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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[1]

Atiya Abbasi[2] and Ghosia Lutfullah[3]
International Centre for Chemical Sciences, HEJ Research Institute of Chemistry, University of Karachi, Karachi 75270, Pakistan

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The primary structure of the major hemoglobin component, HbA (α^A- and β-chain), from Tufted duck (*Aythya fuligula*) is presented. The separation of the globin subunits was achieved by ion exchange chromatography on CM-cellulose in 8 M urea. The amino acid sequence was determined by automatic Edman degradation of native chains as well as tryptic and hydrolytic peptides in a gas-phase sequencer. The automated homology model was generated by the protein structure modeling package WHAT IF using the crystal structure coordinates of Bar-headed goose hemoglobin. The 3D structure prediction enables α99Arg and β101Glu to emerge as a new intersubunit contact site not found in the hemoglobin structure of any other species. α99Arg forms a complex salt bridge network involving α99Arg–β101Glu–β104Arg–β108Asp. Also the substitution at α34 → Ile, α38 → Gln and β55 → Leu serves to stabilize the oxy-structure, leading to higher oxygen affinity.

Key Words: **primary structure; hemoglobin; Anseriformes; homology modeling; Tufted duck; birds.**

Among vertebrates, birds are well known for their ability to survive under conditions of reduced oxygen supply either during migration to long distances when they undertake flights at high altitudes or during swimming/diving for obtaining food in a deep sea (1).

Hemoglobin is the major oxygen transport protein found in all vertebrates with the exception of a few Antarctic deep-sea fishes. It is unique in that it acts as a regulatory switch having remarkable ability to "perceive" information from the environment and adjust its function accordingly. A number of investigations have been carried out at the molecular level to unravel the underlying mechanism and some clues have already been found. In continuation with our earlier investigations aimed at understanding the molecular basis of respiration in birds (2–7), we have analyzed the hemoglobin from Tufted duck (*Aythya fuligula*) and report here the structural characteristics of the major component HbA.

Tufted duck (*Aythya fuligula*) belongs to the avian order Anseriformes, family Anatidae (8). It is a winter migratory bird in Pakistan with distribution all over the country (9). Tufted ducks depend on benthic organisms and obtain their food by diving. The diving depth and time, swimming, voluntary and involuntary diving behavior with respiratory, cardiovascular and metabolic adjustments have been studied in great detail by Butler and colleagues (10–17).

MATERIALS AND METHODS

Isolation of hemoglobin. Blood was obtained from Tufted duck using heparin as an anticoagulant. The plasma was removed by centrifugation and packed erythrocytes were washed three times with physiological saline. The packed cells were then lysed with distilled water and globin precipitation carried out according to the method of Rossi-Fanelli and Antonini (18). The precipitated globin was separated by centrifugation, lyophilized and stored until further use.

Electrophoresis. Hemolyzate was analyzed by polyacrylamide disk electrophoresis using 10% gel and Tris-glycine buffer (19). The purity of the globin chains was analyzed under dissociating conditions using 12% gel containing 8 M urea and Triton X-100 (20).

Separation of globin chains. Globin sample reduced with DTE 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 (21). 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.

[1] Dedicated to the memory of Professor Zafar Zaidi (1939–2001).

[2] To whom correspondence and reprint requests should be addressed. Fax: +92-21-924-3190. E-mail: abbasi@global.net.pk.

[3] Present address: Department of Chemistry, University of Peshawar, Peshawar 21250, NWFP, Pakistan.

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Enzymatic cleavage. A known amount of the purified native/oxidized α^A- and β-chains was dissolved in 10 mM ammonium bicarbonate, adjusted to pH 8.5, and treated with TPCK–trypsin (Worthington, Freehold, NJ) (22) for 3 h at 37°C. 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. A known amount of the protein was incubated with 70% formic acid containing 6 M guanidinium hydrochloride at 42°C for 65 h (23). Peptides generated were lyophilized and then separated by HPLC.

HPLC fractionation. 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 (24).

Mass spectrometry. Molecular masses of the intact protein as well as peptides obtained from chemical and enzymatic cleavages were determined by electrospray ionization (25) 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 were 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 200–2000 Da. Molecular weight estimations had an accuracy of 0.01% corresponding to ±2 Da for a 20 kDa for 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% was collected for further analysis after measurement of UV absorbance at 214 nm.

Amino acid analysis. 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 and Stein on an automatic amino acid analyzer (Biotronik LC-6001, Pucheim, Germany).

Sequence analysis. Purified polypeptide subunits as well as all tryptic and hydrolytic peptides were sequenced by automatic Edman degradation (26) in a gas-phase sequencer (Applied Biosystems 470A, USA) (27) using an online PTH analyzer (Applied Biosystems 120A, USA).

Molecular modeling. The automated homology model building was carried out using structure-modeling package WHAT IF as described by Vriend (28). The three-dimensional model of HbA was derived using crystal structure coordinates of Bar-headed goose hemoglobin HbA (PDB code = IA4F) (29). The inputs for WHAT IF consisted of the aligned sequences of the targets to be modeled and the template structure obtained from Protein Data Bank [http://www.rcsb.org/]. Web Lab Viewer Pro version 3.5 was used for visualization and display of graphic. Model of HbA of Tufted duck built with WHAT IF was evaluated by protein structure verification software PROCHECK (30).

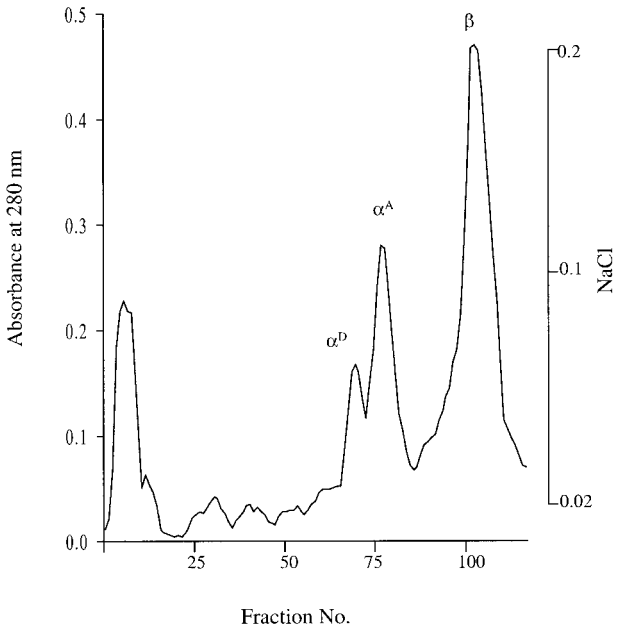

FIG. 1. Separation profile of Tufted duck globin chains on CM-cellulose (CM 52) (2.5 × 10 cm) under denaturing conditions. Buffer: 25 mM sodium acetate containing 8 M urea and 0.2% β-mercaptoethanol, pH 6.5. Gradient, 0.02–0.2 M NaCl (400:400 ml); flow rate, 20 ml/h; fraction volume, 6 ml.

RESULTS

Polyacrylamide gel electrophoresis of hemolysate revealed the presence of a major component HbA and the minor component HbD. Electrophoresis of the globin chains under dissociating conditions showed the presence of three subunits, i.e., α^A, α^D, and β. The crude globin chromatographed on carboxymethyl cellulose resulted in three peaks (Fig. 1) corresponding to α^A-, α^D-, and β-chains. Electrospray ionization mass spectroscopy revealed a molecular mass of 15391.98 ± 1.56 for α^A and 16321.19 ± 1.16 for the β-chain. Tryptic peptides obtained from cleavage of native/oxidized α^A and β-chain were purified by RP-HPLC. The molecular mass of the isolated peptides is shown in Tables 1 and 2. Acid hydrolysis of α^A-chain using 70% formic acid resulted in cleavage at Asp-Pro bond. The cleaved peptides were isolated by gel filtration. Amino acid composition of the tryptic peptides is presented in (Tables 3 and 4). The complete amino acid sequence of the major hemoglobin component is presented in Fig. 2.

DISCUSSION

Tufted duck hemoglobin contains a major component HbA and a minor component HbD, accounting for 90 and 10%, respectively, like all other anseriform representatives. The molecular mass (tetramer) of the two components as determined by ESI-MS is 63,426.34 and 64,131.78 for HbA and HbD, respectively.

TABLE 1

ESI-MS of Tryptic Peptides of α^A-Chain from Tufted Duck (*Aythya fuligula*)

Residue No.	Sequence	Mass calculated M + H	Mass observed M + H
1–7	VLSAADK	703.3	703.5
8–11	TNVK	461.2	461.2
12–16	GIFSK	551.3	551.4
17–31	IGGHAEEYGAETLER	1631.7	1631.8
32–40	MFITYPQTK	1128.6	1128.5
41–56	TYFPHFDLQHGSAQIK	1888.9	1889.1
57–61	AHGKK	540.4	540.3
62–82	VAAALVEAVNHIDDIAGALSK	2077.1	2077.20
83–90	LSDLHAQK	911.4	911.5
91–92	LR	288.2	288.0
93–99	VDPVNFR	846.4	846.5
100–127	FLGHCFLVVLAIHHPSALTPEVHAS	3051.6	3051.9
128–139	LDK	1259.7	1260.1
140–141	FMCAVGATLTAK (oxidized) YR	338.2	338.0

The functional characteristics of hemoglobin are basically derived from subunit contacts, i.e., $\alpha_1\beta_1$ and $\alpha_1\beta_2$ as well as its interaction with effector molecules like Cl^-, CO_2, and organic phosphates like ATP, DPG, or IPP, etc. (31). Studies on the primary structure of Tufted duck hemoglobin indicate key mutations at the subunit interface, i.e., α34Ile, α38Gln, and β55Leu. In addition, substitution at α99Lys → Arg is unique to Tufted duck hemoglobin and seems to be contributing toward a new $\alpha_1\beta_1$ subunit contact site, i.e., α99Arg → β101Glu.

The Tufted duck hemoglobin HbA model has been generated using the coordinates of the crystal structure of oxy-hemoglobin of Bar-headed goose $\alpha_1\beta_1$ dimer (PDB code = 1A4F) determined at a resolution of 2.0 Å (27). At present this is the only available crystal structure of the major avian hemoglobin component. The α^A- and β-chain of Tufted duck HbA show 92 and 96% sequence identity respectively with the corresponding chains of Bar-headed goose HbA. Correspondingly, the model shows a high degree of structural similarity. The 3D-folding pattern of α and β subunits are almost identical to that of human hemoglobin and consist of seven and eight helical segments, respectively, along with interhelical regions. Superimposition of the α^A- and β-subunits from Tufted duck shows that despite remarkable similarity in the overall fold, the hemoglobin differs when compared with Bar-headed goose and human hemoglobin.

Among the substitutions observed α^A99Lys → Arg is unique among birds, mammals and reptiles analyzed so far. Although both Lys and Arg are basic in character, the larger side-chain of Arg with three nitrogen atoms (i.e., NH_1, NH_2, and Nϵ) in its guanidinium group enables it to be associated with more groups in the neighborhood through salt bridges and/or hydrogen bonds. From the homology model of Tufted duck HbA, it can be predicted that the residue α^A99Arg (G6) is exposed at the surface of the G-helix. The extended side-chain of α^A99Arg approaches the G-Helix of the corresponding β-chain forming a salt bridge with the carboxyl group of β101Glu (G3). The β101Glu also shows ionic interaction with β104Arg (G6) which in turn is associated with another acidic residue β108Asp (G10) by salt bridge formation via its carboxylic side chain. These interactions result in the formation of a complex network of α^A99Arg–β101Glu–β104Arg–β108Asp as shown in Fig. 3A. The residues β101Glu (G3) and β104Arg (G6) are also involved in formation of $\alpha_1\beta_1$ and $\alpha_1\beta_2$ subunit contacts respectively. β101Glu, β104Arg and β108Asp are conserved in all avian hemoglobin, whereas in human Hb Asn is present at position β108. The formation of this new salt bridge network is a unique feature of Tufted duck HbA and enables α^A99Arg (G6) and β101Glu (G3) to emerge as a new $\alpha_1\beta_1$ contact site, not found in the hemoglobin structure of any other species. This network seems to stabilize the R-structure with higher oxygen affinity, which favors cooperative binding. Similar interactions can be seen in the crystal structure of Bar-headed goose HbA (PDB code = 1A4F), where Nz of α^A99Lys (G6) interacts with a water molecule which in turn is linked to the side chain of β104 Arg (NH1). The other nitrogen (NH2) interacts with the carboxylic side chain of β101Glu (OE2) resulting in an indirect interaction of α^A99Lys with β104Arg (G6) and β101Glu (G3) mediated by a water molecule (Fig. 3B). A direct interaction between the α^A99Lys (G6) with β104Arg (G6) is, however, not possible because of the shorter length of α^A99Lys. The structural features of α^A99 binding site demonstrates the contribution of side chain of Arg/Lys, i.e., the direct/indirect contact with the same residues of partner β subunits. Bar-headed goose HbA shows

TABLE 2

ESI-MS of Tryptic Peptides from β-Chain from Tufted Duck (*Aythya fuligula*)

Residue No.	Sequence	Mass calculated M + H	Mass observed M + H
1–8	VHWTAEEK	999.5	999.4
9–17	QIITGLWGK	1015.6	1015.6
18–30	VNVADCGAEALAR	1288.6	1288.6
31–40	LLIVYPWTQR	1288.7	1288.8
41–61	FFSSFGNLSSPTAILGNPMVR	2242.1	2242.2
62–66	AHGKK	540.3	540.2
67–76	VLTSFGDAVK	1036.6	1036.5
77–82	NLDNIK	716.4	716.5
83–104	NTFAQLSELHCDKLHVDPENFR	2613.2	2613.5
105–120	LLGDILIVVLAAHFSK	1709.1	1709.4
121–135	EFTPECQAAWQKLVR	1805.9	1806.2
136–143	VVAHALAR	836.5	836.4
144–146	KYH	447.2	447.2

TABLE 3

Amino Acid Composition of Tryptic Peptides (Tp) of α^A-Chain of Tufted Duck (*Aythya fuligula*)

Peptides: Amino acids:	Tp1 1–7	Tp2 8–11	Tp3 12–16	Tp4 17–31	Tp5 32–40	Tp6 41–56	Tp7/8 57–61	Tp9a 62–82	Tp9b 83–90	Tp10 91–92	Tp11 93–99	Tp12 100–127	Tp13 128–139	Tp14 140–141
Asx	0.73 (1)	1.15 (1)	—	—	—	0.95 (1)	—	3.07 (3)	1.21 (1)	—	2.13 (2)	1.13 (1)	—	—
Thr	—	1.10 (1)	—	0.78 (1)	1.8 (2)	0.83 (1)	—	—	—	—	—	0.91 (1)	1.90 (2)	—
Ser	0.70 (1)	—	0.6 (1)	—	—	0.80 (1)	—	0.68 (1)	0.81 (1)	—	—	1.65 (2)	—	—
Glx	—	—	—	3.8 (4)	1.18 (1)	2.31 (2)	—	1.20 (1)	1.15 (1)	—	—	1.13 (1)	—	—
Pro	—	—	—	—	1.11 (1)	0.92 (1)	—	—	—	—	1.12 (1)	2.12 (2)	—	—
Gly	—	—	1.1 (1)	3.3 (3)	—	1.15 (1)	1.1 (1)	1.11 (1)	—	—	—	1.08 (1)	1.20 (1)	—
Ala	1.89 (2)	—	—	2.0 (2)	—	1.11 (1)	1.1 (1)	5.66 (6)	0.78 (1)	—	—	2.81 (3)	3.11 (3)	—
Val	1.20 (1)	0.80 (1)	—	—	—	—	—	2.65 (3)	—	—	2.14 (2)	2.74 (3)	0.91 (1)	—
Met	—	—	—	—	0.78 (1)	—	—	—	—	—	—	—	0.85 (1)	—
Ile	—	—	1.1 (1)	0.9 (1)	0.86 (1)	0.87 (1)	—	1.12 (2)	—	—	—	1.01 (1)	—	—
Leu	1.13 (1)	—	—	1.2 (1)	—	1.07 (1)	—	1.85 (2)	1.81 (2)	0.86 (1)	—	4.88 (5)	1.05 (1)	—
Tyr	—	—	—	1.0 (1)	0.90 (1)	0.66 (1)	—	—	—	—	—	—	—	1.02 (1)
Phe	—	—	0.9 (1)	—	0.96 (1)	1.68 (2)	—	—	—	—	0.86 (1)	2.2 (2)	1.05 (1)	—
Lys	1.07 (1)	1.10 (1)	1.2 (1)	—	1.1 (1)	1.3 (1)	1.1 (2)	0.85 (1)	0.92 (1)	—	—	1.13 (1)	1.02 (1)	—
His	—	—	—	1.1 (1)	—	1.9 (2)	0.8 (1)	1.01 (1)	1.13 (1)	—	—	4.15 (4)	—	—
Arg	—	—	—	1.0 (1)	—	—	—	—	—	1.02 (1)	0.88 (1)	—	—	1.01 (1)
Cys								—	—	—	—	0.65 (1)	0.61 (1)	—
Sum	7	4	5	15	9	16	5	21	8	2	7	28	12	2

Note. Numbers in parentheses denote amino acid residues according to sequence analysis.

greater intrinsic oxygen affinity mainly due to the loss of one $\alpha_1\beta_1$ subunit contact (28). Although direct contacts are much more definite and stronger than an indirect contact, interaction via a water molecule in this case does seem to play its role. It is plausible to conclude that the presence of α^A99Arg forming new salt bridge network represents an adaptation stabilizing the R-structure in Tufted duck HbA.

Among other substitutions α34 is an $\alpha_1\beta_1$ contact point. In human Hb position α34, interacts with β128Ala, β125Pro and β124Pro in the oxy structure (R-structure). Most birds including Bar-headed goose possess threonine at this position forming hydrogen bond with β125, which stabilizes the T-structure thereby lowering the oxygen affinity (33). In Tufted duck hemoglobin isoleucine is present at this position which results in loss of a hydrogen bond. Interestingly, α34Ile has also been observed in the minor hemoglobin component HbD in falconiform representatives (33–35) and is considered to be responsible for the stabilization of R-structure thereby accounting for the higher oxygen affinity. Additionally mutations such as α34Ile

TABLE 4

Amino Acid Composition of Tryptic Peptides (Tp) of β-Chain of Tufted Duck (*Aythya fuligula*)

Peptides: Amino acids:	Tp1 1–8	Tp2 9–17	Tp3 18–30	Tp4 31–40	Tp5/6 41–61	Tp7/8 62–66	Tp9a 67–76	Tp9b 77–82	Tp10/11 83–104	Tp12 105–120	Tp13/14a 121–135	Tp14b 136–143	Tp15 145–146
Asx	—	—	1.9 (2)	—	1.9 (2)	—	1.0 (1)	2.6 (3)	3.8 (4)	1.0 (1)	—	—	—
Thr	0.7 (1)	0.8 (1)	—	0.7 (1)	0.8 (1)	—	0.7 (1)	—	1.1 (1)	—	0.8 (1)	—	—
Ser	—	—	—	—	3.5 (4)	—	0.5 (1)	—	1.0 (1)	0.6 (1)	—	—	—
Glx	2.2 (2)	0.9 (1)	1.2 (1)	1.0 (1)	—	—	—	—	3.2 (3)	—	3.9 (4)	—	—
Pro	—	—	—	1.1 (1)	2.1 (2)	—	—	—	1.0 (1)	—	1.0 (1)	—	—
Gly	—	2.2 (2)	0.9 (1)	—	1.9 (2)	1.2 (1)	1.0 (1)	—	—	1.1 (1)	—	—	—
Ala	1.1 (1)	—	3.7 (4)	—	1.0 (1)	1.3 (1)	1.1 (1)	—	1.2 (1)	2.1 (2)	2.0 (2)	3.2 (3)	—
Val	1.0 (1)	—	1.8 (2)	0.7 (1)	0.9 (1)	—	1.7 (2)	—	1.1 (1)	1.8 (2)	1.0 (1)	2.0 (2)	—
Met	—	—	—	—	0.8 (1)	—	—	—	—	—	—	—	—
Ile	—	1.8 (2)	—	0.7 (1)	1.0 (1)	—	—	0.9 (1)	—	2.1 (2)	—	—	—
Leu	—	1.1 (1)	1.0 (1)	1.7 (2)	2.2 (2)	—	0.9 (1)	1.0 (1)	3.2 (3)	4.1 (4)	1.2 (1)	1.3 (1)	—
Tyr	—	—	—	0.8 (1)	—	—	—	—	—	—	—	—	1.0 (1)
Phe	—	—	—	—	2.7 (3)	—	0.9 (1)	—	1.9 (2)	1.1 (1)	0.8 (1)	—	—
Lys	1.0 (1)	1.1 (1)	—	—	—	1.9 (2)	1.1 (1)	1.0 (1)	1.2 (1)	0.9 (1)	1.1 (1)	—	0.9 (1)
His	0.8 (1)	—	—	—	—	1.1 (1)	—	—	1.8 (2)	1.0 (1)	—	1.2 (1)	1.1 (1)
Arg	—	—	1.1 (1)	0.9 (1)	0.9 (1)	—	—	—	0.9 (1)	—	1.0 (1)	1.1 (1)	—
Cys	—	—	— (1)	—	—	—	—	—	1.1 (1)	—	1.2 (1)	—	—
Trp	— (1)	— (1)	—	— (1)	—	—	—	—	—	—	0.5 (1)	—	—
Sum	8	9	13	10	21	5	10	6	22	16	15	8	3

Note. Numbers in parantheses denote amino acid residues according to sequence analysis.

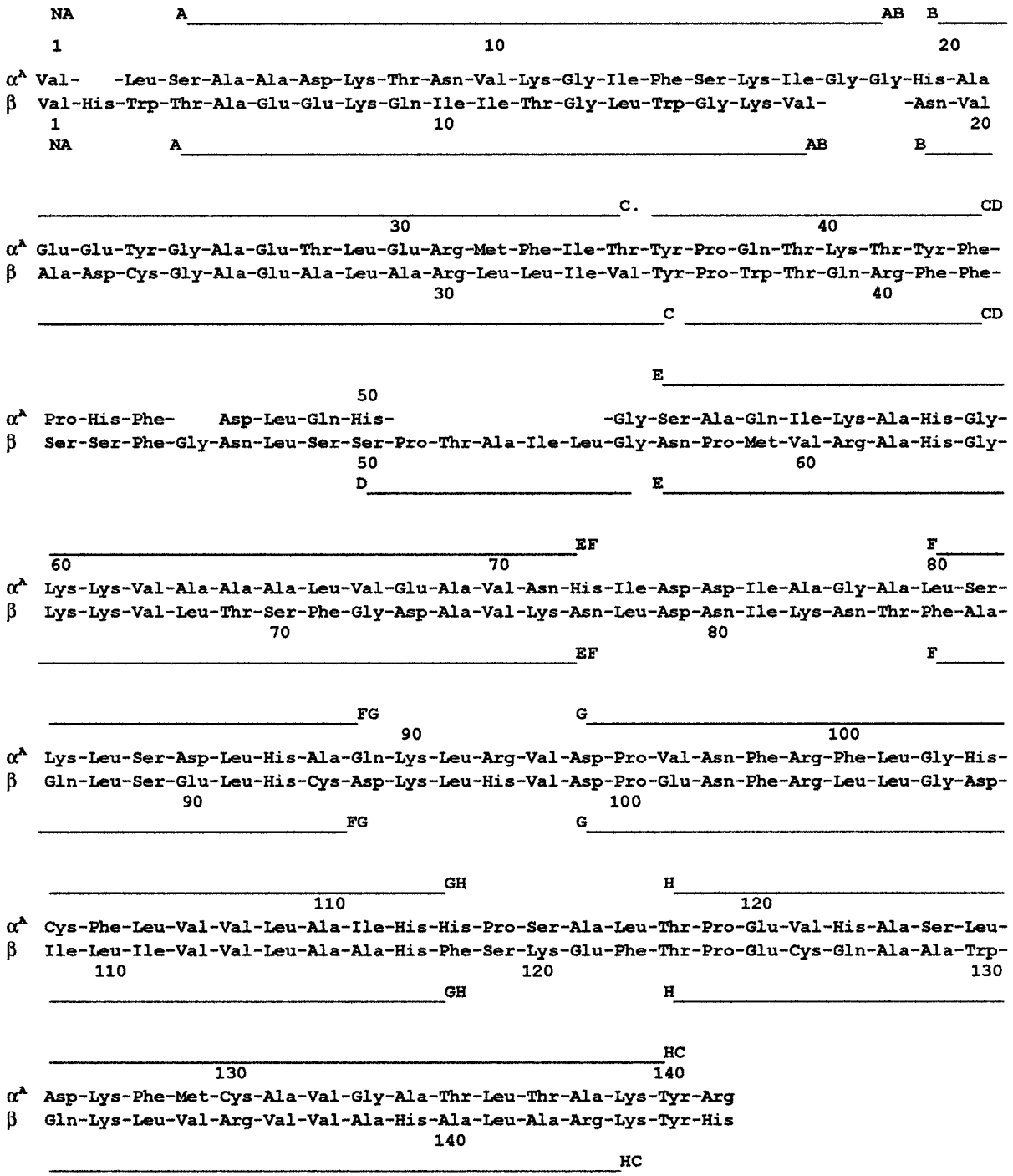

FIG. 2. Amino acid sequence of the major hemoglobin component (HbA) from Tufted duck (*Aythya fuligula*). Bars (A, B, C, etc.) indicate helices.

provide clear evidence for functional adaptation favoring oxygen binding under extreme hypoxic environment (36). Molecular modeling studies indicate that isoleucine in Tufted duck HbA is more in contact with β124Pro due to the dominating van der Waals interaction as compared to β125Glu and β128Ala (Fig. 4). This may be due to the conformational change arising from the presence of hydrophilic glutamic acid residue at β125 position. Stereo-view shows that the side chain of β125Glu is oriented away from the binding site. In case of Bar-headed goose HbA, the threonine residue at position α34 forms a hydrogen bond with β125 Asp. Also α34Thr is at an equal distance with β124Pro, β125Asp and β128Ala. However, these interactions are

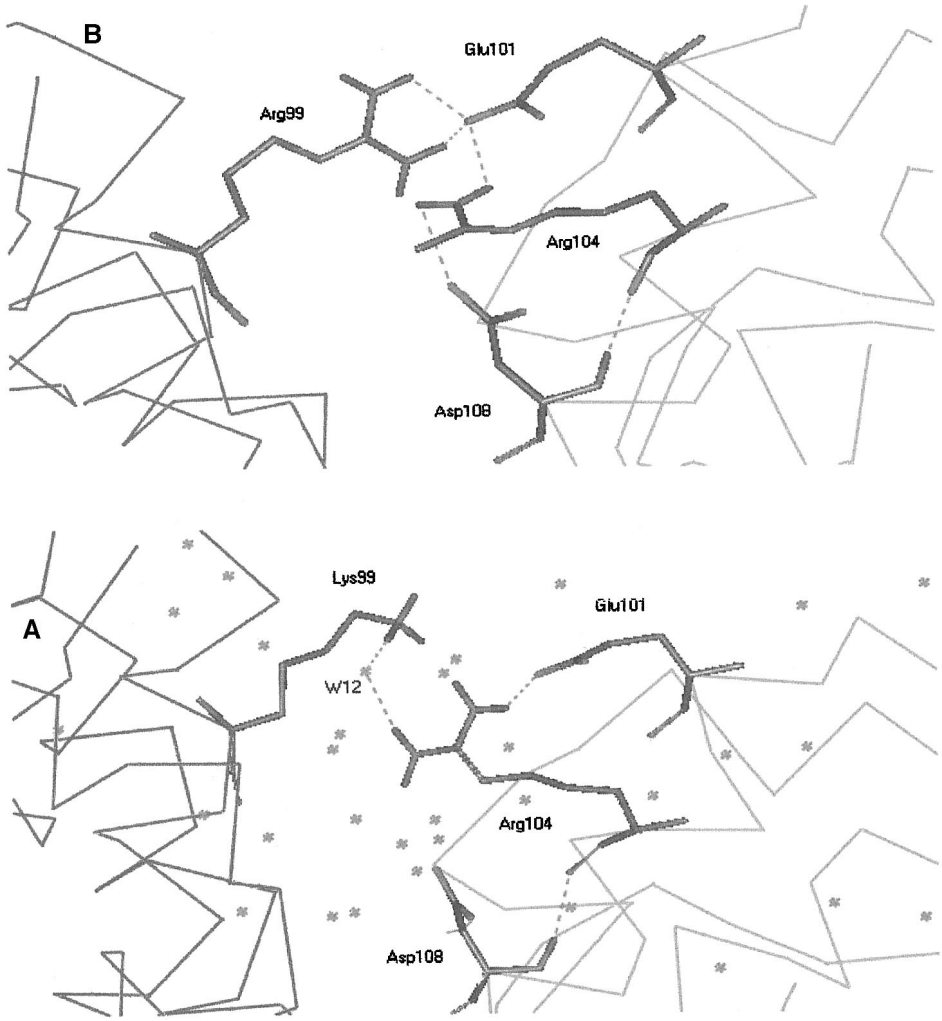

FIG. 3. (A) The salt bridge network of α^{A}99Arg–β101Glu–β104Arg–β108Asp in homology model of Tufted duck HbA. Hydrogen bonds are indicated as dashed lines. The backbones of α^{A} and β subunits are shown by C_{α} wires on the left and right, respectively. (B) The interactions between α^{A}99Lys–β104Arg–β101Glu–β108Asp via water molecule, in the crystal structure of Bar-headed goose HbA (PDB id = 1A4F). Hydrogen bonds are indicated as dashed lines. The backbones of α^{A} and β subunits are shown by C_{α} wires on the left and right, respectively.

weaker than those observed in Tufted duck, which may be attributed to shorter length of the side chain in Bar-headed goose Hb, i.e., α34Thr as compared to α34Ile in Tufted duck HbA.

Substitution at α38 Gln, an $\alpha_1\beta_1$ binding site in the α^{A}-chain, is a common feature of all species of the order anseriformes, cormorant (37), a diving bird, and in *Gyps rueppellii* hemoglobin HbD. It has been postulated that Gln at this position stabilizes the oxy-structure (38) by two hydrogen bonds (β99 and β97) in comparison to the deoxy structure, where only one hydrogen bond is possible. Thus, the major hemoglobin component of Tufted duck, i.e., HbA shows a higher oxygen affinity, loading fully even at low oxygen tension. X-ray diffraction studies indicate a substantial change at $\alpha_1\beta_2$ interface, which is responsible for the observed oxygenation characteristic (38). Also even small changes at the $\alpha_1\beta_1$ interface between tightly packed surfaces seems to be energetically effective at inducing affinity changes (39). The present results provide supporting evidence on the significance of these residues, which play a key role in regulating species-specific variation in intrinsic oxygen affinity in high altitude tolerant birds.

β55Met is another $\alpha_1\beta_1$ contact site unique among birds and mammals and is known to be contributing towards high altitude adaptation. In human hemoglobin β55Met forms a van der Waals contact with α119Pro. In Bar-headed goose and Andean goose, this residue pair is mutated to smaller residues, i.e., β55Leu and α119Ala in Bar-headed goose, and β55Ser and α119Pro in Andean goose, which results in loss of van der Waals interaction and in turn an increase in oxygen affinity (32, 39, 40). In human hemoglobin the distance between α119Pro and β55 Met is approximately 4 Å allowing van der Waals interaction to occur

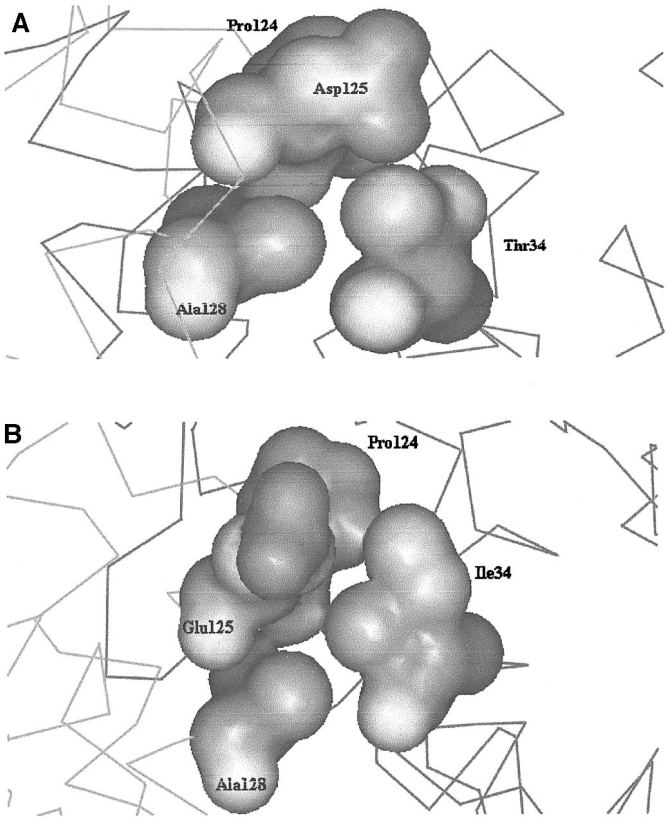

FIG. 4. (A) The $\alpha_1\beta_1$ binding site; the van der Waals interaction of α34 Thr with β124Pro β125Asp and β128Ala in the crystal structure of Bar-headed goose (PDB id = 1A4F). (B) The $\alpha_1\beta_1$ binding site; the van der waals interaction of α34 Ile with β124Pro β125Glu and β128Ala in the crystal structure of Bar-headed goose.

which stabilizes the T-structure (Fig. 5A). In Bar-headed goose, this contact is absent since a shorter amino acid, i.e., β55Leu can not establish an effective van der Waals contact with α119Ala which lies at a distance of 5 Å (Fig. 5B). Likewise the high oxygen affinity in Andean goose (α119 Pro and β55Ser) has been correlated with a gap arising from the substitution of β55Ser, which is much smaller compared to methionine. In Tufted duck HbA, position β55 is also occupied by a smaller residue, i.e., Leu (Fig. 5C). Homology modeling studies of Tufted duck hemoglobin shows a distance of 4.35 Å between α119Pro and β55Leu of the partner β-chain, which results in loss of van der Waals interaction. The loss of this intersubunit contact seems to contribute towards the destabilization of the T-structure with a resultant increase in intrinsic oxygen affinity leading to an increased tolerance to extreme hypoxia.

Thus, the key substitutions observed in Tufted duck HbA at α34Ile, α38Gln, α99Arg and β55Leu may be involved in adaptation to hypoxia both in terms of the efficient diving characteristics as well as high altitude flights in the Tufted duck.

Physiological and Phylogenetic Relationship

Comparison of α^A and β-chains of Tufted duck hemoglobin with the corresponding chains of other anseriform representative indicates a high degree of homology. Alignment of the sequence data also present a unique status for Magpie goose which may be considered as an ancestral (highest mutation rate) link between the different anseriform subfamilies.

Comparison of the primary structure with other representatives from 15 different avian order indicates

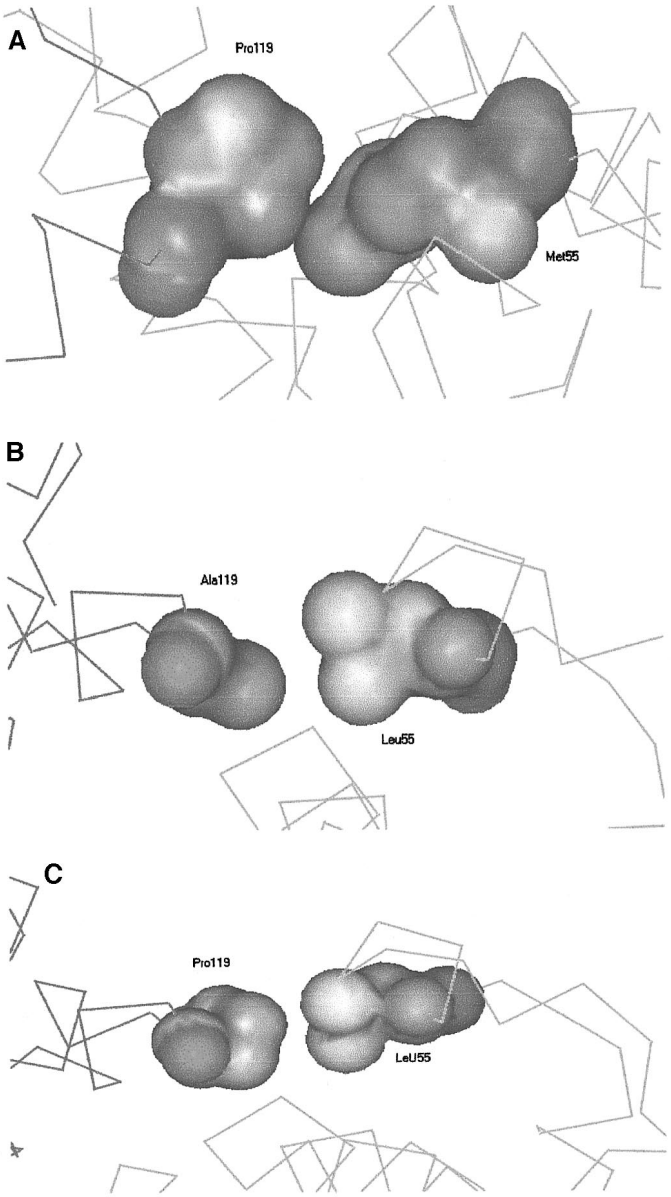

FIG. 5. (A) The $\alpha_1\beta_1$ contact point van der Waal radii of α119 Pro and β55Met in human hemoglobin (PDB id = 1HHO). (B) The $\alpha_1\beta_1$ contact point van der Waal radii of α119 Ala and β55Leu in Bar-headed goose hemoglobin (PDB id = 1A4F). (C) The $\alpha_1\beta_1$ contact point van der Waal radii of α119 Pro and β55Leu in Tufted duck hemoglobin HbA.

TABLE 5

Overall Comparison of Differences between Different Orders of Birds with Tufted Duck α^A-, α^D- and β-Chains

Variation with different orders	α^A-chain	α^D-chain	β-chain
Anseriformes	6–16	10–13	4–8
Apodiformes	25	25	15
Cathartiformes	20	21	12
Charadriiformes	25	—	10
Ciconiiformes	30	—	18
Columbiformes	22–26	—	14–16
Cuculiformes	22	—	—
Falconiformes	18–22	22–27	10–12
Galliformes	18–22	22–29	6–8
Passeriformes	22–27	37–43	13–15
Pelecaniformes	18	41	12
Phoenicopteriformes	27	—	8
Psittaciformes	21–27	—	7–15
Rheiformes	24	24	6
Sphenisciformes	19–21	—	10–12
Struthioniformes	14	19	7

closer relationship with struthioniformes, sphensciformes, pelecaniformes, galliformes, falconiformes and cathartiformes (Table 5). Interestingly, both ostrich and penguins are flightless birds and have evolved roughly at the same time i.e. during the eocene period. Among other orders falconiformes representatives seem to have no obvious evolutionary link with other birds and have currently been placed between ducks and gallinaceous birds (42). However, molecular sequence data indicates slightly different placement, i.e., gallinaceous birds to be in between ducks and falconiform representative. The order pelecaniformes (represented by cormorant), which also shows homology with Tufted duck, is known from the Cretaceous period. Physiologically, cormorants are more similar to diving birds. Their body has a relatively large blood volume like Tufted duck so that the oxygen supply is greatly enhanced supporting long submergence at greater depths. Furthermore, the increased blood volume also provides the energy required for strong migratory flights. The observed homology in the structure of hemoglobin thus, indicates not only their shared ancestral origin but also adaptation at the molecular level for survival under extreme atmospheric conditions.

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