... VHWTAE....	.TG..G..N..D..A...A..L.....TQ.	F.AS..NL.S		
FAL Aquila chrysaes	474	.GN...A... VHWTAE....	.TG..G..N..D..A...A..L.....TQ.	F.AS..NL.S		
FAL Accipiter gent	474	.GN...A... VQWAAE....	.TG..G..N..D..A...A..L.....TQ.	F.AS..NL.S		
FAL Aegypius monac	474	.GN...A... VHWTAE....	.TG..G..N..D..A...A..L.....TQ.	F.AS..NL.S		
FAL Gyps rueppelli	472	.GN...A... VHWTAE....	.TG..G..N..D..A...A..L.....TQ.	F.AS..NL.S		
GAL Gallus gallus	478	.GT...A... VHWTAE....	.TG..G..N..E..A...A..L.....TQ.	F.AS..NL.S		
GAL Coturnix cotur	478	.GT...A... VHWSAE....	.TG..G..N..E..A...A..L.....TQ.	F.AS..NL.S		
GAL Phasianus colic	478	.GT...A... VHWSAE....	.TG..G..N..D..A...A..L.....TQ.	F.AS..NL.S		
GAL Francolinus af	478	.GN...A... VHWTAE....	.TG..G..N..E..A...A..L.....TQ.	F.AS..NL.S		
GAL Meleagris gall	478	.GT...A... VHWSAE....	.TG..G..N..D..A...A..L.....TQ.	F.AS..NL.S		
GRU Grus grus	478	.GN...A... VHWSAE....	.TS..G..N..D..A...A..L.....TQ.	F.SA..NL.S		
FAS Taeniopygia gu	478	.DT...A... VQWTAE....	.TG..G..N..E.....A..L.....TQ.	F.AS..NL.S		
FAS Turdus merula	464	.GT...A... VQWTAE....	.TG..G..N..E..A...A..L.....TQ.	F.AS..NL.S		
FAS Sturnus vulgar	464	.GT...A... VQWTAE....	.TG..G..N..E..A...A..L.....TQ.	F.AS..NL.S		
FAS Passer montanu	478	.GT...A... VQWTAE....	.TG..G..N..E.....A..L.....TQ.	F.AS..NL.S		
FEL Phalacrocorax	243	.AKE...A... VHWTAE....	.TG..G..N..E..A...A..L.....TQ.	F.AS..NL.S		
FHO Phoenicopterus	438	.GT...A... VHWSAE....	.TS..G..N..D..A...A..L.....TQ.	F.AS..NL.S		
PSI Psittacula kra	409	.GL...A... VHWSAE....	.TG..G..N..E..A...A..L.....TQ.	F.TS..NL.S		
PSI Ara ararauna	465	.GN...A... VHWTAE....	.TG..G..N..D..A...A..L.....TQ.	F.AS..NL.S		
RHE Rhea americana	477	.GA...A... VQWTAE....	.TG..G..N..D..A...A..L.....TQ.	F.AS..NL.S		
SPH Eudiptes chrys	479	.GN..... VHWSAE....	.TG..G..N..Q.....A..L.....TQ.	F.SS..NL.S		
SPH Aptenodytes fo	468	.GN..... VHWSAE....	.TG..G..N..E..A...A..L.....TQ.	F.AS..NL.S		
STR Struthio camel	477	.GA...A... VQWSAE....	.SG..G..N..D..A...A..L.....TQ.	F.AS..NL.S		

	550	560	570	580	590	600
<i>Crocodylus nilotic</i>	539	AQAIMHNEKV	QAHGKKVLAS	FGEAVCHLDG	IRAHFANLSK	LHCEKLHVDP ENFKLLGDII
ANS Anser anser	538	PT..LG.PM.	R.....T.	..D..KN..N	.KNT..Q..E	...D.....R.....L
ANS Aythya fuligul	539	PT..LG.PM.	R.....T.	..D..KN..N	.KNT..Q..E	...D.....R.....L
ANS Anser indicus	539	PT..LG.PM.	R.....T.	..D..KN..N	.KNT..Q..E	...D.....R.....L
ANS Anseranas semi	538	PT..IG.PM.	R.....T.	KN..N	.KNT..Q..E	...D.....R.....L
ANS Chloephaga pic	539	PT..SG.PM.	R.....T.	..D..KN..N	.KNT..SQ..E	...D.....R.....L
ANS Anas platyrhyn	539	PT..LG.PM.	R.....T.	..D..KN..N	.KNT..Q..E	...D.....R.....L
ANS Branta canadensis	538	PT..LG.PM.	R.....T.	..D..KN..N	.KNT..Q..E	...D.....R.....L
ANS Cairina moscha	539	PT..LG.PM.	R.....T.	..D..KN..N	.KNT..Q..E	...D.....R.....L
ANS Cygnus olor	537	PT..LG.PM.	R.....T.	..D..KN..N	.KNT..Q..E	...D.....R.....L
APO Apus apus	538	.T.VIG.PM.	R.....T.	KN..S	.KST..Q..E	...D.....R.....L
CHA Larus ridibund	539	PT..NG.PM.	R.....T.	KN..N	.KNT..Q..E	...D.....R.....L
CHA Catharacta mac	532	PT..IG.PM.	R.....T.	KN..N	.KNT..Q..E	...D.....R.....L
CIC Ciconia ciconi	538	.S..TG.AM.	H.....T.	KN..N	.KNT..Q..E	...D.....R.....L
CIC Vultur gryphus	534	PT..IG.PM.	R.....T.	KN..N	.KNT..Q..E	...D.....R.....L
CIM Columba livia	538	.T..SG.PN.	K.....T.	..D..KN..N	.KGT..Q..E	...D.....R.....L
CIM Streptopelia o	539	.T..SG.PN.	K.....T.	..D..KN..N	.KGT..Q..E	...D.....R.....L
FAL Trigoniceps oc	534	PT..IG.PM.	R.....T.	KN..N	.KNT..Q..E	...D.....R.....L
FAL Aquila chrysaes	534	PT..IG.PM.	R.....T.	KN..N	.KNT..Q..E	...D.....R.....L
FAL Accipiter gent	534	.T.VLG.PM.	R.....T.	KN..N	.KNT..Q..E	...D.....R.....L
FAL Aegypius monac	534	PT..IG.PM.	R.....T.	KN..N	.KNT..Q..E	...D.....R.....L
FAL Gyps rueppelli	532	PT..IG.PM.	R.....T.	KN..N	.KNT..Q..E	...D.....R.....L
GAL Gallus gallus	538	PT..LG.PM.	R.....T.	..D..KN..N	.KNT..SQ..E	...D.....R.....L
GAL Coturnix cotur	538	PT..LG.PM.	R.....T.	..D..KN..N	.KNT..SQ..E	...D.....R.....L
GAL Phasianus colic	538	PT..LG.PM.	R.....T.	..D..KN..N	.KNT..SQ..E	...D.....R.....L
GAL Francolinus af	538	PT..LG.PM.	R.....T.	..D..KN..N	.KNT..SQ..E	...D.....R.....L
GAL Meleagris gall	538	PT..LG.PM.	R.....T.	..D..KN..N	.KNT..SQ..E	...D.....R.....L
GRU Grus grus	538	PT..IG.PM.	R.....T.	KN..N	.KNT..Q..E	...D.....R.....L
PAS Taeniopygia gu	538	PT..LLG.P..	T.	KN..S	.KNT..SQ..E	...D.....R.....L
PAS Turdus merula	524	PT..VLG.P..	T.	KN..S	.KGT..Q..E	...D.....R.....L
PAS Sturnus vulgar	524	PT..VLG.P..	T.	..D..KN..S	.KNT..SQ..E	...D.....R.....L
PAS Passer montanu	538	PT..VLG.P..	T.	KN..S	.KNT..SQ..E	...D.....R.....L
PEL Phalacrocorax	303	.T..TG.PM.	R.....T.	KN..N	.K.T..Q..E	...D.....R.....L
PHO Phoenixopterus	498	PT..LG.PM.	R.....T.	KN..N	.KNT..Q..E	...D.....R.....L
PSI Psittacula kra	469	.S..VLG.PN.	R.....T.	KN..N	.KNT..Q..E	...D.....R.....L
PSI Ara ararauna	525	PT..LG.PM.	R.....T.	KN..N	.KNT..Q..E	...D.....R.....L
RHE Rhea americana	537	PT..LG.PM.	R.....T.	..D..KN..N	.KNT..Q..E	...D.....R.....L
SPH Eudiptes chrys	539	PS..LG.PM.	R.....T.	..D..KNM.N	.KNT..Q..E	...D.....R.....L
SPH Aptenodytes fo	528	PA.VLA.PM.	R.....T.	..D..KNM.N	.KNT..Q..E	...D.....R.....L
STR Struthio camel	537	PT..LG.PM.	R.....T.	..D..KN..N	.KNT..Q..E	...D.....R.....L

	610	620	630
Crocodylus nilotic	599	IIIVLAAHYPK DFGLECHAAY	QKLVQRQVAAA LAAEYH
ANS Anser anser	598	FA. E.TP..Q..W	V..H. ..RK..
ANS Aythya fuligul	599	.V.....FS. E.TP..Q..W	V..H. ..RK..
ANS Anser indicus	599	FA. E.TPD.Q..W	V..H. ..RK..
ANS Anseranas semi	598	FS. ..TPD.Q..W	V..H. ..RK..
ANS Chloephaga pic	599	FT. ..TPD.Q..W	V..H. ..RK..
ANS Anas platyrhyn	599	FT. ..TP..Q..W	V..H. ..RK..
ANS Branta canadensis	598	FA. ..TPD.Q..W	V..H. ..RK..
ANS Cairina moscha	599	FT. ..TPD.Q..W	V..H. ..RK..
ANS Cygnus olor	597	FA. ..TPD.Q..W	V..H. ..RK..
APO Apus apus	598	FS. ..TP.AQQ.W	A....A..H. ..RK..
CHA Larus ridibund	599	FA. ..TPDSQ..W	V..H. ..RK..
CHA Catharacta mac	592	FA. E.TPD.Q..W	V..H. ..RK..
CIC Ciconia ciconi	598	FG. ..TPD.Q..W	K....V..H. ..RK..
CIC Vultur gryphus	594	FA. ..TPD.Q..W	A..H. ..RK..
CIM Columba livia	598	V.I....FG. ..TP..Q..W	V..H. ..RK..
CIM Streptopelia o	599	V.....FG. ..TP..Q..W	V..H. ..RK..
FAL Trigoniceps oc	594	FG. ..SPD.Q..W	A..H. ..RK..
FAL Aquila chrysaes	594	FT. ..SPD.Q..W	A..H. ..RK..
FAL Accipiter gent	594	.V.....FG. ..SPD.Q..W	A..H. ..RK..
FAL Aegypius monac	594	FG. ..SPD.Q..W	A..H. ..RK..
FAL Gyps rueppelli	592	FG. ..SPD.Q..W	A..H. ..RK..
GAL Gallus gallus	598	FS. ..TP..Q..W	V..H. ..RK..
GAL Coturnix coturn	598	FT. ..TP..Q..W	V..H. ..RK..
GAL Phasianus colch	598	FS. ..TP..Q..W	V..H. ..RK..
GAL Francolinus af	598	FS. ..TPD.Q..W	V..H. ..RK..
GAL Meleagris gall	598	FS. ..TP..Q..W	V..H. ..RK..
GRU Grus grus	598	FA. ..TPD.Q..W	V..H. ..RK..
PAS Taeniopygia gu	598	VV.....FG. ..TPD.Q..W	V..H. ..RK..
PAS Turdus merula	584	VV.....FG. ..TPD.Q..W	V..H. ..RK..
PAS Sturnus vulgar	584	VV.....FG. ..TPD.Q..W	V..H. ..RK..
PAS Passer montanus	598	VV.....FG. ..TPD.Q..W	V..H. ..RK..
PEL Phalacrocorax	363	FA. ..TP..Q..W	GA..H. ..RK..
PHO Phoenicopterus	558	FA. ..TP..Q..W	V..H. ..RK..
PSI Psittacula kra	529	G.FG. ..TPD.Q..W	A..H. ..RK..
PSI Ara ararauna	585	FG. ..TP..Q..L	V..H. ..RK..
RHE Rhea americana	597	FA. ..TP..Q..W	V..H. ..RK..
SPH Eudytes chrys	599	FA. ..TP..Q..W	E....V..H. ..RK..
SPH Aptenodytes fo	588	FS. ..TPD.Q..A	V..H. ..RK..
STR Struthio camel	597	FT. E.TP..Q..W	V..H. ..RK..