Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 326 (2005) 123–130
BBRC
www.elsevier.com/locate/ybbrc

Molecular mechanism of high altitude respiration: primary structure of a minor hemoglobin component from Tufted duck (*Aythya fuligula*, Anseriformes)

Ghosia Lutfullah[1], Syed Abid Ali, Atiya Abbasi*

International Centre for Chemical Sciences, HEJ Research Institute of Chemistry, University of Karachi, Karachi 75270, Pakistan

Received 18 October 2004

Abstract

Avian hemoglobins have attracted much attention in view of the unique oxygen transport characteristics. The present study describes the primary structure of minor hemoglobin component HbD from Tufted duck (*Aythya fuligula*), a migratory bird seen in Pakistan during the winter season. Separation of the polypeptide subunits was achieved by ion exchange chromatography in the presence of 8 M urea. Molecular masses of the intact protein as well as peptides obtained from chemical and enzymatic cleavages were determined by electrospray ionization mass spectrometry. The sequence was studied by automatic Edman degradation of the native chains and their tryptic/hydrolytic fragments in a gas-phase sequencer. Comparison of the hemoglobin sequence with the corresponding sequences of Anseriform representatives and other avian species shows residues like $\alpha^{D}23$ Asp, $\alpha^{D}120$ Asp as being specific to Tufted duck. The three-dimensional structure analyzed with the protein structure modeling package, WHAT IF, using the crystal structure coordinates of chicken hemoglobin (PDB code = 1hbr) shows $\alpha^{D}34$ Val, $\alpha^{D}38$ Gln, and $\alpha^{D}94$ Asp as possible mediators offering alternate pathway for oxygen uptake and release thereby leading to distinct hypoxia tolerance in the Tufted ducks. Results are discussed with reference to function and evolution in the Anseriform representatives.

Keywords: Primary structure; α^{D}-Chain; β-Chain; Hemoglobin; Anseriformes; Homology modeling; Amino acid sequence; Tufted duck; *Aythya fuligula*; Birds; Evolution

Hemoglobin, the key component of oxygen storage and regulation system, is widely distributed in all living organism including animals, plants, bacteria, yeast, etc. [1]. It is unique in its adaptability to a wide range of environmental conditions and has successfully survived the evolutionary pressures. The protein is a tetramer with each subunit being linked to a prosthetic group—the heme. The coordinate relationship between the subunits is governed allosterically in the presence of ligand and effector molecules like ATP, DPG or IPP, chloride, CO_2, and H^+. Binding of oxygen with hemoglobin is cooperative leading into a fully functional and efficient transport system [2].

Among vertebrates, birds occupy a unique position in terms of their ability to maintain an efficient oxygen supply to the brain during severe hypoxia, an important adaptation contributing to exceptional tolerance at extreme altitudes [3]. Hypoxia affects oxygen transport properties of hemoglobin and alters oxygen affinity by several mechanisms. All modifications adopted by animals appear to be optimizing both arterial oxygen loading and peripheral unloading [4]. The hemoglobin affinity for oxygen allows rapid adjustments of oxygen

* Corresponding author. Fax: +92 21 9243190.
E-mail address: atiya786@super.net.pk (A. Abbasi).
[1] Present address: Department of Chemistry, University of Peshawar, Peshawar 21250, NWFP, Pakistan.

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.10.209

binding and release since the process is far less energy demanding than an increase in cardiac output. This adaptation has been attributed to changes in the primary structure of globin chains, which modulates oxygen uptake and delivery to the tissues.

Birds are typically characterized by the presence of multiple hemoglobins, i.e., an adult hemoglobin or HbA and one or more embryonic (or developmental) hemoglobin(s) such as HbD. In continuation of our investigations aimed at understanding the molecular basis of respiration in birds [5–10], we have studied the hemoglobin of Tufted duck and report the primary structure of minor hemoglobin component HbD.

Tufted duck (*Aythya fuligula*) belongs to the avian order Anseriformes [11]. Members of this order are well known for their exceptional tolerance to hypoxia [3]. Tufted duck is a winter visitor in Pakistan and arrives through "flyway No.4," also known as the "*Green Route*." Over 2 million waterfowls of 169 species (including some rare ones) have been following this specific, well-defined route for thousands of years [12]. Tufted ducks feed on benthic organisms and obtain their food by diving. The preferred diving depth is approximately 1–3 m dive for 10–25 s but extended dives at the depth of 6 m for food have also been observed with a maximum submergence time of 51 s [13].

This study on Tufted duck hemoglobin provides an insight into the functional adaptations seen in avian hemoglobin as well as evolutionary status of the present-day representative of the order Anseriformes.

Materials and methods

Isolation and characterization of hemoglobin. Tufted duck erythrocytes were isolated from heparinized blood. The packed erythrocytes were washed three times with physiological saline, lysed with distilled water, and globin was precipitated according to the method of Rossi-Fanelli et al. [14]. The precipitated globin was separated by centrifugation, lyophilized, and stored until further use.

Hemolyzate was analyzed by polyacrylamide disc electrophoresis using 10% gel and Tris–glycine buffer [15]. The purity of the globin chains was analyzed under dissociating conditions using 12% gel containing 8 M urea and Triton X-100 [16].

DTE reduced globin sample was applied to a column of CM-cellulose (2.5 × 11 cm), previously equilibrated with 25 mM sodium acetate buffer containing 8 M urea and 0.2% β-mercaptoethanol, pH 6.5 [17]. Globin chains were eluted using a linear gradient of 0.02–0.2 M NaCl. The subunits were desalted by gel filtration on Sephadex G-25 (2.5 × 70 cm) column.

Enzymatic and chemical cleavages and separation of peptides. A known amount of the purified native/oxidized α^D-chain was dissolved in 10 mM ammonium bicarbonate, adjusted to pH 8.5, and treated with TPCK-Trypsin (Worthington, Freehold, New Jersey) for 3 h at 37 °C [18]. The enzyme was added in two steps at an enzyme to substrate ratio of 1:50 (w/w). The digested sample was finally adjusted to pH 4.5, centrifuged, and subjected to chromatographic separation.

Chemical cleavage at Asp–Pro bond was performed by incubating the protein with 70% formic acid containing 6 M guanidinium hydrochloride at 42 °C for 65 h [19]. Peptides generated were lyophilized and then separated by HPLC.

The tryptic peptides were fractionated using reversed-phase high-performance liquid chromatography on a RP-C4 or RP-Select B column. Peptides were eluted using a gradient of 0.1% TFA and acetonitrile. The hydrolytic peptides were separated by gel filtration using TSK 2000 column equilibrated and eluted with 0.1% TFA. The isolated peptides were freed of acetonitrile and lyophilized [20].

Mass spectrometry. Molecular masses of the intact protein as well as peptides obtained from chemical and enzymatic cleavages were determined by ESI MS [21] on VG platform II (Micromass, USA). The solvent (acetonitrile:water, 1:1) flow was maintained at 6 μl/min. Samples were acidified with 1% formic acid prior to analysis and approximately 10–15 pmol was used. The instrument was calibrated using myoglobin solution (2.5 pmol/μl) from horse skeletal muscle for protein estimations whereas multiple peaks of NaI were used for the analysis of peptides. The skimmer potential was kept at 50 V. A scan time of 6–8 s was used and 10–15 scans were combined to obtain the representative spectra. Proteins were scanned in the range of 700–1800 Da whereas peptide scanning was from 200 to 2000 Da. Molecular weight estimations had an accuracy of 0.01% corresponding to ±2 Da for a 20 kDa protein and ±0.3 Da for peptides. The data were analyzed using the Masslynx software program.

LC/MS experiments were performed using a Shimadzu HPLC system with dual syringe pumps, a 75 μl dynamic mixer, and 50 μl replaceable loop. Microbore separations were performed using a C4 or C18 (50 × 1 mm) column at a flow rate of 50 μl/min. Elution was performed using 0.1% TFA and acetonitrile gradients. The effluent was split using an Upchurch low dead volume tee with 10% being directed to the mass spectrometer and 90% collected for further analysis after measurement of UV absorbance at 214 nm.

Amino acid analysis and sequence determination. The purified native proteins and separated peptides were hydrolyzed with 6 N HCl at 110 °C for 24 h. Amino acid analysis was performed according to Moore & Stein on an automatic amino acid analyzer (Biotronik LC-6001, Pucheim, FRG).

Purified polypeptide subunits as well as all tryptic and hydrolytic peptides were sequenced by automatic Edman degradation [22] in a gas-phase sequencer [23] (Applied Biosystems 470A, USA) using an online PTH analyzer (Applied Biosystems 120A, USA).

Bioinformatics analysis. All known hemoglobin sequences were extracted from Protein Data Banks, i.e., SWISSPROT (http://www.expasy.ch), PIR (http://www-nbrf.georgetown.edu/pir), and PDB (http://www.rcsb.org/pdb/cgi/) and analyzed using BLAST/PSI-BLAST (http://www.ncbi.nlm.nih.gov/blast/psiblast). Multiple sequence alignment of the established protein sequence with other closely related ones was performed using ClustalX (http://www2.ebi.ac.uk/clustalw/).

Phylogeny lineage of Tufted duck HbD-α^D and β-subunits was traced from the available embryonic hemoglobin sequences of all Avian representatives using PHYLIP (Phylogeny Inference Package) version 3.5c [24].

The three-dimensional structure predictions were performed by homology modeling package WHAT IF [25]. The homology model of HbD was derived using crystal structure coordinates of chicken hemoglobin (PDB code = 1hbr) obtained from Protein Data Bank [http://www.rcsb.org/] [26]. The inputs for WHAT IF consisted of aligned sequences of the target and the template structure. WebLab ViewerPro Version 3.5 was used for visualization of the protein structure. Reliability was assessed by the FULCHK command of the WHAT IF. The predicted structure of Tufted duck hemoglobin HbD was also evaluated by protein structure verification software PROCHECK [27]. Root mean square deviation (RMSD) between the predicted target structure and the template was obtained by superposition of Cα traces of the backbone atoms of the predicted model onto the template structures using SUPERPOSE command of MODELLER 6 [28].

Results and discussion

Analysis of Tufted duck hemolyzate by native polyacrylamide gel electrophoresis revealed the presence of two hemoglobin components—a major component, HbA, and a minor component, HbD. The crude globin subunits separated by chromatography on CM-cellulose showed three peaks corresponding to α^A, α^D, and β-chains [29]. Electrospray ionization mass spectrometry profile of the native polypeptides of Tufted duck hemoglobin α^D and β-chains revealed a molecular mass of 15,745 and 16,321 Da, respectively (Fig. 1). Amino acid composition of the native α^D and its enzymatic/hydrolytic fragments is shown in Table 1. Molecular mass library of the tryptic peptides determined by ESI-MS is summarized in Table 2. The purified α^D and β-chains were analyzed for the amino terminal sequence by automatic Edman degradation. The complete amino acid sequence of α^D-chain aligned with the corresponding subunit from other avian representatives is presented in Fig. 2A. The phylogenetic analysis of these proteins is presented in Fig. 2B.

Embryonic/developmental hemoglobin

Hemoglobin of Tufted duck (*A. fuligula*) contains a major component HbA ($\alpha_2^A\beta_2$) (90%) and a minor component HbD ($\alpha_2^D\beta_2$) (10%). The minor hemoglobin component, i.e., HbD is expressed in most avian species with the exception of pigeon, penguins, parrot, white stork, swift, dove, and blue-yellow macaw. Among the different species of adult birds expression of α^D-chain varies and is usually greatly reduced as compared to α^A-chains. Although the importance and exact biological function of α^D-chain remain obscure, investigators believe that it might share some functional or genomic properties leading to a decrease in the evolutionary rate [30].

α^D-Chain shows structural and functional similarities with the embryonic avian α-chain. Czelusniak et al. [31] pointed out that the α^D-chain is more closely related to the embryonic chains as compared to adult α^A-chain and proposed that the α^D gene arose shortly before duplication of ancestral gene for embryonic α-like chains. The most important advantage for the multiplicity of hemoglobin resides in the differential binding of oxygen and its transport adaptability with respect to environmental and physiological needs. Thus, the functional heterogeneity also ensures a division of labor between individual hemoglobin components, by extending the range of conditions required for oxygen transport [32]. These hemoglobins also possess different isoelectric point (p*I*) and modulate the cellular pH range for effective functioning as an oxygen transporter.

Evaluation of the predicted 3D structure

Analysis of the predicted structure of Tufted duck HbD shows that all stereo-chemical and geometrical parameters implicated in PROCHECK have been satisfied in the predicted structure. The Ramachandran plot shows a good distribution for psi/phi angles of tetrameric form of HbD from Tufted duck. α^D and β-sub-

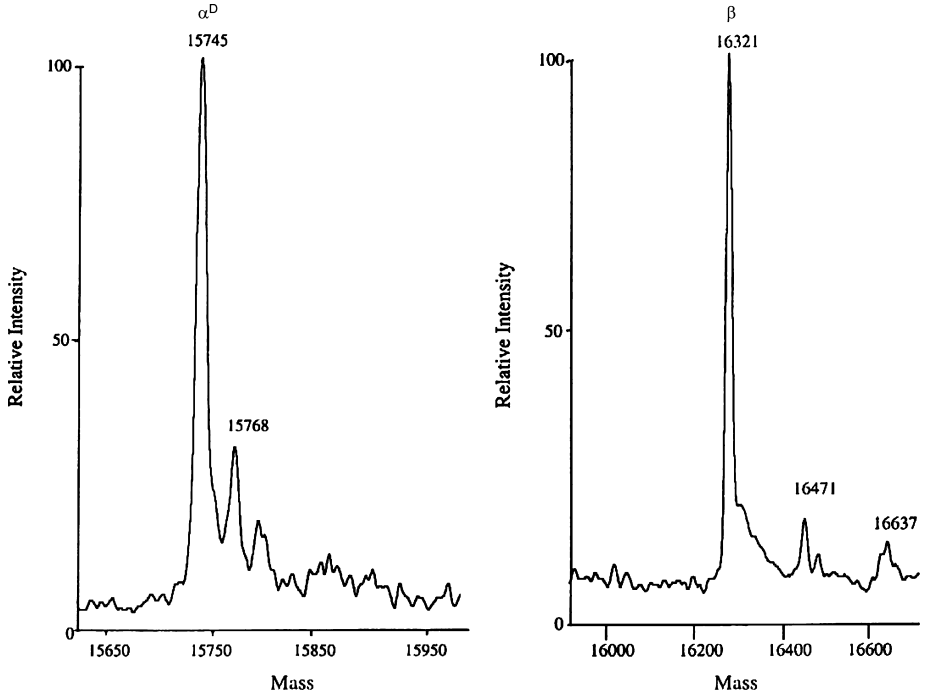

Fig. 1. Electrospray ionization mass (ESI) spectrum of purified subunits of minor hemoglobin component from Tufted dock *Aythya fuligula*.

Table 1
Amino acid composition of tryptic peptides (Tp) of α^D-chain of Tufted duck (*Aythya fuligula*)

Peptides Amino acids	Tp1/2a 1–8	Tp2b/3 12–16	Tp4 17–31	Tp5 32–40	Tp6 41–56	Tp7/8 57–61	Tp9a 62–71	Tp9b/10 72–92	Tp11 93–99	Tp12a 100–115	Tp12b 116–127	Tp13 128–139	Tp14 140–141	Total
Asx	0.9(1)	—	2.8(3)	—	1.1(1)	—	1.1(1)	3.9(4)	2.1(2)	—	2.9(3)	—	—	14.66 (15)
Thr	0.8(1)	0.9(1)	—	1.8(2)	1.0(1)	—	—	—	—	—	0.9(1)	—	—	5.5 (06)
Ser	—	—	—	—	1.1(1)	—	—	3.6(4)	—	—	—	0.8(1)	—	5.05 (06)
Glx	1.0(1)	2.1(2)	2.8(3)	0.8(1)	2.1(2)	—	—	2.0(2)	—	1.8(2)	—	1.1(1)	—	13.8 (14)
Pro	—	—	—	1.1(1)	2.0(2)	—	—	—	1.0(1)	—	1.0(1)	—	—	4.6 (05)
Gly	—	—	2.1(2)	—	1.1(1)	2.1(2)	1.1(1)	—	—	1.0(1)	—	—	—	6.8 (07)
Ala	0.9(1)	—	2.0(2)	—	—	—	3.6(4)	1.9(2)	—	3.1(3)	1.9(2)	3.8(4)	—	17.89 (18)
Val	—	—	0.8(1)	0.8(1)	0.8(1)	—	1.9(2)	—	2.0(2)	2.1(2)	—	2.1(2)	—	10.5 (11)
Met	0.7(1)	—	—	0.9(1)	—	—	—	—	—	—	0.8(1)	—	—	2.65 (03)
IIe	—	1.1(1)	—	—	—	—	—	1.0(1)	—	—	—	—	—	1.7 (02)
Leu	0.8(1)	1.9(2)	1.2(1)	—	1.1(1)	—	1.0(1)	4.9(5)	—	3.8(4)	—	2.0(2)	—	16.6 (17)
Tyr	—	—	—	1.0(1)	0.9(1)	—	—	0.9(1)	—	—	1.0(1)	—	1.0(1)	5.0 (05)
Phe	—	—	1.0(1)	1.0(1)	2.0(2)	—	—	—	—	1.1(1)	1.1(1)	1.0(1)	—	7.1 (07)
Lys	2.1(2)	1.2(1)	—	1.1(1)	—	2.1(2)	1.0(1)	—	1.1(1)	1.2(1)	0.9(1)	1.0(1)	—	11.1 (11)
His	—	—	0.9(1)	—	2.3(2)	0.8(1)	—	0.9(1)	0.9(1)	1.0(1)	1.0(1)	—	—	7.6 (08)
Arg	—	—	1.0(1)	—	0.9(1)	—	—	0.9(1)	—	—	—	—	1.1(1)	4.1 (04)
Cys	—	—	—	—	—	—	—	—	—	1.1(1)	—	—	—	0.8 (01)
Trp	—	0.8(1)	—	—	—	—	—	—	—	—	—		—	ND (01)
Sum	8	8	15	9	16	5	10	21	7	16	12	12	2	141

Number in parentheses denotes amino acid residues according to sequence analysis; ND, not determined.

Table 2
FAB-MS of tryptic peptides of α^D-chain from Tufted duck (*Aythya fuligula*)

Residue No.	Sequence	Mass calculated	Mass observed
1–7	MLTAEDKK	935.5	935.3
9–16	LITQLWEK	1030.6	1029.3
17–31	VAGHQDDFGNEALQR	1657.8	1657.7
32–40	MFVTYPQTK	1114.6	1114.6
41–56	TYFPHFDLHPGSEQVR	1930.16	1929.9
57–60	GHGKK	526.3	525.7
62–71	VAAALGNAVK	913.11	913.5
72–92	SLDNISQALSELSNLHAYNLR	2358.6	2358.6
93–99	VDPVNFK	818.4	818.4
100–115	LLAQCFQVVLAAHLGK	1711.15	1710.8
116–127	DYTPDMHAAFDK	1410.6	1410.7
128–139	FLSAVAAVLAEK	1218.7	1218.5
140–141	YR	338.2	338

units of the predicted structure superimpose well on the corresponding subunits of template as well as on human HbA. The RMSD between crystal structures of chicken oxy HbD [26], human oxy HbA [33], and the predicted structure Tufted duck HbD was 0.21 and 0.69, respectively. The small difference in the RMSD of predicted vs. experimentally derived structure reflects the presence of strong restraints in most regions and emphasize a very similar folding pattern among hemoglobin. The Tufted duck HbD model has been calculated using crystal structure coordinates of chicken HbD (oxy form) (PDB code = 1hbr) known at a resolution of 2.3 Å. Tufted duck α^D and β-subunits show 82% and 95% identity to the corresponding subunits of chicken HbD. In the predicted structure, each heme is ligated to the $N \in -2$ of the proximal His, i.e., His 87 in the α-subunit and His 92 in the β-subunit. The distal residues in contact with the ligand, i.e., E7 His and E11 Val of Tufted duck hemoglobin have very similar conformation and environment as observed in human hemoglobin. All amino acid residues present in the vicinity of heme in Tufted duck hemoglobin are well conserved.

Alteration of the functionally important residues (α34 and α38) strongly influences the oxygen affinity of the blood. Our results support this observation as the primary structure of HbD shows amino acid substitution in the α^D-chain at these key intra-molecular contacts. In human hemoglobin position α34, an $\alpha_1\beta_1$ contact point, interacts with β128, β125, and β124 in the oxy structure (R-structure). Most birds including chicken HbD possess Thr at this position forming hydrogen bond with β125, which stabilizes the T-structure thereby lowering the oxygen affinity. In Tufted duck, position α34 is occupied by Val instead of Thr, which results in

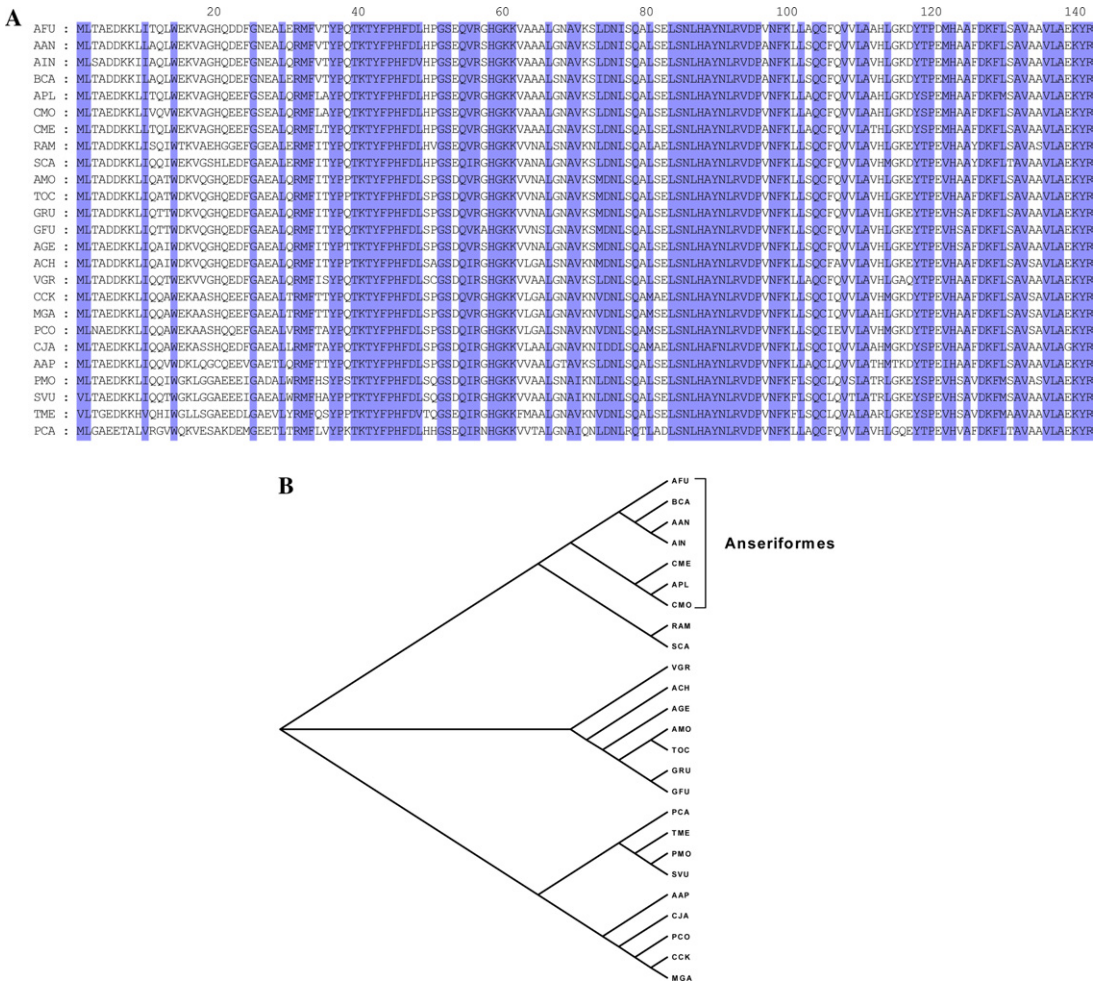

Fig. 2. (A) Multiple sequence alignment of α^D-chain from Tufted duck hemoglobin. Conserved residues have been high-lighted. (B) Phylogenetic lineage of avian embryonic hemoglobin derived from α^D subunit. Abbreviations: AFU - Tufted Duck (*Aythya fuligula*), PCA - Cormorant (*Phalacrocorax carbo*), TOC - White-headed Vulture (*Trigonoceps occipitalis*), AMO - Black Vulture (*Aegypius monachus*), GRU - Ruppell's Griffon (*Gyps rueppellii*), AGE - Adult Goshawk (*Accipiter gentilis*), ACH - Golden Eagle (*Aquila chrysactors*), GFU - Griffon Vulture (*Gyps fulvus*), RAM - American Rhea (*Rhea Americana*), CJA - Japanese Quail (*Coturnix coturnix Japonica*), PCO - Pheasant (*Phasianus colchicus colchicus*), CCK - Chicken (*Gallus gallus*), MGA -Turkey (*Meleagris gallopavo*), SCA Ostrich (*Struthio camelus*), VGR - Andean Condor (*Vulture gryphus*), BCA - Canada Goose (*Branta canadensis*), AIN - Bar-headed Goose (*Anser indicus*), AAN - Graylag Goose (*Anser anser*), CMO - Muscovy Duck (*Cairina moschata*), APL - Domestic Duck (*Anas platyrhynchos*), CME -Andean Goose (*Chloephaga melanoptera*), SVU - Starling (*Sturnus vulgaris*), TME - Blackbird (*Turdus merula*), AAP - Swift (*Apus apus*).

the loss of hydrogen bond and shows weak van der Waals interaction compared to human hemoglobin (Fig. 3). The predicted 3D structure of Tufted duck HbD also indicates stronger van der Waals interaction between α34 Val and β124 Pro as compared to β125 Glu and β128 Ala.

Another important substitution at $\alpha_1\beta_2$ interface is α38, which is occupied by glutamine in Tufted duck HbD like chicken HbD. Glutamine at α38 is a common feature of all species of the order Anseriformes, as well as Cormorant [34], a diving bird, and *Gyps rueppellii* [35] which is known to fly at an altitude of 11,000 m. In the case of human hemoglobin, the hydroxyl group of α38 Thr forms a hydrogen bond with main chain carboxyl oxygen of β97 His in the R-state. The predicted structure also shows that the side chain of α38 Gln in Tufted duck HbD does not form the same bond, thus reducing the stability of the $\alpha_1\beta_2$ interface. It is postulated that glutamine at this position stabilizes the oxy-structure by two hydrogen bonds (β99 and β97) in comparison to the deoxy-structure, where only one hydrogen bond is possible [35]. The loss of a hydrogen bond due to this substitution serves to increase the oxygen affinity in HbD in both Tufted duck and chicken. Thus, the minor hemoglobin component HbD in the Tufted duck seems to possess higher oxygen affinity, loading fully even at low oxygen tension.

Like chicken HbD, the predicted structure of Tufted duck HbD also shows a hydrogen bond between α^D94

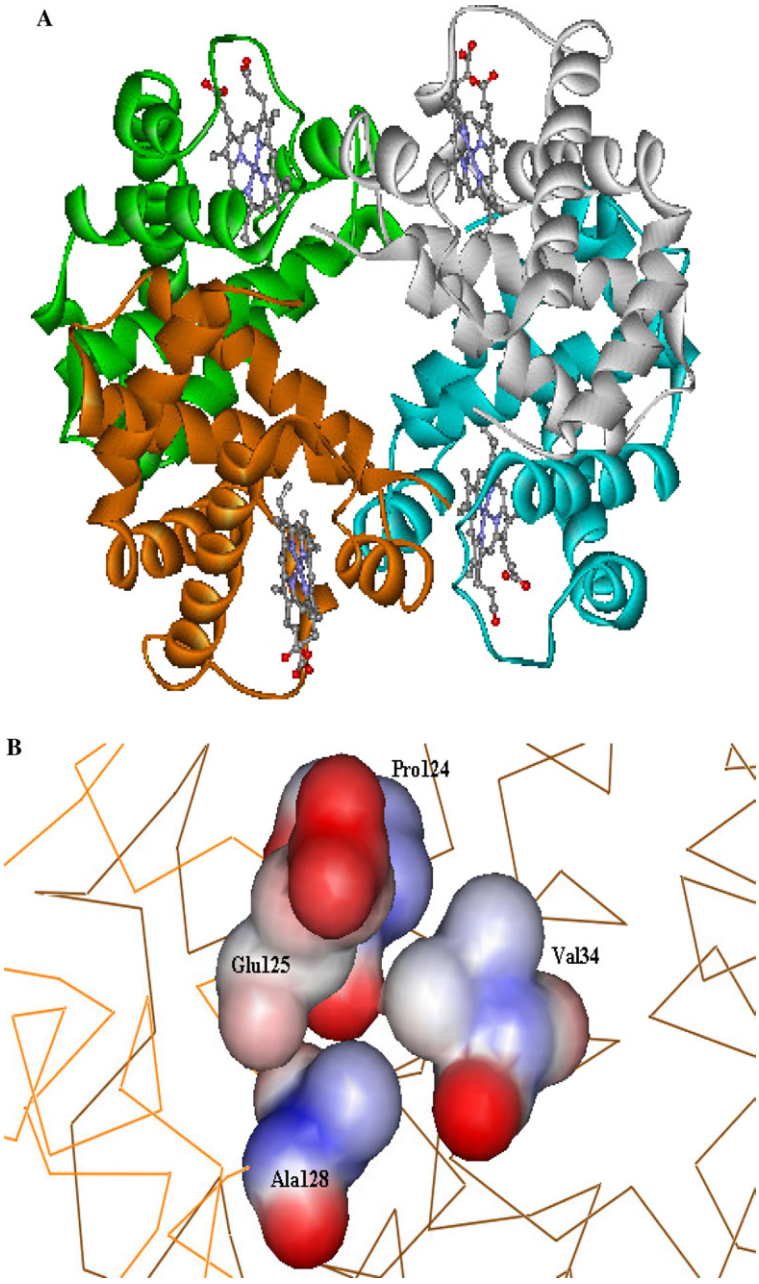

Fig. 3. (A) Schematic representation of the tetrameric structure of Tufted duck. HbD homology model constructed using the crystal structural coordinates of Chicken (PDB id = 1hbr). Heme is depicted in ball–stick representation whereas globin chains are shown as colored solid ribbon. (B) The a34 albl binding site, van der Waals interactions of aD Val34 with b124Pro, b125 Glu, and b128Ala in the predicted structure of Tufted duck HbD. The backbone of α and β subunits is shown by Ca wires in dark and light brown colors, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Asp and β102 Asn whereas a hydrogen bond between $\alpha^{D}97$ Asn and β99 Asp present in human hemoglobin is lacking in both species. The key substitution observed in the sequence of Tufted duck HbD at $\alpha^{D}34$ Val, $\alpha^{D}38$ Gln, and $\alpha^{D}94$ Asp may be involved in adaptation to hypoxia. The present studies also provide evidence that modification of $\alpha_1\beta_1$ and $\alpha_1\beta_2$ contacts may be responsible for the efficient diving characteristics and high altitude flights in Tufted duck.

Physiological and evolutionary characteristics

Tufted duck belongs to the order Anseriformes. Order Anseriformes comprises of two families, namely Anhimidae and Anatidae. The family Anhimidae has not been studied as yet so molecular sequence data area available only for representatives of the family Anatidae. Tufted duck is well known for its strong flights as well as diving characteristics. The bird shows a number

of adaptations like greater volumes of oxygen in the lungs and air sac system, high concentration of myoglobin in the skeletal and cardiac muscles or thermoregulatory capabilities [36–38]. All these adaptations are known to support survival under extreme environmental conditions. Alignment of the α^D- and β-chain sequences of Tufted duck with representatives of the order Anseriformes shows a high degree of homology indicating a very close relationship.

Comparison of the subunit sequences from different species shows the highest mutation rate for the α^D-chain while a low mutation rate has been observed for the β-chain indicating higher evolutionary constraints. Alignment of α^D- and β-chains from all avian species reveals 65 and 104 invariant positions, respectively, corresponding to 46.1% and 71.23% homology. Comparison of the primary structure of Tufted duck minor hemoglobin component with those of other representatives from nine different avian orders indicates a closer relationship with order struthioniformes, galliformes, falconiformes, and cathartiformes. Interestingly, α^D- and β-chains of Tufted duck hemoglobin show highest homology with ostrich (order struthioniformes), i.e., 86.52% and 95.21% (19 and 7 amino acid exchanges), respectively. Ostrich is a flightless bird and had evolved during the Eocene period. Among other orders falconiforme representatives seem to have no obvious evolutionary link with other birds and have currently been placed between ducks and gallinaceous birds [39]. However, molecular sequence data indicate a slightly different placement, i.e., gallinaceous birds to be in between ducks and falconiforme representative.

Our studies and the available sequence data also indicate that despite the wide variety of present-day Anatidae, the family is genetically homogeneous. All members of this family display two hemoglobin components, i.e., HbA (the major component) and HbD (the minor component). The evidence from serological investigations and physico-chemical examination of egg-white proteins and egg-shell structure also supports this conclusion. Although the fossil record is inadequate to explain the origin and evolution of Anseriformes, the ancestral forms were believed to be the Galliform Curassows and Ciconiiform Flamingos [40]. Thus, the ability of Tufted duck to fly continuously over long distances during migration as well as the ability to dive is not solely a genotypic molecular adaptation rather it seems to be a coordination of organismic, cellular, and molecular adaptation each of which becomes critical under extreme hypoxia.

Acknowledgments

Authors are also grateful to Professor D.L. Smith, Department of Chemistry, University of Nebraska, USA, for allowing one of us (A. Abbasi) to use the electrospray ionization mass spectrometer.

References

[1] R. Hardison, Hemoglobins from bacteria to man: evolution of different patterns of gene expression, J. Exp. Biol. 201 (1998) 1099–1117.
[2] M.F. Perutz, Mechanisms of cooperativity and allosteric regulation in proteins, Q. Rev. Biophys. 22 (1989) 139–236.
[3] R.E. Weber, Hemoglobin adaptation to hypoxia and altitude—the phylogenetic perspective, in: J.R. Sutton, C.S. Houston, G. Coates (Eds.), Hypoxia and the Brain, Queen City Printers, USA, 1995, pp. 31–44.
[4] H. Mairbaurl, Red blood cell function in hypoxia at altitude exercise, Int. J. Sports Med. 15 (1994) 51–63.
[5] A. Abbasi, G. Braunitzer, High altitude respiration in birds: primary structure of hemoglobin from griffon vulture (*Gyps fulvus*), in: Z.H. Zaidi, A. Abbasi, D.L. Smith (Eds.), Proceeding of 2nd International Symposium on Protein Structure Function, TWEL Publ., 1990, pp. 277–286.
[6] C. Sultana, A. Abbasi, Z.H. Zaidi, Primary structure of hemoglobin α-chain of *Columba livia* (grey wild pigeon), J. Protein Chem. 8 (1989) 629–645.
[7] C. Sultana, A. Abbasi, Z.H. Zaidi, Primary structure of hemoglobin β-chain of *Columba livia* (grey wild pigeon), J. Protein Chem. 10 (1991) 145–149.
[8] A. Abbasi, Z.H. Zaidi, Primary structure of hemoglobin from gray partridge (*Francolinus pondacerianus*, Galliformes) hemoglobin, J. Protein Chem. 8 (1989) 647–652.
[9] A. Abbasi, Z.H. Zaidi, Primary structure of hemoglobin α-chain from cuckoo (*Eudynamys scolopacea*, Cuculiformes), J. Protein Chem. 10 (2) (1991) 139–144.
[10] A. Abbasi, Z.H. Zaidi, unpublished results—Sequence submitted to JIPID.
[11] H.C.B. Grzimek, in: Grzimek's Animal Life Encyclopedia, vol. 7, van Nostrand Reinhold Co., 1979, pp. 253–326 and 503–506.
[12] M.F. Ahmed, S.A. Ghalib, Field Guide to the Ducks, Geese, Swans of Pakistan, 1986.
[13] P.J. Butler, Physiology of diving ducks and other aquatic birds (excluding penguins), in: Acta XX Congressus International Ornithologici, Christchurch, New Zealand, vol. 111, 1990, pp. 1886–1895.
[14] A. Rossi-Fanelli, E. Antonini, A. Caputo, Studies on the structure of hemoglobin I. Physicochemical properties of human globin, Biochem. Biophys. Acta 30 (1958) 608–615.
[15] H.R. Maurer, Disc Electrophoresis, Walter de Gruyter, Berlin, 1971.
[16] B.P. Alter, S.O. Goff, G.D. Efremov, M.E. Gravely, T.H. Huisman, Globin chain electrophoresis: a new approach to the determination of γ^G/γ^A ratio in fetal hemoglobin and to the studies on globin synthesis, Br. J. Haematol. 44 (1980) 527–534.
[17] J.B. Clegg, M.A. Naughton, D.J. Weather-Ball, Abnormal human hemoglobins: separation and characterization of the α and β-chain by chromatography and the determination of two new variants Hb Chesapeak and HbJ (Bangkok), J. Mol. Biol. 19 (1996) 91–108.
[18] C.H.W. Hirs (Ed.), Enzyme Structure, Methods in Enzymology, vol. XI, 1967, Academic Press, New York.
[19] J. Jauregui-Adell, J. Marti, Acidic cleavage of the aspartyl-proline bond and the limitations of the reaction, Anal. Biochem. 69 (1975) 468–473.
[20] M.T.W. Hearn, W.S. Hancock, in: C.L. Hawk (Ed.), Biological and Biomedical Applications of Liquid Chromatography, Marcel Dekker, New York, 1997, pp. 243–271.

[21] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong, Electrospray ionization—principles and practice, Mass Spectrom. Rev. 9 (1990) 37–70.
[22] P. Edman, G. Begg, A protein sequenator, Eur. J. Biochem. 1 (1967) 80–91.
[23] R.M. Hewick, M.W. Hunkapillar, L.E. Hood, W.J. Dreyer, A gas-liquid solid phase peptide and protein sequenator, J. Biol. Chem. 256 (1981) 7990–7997.
[24] J. Felsenstein, PHYLYP (Phylogeny Inference Package) version 3.5C, Distributed by the author, Department of Genetics, University of Washington, Seattle.
[25] G. Vriend, WHAT IF: A molecular modeling and drug design program, J. Mol. Graph. 8 (1990) 52–55.
[26] J.E. Knapp, A. Oliveira, Q. Xie, S.R. Ernst, A.F. Riggs, M.L. Hackert, The structural and functional analysis of the hemoglobin D component from chicken, J. Biol. Chem. 274 (1999) 6411–6420.
[27] R.A. Laskowski, Computer programs: PROCHECK: a program to check the stereochemical quality of protein structures, J. Appl. Crystallogr. 26 (1993) 283–291.
[28] A. Sali, T.L. Blundell, Comparative protein modeling by satisfaction of spatial restraints, J. Mol. Biol. 234 (1993) 799–815.
[29] A. Abbasi, G. Lutfullah, Molecular basis of bird respiration: primary hemoglobin structure component from Tufted duck (*Aythya fuligula*, Anseriformes)—role of α99Arg in formation of a complex salt bridge network, Biochem. Biophys. Res. Commun. 291 (2002) 176–184.
[30] I. Heibl, D. Schneeganss, F. Grimm, J. Kosters, G. Braunitzer, High altitude respiration of birds: the primary structure of major and minor hemoglobin component of adult european black vulture (*Aegypius monachus*, Aegypiinae), Biol. Chem. Hoppe-Seyler 368 (1987) 11–18.
[31] J.O. Czelusniak, M. Goodman, D. Hewett-Emmett, M.L. Weiss, P.J. Venta, R.E. Tashian, Phylogenetic origins and adaptive evolution of avian and mammalian hemoglobin genes, Nature 298 (1982) 297–300.
[32] R.E. Weber, in: J.P. Truchot, B. Lahlou (Eds.), Animal Nutrition and Transport Processes, vol. 2, Comp. Physiol. 6 (1990) 58–75.
[33] B. Shaanan, Structure of human oxyhemoglobin at 2.1 Å resolution, J. Mol. Biol. 171 (1983) 31–59.
[34] K. Huber, G. Braunitzer, D. Schneeganss, J. Kosters, F. Grimm, The primary structure of the hemoglobin of cormorant (*Phalacrocorax carbo*, Pelecaniformes), Biol. Chem. Hoppe-Seyler 369 (1988) 1251–1256.
[35] I. Hiebl, R.E. Weber, D. Schneeganss, J. Kosters, G. Braunitzer, High altitude respiration of birds: structural adaptations in the major and minor hemoglobin components of adult Ruppell's griffon (*Gyps rueppellii*, Aegypiinae), Biol. Chem. Hoppe-Seyler 369 (1988) 217–232.
[36] R. Stephenson, D.L. Turner, P.J. Butler, The relationship between diving activity and oxygen storage capacity in the Tufted duck (*Aythya fuligula*), J. Exp. Biol. 141 (1989) 265–275.
[37] E. Keijar, P.J. Butler, Volumes of the respiratory and circulatory systems I tufted duck and mallard ducks, J. Exp. Biol. 101 (1982) 213–220.
[38] R.M. Bevan, P.J. Butler, Oxygen consumption during voluntary diving in Tufted duck, *Aythya fuligula*, acclimated to summer and winter temperature, J. Physiol. 418 (1989) 132–139.
[39] H.F. Pough, J.B. Heiser, K.N. Mcfarland, Vertebrate life, third ed., Maxwell Macmillian, New York, 1990.
[40] J. Godovac-Zimmerman, G. Braunitzer, The amino acid sequence of α^{A} and β-chains from the major hemoglobin component of american flamingo (*Phoenicopterus ruber ruber*), Hoppe-Seyler's Z. Physiol. Chem. 365 (1984) 437–443.