doi: 10.4149/gpb_2014019

The crystal structure of oxy hemoglobin from high oxygen affinity bird emu (*Dromaius novaehollandiae*)

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Abstract. Hemoglobin is an honorary enzyme, a two-way respiratory carrier, transporting oxygen from the lungs to the tissues and facilitating the return transport of carbon dioxide. Hemoglobin has high affinity for oxygen and low affinity for carbon dioxide and other substances in the arterial circulation, whereas in the venous circulation these relative affinities are upturned. The oxygen affinity of hemoglobin increases with the fall in temperature and decreases with the increase in pH and 2, 3-bisphosphoglycerate; point mutations also affect the tetrameric arrangement and alter the oxygen affinity. Though several studies have revealed the specific reasons for the adaptation of increased oxygen affinity of avian hemoglobins at high-altitudes, further structural insights on hemoglobins from high oxygen affinity species are required to understand the detailed oxygen adaptation at the molecular level. Herein, we describe the structural investigation of hemoglobin from emu (*Dromaius novaehollandiae*), a high oxygen affinity bird. Hemoglobin from emu was purified using anion-exchange chromatography, crystallized and determined the structure in the oxy form at a resolution of 2.3 Å; the R-factor of the model was 19.2%. The structure was compared with other oxy hemoglobins of high oxygen affinity avian species; significant changes are noted at intra-subunit contacts which provide the clues for increased oxygen affinity of emu hemoglobin.

Key words: Hemoglobin — Crystal structure — Oxygen affinity — pH

Introduction

Hemoglobin (Hb) is functionally similar in all species, which carries oxygen from lungs to tissues and direct the return transport of carbon dioxide from tissues to the lungs. The oxygen transport mechanism of hemoglobin provides the understanding of the respiratory system and environmental adaptation characteristics (Wells 1999). Hemoglobin molecule consists of four subunits α1-β1 and α2-β2 arranged as dimers in a fashion having pseudo two-fold symmetry and each subunit carries a specific functional prosthetic heme group attached to a Fe^{2+} ion. Earlier investigations clearly demonstrated that the two-state models of hemoglobin exists in equilibrium between the tense "T" state and the relaxed "R" state (Monod et al. 1965) mediated by the allosteric transition between the interfaces in the subunits (Perutz et al. 1987). Further, intermediate conformational states between T and R (R3, RR2, R2) were also observed from several crystallographic studies (Smith et al. 1991; Silva et al. 1992; Smith and Simmons 1994; Schumacher et al. 1997; Safo and Abraham 2005).

Of the various species, birds need high energy which is fulfilled with the increased supply of oxygen through metabolic pathways (Perutz 1983; Weber 2007). The respiratory system of avian species differ from the mammals and a number of investigations showed that oxygen affinity of

Electronic supplementary material. The online version of this article (doi: 10.4149/gpb_2014019) contains supplementary material.

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avian species is higher than the mammals (Faraci 1991; Liang et al. 2001a, 2001b). However, the hemoglobin from birds is functionally similar to mammalians and regulated by inositol pentaphosphate (IPP) in place of 2,3-diphosphoglycerate (DPG) as an allosteric effector (Lenfant et al. 1969; Torrance and Bartlett 1970). Further studies on the hemoglobin structures show the altered oxygen affinity upon amino acid substitutions at heme-protein contacts and the inter-subunit contacts which orchestrates the oxy and deoxy transition states of the tetramer (Jessen et al. 1991; Vallone et al. 1996; Thom et al. 2013). It has been shown that the waterfowl species, the bar-headed goose is characterized by a remarkably higher Hb–O_2 affinity than greylag goose (Petschow et al. 1977; Rollema and Bauer 1979), because of the Pro α119(H2) → Ala substitution which disrupts an important intradimer van der Waals contact (Perutz 1983).

Site-directed mutagenesis studies on human hemoglobin representing the mutations observed in the high altitude geese [bar-headed goose (Pro α119 → Ala) and Andean goose (Leu β55 → Ser)] show that engineered Hb mutant was characterized by higher oxygen binding affinity than native human Hb and confirm that the difference in the affinity was primarily due to the loss of a single α1-β1 interchain contact (Jessen et al. 1991; Weber et al. 1993). Similarly, the regulation of higher oxygen affinity of vulture, a very high altitude bird, was also evidenced by the substitutions at the α_1-β_1 and α_1-β_2 interfaces by the mutations Thr α34→Ile and Gln α38→Pro contacts (Hiebl et al. 1988). Further studies show the substitutions involving the C-terminal residues of the β chain or of the BPG (2,3-bisphosphoglyceric acid) binding sites favor the oxygenated conformation and cause a left shift of the oxygen dissociation curve, which reflects an increased blood affinity for oxygen (Marengo-Rowe 2006). The emu bird (*D. novaehollandiae*) is the second largest living ratite in avian species and its hemoglobin encompass high oxygen affinity to run at a high speed of about 48 km/h (Davies 2003). They travel long distances to find food; they feed on a variety of plants and insects, but have been known to go weeks without food. Emu's hemoglobin plays an important role for their tremendous metabolic rate and adaptation of irregular hypoxic environment (Maloney et al. 1998). The amino acid sequence of bird Hbs always shows certain distinctive patterns and it is therefore of interest whether these lead to altered structures. Further, the detailed structures of avian Hbs, particularly high oxygen affinity Hbs are limited (Zhang et al. 1996; Liang et al. 2001b; Liu et al. 2001). In the present study we focus our investigations on three-dimensional structure of emu hemoglobin and the conformational changes among the high altitude adaptation of birds. This study provides some valuable clues on higher oxygen- affinity of the bird emu. Herein, we have performed the purification and crystallization of hemoglobin under unbuffered condition which reflect the physiological pH of hemoglobin. The three-dimensional structure was determined using molecular replacement procedure and compared with the hemoglobins of high oxygen affinity species.

Materials and Methods

Isolation and purification

Emu hemoglobin was purified and crystallized according to our previously established procedure (Mohamed Abubakkar et al. 2013). Fresh whole blood from healthy adult emu bird was collected and 10 ml of the blood was subsequently treated with 3 ml of 0.9% (w/v) saline solution containing 0.5 g EDTA to avoid clotting and incubated at 4°C for 30 minutes. Then the red blood cells (RBC) were isolated from the whole blood by centrifugation for 30 min at 10000 rpm at 4°C. The isolated RBC pellet was washed three times in two volumes of 0.9% (w/v) isotonic saline solution and hemolysed by the addition of three times the volume of triple-distilled water. Maximum yield of cell-free hemoglobin solution as the supernatant was achieved after 90 min of hemolysation and subsequent centrifugation at 10000 rpm at 4°C for 1 h. The hemoglobin solution was carefully removed by suction and extensively dialyzed in distilled water for 48 h, changing the distilled water for once in 12 h. Finally, the sample was lyophilized and stored at 4°C.

The partially purified emu hemoglobin sample was reconstituted with distilled water and loaded onto a DEAE-Cellulose anion exchange chromatographic column (10 × 1.5 cm) equilibrated with water (Knapp et al. 1999). The column was initially eluted with water, followed by sodium chloride concentration gradient (0.2–1.0 M, at a step of 0.1 M) at a flow rate of 3 ml/min. The purified sample corresponding to a single peak was obtained at 0.2 M NaCl. The fractions showing single peak were pooled and the homogeneity of the purified emu hemoglobin was confirmed by 10% native-PAGE (Davis 1964) and UV absorption peak at 278 nm (Fig. 1A, B). Protein concentration was estimated as 20 mg/ml using Bradford absorption method (Bradford 1976) using BSA as standard. Further the purified hemoglobin sample was dialyzed in distilled water and used for crystallization to perform X-ray diffraction studies.

Crystallization

The purified emu hemoglobin was crystallized at 20°C using hanging-drop vapour-diffusion method. The purified sample was further concentrated using Millipore centrifugal concentrator devices and the protein concentration of 20 mg/ml was used in the initial crystallization screen using different precipitants such as 2-methyl-2,4-pentanediol

(MPD) and polyethylene glycol (PEG; molecular weight ranges between 400 and 8000) as well as varying the protein concentration and pH. The crystals appeared in different buffer conditions; however the crystals grown in unbuffered condition diffracts better, in which the crystallization drop was prepared by mixing 3 µl of protein solution (20 mg/ml) and 1 µl of precipitant solution (30% PEG 3350), equilibrated against 1 ml reservoir solution comprising 30% PEG 3350 without any buffer or additives. The crystals that appeared in buffered reservoir solution (25% PEG 3350+sodium phosphate buffer, pH 6.3; 25% PEG 3350+HEPES, pH 8.0) were highly mosaic and diffracted poorly (Supplementary Figure 1). The pH of unbuffered reservoir solution and equilibrated drop were observed as 7.0 (using electrode and pH-indicator strips (Whatman) respectively), which is similar to the physiological pH of hemoglobin found in the peripheral tissues (Voet et al. 2008). The crystals appeared overnight and grew within two days to dimensions of 0.4 × 0.3 × 0.2 mm. The microscopic images of the crystals are shown in Fig. 1C.

Data collection and processing

X-ray diffraction data were collected at the in-house G. N. Ramachandran X-ray facility using MAR345dtb image plate detector and a Bruker Microstar copper rotating-anode generator operating at 45 kV and 60 mA. To collect the diffraction data under cryogenic conditions, crystals of emu hemoglobin were soaked for 30–40 seconds in a cryoprotectant solution containing the reservoir solution supplemented with 25% (v/v) glycerol before flash-cooling in a stream of liquid nitrogen at −173°C. Data were processed and scaled using *AUTOMAR* software suite (Klein 2009). The crystals diffracted to 2.3 Å resolution, and statistics of the crystallographic data collection and processing are presented in Table 1.

Structure determination, model building and refinement

The diffracted crystal belongs to orthorhombic space group which accommodates one whole biological molecule (α1β1, α2β2) in the asymmetric unit with a solvent content of 47% (Matthews 1968; Kantardjieff and Rupp 2003). The structure was solved by the molecular replacement method using the program Phaser (McCoy et al. 2007) from the CCP4 program suite (Potterton et al. 2003; Winn et al. 2011) using ostrich hemoglobin (Sundaresan et al. 2009; PDB id: 3FS4) as a starting model. The structure solution showed no steric clashes between symmetry-related molecules and good crystal packing with the likelihood gain of 2225. As the amino acid sequence of β subunit of emu hemoglobin is not available, the model building of β subunit is performed by identifying the consensus residues using multiple sequence

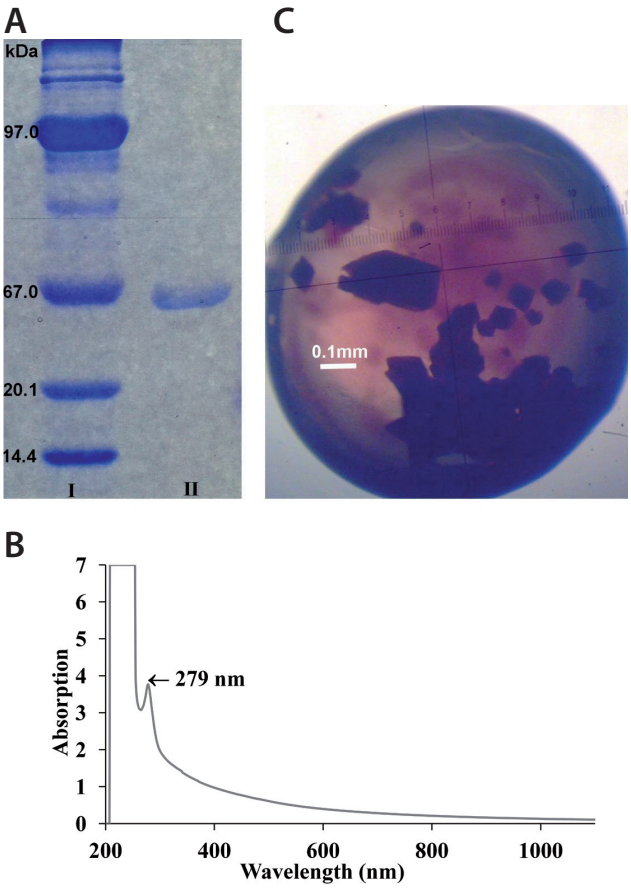

Figure 1. **A.** Native PAGE showing purified emu hemoglobin in lane-II (mw: 66.5 kDa) and the molecular weight standards in lane-I. **B.** The UV absorption spectra of the purified hemoglobin showed a single peak at 279 nm. **C.** Crystals of emu hemoglobin grown under unbuffered condition.

alignment of 149 avian sequences including 2 ratites (Supplementary Data 1). Interestingly, the amino acid sequence deduced from the β subunit of the model shares high similarity (95% identity) with the hemoglobins of high oxygen affinity species, vulture and geese, suggesting that the evolution of increased oxygen affinity can be attributed to similar changes with similar mechanism. Indirectly, the consistency between the electron density and amino acids derived through multiple sequence alignment indicates that electron density at 2.3 Å resolution is clear and reliable enough. Also, in α1-β1 dimer, the electron density clearly resolves the side chain orientations of twenty-five substitutions with respect to ostrich hemoglobin (starting model). The structures were refined using Refmac5 (Murshudov et al. 1997) through CCP4i interface (Potterton et al. 2003), with the maximum likelihood target function and using anisotropic B factors. Five percent of the total reflections were flagged randomly for cross validation before refine-

Table 1. Crystallographic data-collection and model refinement statistics of Hb from *D. novaehollandiae* (values in parentheses correspond to the highest resolution shell)

X-ray diffraction data collection	
X-ray Source	Cu Kα
Wavelength (Å)	1.5417
Crystal dimensions (mm)	0.4 × 0.3 × 0.2
Rotation range per image (°)	1
Total rotation range (°)	180
Exposure time *per* image (s)	60
Space group	$P2_12_12_1$
Unit cell parameters (Å)	a = 66.27, b = 80.01, c = 103.55
Mosaicity (°)	0.20
Resolution (Å)	30.0–2.3 (2.35–2.30)
No. of measured reflections	178094
No. of unique reflections	25129
Data completeness (%)	99.5 (100)
Multiplicity	7.0
R_{merge} (%)[a]	6.1 (26.2)
<I/σ(I)>	6.4 (2.0)
Refinement	
Resolution range (Å)	30.0–2.3 (2.35–2.30)
R_{model} (%)[b]	19.2 (21.8)
R_{free} (%)[c]	25.6 (33.0)
Residues in the asymmetric unit (water molecules)	568 (360)
Mean B factor (Å^2) [protein (water)]	20.7 (26.9)
r.m.s deviation: bond lengths (Å)	0.018
r.m.s deviation: bond angles (°)	1.579
Ramachandran plot [d]	
most favored (%)	91.7
additionally allowed (%)	7.8
generously allowed (%)	0.6

[a] $R_{merge} = \Sigma_{hkl} \Sigma_i |I(hkl)_i - <I(hkl)>| / \Sigma_{hkl} \Sigma_i < I(hkl)_i >$; [b] $R_{model} = \Sigma_{hkl} |F_o(hkl) - F_c(hkl)| / \Sigma_{hkl} |F_o(hkl)|$, where F_o and F_c are the observed and calculated structure factors, respectively. [c] R_{free} was calculated using 5.0% of the total reflections. [d] Calculated with PROCHECK (Laskowski et al. 1993).

ment. TLS (Translation-Libration-Screw) parametrization was used in the latter stage of refinement, with one set of parameters for each chain (Winn et al. 2001). Structures were visualized and modified using Coot5 (Emsley and Cowtan 2004). The water molecules in the structure, including heme coordinated oxygens were identified and accounted by electron density peaks greater than 3σ in Fo-Fc map and 1σ in 2Fo-Fc map. The stereochemistry of the model was evaluated with PROCHECK (Laskowski et al. 1993) and the structural alignments were made using the SSM algorithm (Krissinel and Henrick 2004) as implemented in Coot5. Refinement and geometrical statistics of present model are summarized in Table 1. The atomic coordinates and structure factors of emu Hb have been deposited in the RCSB Protein Data Bank (www.pdb.org) as an entry code 3WTG.

Results and Discussion

Overall structure

The final refined model of emu Hb contains one tetrameric unit of Hb having two α chains (α1, α2) and two β chains (β1, β2). There are seven and eight helices in α and β chains, respectively, comprising 568 Hb amino acid residues, 4 heme groups, 4 oxygen molecules and 360 water molecules. The 2Fo-Fc electron density map at 1.0σ shows continuous density for the entire polypeptide main chain and side chain atoms. The final model confirms the oxy form of emu hemoglobin. In emu Hb, all four heme groups, as well as the O_2 molecules are well-defined by electron density in both 2Fo-Fc and omit maps. The individual α, β subunits and the overall model are similar to avian Hbs; and comparable to oxy Hbs of the high oxygen affinity species, namely bar-headed goose (Zhang et al. 1996) and greylag goose (Liang et al. 2001b), with the r.m.s.d (root-mean-square deviation) values of 0.64 and 0.63 Å, respectively (Fig. 3A). However, α subunit shows larger deviation than in β subunits (r.m.s.d. of 0.65 and 0.55 Å with bar-headed and greylag goose oxy

Table 2. Comparison of the overall structure of emu oxy Hb with oxy Hbs from bar-headed goose (PDB id: 1A4F), greylag goose (PDB id: 1FAW), ostrich (PDB id: 3FS4) and human (PDB id: 1HHO)

Subunits	r.m.s.d. values (in Å) between Emu and:			
	Bar-headed goose	Grey lag goose	Ostrich	Human
α subunit	0.650	0.545	0.612	0.781
β subunit	0.424	0.395	0.272	0.885
α1β1 dimer	0.667	0.552	0.669	0.889
tetramer	0.635	0.628	0.663	1.053

Root mean square deviation (r.m.s.d) was calculated by least-squares superposition of protein main chain atoms using Superpose algorithm (Krissinel and Henrick 2004).

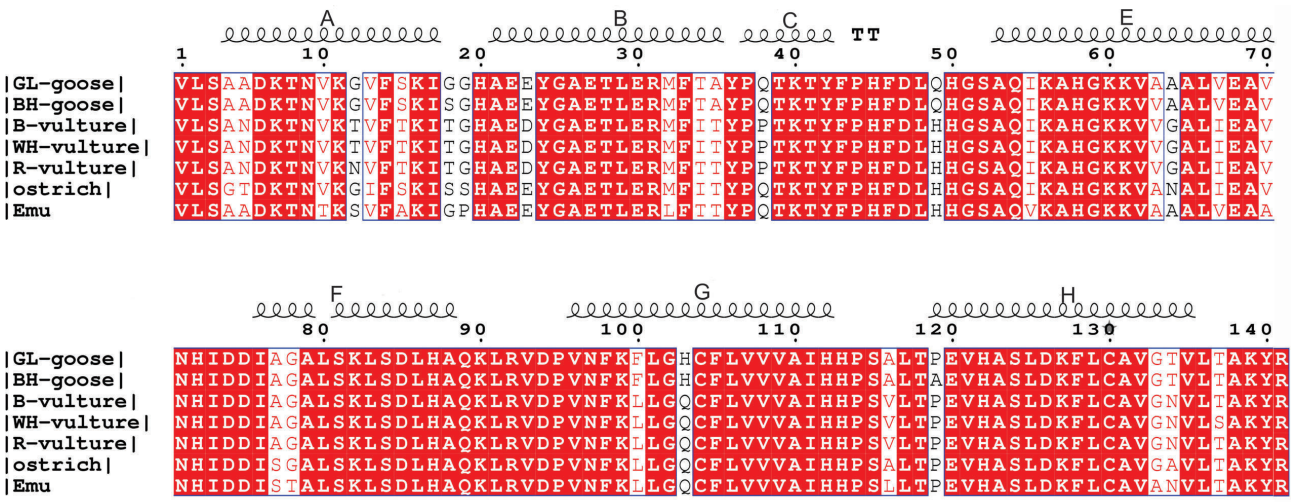

Figure 2. Multiple sequence alignment of the emu Hb (α subunit) with Hbs from high altitude birds (GL-goose, greylag goose; BH-goose, bar-headed goose; B-vulture, black vulture; WH-vulture, white-headed vulture; R-vulture, Rüppell's vulture and ostrich). The alignment was made using ClustalW algorithm (Larkin et al. 2007). The α- helical segments are denoted.

Hbs, respectively) due to the effect of more amino acid substitutions in this subunits. Table 2 reveals the r.m.s. deviations between emu oxy Hb and other oxy Hbs. The amino acid sequence comparison of high altitude birds shows that α subunit of emu's hemoglobin shares 88% and 86% similarity with the oxy Hbs of vulture and geese, respectively (Fig. 2); particularly, of the 141 amino acids in α subunit, 19 substitutions occurred at the helices A, B, E, F, G and H. With respect to human Hbs, the α and β subunits of emu shares 69% and 68% sequence identity, respectively.

Table 3. Geometry of the heme groups and its environment residues in oxy hemoglobins (oxy Hb)

Oxy Hb	Subunit	Fe-His(E7)NE2 (Å)	Fe-Val(E11)CG2 (Å)	Fe-Phe(CD1)CZ (Å)	Fe-His(F8)NE2 (Å)	Fe-Plane distance* (Å)
Emu	α1	4.44	4.86	5.58	1.96	0.14
	β1	4.34	4.65	5.46	2.08	0.19
	α2	4.54	4.81	5.36	2.07	0.12
	β2	4.37	4.82	5.56	2.01	0.13
Bar-headed goose	α1	4.52	5.06	5.66	2.10	0.21
	β1	4.51	4.90	5.64	2.07	0.13
	α2	4.52	5.06	5.66	2.10	0.21
	β2	4.51	4.90	5.64	2.07	0.13
Greylag goose	α1	4.49	5.19	5.60	2.19	0.26
	β1	4.45	4.89	5.63	2.14	0.16
	α2	4.49	5.19	5.60	2.19	0.26
	β2	4.49	4.89	5.63	2.14	0.16
Ostrich	α1	4.27	4.99	5.56	2.12	0.19
	β1	4.14	4.70	5.44	2.17	0.14
	α2	4.40	4.79	5.54	1.96	0.13
	β2	4.19	4.66	5.27	2.12	0.12
Human	α1	4.39	4.79	5.51	1.94	0.16
	β1	4.19	4.66	5.66	2.07	0.06
	α2	4.39	4.79	5.51	1.94	0.16
	β2	4.19	4.66	5.66	2.07	0.06

* The plane is defined as that passing through all heme atoms excluding the side chains and the Fe atom.

Heme environment

The final model shows a clear 2Fo-Fc electron density at 1.0 σ level at the distal side of heme plane in all four subunits in the asymmetric unit, which authenticates the oxy form of emu hemoglobin. The heme pockets of emu Hb are similar to those of human Hb (Shaanan 1983) and high oxygen affinity birds (greylag goose, bar-headed goose and ostrich) and there are no mutations in the heme vicinity. The heme regions of the molecule are well conserved and the equivalent residues overlap closely. The heme and the residues around it in the emu oxy Hb structure fit well to electron densities. Figure 3B shows the fitted superposition of heme pockets of emu and other species Hbs refined through least-squares procedures. A comparison of the geometries around the heme group between the emu oxy Hb and oxy Hbs is given in Table 3.

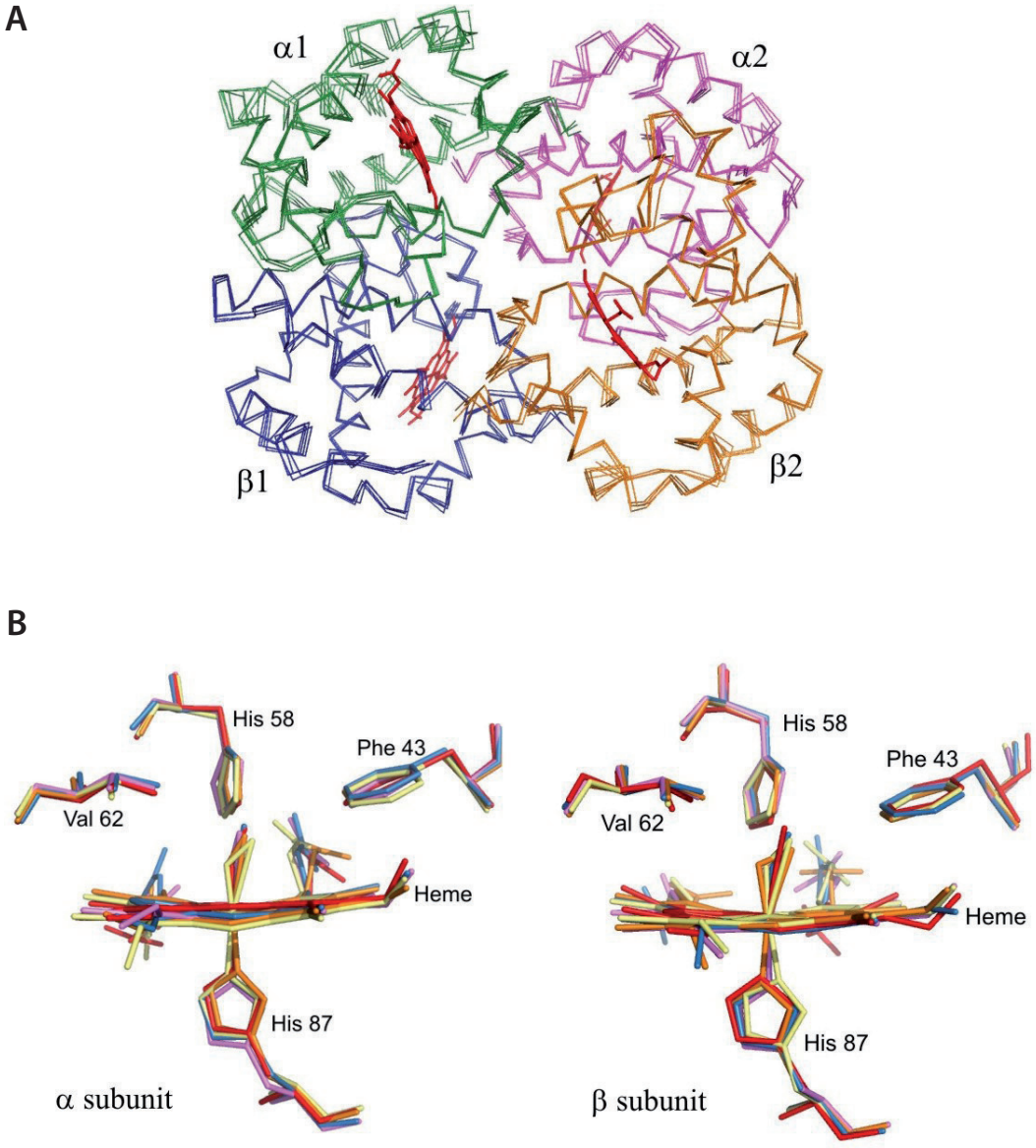

Figure 3. A. Structural superposition of the Cα traces of oxy Hbs of emu, bar-headed goose (PDB id: 1A4F), greylag goose (PDB id: 1FAW), ostrich (PDB id: 3FS4) and human (PDB id: 1HHO) showing overall similarity. The molecules are shown in thin lines and the heme is shown in stick representation. **B.** Structural superposition of the heme pocket of the emu oxy Hb (black/orange) on oxy Hbs of bar-headed goose (grey/blue), greylag goose (white/pink), ostrich (grey-thin sticks/yellow) and human (white-thin sticks/red). [Molecule colors according to B&W/color version]

Inter and intra subunit interactions

In common with other Hbs, emu hemoglobin structure has extensive intradimer (α1-β1/α2-β2) packing contacts amounting to 29 residues, whereas interdimer (α1-β2 / α2-β1) sliding contacts were less with the involvement of 13 residues. In order to maintain the free-energy difference between the T- and R-states (Pettigrew et al. 1982; Dickerson and Geis 1983), the highly conserved inter-subunit slide contacts in emu Hb are also similar to other vertebrate Hbs.

The α1-β1/α2-β2 dimer interface of emu oxy Hb contains similar non-covalent interactions as in the case of oxy state Hb structures of high altitude/high oxygen affinity birds, bar-headed goose (Zhang et al. 1996), greylag goose (Liang et al. 2001b) and ostrich (Sundaresan et al. 2009). In fact, the residues involved in hydrogen bonding and ion pair interactions at this interface are conserved in these four Hb structures, except the substitutions in geese at Thr α35(B16) →Ala, Gln α103(G10) →His. In particular, the amino acids responsible for the elevated O_2 affinity of adult Hbs of the geese and vulture [Thr α34(B15), Gln α38(C3), Pro α119(H2) (except bar-headed goose)] are highly similar in the emu Hb structure (Hiebl et al. 1988; Jessen et al. 1991; Weber et al. 1993), where as in human Hb, 34 and 38 positions were substituted with Leu and Thr, respectively.

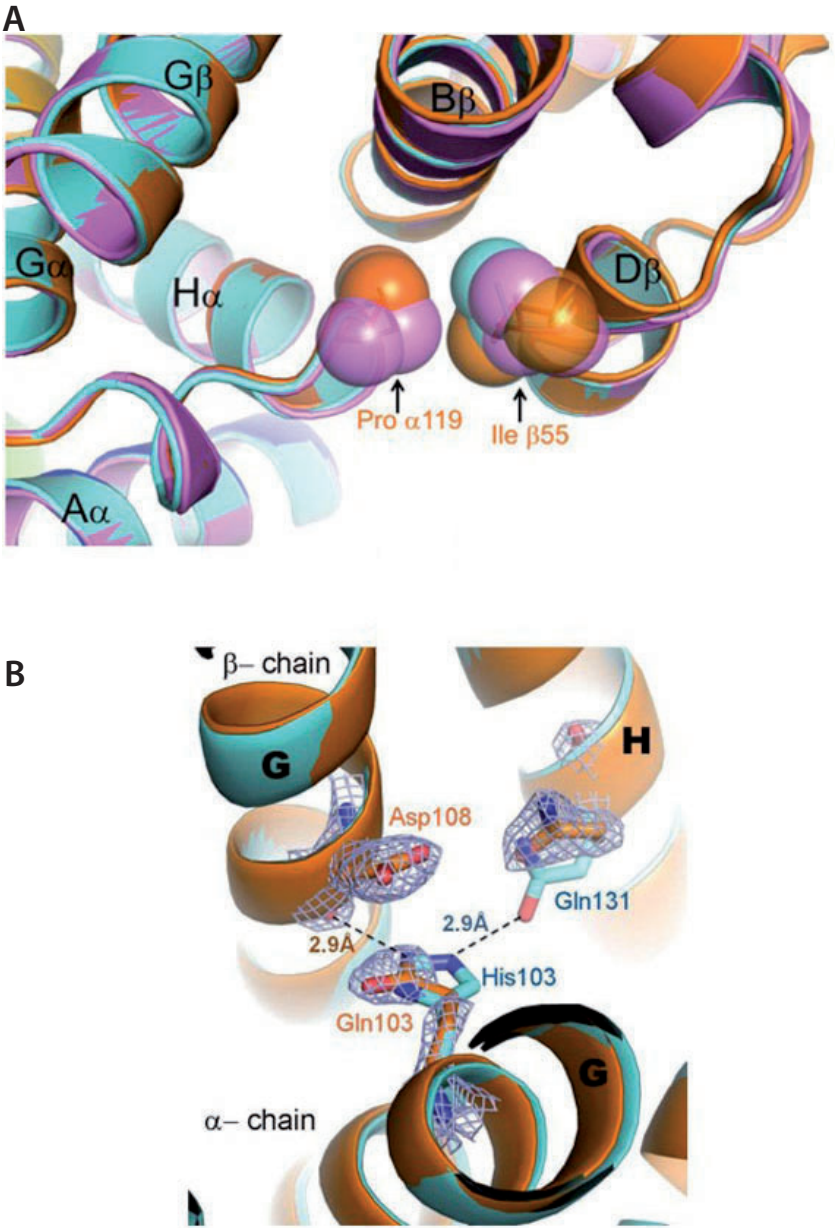

Figure 4. A. Variation in the van der Waals contacts between α119(H2) and β55(D6) in oxy Hbs of emu (black/orange), bar-headed goose (grey/blue) and greylag goose (white/pink). van der Waals radius of side chain atoms are shown in spheres. In bar-headed goose Hb, the contact disrupts due to Pro α119(H2) → Ala substitution, where as in emu Hb, the substitution in β subunit, Leu β55(D6) → Ile, triggers similar disruption compared to greylag goose's Hb. **B.** Structural comparison of hemoglobin of emu (black/orange) and bar-headed goose (grey/blue) (Zhang et al. 1996; PDB id: 1A4F) showing the compensation of inter-subunit interaction due to the variation in the amino acid sequence. In bar-headed goose (also in greylag goose), the association of α1-β1 subunit is stabilized by a hydrogen bond interaction between His103 (α1) and Gln131 (β1), whereas emu structure is stabilized by a hydrogen bond between Gln103 (α1) and Asp108 (β1). The 2Fo-Fc electron density map is shown around the emu hemoglobin residues contoured at 1.0σ level. [Molecule colors according to B&W/color version]

Here in, Pro α119(H2) → Ala substitution is found to be interesting in the avian Hbs as it disrupts an important intradimer van der Waals contact between Pro α119(H2) and Leu β55(D6), which is responsible for the higher O_2 affinity of bar-headed goose Hb than greylag goose and vulture (Perutz 1983). Although Pro α119(H2) is unaltered in the emu oxy Hb structure, the electron density clearly displays the change Leu β55(D6)→Ile, as in vultures, chicken, common swift, Andean condor etc. (Supplementary Figure 2 and Supplementary Data 1); as an effect of this, the significant variation in van der Waals contacts are marked in Figure 4A. The closest distance observed between Pro α119 and Ile β55 in emu oxy Hb is 4.31 Å, the distance comparable to bar-headed goose between Ala α119 and Ile β55 (4.56 Å), whereas the distance is shorter in greylag goose and ostrich oxy Hbs (3.79 and 3.86 Å, respectively). The disruption of this contact due to the substitution in β subunit (Leu β55(D6)→Ile) clearly dictates the reasons for the higher oxygen affinity of emu oxy Hb. This β subunit substitution resembles the change found in Andean goose Hb (β55(D6)→Ser) which shows increased O_2 affinity when engineered in the human Hb (Metβ55Ser) (Weber et al. 1993). These results shed the light in such a way that the change in α or β subunits (Proα119Al or Metβ55Ser mutation) can elevate the oxygen affinity. However, the availability of complete amino acid sequence of β subunit of emu Hb and protein-engineering experiments can strengthen this observation. Further, due to the local backbone perturbation of Pro α119(H2) → Ala substitution leads to loss of a hydrogen bond between Pro α119 (H2) and Arg β30 (B12) in bar-headed goose Hb, a notable feature preserved in emu and greylag goose Hbs.

On the other side of the interface, the new orientation of His α50 (CE8) in the emu oxy Hb stabilizes the CE loop of α subunit with H-helix of β subunit through a salt bridge with Glu β125(H3) at a distance of 3.30 Å, which is not seen in ostrich Hb (Supplementary Figure 3). Interestingly, the greylag goose oxy Hb structure proposes the possibility of such a salt bridge in one of the alternative conformations of His α50, at distance of 3.75 Å (Liang et al. 2001b). In fact this inference is valid if the side-chain flexibility of Glu and His are considered, whereas the present emu oxy Hb structure authenticates the existence of such His α50 conformation and thus the salt bridge. In contrast to this, the mutation in bar-headed goose oxy Hb, Glu β125(H3) → Asp separates His α50 far away (8.2 Å), indicating the absence of van derWaals contact, another contributing factor to the higher oxygen affinity (Zhang et al. 1996; Liang et al. 2001b). Therefore, the observed intra-subunit linkage *via* the salt bridge in emu oxy Hb suggests its O_2 affinity is comparable to bar-headed goose oxy Hb.

In addition, an intra-subunit hydrogen bond between Thr α35 (B16) and Gln β127 (H7) is observed in emu and ostrich Hbs whereas the substitution Thr α35 (B16) →Ala in geese prevents such interaction. Further, the substitution His α103→Gln in emu Hb, compensates the contact between His α103 (G10) and Gln β131(H9) observed in the geese structure, through hydrogen bonding between Gln α103(G10) and Asp β108(G10) (Fig. 4B). Also the substitution Gln α103(G10) pushes away the side chain of Gln β131(H9), which is slightly different from the geese Hb's conformation.

Conclusions

In the present study, the crystals of emu hemoglobin were obtained at 20°C under neutral pH (unbuffered) condition and the three-dimensional structure was determined at 2.3 Å resolution. Although sequence information is not available for the β subunit of emu, the present structure is reliable as the low R and R_{free} values provide clear information on the electron density map throughout the model. The three high oxygen affinity avian oxy Hb structures solved so far, bar-headed goose, greylag goose and ostrich have been compared with the emu Hb structure to understand the reasons behind high O_2 affinity of emu Hb. Despite the overall structural similarity, the two structural hot spots (mutations at α119-β55 and mutations α50-β125) are responsible for high O_2 affinity and reveal the contrasting changes in the present emu oxy Hb (Weber et al. 1993; Zhang et al. 1996; Liang et al. 2001b). The Leu β55→ Ile substitution producing a gap between α199 and β55 as in the bar-headed goose Hb, indicates the elevated O_2 affinity compared to greylag goose and ostrich; whereas the strong salt bridge between His α50-Glu β125, due to the new orientation of His α50, can reduce considerable affinity compared to bar-headed goose Hb. Further O_2 affinity and protein engineering experiments can provide more valuable information on the exact affinity of emu Hb towards oxygen.

Acknowledgement. The authors thank the anonymous reviewers for their constructive comments. The authors gratefully acknowledge the Department of Biotechnology (DBT), Govt. of India for financial support to create the in-house G. N. Ramachandran X-ray facility in CAS in Crystallography and Biophysics, University of Madras, Chennai, India. Further KS gratefully acknowledges Prof. T.R. Rajagopalan Memorial Research Grant, SASTRA University and DST-SERB, Government of India for financial support through Fast Track scheme for Young Scientists.

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Received: January 23, 2014
Final version accepted: June 11, 2014
First published online: August 22, 2014