doi:10.1006/jmbi.2001.5028 available online at http://www.idealibrary.com on IDEAL® J. Mol. Biol. (2001) 313, 123–137

JMB

The Crystal Structure of Bar-headed Goose Hemoglobin in Deoxy Form: The Allosteric Mechanism of a Hemoglobin Species with High Oxygen Affinity

Yuhe Liang, Ziqian Hua*, Xue Liang, Qian Xu and Guangying Lu

National Laboratory of Protein Engineering and Plant Genetic Engineering, College of Life Sciences, Peking University Beijing 100871, China

*Corresponding author

The crystal structure of a high oxygen affinity species of hemoglobin, bar-headed goose hemoglobin in deoxy form, has been determined to a resolution of 2.8 Å. The R and R_{free} factor of the model are 0.197 and 0.243, respectively. The structure reported here is a special deoxy state of hemoglobin and indicates the differences in allosteric mechanisms between the goose and human hemoglobins. The quaternary structure of the goose deoxy hemoglobin shows obvious differences from that of human deoxy hemoglobin. The rotation angle of one αβ dimer relative to its partner in a tetramer molecule from the goose oxy to deoxy hemoglobin is only 4.6°, and the translation is only 0.3 Å, which are much smaller than those in human hemoglobin. In the $\alpha_1\beta_2$ switch region of the goose deoxy hemoglobin, the imidazole ring of His $\beta_2$97 does not span the side-chain of Thr $\alpha_1$41 relative to the oxy hemoglobin as in human hemoglobin. And the tertiary structure changes of heme pocket and FG corner are also smaller than that in human hemoglobin. A unique mutation among avian and mammalian Hbs of α119 from proline to alanine at the $\alpha_1\beta_1$ interface in bar-headed goose hemoglobin brings a gap between Ala α119 and Leu β55, the minimum distance between the two residues is 4.66 Å. At the entrance to the central cavity around the molecular dyad, some residues of two β chains form a positively charged groove where the inositol pentaphosphate binds to the hemoglobin. The His β146 is at the inositol pentaphosphate binding site and the salt-bridge between His β146 and Asp β94 does not exist in the deoxy hemoglobin, which brings the weak chloride-independent Bohr effect to bar-headed goose hemoglobin.

Keywords: crystal structure; hemoglobin; bar-headed goose; allosteric mechanism; high-altitude adaptation

Introduction

Bar-headed goose, a migratory bird residing at Qinghai Lake in China, migrates annually across the Himalayas to Bengal Gulf. As a favorable model in the research of high-altitude adaptation of hemoglobin (Hb), the bar-headed goose Hb has been researched deeply in physiology, biochemistry, molecular biology and so on. The bird can fly over an altitude of 9000 m, showing high adaptation to high-altitude and extreme hypoxic environment; this is mainly due to the high oxygen affinity of its hemoglobin.[1,2] Compared to its closely related lowland species, greylag goose, the stripped Hb from the bar-headed goose shows a slightly higher oxygen affinity, the P_{50} values of the two Hbs are 2.8 and 2.0 Torr (at pH 7.2 and 25°C, with 100 mM chloride), respectively, these values increase to 30.9 and 20.4 Torr in the presence of inositol pentaphosphate (IPP), an allosteric effector in the avian red cells. Since the affinities for IPP of the two deoxyHbs are similar, and the concentrations of IPP in the red cells of both

Present addresses: X. Liang, 218 University Park, Rochester 14620, NY, USA; Q. Xu, Department of Physiology and Biophysics, Boston University School of Medicine, 715 Albany Street, Boston, MA 02118-2526, USA.

Abbreviations used: Hb, hemoglobin; IPP, inosital pentaphosphate; DPG, 2,3-diphosphoglycerate; PEG, polyethyleneglycol; r.m.s.d., root mean square deviation.

E-mail address of the corresponding author: huazq@yahoo.com.cn

0022-2836/01/010123–15 $35.00/0

species are in the same level, the functional changes of the two Hbs must come from their intrinsic structural differences.[3,4]

There are only four amino acid substitutions between Hb A of greylag goose and bar-headed goose,[5] which are Gly α18 to Ser, Ala α63 to Val, Pro α119 to Ala and Glu β125 to Asp. The substitutions at α18 and α63 locate near the surface of the molecule, while α119 and β125 are at the $\alpha_1\beta_1$ interface. From the sequential homology research of Hb and the abnormal human Hb research,[6–9] it was recognized that the mutation Pro α119 to Ala at the $\alpha_1\beta_1$ interface in bar-headed goose Hb removes a hydrophobic contact between α_1 and β_1 subunits that exists in greylag goose Hb, weakens the $\alpha_1\beta_1$ interface, and causes the relative higher oxygen affinity of bar-headed goose Hb. Introducing the mutation Pro α119 to Ala and Met β55 to Ser into human Hb by genetic engineering, respectively, also causes an increase in oxygen affinity at the same level with that between the two goose Hb species, which supports the hypothesis.[10,11]

The crystal structure at 2.0 Å resolution of bar-headed goose Hb in oxy form has been determined.[12] The tertiary structure of the goose oxyHb is very similar to that of human oxyHb. But the quaternary structures of the two Hbs are somewhat different, there is a 4.3° difference of the orientation of $\alpha_1\beta_1$ dimer relative to $\alpha_2\beta_2$ dimer in a tetramer molecule between the two Hbs. The α119-β55 contact does not exist in the goose oxyHb, which agrees with what Perutz predicted,[8] but α119 does contact with β30 and β33 as in human oxyHb. The Ala α119 in the goose Hb takes a conformation similar to Pro in the human Hb, and does not affect the main chain structure of the protein. The structure also shows the IPP binding site of avian Hbs in the entrance to the central cavity of the molecule, where exists the well-ordered conformation and the three-dimensional distribution of positive charges. But there are lots of questions left, which are impossible to be answered without the structure of the goose dexoyHb, such as whether the contacts of Ala α119 with β55, β30 and β33 exist in the goose deoxyHb, what the detailed conformation of the IPP binding site is, whether β146 takes part in the contact with IPP and what the allosteric mechanism of this avian Hb is. We determined the crystal structure of bar-headed goose deoxyHb and compared it with its oxy form, human oxy and deoxy Hbs, as well as tried to give an elucidation of the allosteric mechanism and the high oxygen affinity mechanism of the bar-headed goose Hb.

Results

Overall structure

As with human Hb, the bar-headed goose Hb tetramer molecule contains two α chains and two β chains, and there are seven and eight helices in α and β chains, respectively. Superimposing the goose deoxyHb on oxyHb using BGH reference frame ($\alpha_1$30-36, $\alpha_1$102-113, $\alpha_1$117-127, $\beta_1$26-37, $\beta_1$51-55, $\beta_1$107-132), the r.m.s.d. (root mean square deviation) is 0.3714 for the main-chain atoms. Overlapping the two forms of Hb on the reference frame defined by Zhang *et al.*,[12] named as Zhang frame, the r.m.s.d. of the overlapping frame is 0.4303. From the C^α traces of the goose Hb in the deoxy and oxy forms (Figure 1), which is least-squares fitted on the C^α atoms of BGH frame, and from the plot of r.m.s.d. of individual residues along the peptide chains (Figure 2), it can be seen that the main differences in tertiary structure of the two forms of goose Hb occur at AB and CD corners and F and H helices of α chain, and CD and EF corners and C termini of the β chain. Superimposing the goose deoxyHb on human oxyHb (PDB ID: 1hho) and deoxyHb (3hhb) were also done, the Zhang frame was used in the overlapping since the structural differences between the goose and human Hbs make the BGH frame less suitable for superimposing the coordinates.[12] The r.m.s.d. of

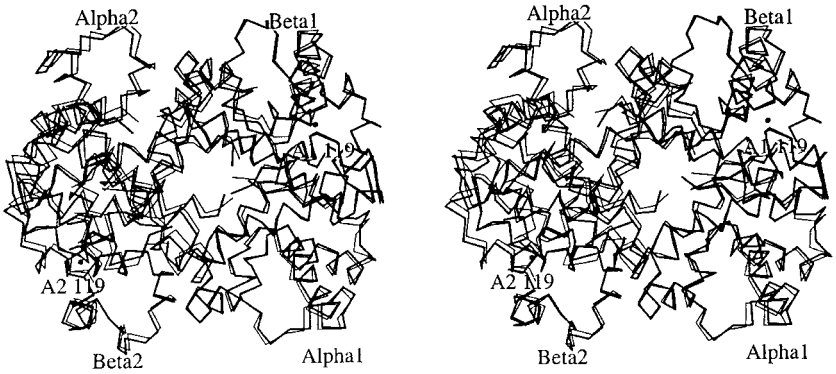


Figure 1. Overlap of the C^α traces of bar-headed goose Hb in oxy (thin line) and deoxy (thick line) forms on BGH frame of $\alpha_1\beta_1$ dimer. The α119 mutation site is highlighted.

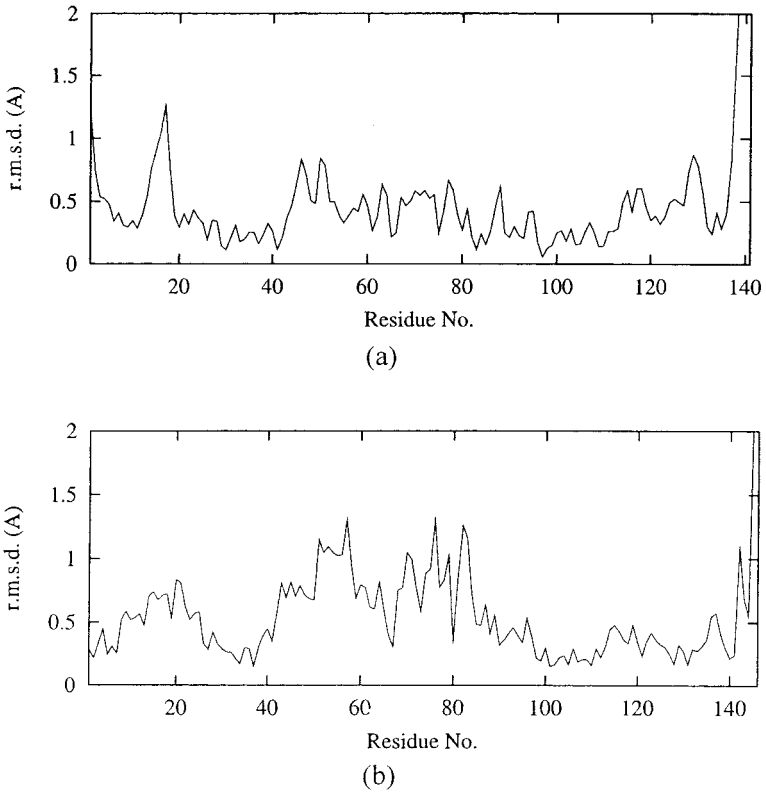


Figure 2. The r.m.s.d. of main-chain atoms between bar-headed goose Hb in oxy and deoxy forms, overlapped on BGH frame. (a) α Subunit, (b) β subunit.

main-chain atoms between the goose deoxyHb and human Hb in the oxy and deoxy forms are 0.6152 Å and 0.6148 Å, respectively, indicating remarkable differences in tertiary structure between the goose deoxyHb and human Hbs.

Compared to bar-headed goose oxyHb, the $\alpha_1\beta_1$ dimer rotates 4.6° relative to the $\alpha_2\beta_2$ dimer in a tetramer molecule of the deoxyHb, and translates 0.3 Å along the axis. These values are much smaller compared to 13.0° and 1.4 Å in human Hb. The rotation and translation between the goose deoxyHb and human Hb in the deoxy and oxy forms are 15.4°, 3.3 Å and 7.2°, 1.7 Å, respectively. These indicate that the goose deoxyHb takes a quaternary structure obviously different from that of human oxyHb and deoxyHb. Furthermore, the goose deoxyHb does not take the Y quaternary structure of human Hb[13,14] (PDB ID: 1cmy), the packing difference of dimers in the tetramer between the two Hbs are 8.1°, 0.3 Å, which is remarkable. Superimposing one dimer of the goose deoxyHb on that of human oxyHb and deoxyHb using the Zhang frame, respectively, the same conclusion can be drawn from the plot of r.m.s.d. of corresponding residues along the four peptide chains in the tetramer (Figure 3). And the salt-bridges stabilizing the T quaternary structure in human deoxyHb were not found in the goose deoxyHb. All these indicate that the bar-headed goose deoxyHb takes a special quaternary structure, and we name it the "T_B" state.

Heme group region

The ligand state and location of Fe

There are no densities at the distal side of heme plane in all of the four α and four β subunits of the two Hb molecules in an asymmetric unit in the $2F_o - F_c$ electron density map of 1σ level (Figure 4), and no density or very low density in the $F_o - F_c$ electron map of 3σ level. To check further the ligand state of Fe, the oxygen molecule was put on the distal side of the heme plane, and then the $F_o - F_c$ density map was calculated. There are negative densities at this region, indicating the Fe is unliganded. To determine the exact location of Fe, no geometric energy restriction was added on Fe in the refinement procedure. The Fe coordinate was checked in the $F_o - F_c$ omit maps of 3σ level in which heme and Fe were omitted, respectively. The heme stereochemistry parameters in bar-headed goose and human Hbs are listed in Table 1. Although it is difficult to determine the exact extent of the Fe atom movement from the heme plane at 2.8 Å resolution, it can be seen in Table 1 that the distance between the Fe atom and the porphyrin plane varies less in the goose Hb than that

Table 1. The heme stereochemistry parameters of bar-headed goose and human hemoglobins

		Distances to planes (Å)					Distances to Fe (Å)		His F8 atoms to porphyrin N atoms (Å)				NA-Fe-NC(deg.)
		Fe-Ph[a]	Fe-Pn[a]	Fe-Pc[a]	FG3-Ph	FG5-Ph	Fe-F8 N^{ε}[b]	Fe-E7 N^{ε}	N^{ε}-NA[b]	N^{ε}-NC[b]	C^{ε}[b]-NA	C^{δ}[b]-NC	
Bhg-deoxy[c]	Alpha	0.044	0.076	0.032	6.360	5.470	2.301	4.301	2.81	3.01	2.99	3.19	172.31
	Beta	0.073	0.087	0.070	6.180	5.392	2.372	4.195	3.28	2.91	3.23	3.32	175.74
Bhg-oxy[c]	Alpha	0.211	0.156	0.222	6.058	5.265	2.103	4.516	2.74	3.24	3.23	3.20	180.00
	Beta	0.132	0.034	0.152	5.943	5.118	2.074	4.506	2.89	2.86	3.06	3.01	180.00
Hum-deoxy[c]	Alpha	0.528	0.401	0.554	5.699	4.931	2.112	4.448	3.13	3.33	2.91	3.24	168.90
	Beta	0.443	0.358	0.460	5.809	5.102	2.058	4.266	3.04	3.27	3.00	3.12	180.00
Hum-oxy[c]	Alpha	0.155	0.121	0.162	6.169	5.073	1.937	4.310	2.87	2.92	3.21	3.79	157.84
	Beta	0.058	0.110	0.048	6.088	5.178	2.068	4.193	2.67	2.98	3.10	3.74	160.22

[a] Pn: Ph, the heme plane excluding side-chain and Fe atoms; the pyrrole nitrogen atoms plane of heme; Pc, the pyrrole carbon atoms plane of heme.
[b] N^{ε}, ε nitrogen atom of His; C^{δ}, C^{ε}, δ and ε carbon atoms of His; NA, NC, A and C site nitrogen of porphyin.
[c] bhg-deoxy, bar-headed goose deoxyHb; bhg-oxy, bar-headed goose oxyHb; hum-deoxy, human deoxyHb; hum-oxy, human oxyHb.

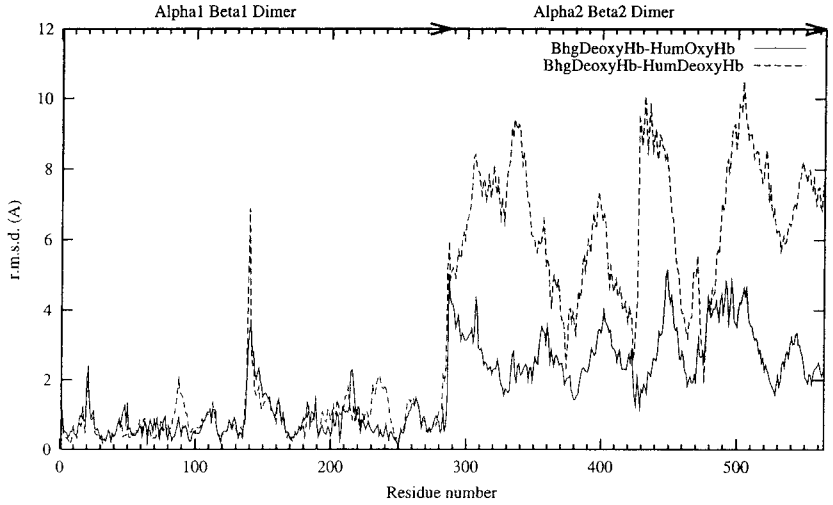


Figure 3. The r.m.s.d. of main-chain atoms between bar-headed goose deoxyHb (BhgDeoxyHb) and human Hb tetramer (HumOxyHb and HumDeoxyHb), overlapped on ZJ frame in $\alpha_1\beta_1$ dimer (continuous line, with human oxyHb; broken line, with human deoxyHb), the residue number of α_1, β_1, α_2 and β_2 subunits are represented by 1 to 141, 142 to 287, 288 to 428 and 429 to 574 along abscissa, respectively.

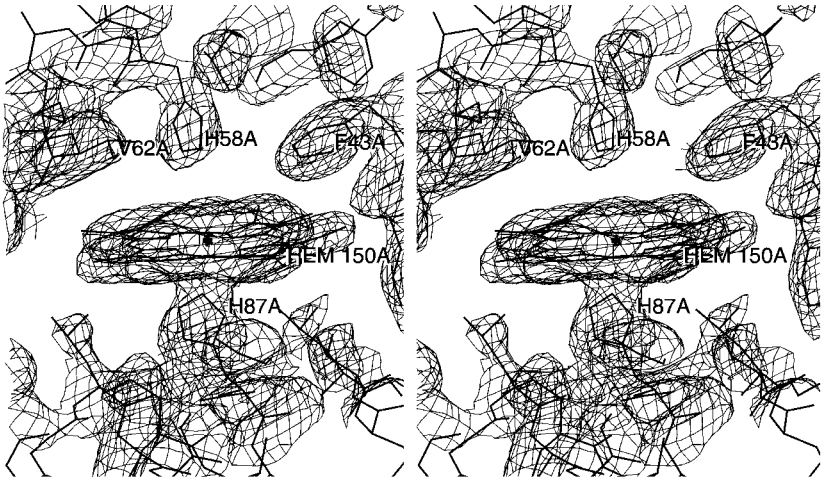
in human Hb, so the tension in the Fe movement would be smaller in the goose Hb.

The residues in heme pockets

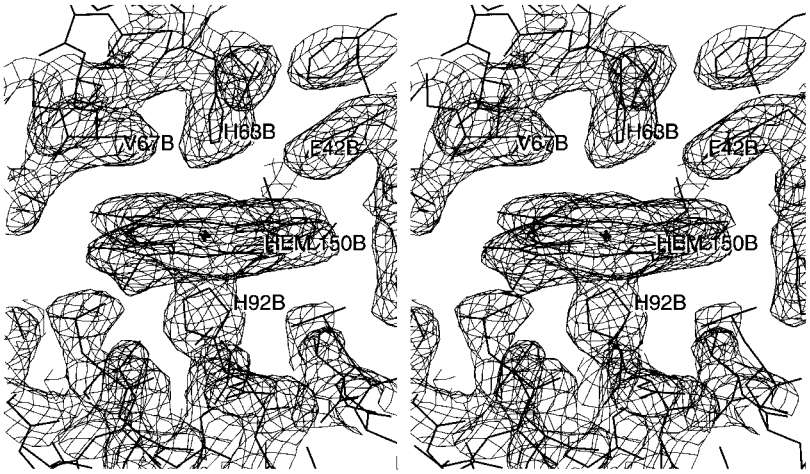
To compare the residue conformations in the heme pocket of bar-headed goose Hb in deoxy and oxy forms, three reference frames were used for least-squares overlapping of the two forms of Hb, respectively, which are BGH frame, heme frame (excluding Fe and side-chain atoms of heme), and F helix frame (F5-F8).

α Heme pocket. When the two forms of the goose Hb are overlapped on the three frames, respectively, there are only slight displacements of proximal residues. The distances from N^{ε} atom of the proximal His α87 to NA and NC atoms of heme and from C^{ε} and C^{δ} atoms of the proximal His to NA and NC atoms of heme are listed in Table 1. There are no remarkable changes in the symmetrical position of proximal His relative to the heme axis between the two forms, which would be more favorable to the ligation of Fe than that in human Hb.[12] Overlapping the two forms on BGH frame, it can be seen that there are differences in the distal side of the heme plane between the two forms, Val α62 (E11) and His α58 (E7) in bar-headed goose deoxyHb shift away from the $\alpha_1\beta_1$ interface (Figure 5(a)), but the differences at the F helix and FG corner are much smaller than that in human Hbs. When overlapped on the heme frame, the imidazole ring of the distal His α58 (E7) in deoxyHb turns about 30° relative to that in oxyHb, the distance from N^{ε} atom of His α58 (E7) to heme Fe atom reduces to 4.301 Å in deoxyHb from 4.516 Å in oxyHb, and the Phe α43 (CD1) moves towards the heme plane (Figure 5(b)) in the deoxy form. In human Hbs, overlapping the two forms on α F helix frame, the two pyrrole rings above the FG corner move away from the FG corner obviously in deoxyHb, while no remarkable changes in two pyrrole rings above the F helix,[15] the tilt and translation of α F helix relative to heme plane make the Fe atom displace from the heme plane to the proximal side in deoxyHb. While overlapping bar-headed goose deoxyHb and oxyHb on the α F helix frame (Figure 5(c)), the two pyrrole rings above the FG corner in deoxyHb does move in the same direction as in human deoxyHb, but the extent is much smaller than that in human Hb, the porphyrin is not domed as much as human deoxyHb. Besides, the heme plane of bar-headed goose deoxyHb makes a shift of 0.60 Å. Using Leu α91 (FG3) and Val α93 (FG5) as references of the position of the FG corner relative to the heme plane, the distances from their C^{α} atoms to the heme plane changed 0.30 Å and 0.22 Å in deoxyHb compared to that in oxyHb, respectively. These values in human Hb are 0.47 Å and 0.14 Å, respectively. These movements are caused by the tilt and translation of the F helix relative to the heme plane during the deoxygenation of bar-headed goose Hb, but the extents are much smaller than that in human Hb, and these movements do not distort the heme plane so markedly as in human deoxyHb.

β Heme pocket. The β heme pocket of the bar-headed goose Hb changes to a larger extent from oxy to deoxy forms, compared to the α heme pocket, which is consistent with there being tighter packing and smaller geometric changes in α heme region than in β heme region.[16,17] Overlapping the two forms on the BGH frame, there are remarkable translations of F helix and heme plane in deoxyHb, to an extent of 0.4 Å (Figure 6(a)), indicating that β heme moves further from the $\alpha_1\beta_1$ interface than α heme. Overlapped on β heme plane, the imidazole ring of the distal His β63 (E7) moves towards the center of porphyrin in deoxyHb with a 0.61 Å shift of N^{ε} atom of His β63 (E7), and partially fills the space held by oxygen molecule in oxyHb. And the benzene ring of Phe β42(CD1) rotates about 30° in



(a)



(b)

Figure 4. The σ_A-weighted $2F_o - F_c$ map at 1σ level around the heme pockets in the α (a) and β (b) subunits of bar-headed goose deoxyHb.

the deoxyHb relative to oxyHb. But the Val β67 (E11) does not move towards the heme plane as much as in human deoxyHb, which forms the steric hindrance for oxygen loading in human deoxyHb. (Figure 6(b)). While overlapped on F helix frame (Figure 6(c)), the heme plane translates with a Fe shift of 0.68 Å in deoxyHb compared with oxyHb, reflecting the tilt of F helix relative to heme plane. Meanwhile, there is a shift of main-chain atoms from Cys β93 (F9) to the FG corner, which causes FG corner residues to move further from the heme plane, the distances from C^{α} atoms of the Leu β96 (FG3) and Val β98 (FG5) to heme plane in deoxyHb change 0.24 Å and 0.27 Å from that in oxyHb, respectively, while these values in human Hb are 0.29 Å and 0.08 Å, correspondingly. And it can be seen that in human Hb, while overlapped on F helix frame, the nitrogen atoms of porphyrin ring move towards the proximal side of the heme plane in deoxyHb compared with oxyHb, but not seen in the goose deoxyHb.

From the comparisons above, there are some conformational changes following the loss of ligand of bar-headed goose Hb. The orientations of side-chains of some distal residues of the goose deoxyHb shift and move towards the heme plane, and the proximal F helix tilts and translates relative to the heme compared with oxyHb; all these movements are similar to that in human Hb. In human Hb, the distance between C^{α} atoms of FG1α_1 to

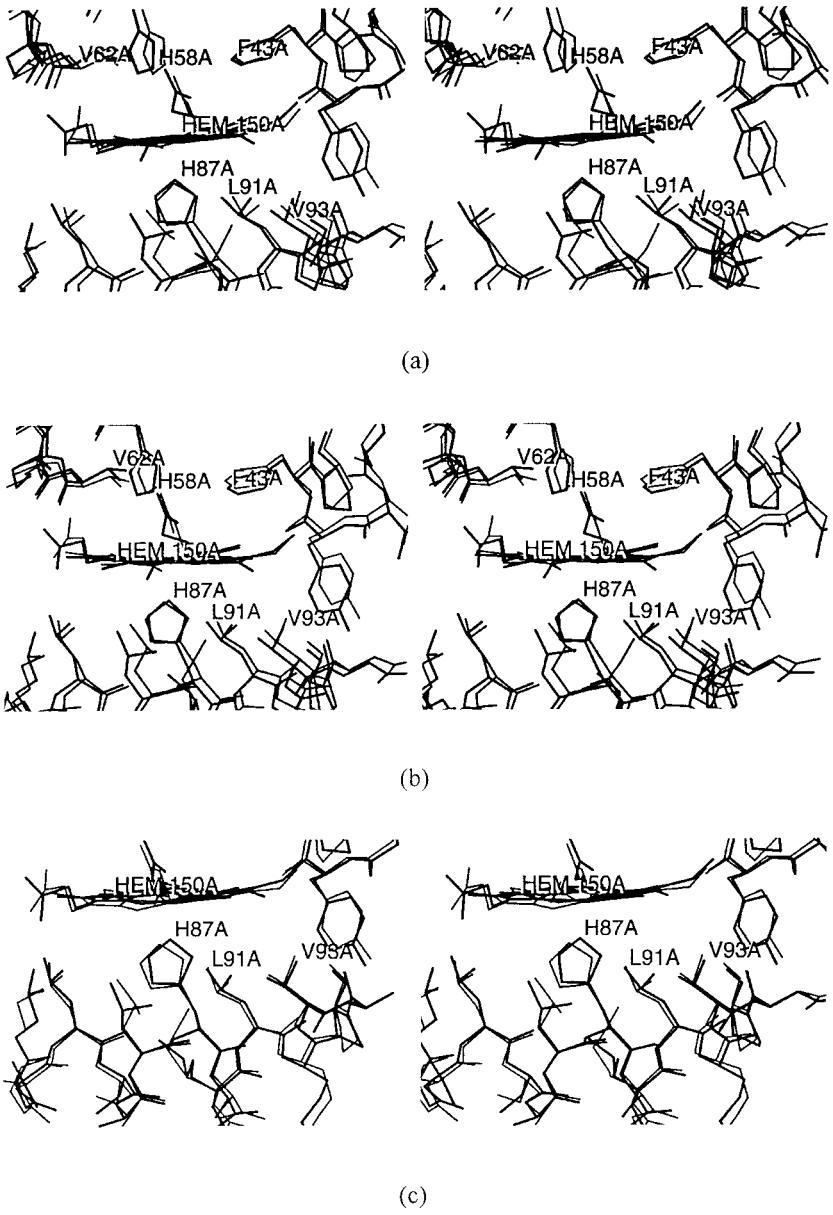


Figure 5. Overlap of the α heme pocket of the goose Hb in oxy (thin line) and deoxy (thick line) forms on (a) BGH frame, (b) heme frame, (c) F helix frame.

FG1β_1 shrinks from 45.7 Å to 41.3 Å during oxygenation of human deoxyHb, while these values are 42.2 Å and 41.5 Å in the goose Hbs, respectively. And, the amounts of contacts between globin and heme in the goose deoxyHb are less than in oxyHb (α, 73:86; β, 63:79), similar to that in human deoxy and oxy Hbs (α, 83:99; β, 72:100). It also shows that such contacts in the goose Hb are less than in human Hbs, indicating that the heme pocket in the goose Hb is relaxed compared to that in human Hb. Therefore there may be less steric hindrance in the ligand binding or releasing of heme in the goose Hb. It may be necessary for the Hb species adapted to high altitude hypoxic environment.

Subunit contacts

$\alpha_1\beta_1$ *interface*

It was concluded that the $\alpha_1\beta_1$ interface of Hb remains nearly unaltered in the transition between R and T states, and involves conservative residues in different species.[18] But it is indicated from the statistic results of human abnormal Hbs that the substitutions of residues in the $\alpha_1\beta_1$ interface are important to the oxygen affinity of Hbs.[19] Perutz suggested[8] that the substitution of Pro $\alpha_1$119 in greylag goose Hb by Ala in bar-headed goose Hb removes a hydrophobic contact between $\alpha_1$119 and $\beta_1$55, weakens the $\alpha_1\beta_1$ interface and hence results

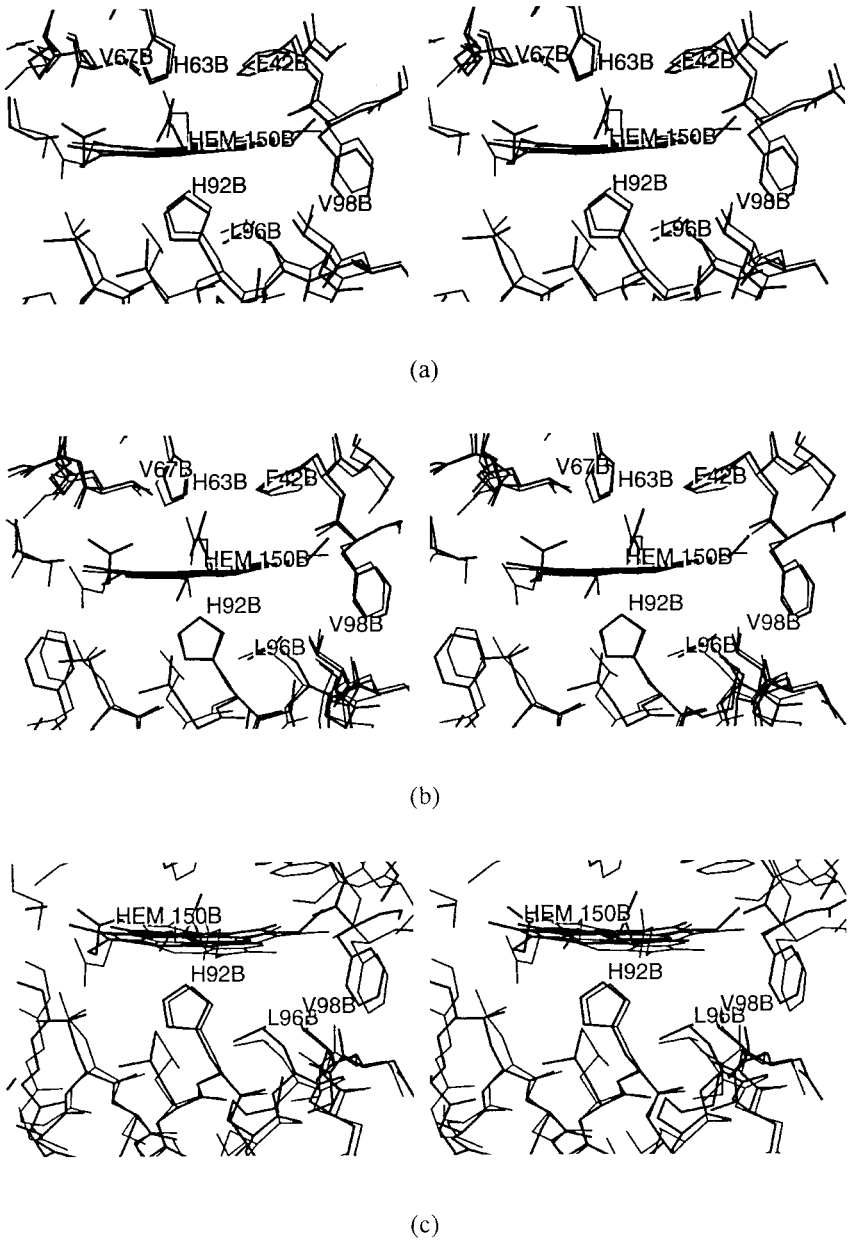


Figure 6. Overlap of the β heme pocket of the goose Hb in oxy (thin line) and deoxy (thick line) forms on (a) BGH frame, (b) heme frame, (c) F helix frame.

in the high oxygen affinity of bar-headed goose Hb. In the goose oxyHb, the minimum distance between $\alpha_1$119 and $\beta_1$55 residues is 4.56 Å, indicating that there are no contacts between them, but the contacts between $\alpha_1$119 and $\beta_1$30, $\beta_1$33 still exist as in human Hb, the minimum distances are 3.58 Å and 3.77 Å, respectively. In the model of the goose deoxyHb, the α119 does not interact with β55 as predicted, the minimum distance is 4.66 Å, and $\alpha_1$119 still interacts with $\beta_1$30 and $\beta_1$33 at distances of 3.16 Å and 3.92 Å, respectively (Figure 7). And there is an extra contact of 3.56 Å between α118 and β30 in bar-headed goose deoxyHb, which does not exist in the oxyHb, but the contact between $\alpha_1$120 and $\beta_1$51 in the oxyHb disappears in the deoxyHb. The immediate environment around α119 and β55 residues has not been changed and the gap between the two residues still exists in the $\alpha_1\beta_1$ interface of bar-headed goose deoxyHb. The number of $\alpha_1\beta_1$ contacts involving $\alpha_1$119 in the bar-headed goose Hbs is four to five fewer than in human Hbs, indicating a relaxation in this region of $\alpha_1\beta_1$ interface of the goose Hb.

Another mutation Glu $\beta_1$125 to Asp from greylag goose Hb to bar-headed goose Hb is also at the $\alpha_1\beta_1$ interface. There is a van der Waals contact between $\beta_1$125 and $\alpha_1$34 in bar-headed goose oxyHb, the minimum distance is 3.49 Å, while in

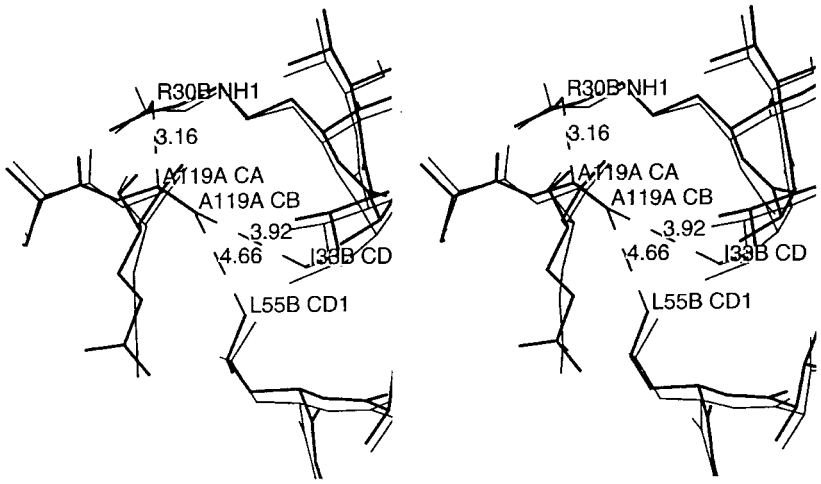


Figure 7. Overlapping the goose oxyHb (thin line) and deoxyHb (thick line) on α116-125. Ala α119 contacts with Arg β30 and Ile β33, but has no contacts with Leu β55 in the goose deoxyHb.

the deoxyHb, this value changes to 4.19 Å and $\beta_1$125 does not form any contact with the α_1 subunit. In human oxyHb, there are four contacts between $\beta_1$125 and $\alpha_1$34, thus the contact between α_1 and β_1 is tighter than that in the goose Hbs. It can be concluded from the descriptions above, that the mutations at α119 and β125 both have an effect on the relaxation of the $\alpha_1\beta_1$ interface, but the Ala at $\alpha_1$119 of the bar-headed goose Hb is more crucial, which is a unique substitution among avian and mammalian Hbs[20] and brings a gap in $\alpha_1\beta_1$ interface to both deoxyHb and oxyHb of bar-headed goose and hence leads to the high oxygen affinity of the goose Hb.

$\alpha_1\beta_2$ *interface*

$\alpha_1\beta_2$ interface is a principal region controlling the transition between the R and T states of Hb[21] in which the contact region of α_1 FG corner with β_2 C helix, named as flexible region, involves nearly the same residues and van der Waals contacts in the two quaternary states, while the contact region of α_1 C helix with β_2 FG corner has significantly different contact patterns in the two quaternary states and is named as switch region. During the transition from T state to R state in human Hb, the β_2 FG corner shifts about 6 Å relative to the α_1C helix, and the imidazole ring of His $\beta_2$97 spans the side-chain of Thr $\alpha_1$41, points to the central position between Thr $\alpha_1$38 and Pro $\alpha_1$41 in R state from the position between Gln $\alpha_1$41 and Thr $\alpha_1$44 in T state.[18] At the same time during the transition from oxy form to deoxy form in bar-headed goose, the shift of β_2 FG corner relative to α_1 C helix is only 0.9 Å and the His $\beta_2$97 does not span the side-chain of Thr $\alpha_1$41 (Figure 8). In the model of the goose oxyHb, Gln $\alpha_1$38 hydrogen bonds to Asp $\beta_2$99 *via* a water molecule,[12] the minimum distances of Gln $\alpha_1$38 with His $\beta_2$97 and Asp $\beta_2$99 are 4.03 Å and 4.82 Å, respectively. While in the deoxyHb, these distances are 3.96 Å and 5.21 Å, respectively, and the water-bridge between Gln $\alpha_1$38 and Asp $\beta_2$99 was not found. The Thr $\alpha_1$41 interacts with His $\beta_2$97 at a distance of 3.74 Å in the goose oxyHb, while the distance is 4.50 Å in the deoxyHb, so there is a little relaxation in this region of $\alpha_1\beta_2$ interface in the goose deoxyHb. The structural basis of switch region changes in bar-headed goose deoxyHb may be the sequential difference here among the goose Hb and human Hb. Instead of Thr in human Hb, the α38 of the goose Hb is Gln, which has a bigger side-chain and widens the space of this region between α_1 and β_2 subunits. The $\alpha_1$38 interacts with $\beta_2$97 in human oxyHb at the distance of 3.22 Å, while in the goose oxyHb the distance is 3.96 Å, which allows the side-chain of His $\beta_2$97 to move more freely and increases the flexibility here. The hydrogen bonds between $\alpha_1$94 and $\beta_2$102 in the oxy form and between $\alpha_1$42 and $\beta_2$99 in deoxy form of human Hb stabilize both mammalian quaternary structures.[18] The minimum distance of $\alpha_1$94 to $\beta_2$102 is 2.98 Å in the goose oxyHb, and the contact weakens in the deoxyHb, the distance is 3.25 Å. The $\alpha_1$42 and $\beta_2$99 do not contact in both the goose oxy and deoxy forms, the distances are about 8 Å. The contacts between Tyr $\alpha_1$140 and $\beta_2$36-37 of the goose deoxyHb are fewer than in oxyHb, which is consistent with the changes in human Hbs, but the interactions between Arg $\alpha_1$141 and $\beta_2$35-37 and between $\beta_2$145-146 and $\alpha_1$38-41 in human deoxyHb were not found in the goose deoxyHb.

IPP binding region and Bohr effect

IPP is the organic phosphate allosteric effector in avian red cells. The organic phosphate binds to the avian Hbs more tightly than to the mammalian Hbs, and avian Hbs can bind to IPP in both oxy

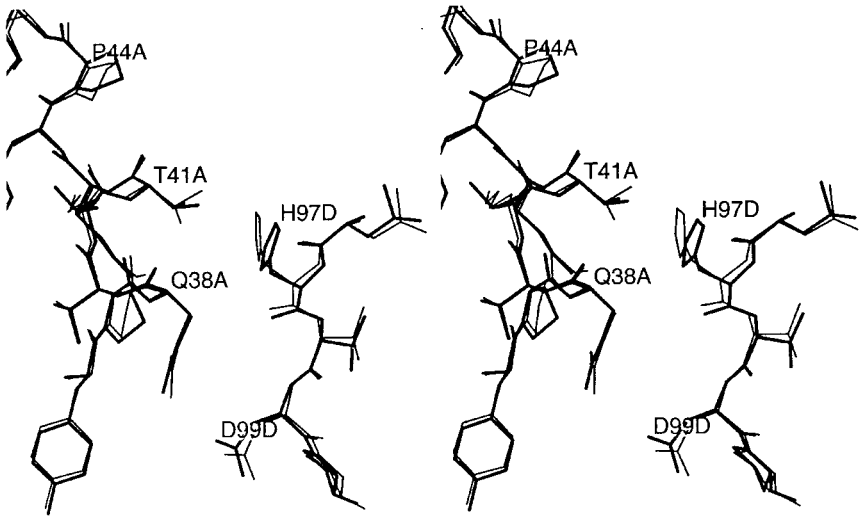


Figure 8. The small shift of (FG turn at switch region, the oxy (thin line) and deoxy (thick line) forms of the goose Hb are overlapped on α C helix.

and deoxy forms, with a higher affinity in deoxy form,[4] while the human Hb binds to its organic phosphate allosteric effector, 2,3-bisphosphoglycerate (DPG) much weaker than avian Hb.[22] There may be differences between the effects of IPP on avian Hb and that of DPG on human Hb. Although the electron density around β143-146 is not very clear in the model of bar-headed deoxyHb, we can identify the positively charged groove at the entrance to the central cavity between the two β chains, which should be the binding site of IPP. The groove is formed by Val β1, His β2, Lys β82, Arg β104, Arg β135, His β139, Arg β143, Lys β144, His β146 residues from two β chains. Among these residues, His β139, Arg β143 and His β146 are at sites suitable for IPP binding and may interact with IPP directly (Table 2, Figure 9), the other residues may provide a positively charged environment around IPP. The main structural differences of the goose Hb in two forms at this region are the positions of His β146 and Arg β143. The minimum distances are 7.85 Å between two Arg β143 and 8.83 Å between two His β146 in oxyHb, while they are enlarged to 10.54 Å and 11.75 Å in deoxyHb, respectively. The orientations of imidazoles in the two His β146 also change; they point to the groove in oxyHb, but turn outside in deoxyHb (Figure 9). It is indicated that the groove of oxyHb is narrower than that of deoxyHb, which may be the reason why it is disadvantageous for IPP binding to the oxyHb, while the groove stereochemistry of deoxyHb is more suitable for IPP binding. This may be the structural basis of higher IPP affinity of deoxyHb than oxyHb.

Table 2. The minimum distances (Å) between residues of IPP-binding site in bar-headed goose Hbs

	OxyHb	DeoxyHb
$\beta_1$1-$\beta_2$1	18.38	18.24
$\beta_1$2-$\beta_2$2	25.02	24.98
$\beta_1$82-$\beta_2$82	12.15	14.08
$\beta_1$104-$\beta_2$104	10.55	10.84
$\beta_1$135-$\beta_2$135	15.52	16.25
$\beta_1$139-$\beta_2$139	3.75	3.89
$\beta_1$143-$\beta_2$143	7.85	10.95
$\beta_1$144-$\beta_2$144	16.94	15.96
$\beta_1$146-$\beta_2$146	8.83	12.07

The His β146 of bar-headed goose deoxyHb is involved in the binding to IPP, and does not form a salt-bridge with Asp β94, so its role in the Bohr effect may change a lot compared to that in human Hb. In human Hb, the chloride-independent Bohr effect arises from a salt-bridge formed between His $\beta_1$146 and Asp $\beta_1$94 in the T state which is broken in the R state.[23–26] The Bohr effect of bar-headed goose and greylag goose Hbs are lower than human Hb, and the chloride-independent Bohr effects are very weak.[4] From the model of bar-headed goose deoxyHb, it can be seen that the reason may lie in the inexistence of a $\beta_1$146-$\beta_1$94 salt-bridge in the deoxy state; the minimum distance between the two residues is 7.74 Å, this may reflect the difference of Bohr effect between human and avian Hbs. Besides, from the statistic results of abnormal human Hbs, the mutations at the $\alpha_1\beta_1$ interface may cause instability, low cooperativity and low alkaline Bohr effect in Hbs.[19] But the genetic engineering of α119 Pro to Ala into human Hb has only little influence on the Bohr effect,[10] the influence of β125 on Bohr effect is still unknown.

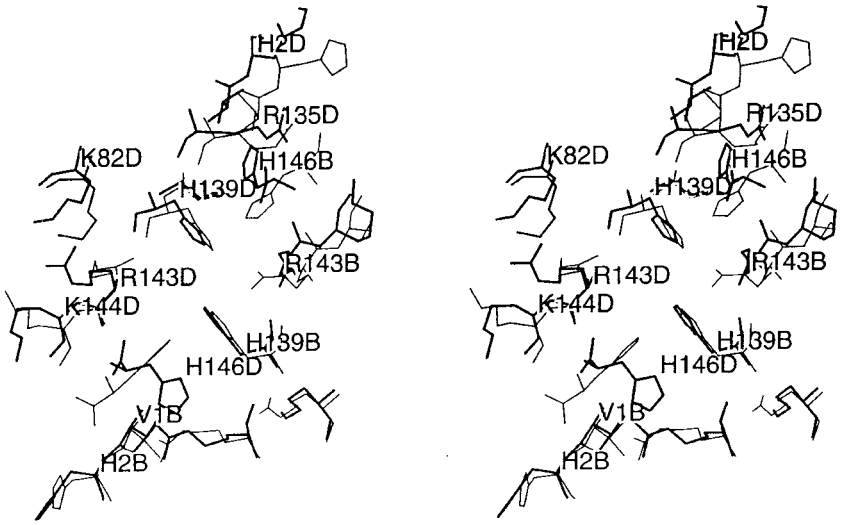


Figure 9. The IPP binding site of bar-headed goose oxyHb (thin line) and deoxyHb (thick line), overlapped on BGH frame.

Discussion

The structural characteristic of bar-headed goose deoxyHb and the allosteric mechanism of the goose Hb

The typical T state and R state models of Hb and the transition mechanism between them have been well known from a number of crystallographic studies of mammalian Hbs in different forms. The transition between R and T state can be described as a rotation and translation of the $\alpha_1\beta_1$ dimer relative to $\alpha_2\beta_2$ dimer accompanied by shifts at the $\alpha_1\beta_2$ and $\alpha_2\beta_1$ contacts.[18] Loss of ligand at the oxyHb results in movement of the heme and FG corners, then α_1C-β_2FG interface undergoes much larger changes, in particular His β97 switches to other side of the Thr α41. But the structure of bar-headed goose deoxyHb is distinguished from the typical human T state Hb and close to the structure of the goose oxyHb. The relative packing of the two αβ dimers in the goose deoxyHb tetramer differs from that in both typical T and R state human Hbs, the rotation angle of one αβ dimer relative to its partner between oxy and deoxy states of the goose Hb is only 4.6°, and the translation is only 0.3 Å. At the switch region in $\alpha_1\beta_2$ interface, the His $\beta_2$97 of the goose deoxyHb moves only about 0.9 Å relative to its position in oxyHb, which is much smaller than 7 Å in human Hb. And the imidazole ring of His $\beta_2$97 of the goose Hb does not span Thr $\alpha_1$41 as in the human Hb during transition from oxy to deoxy forms. We tried to find out the relation between the α119 mutation in the $\alpha_1\beta_1$ interface and the altered T-state quaternary structure, although we have not yet found any straightforward structural relations between them, but in the comparison of structures of oxyHbs of bar-headed goose and grelag goose, it is found that the quaternary structural differences between the two oxyHbs are due partially to the mutations at α18 and α63.[27] So far, only three avian Hb species in R state structure have been reported, the greylag goose oxyHb[27] and chicken oxyHbD[27] have the typical R-state structure of human Hb, while bar-headed goose oxy[12] and metHb[29] are in unique R state, indicating that the special R state quaternary structure of bar-headed goose Hb is not common to various avian species. Concerning the T-state deoxyHbs, there is no adequate experimental evidence to determine whether the T-state structure seen in the bar-headed goose is common to various avian species. But according to the unique R-state oxyHb of bar-headed goose, we propose that the special quaternary structure of the goose deoxyHb is not common to various avian species, either, and it may have some special effects on the high oxygen affinity of the goose Hb. Of course, to draw the conclusion, it is needed to compare the high-resolution structures of bar-headed and greylag goose Hbs in T states. There is an additional example, which might help us to the conclusion. The human hemoglobin deoxyHbβ_4 is also in a quaternary structure that very closely resembles the R state, and the T-to-R quaternary structure transition induces some changes in tertiary structure that has high ligand affinity.[30]

In the heme group region of the goose deoxyHb, the stereochemistry of the heme plane changes a little compared to the oxyHb, the displacement of the iron atoms from the plane of the heme are different from that in the human deoxyHb and other hemoglobin, myoglobin and synthetic iron porphyrins. So far, the iron atoms in the reported crystal structures of deoxyHb species are in ''high-spin'' state and locate out of the porphyrin plane. But our careful studies on the structure reported here show that it is an actual appearance of the deoxy state of the bar-headed goose Hb, which we

regard as a reasonable structure. Though the five-coordinated ferrous complexes are usually in high-spin state, there also exist states of intermediate spin and ''low-spin'' in five-coordinated iron porphyrins complex.[31] And the exact extent of the displacement of the iron atom from the heme plane depends not only on the spin states of the iron, but also on the ligation state of the iron atoms, protein structure around the heme and the crystal lattice force.[30,31] Furthermore the 2.8 Å resolution of the bar-headed goose deoxyHb is not high enough to study the details of the stereochemistry of the heme and the iron atoms, and the affecting factors that imposed on them. Although the protein structural differences in the heme pocket between the bar-headed goose oxy and deoxy Hbs are similar to that between huaman oxy and deoxyHbs, the F helix tilts and translates relative to the heme plane only to a smaller extent than that in human deoxyHb. These structural differences may affect the heme stereochemistry and the exact displacement of iron atoms relative to the heme plane.

All results reported here suggest that the structure of the goose deoxyHb is very close to that of the R state of the goose oxyHb, but the distinction between them is clear, and it is a special deoxy state of Hb. Do these characteristics of the model we report reflect the real structure of the goose deoxyHb? As the electron density maps of the goose deoxyHb confirm that all α and β subunits in the asymmetric unit are virtually free of ligand, the Hb structure reported here is in deoxy state undoubtedly. As we use polyethylene glycol (PEG) as precipitant in crystallization of bar-headed goose deoxyHb, and the crystals were obtained at physiological pH condition, this would favor the deoxy form of Hb and will prevent the oxidation of ferrous heme.[15,32] Furthermore, as reported previously, the ferric in the crystals of horse met bis(*N*-maleimidoethyl)ether (BME)-linked Hb could be reduced by $Na_2S_2O_4$ in nitrogen environment, the metHb content in the crystals is no more than 10 % measured by the spectroscopy after X-ray data collection,[33] so using $Na_2S_2O_4$ as reducing agent can also prevent the oxidation of ferrous heme and reduce the metHb to deoxyHb. $Na_2S_2O_4$ was added in preparation of the goose deoxyHb, and the typical deoxyHb spectrum was obtained, so the crystal used for data collection is the goose deoxyHb crystal. Meanwhile, the crystals we obtained were in space-group *P*1, there are two Hb tetramer molecules in one asymmetric unit, so the crystal lattice force would not affect the quaternary packing inside the tetramer. We also noted the structural reports of some allosteric intermediates of T or R state of Hb,[15,33–35] but the intermediates were stabilized either by crystal lattice force or by using cross-linking reagents. The crystals of the goose deoxyHb were obtained in normal condition, so there is no factor in the experiments that could cause intermediate state of the goose Hb. For the reasons above, the model we determined is the real structure of bar-headed goose deoxyHb but a special form of the deoxy state that we named it as T_B state.

Since the bar-headed goose Hb shows the adaptation for hypoxic environment and unusual quaternary structural changes between oxy and deoxy forms, we proposed that it takes a special allosteric mechanism slightly different from that of mammalian Hbs. The structural differences between the goose deoxyHb and oxyHb are small, so the ''journey'' of the transition between deoxy and oxy forms is shorter than that in human Hbs. During the transition from deoxy to oxy form, the Fe atom of α heme need not move from the proximal side to the heme plane as much as in human Hb. But the bond length of Fe to N^{ε} atom of proximal histidine (α87 F8) in the goose oxyHb becomes shorter to an extent of about 0.15 Å than that in the deoxyHb (Table 1), this may relieve the tension in deoxy state and cause the tilt and translation of F helix relative to heme plane, though the movement extent is smaller than that in human Hb. The shift of F helix will affect the conformation of FG corner with a movement of about 0.4 Å, and such a small change still can trigger the quaternary structural transition just as in human Hb, because the steric hindrance between oxy and deoxy state of the goose Hbs is relatively smaller than that in human Hb. There are no salt-bridges firming the deoxy structure, so the α C termini need not move as much as in human Hb, and the conformational transition in the switch region is easily achievable. In the β subunit, the steric hindrance of distal Val β67 (E11) to oxygen binding is weak compared to human Hb, so the oxygenation is also easier. Similar to α heme, the bond of Fe in β heme to proximal His β92 (F8) shortens about 0.2 Å, bringing conformational changes of β F helix and β FG corner as in the α subunit. The small tertiary and quaternary structural changes during transition between deoxyHb and oxyHb of bar-headed goose, mean that the energy constraints of the transition is much smaller than that in human Hb. And by inference, the transition may be much easier and faster than that in human Hb, which may be necessary for both oxygen loading and releasing of the Hb species with high oxygen affinity. Also by inference, this structural characteristic may be the reason for the marked difference of P_{50} value between bar-headed goose and greylag goose Hbs in the presence of IPP, but it cannot be proved from structural scrutiny alone.

The mechanism of the high oxygen affinity of bar-headed goose Hb

The sequential, functional and structural studies of bar-headed goose Hb are consistent with the neutral theory of protein evolution proposed by Kimura,[36] which indicates that the mutations of a small number of residues at key positions would dramatically alter the functions of the protein without affecting the basic bodyplan of the molecule. In the structures of both oxyHb and deoxyHb of the

bar-headed goose, there are no contacts between Ala $\alpha_1$119 and Leu $\beta_1$55 in the $\alpha_1\beta_1$ interface, while in the greylag goose oxyHb, the Pro $\alpha_1$119 does interact with Leu $\beta_1$55 at a distance of 3.79 Å,[27] indicating the substitution of Pro α119 by Ala in bar-headed goose Hb leaves the gap between α119 and β55 residue and relaxes the $\alpha_1\beta_1$ interface significantly. In the structure of human HbMβ55S mutant, there is no remarkable tertiary and quaternary structural alternation relative to human deoxyHbA except for the relaxed $\alpha_1\beta_1$ interface around immediate neighborhoods of the α119 substitution.[11] The exact effect of the α119 or β55 mutations on the oxygen affinity of Hb, is still an unsolved problem. Even in the deoxy structure of bar-headed goose Hb, we have not yet found a reasonable solution to this question. The final solution to the question may depend on the further researches of high-resolution structures of bar-headed goose and greylag goose deoxyHbs. Besides, the mutations at other regions may have some subtle effects on the function of bar-headed goose Hb. The replacement of Glu β125 to Asp is also sited on the $\alpha_1\beta_1$ interface, it may also bring relaxation of the $\alpha_1\beta_1$ interface to some extent in bar-headed goose Hb, but it is not the crucial factor for the high oxygen affinity. The effect on the quaternary structure of the α18 and α63 mutations may also affect the allosteric mechanism of bar-headed goose Hb.

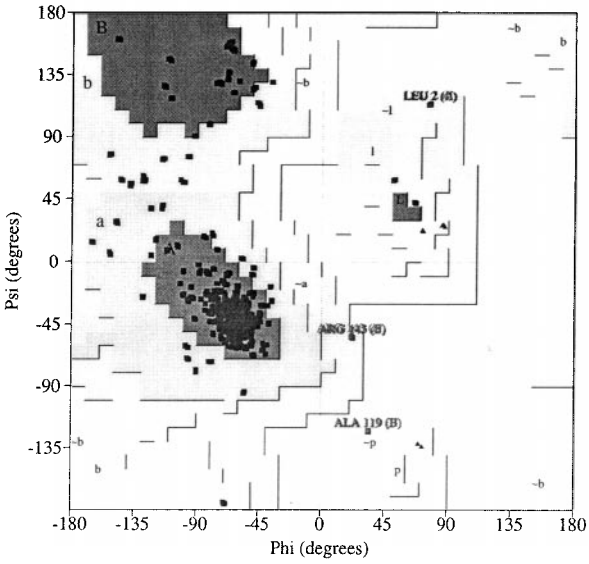


Figure 10. Ramachandran plot for bar-headed goose deoxyHb, drawn with *PROCHECK*, 85.3 % of total residues in most favored regions, 13.6 % in additional allowed regions and 1.2 % in generously allowed regions, no residues in disallowed regions.

Materials and Methods

Crystallization and data collection

The red cells from bar-headed goose were washed in 0.82 % (w/v) NaCl, then lysed with distilled water; cell membrane and other insoluble materials were removed by adding CCl_4 and spinning at 10,000 rpm for one hour. The Hb solution was then dialyzed to distilled water for 72 hours to remove saline, and concentrated to 100 mg/ml. Before crystallization, the Hb solutions were deoxygenated by adding excess $Na_2S_2O_4$ in a 50:1 molar ratio, the characteristic peak of deoxyHb at wavelength of 555 nm can be seen in the optical spectrum, indicating the complete deoxygenation of the Hb. All the crystallization works were under a nitrogen environment, the crystals were grown at 18 °C by hanging drop vapor diffusion method. The drop contains a protein solution of 18 mg/ml and 5 % (w/v) PEG-6000, and was against 30 % PEG-6000, 50 mM potassium phosphate buffer (pH 7.2). The crystals suitable for X-ray diffraction were obtained after ten days, with maximum dimensions of 0.8 mm× 0.8 mm × 0.4 mm. X-ray diffraction data were collected at a Simens X-200B image plate detector, a total of 57,884 observations with 30,654 independent reflections were collected. The crystal diffracts to 2.5 Å but the data were processed to 2.8 Å resolution to give a required completeness. The data were processed with XEN-GEN and gave an R_{merge} of 5.41 %. The crystal is in space-group *P*1 with unit cell dimensions of $a = 70.66$ Å, $b = 94.10$ Å, $c = 59.23$ Å, $\alpha = 71.55°$, $\beta = 65.10°$ and $\gamma = 83.10°$. An asymmetric unit is occupied by two Hb tetramer molecules with a V_m of 0.0026 nm^3/Dalton.

Structure determination

The initial model for refinement was obtained by molecular replacement method using the original model of bar-headed goose oxyHb dimer coordinate (PDB ID: 1a4f[12]). The Hb tetramer was obtained by a 2-fold symmetric operation on the original dimer. Molecular replacement calculations were carried out with *X-PLOR*,[37] using 15 to 4 Å resolution data. The orientation of the first Hb tetramer in the asymmetric unit was obtained by cross-rotation and translation calculations, the orientation of the second tetramer was solved by the self-rotation calculation from the first tetramer, and the relative positions of the two tetramers were solved by translation function calculation. The molecular replacement solution gave an *R* factor of 0.304 after rigid body refinement. The structural refinements were carried out with *CNS*,[38] all of the 27,148 reflections within the resolution range of 36.89 to 2.8 Å were used in refinement, among which 2752 (10 %) reflections were used as test set. The initial *B* factors of all atoms were set to 15 $Å^2$. The starting temperature in simulated annealing was 3500 K, and the cooling rate was 25 K per cycle. The refinement target is a maximum likelihood target. The anisotropic overall *B*-factor correction with a lower resolution limit of 6 Å and bulk solvent corrections were applied to the data. After each refinement cycle, the σ_A weighted $2mF_o - DF_c$ and $mF_o - DF_c$ maps were calculated, and the model was rebuilt manually using *TURBO FRODO*,[39] guided by the electron density maps. Individual *B*-factors were refined after the *R* factor nearly converged. The final *R* factor is 0.197, R_{free} is 0.243, and the average *B* factor is 25.84 $Å^2$ for main-chain atoms and 29.55 $Å^2$ for side-chain atoms. The stereochemistry quality of the final model is reasonable as checked by *PROCHECK*,[340] the Ramachandran plot of the model is shown in

Table 3. Refinement statistics of the model

Parameters	Value
Resolution range (Å)	36.89-2.8
Number of reflections used	27,148
Test set number	2752
R-factor[a]	0.197
R_{free}[b]	0.243
Non-hydrogen protein atoms	8944
Heme atoms	344
Mean *B*-factors (Å)	
All atoms in the model	27.75
Main-chain atoms	25.84
Side-chain atoms	29.55
Heme atoms	30.46
Deviation from ideality	
Bonds (Å)	0.014
Angles (deg.)	1.6
Dihedrals (deg.)	19.8
Improper dihedrals (deg.)	1.25

[a] $R\text{-factor} = \Sigma_h||F_o| - k|F_c||/\Sigma_h\ |F_o|$, where F_o and F_c are the observed and calculated structure factors.
[b] *R*-factor calculated for test set reflections that were always excluded in refinement.

Figure 10, and the statistics of the structural refinement are summarized in Table 3.

RCSB Protein Data Bank accession number

Atomic coordinates and structure factors for bar-headed goose deoxyHb have been deposited with the RCSB Protein Data Bank with the PDB ID of 1hv4.

Acknowledgments

This work was supported by the Chinese National Natural Science Foundation (grant no. 39670161).

References

1. Swan, L. A. (1970). Goose of the Himalayas. *Nat. Hist.* **79**, 68-75.
2. Black, C. P. & Tenney, S. M. (1980). Oxygen transport during progressive hypoxia in high altitude and sea-level waterfowl. *Respir. Physiol.* **39**, 217-239.
3. Petschow, D., Wurdinger, I., Baumann, R., Duhm, J., Braunitzer, G. & Bauer, C. (1977). Cause of high blood O_2 affinity of animals living at high altitude. *J. Appl. Phys.* **42**, 139-143.
4. Rollema, H. S. & Bauer, C. (1979). The interaction of inositol pentaphosphate with haemoglobins of highland and lowland geese. *J. Biol. Chem.* **254**, 12038-12043.
5. Oberthur, W., Braunitzer, G. & Wurdinger, I. (1982). Das hemoglobin der streifengans (*Anser indicus*) primarsruktur und physiologie der atmung, systematic und evolution. *Hoppe-Seyler's Z. Physiol. Chem.* **363**, 581-590.
6. Hiebl, I., Braunitzer, G. & Schneeganss, D. (1987). The primary structure of the major and minor haemoglobin-components of adult Andean goose (*Chloephaga melanoptera*, anatidae): the mutation Leu-Ser in position 55 of the β chains. *Biol. Chem. Hoppe-Seyler,* **368**, 1559-1569.
7. Hiebl, I., Weber, R. E., Schneegans, D. & Braunitzer, G. (1989). High-altitude respiration of falconiformes. The primary structures and functional properties of the major and minor hemoglobin components of the adult white-headed vulture (*Trigonoceps occipitalis, Aegipiinae*). *Biol. Chem. Hoppe-Seyler,* **370**, 699-706.
8. Perutz, M. F. (1983). Species adaptation in a protein molecule. *Mol. Biol. Evol.* **1**, 1-28.
9. Fermi, G. & Perutz, M. F. (1981). *Haemoglobin and Myoglobin, Atlas of Molecular Structure in Biology* (Phillips, D. C. & Richards, F. M., eds), Clarendon Press, Oxford.
10. Weber, R. E., Jessen, T. H., Malte, H. & Tame, J. (1993). Mutant haemoglobins (α^{119}-Ala and β^{55}-Ser): functions related to high-altitude respiration in geese. *J. Appl. Physiol.* **75**, 2646-2655.
11. Jessen, T. H., Weber, R. E., Fermi, G., Tame, J. & Braunitzer, G. (1991). Adaptation of bird haemoglobin to high altitudes: demonstration of molecular mechanism by protein engineering. *Proc. Natl Acad. Sci. USA,* **88**, 6519-6522.
12. Zhang, J., Hua, Z., Tame, J., Lu, G., Zhang, R. & Gu, X. (1996). The crystal structure of a high oxygen affinity species of haemoglobin (Bar-headed Goose haemoglobin in the oxy form). *J. Mol. Biol.* **255**, 484-493.
13. Smith, F. R., Lattman, E. & Carter, C. W., Jr (1991). The mutation β99 Asp-Tyr stabilizes Y - a new, composite quaternary state of human hemoglobin. *Proteins: Struct. Funct. Genet.* **10**, 81-91.
14. Smith, F. R. & Simmons, K. C. (1994). Cyanomet human hemoglobin crystallized under physiological conditions exhibits the Y quaternary structure. *Proteins: Struct. Funct. Genet.* **18**, 295-300.
15. Liddington, R., Derewenda, Z., Dodson, E., Hubbard, R. & Dodson, G. (1992). High resolution crystal structures and comparisons of T-state deoxyhaemoglobin and two liganded T-state haemoglobins: T (α-oxy) haemoglobin and T (met) haemoglobin. *J Mol. Biol.* **228**, 551-579.
16. Shaanan, B. (1983). Structure of human oxyhaemoglobin at 2.1 Å resolution. *J. Mol. Biol.* **171**, 31-59.
17. Matthews, A. J., Rohlfs, R. J., Olson, J. S., Tame, J., Renaud, J-P. & Nagai, K. (1989). The effect of E7 and E11 mutations on the kinetics of ligand binding to R state human haemoglobin. *J. Biol. Chem.* **264**, 16573-16583.
18. Baldwin, J. & Cothia, C. (1979). Haemoglobin: the structural changes related to ligand binding and its allosteric mechanism. *J. Mol. Biol.* **129**, 175-220.
19. International Hemoglobin Information Center (1985). Variants of the alpha chain and variants of the beta chain. *Hemoglobin,* **9**, 229-323.
20. Kleinschmidt, T. & Sgouros, J. G. (1987). Hemoglobin sequences. *Biol. Chem. Hoppe-Seyler,* **368**, 579-615.
21. Perutz, M. F. (1970). Stereochemistry of cooperative effects in haemoglobin. *Nature,* **228**, 726-734.
22. Bruin de, S. H., Rollema, H. S., Lambert, H. M., Van Os, J. & Van Os, G. A. J. (1974). The interaction of 2,3-diphosphoglycerate with human deoxy- and oxyhemoglobin. *Biochem. Biophys. Res. Commun.* **58**, 204-209.
23. Perutz, M. F. (1970). The Bohr effect and combination with organic phosphates. *Nature,* **228**, 734-739.
24. Kilmartin, J. V., Fogg, J. H. & Perutz, M. F. (1980). Role of the C-terminal histidine in the alkaline Bohr effect of human hemoglobin. *Biochemistry,* **19**, 3189-3193.

25. Shih, D. T. & Perutz, M. F. (1987). Influence of Anions and protons on the Adair coefficients of haemoglobin A and Cowtown (HisHC3(146)β → Leu). *J. Mol. Biol.* **195**, 419-422.
26. Shih, D. T., Luisi, B. F., Miyazaki, G. & Perutz, M. F. (1993). A mutagenic study of the allosteric linkage of HisHC3(146)β in haemoglobin. *J. Mol. Biol.* **230**, 1291-1296.
27. Liang, Y. H., Liu, X. Z., Liu, S. H. & Lu, G. Y. (2001). The structure of greylag goose oxy haemoglobin C the roles of four mutations compared with bar-headed goose haemoglobin. *Acta Cryst. Sect. D,* Accepted.
28. Knapp, J. E., Oliveira, M. A., Xie, Q., Erust, S. R., Riggs, A. F. & Hacker, M. L. (1999). The structural and functional analysis of the hemoglobin D component from chicken. *J. Biol. Chem.* **274**, 6411-6420.
29. Liu, X. Z., Li, S. L., Jing, H., Liang, Y. H., Hua, Z. Q. & Lu, G. Y. (2001). Avian hemoglobins and structural basis of high affinity to oxygen: crystal structure of bar-headed goose aquomet hemoglobin. *Acta Crystallog. sect. D,* **57**, 775-783.
30. Borgstahl, G. E. O., Rogers, P. H. & Arnone, A. (1994). The 1.9 Å structure of deoxyβ_4 hemoglobin (analysis of the partitioning of quaternary-associated and ligand-induced changes in tertiary structure). *J. Mol. Biol.* **236**, 831-843.
31. Perutz, M. F. (1979). Regulation of oxygen affinity of hemoglobin: influence of structure of the globin on the heme iron. *Annu. Rev. Biochem.* **48**, 327-386.
32. Ward, K. B., Wishner, B. C., Lattman, E. E. & Love, W. E. (1975). Structure of deoxyhemoglobin A crystals grown from polyethylene glycol solutions. *J. Mol. Biol.* **98**, 161-177.
33. Wilson, J., Phillips, K. & Luisi, B. (1996). The crystal structure of horse deoxyhaemoglobin trapped in the high-affinity (R) state. *J. Mol. Biol.* **264**, 743-756.
34. Paoli, M., Liddington, R., Tame, J., Wilkinson, A. & Dodson, G. (1996). Crystal structure of T state haemoglobin with oxygen bound at all four haems. *J. Mol. Biol.* **256**, 775-792.
35. Fernandez, E. J., Abad-Zapatero, C. & Olsen, K. W. (2000). Crystal structure of Lys$\beta_1$82-Lys$\beta_2$82 cross-linked hemoglobin: a possible allosteric intermediate. *J. Mol. Biol.* **296**, 1245-1256.
36. Kimura, M. (1979). The neutral theory of molecular evolution. *Sci. Am.* **241**, 94-104.
37. Brünger, A. T. (1992). *X-PLOR Version 3.1, A System for X-ray Crystallography and NMR*, Yele University Press, Newheven and London.
38. Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W. *et al*. (1998). Crystallography & NMR system: a new software suite for macromolecular structure determination. *Acta Crystallog. sect. D,* **54**, 905-921.
39. Cambillau, C., Roussel, A., Inisan, A. G. *et al*. (1996). *TURBO-FRODO Version 5.5 User Manual*, University Aix-Marseille II, France.
40. Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. (1993). PROCHECK: a program to check the stereochemical quality of protein structures. *J. Appl. Crystallog.* **26**, 283-291.

Edited by A. Klug

(Received 20 February 2001; received in revised form 20 August 2001; accepted 24 August 2001)