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The Crystal Structure of a High Oxygen Affinity Species of Haemoglobin (Bar-headed Goose Haemoglobin in the Oxy Form)

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We have determined the crystal structure of bar-headed goose haemoglobin in the oxy form to a resolution of 2.0 Å. The *R*-factor of the model is 19.8%. The structure is similar to human HbA, but contacts between the subunits show slightly altered packing of the tetramer. Bar-headed goose blood shows a greatly elevated oxygen affinity compared to closely related species of geese. This is apparently due to a single proline to alanine mutation at the $\alpha_1\beta_1$ interface which destabilises the T state of the protein. The β chain N and C termini are well-localized, and together with other neighbouring basic groups they form a strongly positively charged groove at the entrance to the central cavity around the molecular dyad. The well-ordered conformation and the three-dimensional distribution of positive charges clearly indicate this area to be the inositol pentaphosphate binding site of bird haemoglobins.

Keywords: crystallography; haemoglobin; high-altitude adaptation

Introduction

Bar-headed geese from Central Asia migrate annually across the Himalayas at altitudes of about 9000 m and can fly far higher than closely related lowland species such as the greylag goose (Swan, 1970). This toleration of hypoxic conditions is due to a substantial increase in the oxygen affinity of the blood, allowing the bird to extract sufficient oxygen for flight from the very thin atmosphere five miles above sea-level (Petschow *et al*., 1977). While stripped Hb from the bar-headed goose shows only a slightly higher oxygen affinity than greylag goose Hb, this difference is greatly increased in the presence of inositol pentaphosphate. Birds use inositol pentaphosphate (IPP) as an allosteric effector in the red cell instead of DPG (2,3 diphosphoglycerate) which is used by human Hb. In 100 mM chloride, bar-headed and greylag geese Hbs have p50 values of 2.0 and 2.8 Torr, respectively (at pH 7.2 and 25°C), but these increase to 20.4 and 30.9 Torr in the presence of IPP. Since in the deoxy state both bar-headed and greylag goose Hbs have similar affinities for IPP, which is present at the same level in the red blood cells of both species, the functional change must be the result of an inherent difference in the Hb molecule (Rollema & Bauer, 1979).

There are only four amino acid differences between the major Hb types of these two species, only one of which appears likely to affect oxygen affinity (Oberthuer *et al*., 1982; Hiebl *et al*., 1989; Perutz, 1983). This mutation, α119 Pro to Ala, has been introduced into human Hb by genetic engineering and shown to cause an increase in oxygen affinity close to that found between blood from the two species of goose (Jessen *et al*., 1991; Weber *et al*., 1993). The loss of the proline side-chain removes a small hydrophobic contact between the α_1 and β_1 subunits in the T state, destabilizing the low oxygen affinity form of the protein relative to the high affinity R state. We have determined the structure of bar-headed goose Hb to discover whether additional changes may affect the control of oxygen binding. This is the first structure of any bird haemoglobin to be reported. All bird Hbs show certain distinguishing patterns of amino acid sequence and it is therefore of interest whether these lead to altered structures. In particular, this structure allows us to examine the differences

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Abbreviations used: Hb, haemoglobin; IPP, inositol pentaphosphate; DPG, 2,3 diphosphoglycerate.

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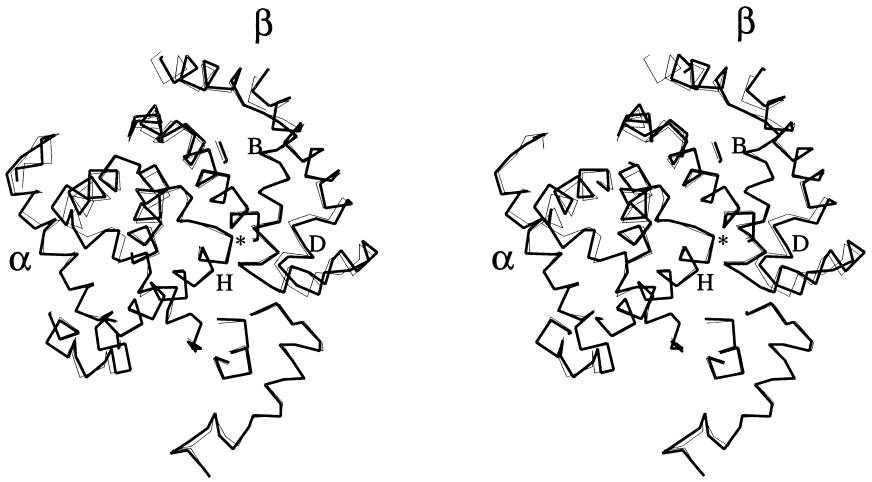

Figure 1. Overlap of the C^{α} traces of oxy bar-headed goose Hb (thick line) and oxy human Hb (thin line) at the $\alpha_1\beta_1$ interface. The structures were overlapped on a subset of residues as described in the text. α119 (H2) is marked with an asterisk. It can be seen that the N terminus of the H helix of the α_1 subunit lies close to the B and D helices of the β_1 subunit. The replacement of proline α119 with alanine has no effect on the C^{α} trace of the protein, but removes a van der Waals' contact with β55.

around the DPG binding site of human Hb and the IPP binding site of bird Hbs. A detailed analysis of the allosteric mechanism and the IPP binding site are not possible however without the deoxy T state structure.

Results

Overall structure

The tertiary and quaternary structures of bar-headed goose oxy Hb are similar to human Hb, having seven helices in the α chain and eight in the β chain. The main differences in structure occur at the subunit termini and surface residues. Superimposing the haem groups of the bird Hb on R state human Hb (excluding the haem side-groups), it can be seen that the A helix and AB and GH corners of the α chain and the A and F helices, and AB, EF and GH corners of the β chain are shifted. The β chain shows larger differences than the α chain, particularly in the A helix. Baldwin & Chothia (1979) identified the $\alpha_1\beta_1$ contact interface as the most suitable reference region for the comparison of different forms of human Hb since it remains relatively unaltered between the T and R states of the protein, but structural differences between goose and human Hb make this region less suitable for overlapping the coordinates. We have therefore chosen a slightly different set of residues to fit human and bar-headed goose Hbs. Residues were chosen whose main-chain conformation and position are very similar in both goose and different forms of human Hb. These residues are α 3 to 13, 31 to 42, 54 to 73, 80 to 84, 97 to 104, 117 to 136 and β 26 to 37, 62 to 68, 106 to 114 and 127 to 139, a total of 117 amino acid residues (76 in the α chain, 41 in the β chain) and 468 atoms. These account for 40.8% of the main-chain atoms in the αβ dimer. Using this frame of reference, the rms deviations of main-chain atoms between the goose haemoglobin and human haemoglobin in the oxy, carbonmonoxy and deoxy forms are 0.450, 0.434 and 0.436 Å, respectively. Figure 1 shows the C^{α} traces of the goose Hb and human Hb, least-squares fitted on the C^{α} atoms of the residues given above.

The main structural difference between the T and R states of human Hb is a rotation of about 15° of one αβ dimer relative to the other. Compared to deoxy human Hb, the αβ dimers of goose oxy-Hb are rotated slightly further (about 4° more) than those of human R state Hb, and translated by 1.2 Å. Generally the $\alpha_1\beta_1$ contact region of bar-headed goose Hb involves the same residues as human Hb, the B, G and H helices of the α subunit and the B, D, G and H helices and GH loop of the β subunit. The major change is the GH loop of the β chain which is displaced relative to the other part of the interface. In the crystal form of the protein described here this loop forms a crystal contact with a neighbouring molecule.

Haem group region

The haem pockets of bar-headed goose Hb are very similar to those of human Hb; these regions of the molecule are particularly well-conserved and the equivalent residues overlap closely. Figure 3 shows the haem pockets of bar-headed and human Hbs least-squares fitted on our reference frame of 117 residues. There are no mutations in the haem vicinity which would be expected to alter ligand affinity either directly or through the haem. Compared with human Hb, the α subunit proximal histidine residues of bar-headed goose Hb adopt slightly more symmetrical positions on the haem axis. This would relieve steric hindrance between the haem and the imidazole CD2 atom as the

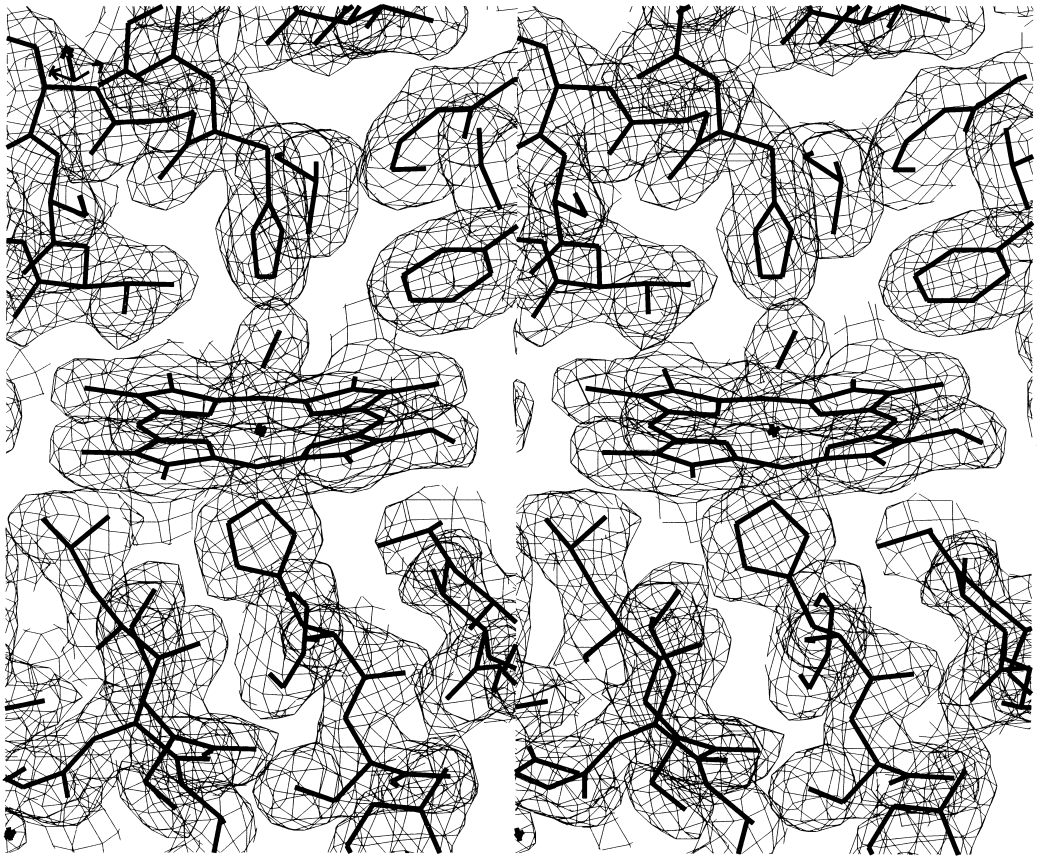

Figure 2. Electron density around the haem pocket of the β subunit. The electron density map is displayed at a level of 1σ. The oxygen ligand can be seen clearly in the density over the haem iron.

side-chain approaches the haem plane on ligation (Gelin & Karplus, 1977). However, the F helix is perfectly conserved between bar-headed and greylag geese, so this slightly altered geometry appears to have no functional significance. (The only residue mutated between human Hb and the geese Hbs in this helix is the surface residue Ala82(F3), which is a Lys in the bird proteins.) The distal histidine residues have very similar positions and environments to those in human Hb. In the model of human oxy Hb a hydrogen bond forms between the oxygen ligand and the distal histidine in the α subunits, but not in the β subunits where the ligand appears to have much greater rotational freedom and higher temperature factors (Shaanan, 1983). A later refinement of this structure carried out paying particular attention to thermal parameters and geometric constraints using reciprocal space refinement led to a model in which the ligand in the β subunits does seem to hydrogen bond to the distal histidine (Emsley, 1991). A clear functional difference between the distal histidine residues of the two subunits has also been observed in studies of engineered Hb mutants (Olson *et al*., 1988). In human Hb it was found that replacing the distal histidine with glycine lowered the oxygen affinity significantly in the α subunit, but not the β subunit. In bar-headed goose Hb however a good hydrogen bond appears to form in both subunit types. Both ligand atoms have similar temperature factors and are well represented in the electron density map, indicating that the oxygen molecule is well-ordered (Figure 2). An omit map was calculated after five cycles of PROLSQ refinement on the final model with the second oxygen atoms (OL2) removal to check the level of oxidation. A substantial peak was found in this difference map over the ligand at the β haem, but a smaller peak at the α haem. We estimated that no more than 10% of the β haems are oxidised, but 20 to 30% of the α haems appear to be in the met form. α Globin is known to oxidise faster than β globin, so the higher proportion of met is expected (Mansouri & Winterhalter, 1993; Demma & Salhany, 1979). This is also consistent with the slightly higher temperature factor of the OL2 atom in the α subunits (Table 1). The OL2 atom has greater rotational and vibrational freedom than OL1, however, so its higher temperature factor is not solely due to met formation in the crystal. Despite the structural conservation of the haem pockets, the ligand is less bent in the α subunit and less tilted in the β subunit of goose Hb than in human Hb. The reason for this apparent change is unclear since the distal residues in contact with the ligand (His E7 and Val E11) adopt very similar positions in both proteins. Given the similarity of

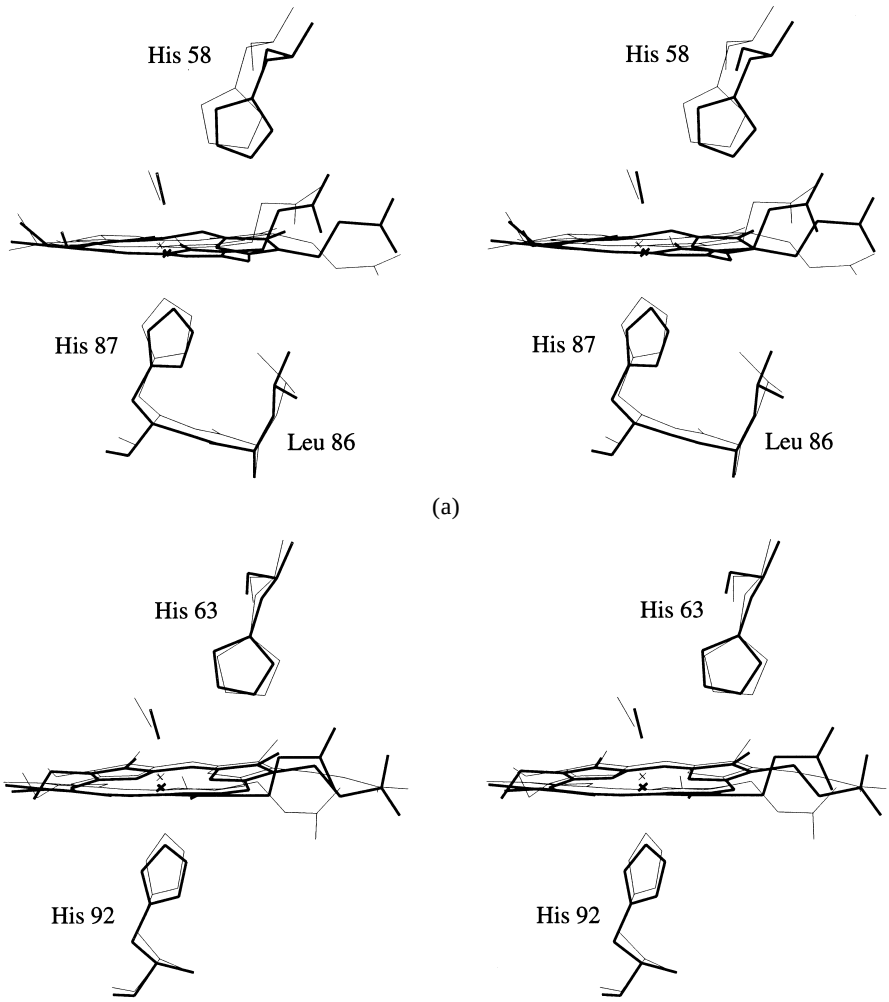

Figure 3. Comparison of the ligand geometry in oxy bar-headed goose Hb (thick line) and oxy human Hb (thin line) in the α (a) and β (b) subunits. In the α subunit the distal histidine is slightly rotated between the two structures, but the oxygen ligand adopts suitable geometry for hydrogen bonding in both. The distal histidine and haem group overlap closely in the β subunit. A hydrogen bond appears to form between the distal histidine and the ligand in the bar-headed goose Hb, but not in the model of oxy human Hb (Shaanan, 1983). The position of the β subunit oxygen ligand in human Hb is somewhat altered in a later refinement which indicates that this bond does form in the human protein as well (Emsley, 1991).

haem pocket sequence and structure between bar-headed goose and human Hb, and the lack of any mutations between bar-headed and greylag goose Hb around the haems, it is unlikely that any change in ligand binding is brought about by altered residues influencing the ligand directly. The differences seen in the geometry of the oxygen ligand of bar-headed goose Hb and human Hb are probably artifacts caused by the different refinement procedures. The high temperature factors of the ligand in the human oxy Hb structure also indicate some level of oxidation.

Table 1. Ligand geometry in oxy bar-headed goose and human Hb

	Goose		Human	
	α	β	α	β
Temperature factor Fe	23.51	19.83	27.37	36.73
Temperature factor O1	18.09	18.79	73.19	51.86
Temperature factor O2	43.77	28.52	60.72	48.20
Fe-O-O angle (°)	170.2	158.7	152.7	158.7
Fe-O distance (Å)	1.78	1.81	1.66	1.86
His F8 N^{ϵ}-Fe distance (Å)	2.10	2.07	1.94	2.07
His E7 N^{ϵ}-O2 distance (Å)	2.53	2.57	2.68	3.49
Val E11 $C^{\gamma 2}$-O2 distance (Å)	3.22	3.25	3.25	3.36

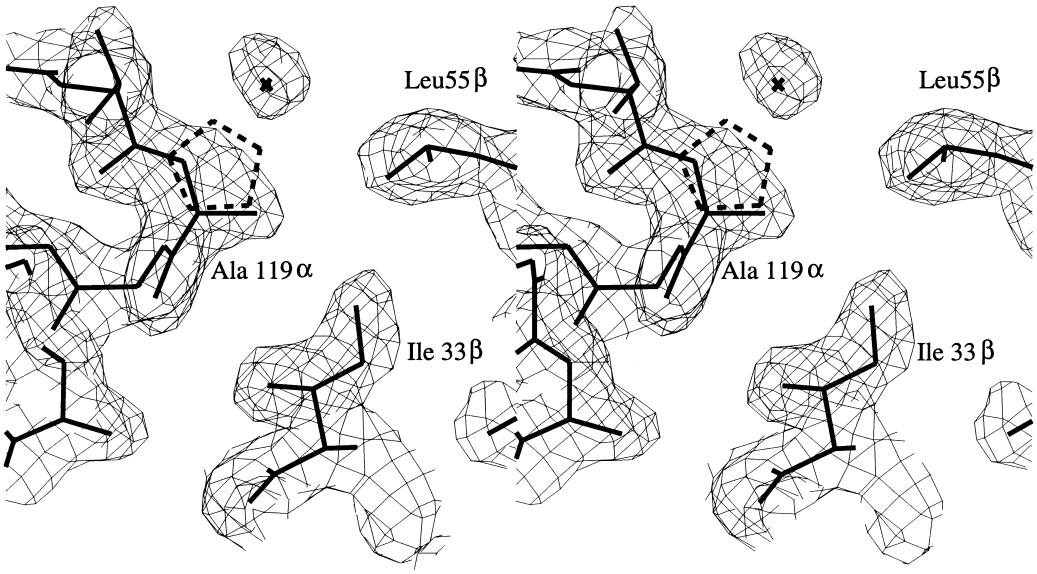

Figure 4. Electron density over Ala α119 and Leu β55, contoured at 1σ. The position of the ring of proline α119 of human Hb superimposed on the goose structure is shown in broken lines.

Subunit contacts

The $\alpha_1\beta_1$ contact region

Of the several amino acid replacements at the $\alpha_1\beta_1$ interface compared to human Hb, only one appears to be of any functional significance, alanine α119 (H2). The substitution of proline 119 is unique amongst birds and mammals (Kleinschmidt & Sgouros, 1987). Figure 4 shows the electron density map over this residue. This region of the map is representative of the whole structure, and all residues are visible in the electron density. Alanine α119 closely mimics the proline found in the human protein, which lies close to methionine β55. The small side-chain in bar-headed goose Hb is however unable to interact with β55 (which is leucine in the goose Hb, in common with other bird Hb sequences) and the loss of this interaction weakens the $\alpha_1\beta_1$ contact. In human Hb, Pro α119 appears to interact as strongly with Arg β30 as it does with Met β55. This arginine side-chain hydrogen bonds to the carbonyl oxygen of Phe α117 (which is leucine in the goose proteins) and lies approximately 4.0 Å from the proline ring. Weber *et al*. (1993) have found however that replacing Pro α119 or Met β55 in human Hb with smaller side-chains gives identical increases in oxygen affinity so the weakening of the T state must come about solely from the loss of the proline-methionine interaction.

The $\alpha_1\beta_1$ interface

The early crystallographic studies of Perutz and co-workers identified the $\alpha_1\beta_2$ interface as the principal switch region controlling the transition between the high and low affinity forms of haemoglobin (Perutz, 1970a). This conclusion has been confirmed by a wide range of studies on mutant proteins and crystallographic analysis of intermediate ligation states (Perutz *et al*., 1987). Amino acid changes in this region of the molecule are therefore prime candidates for detailed scrutiny when analysing a haemoglobin with a profoundly altered oxygen affinity. However, there are no mutations between bar-headed and greylag goose haemoglobin at the $\alpha_1\beta_2$ interface which appear to alter the oxygen affinity significantly. The only amino acid substitutions between the human and goose proteins are α38 Thr (human) to Gln (goose) and α100 Leu (human) to Phe (goose). These differences between human and bar-headed haemoglobins probably reflect differences between mammalian and avian haemoglobins generally (since they are also found in other bird Hb sequences), and are not adaptive to a specific environment. In bar-headed goose oxy Hb, Gln $\alpha_1$38 has no direct contact with β chain residues, but hydrogen bonds to $\beta_2$99 Asp carboxyl group *via* a water molecule. Interestingly, Thr α38 is implicated in the high oxygen affinity of birds of the falconiform family, but apparently by a different mechanism to that of bar-headed goose Hb (Hiebl *et al*., 1989). The contacts between the α and β subunits closely resemble those of human Hb in the R state, and no other altered contacts are apparent which might affect the allosteric equilibrium.

Bohr effect

The alkaline Bohr effect, the lowering of the oxygen affinity of haemoglobin with pH, is a common feature among vertebrate haemoglobins which helps unload oxygen in respiring tissue. The effect can be divided into two components, an intrinsic or chloride-independent part and a chloride-dependent part. In human Hb the chloride-independent Bohr effect arises from a salt-bridge

formed between Asp β94 and His β146 in the T state which is broken in the R state (Perutz, 1970b; Shih *et al*., 1993). This accounts for 40 to 50% of the Bohr effect under physiological conditions. The chloride-dependent effect is due to groups such as the N termini of the α subunits whose p*K* is raised by the binding of chloride ions to the T state. Engineering the α119 proline to alanine mutation into human Hb has little influence on the Bohr effect (Weber *et al*., 1993). The slight changes observed for the mutant human Hb (Ala α119) are almost certainly mediated by the alteration of the allosteric equilibrium. The Bohr effects of bar-headed and greylag geese Hbs are the same, but lower than human Hb. In the presence of 100 mM chloride but the absence of IPP the Bohr effect of both goose Hbs reaches a maximum at pH 7.2. The bird Hbs release 1.2 protons on oxygenation, which is about 40% less than human Hb (Rollema & Bauer, 1979). At low chloride concentration (1 mM), the Bohr effect of the geese Hbs drops to about 15% of that found for human Hb, indicating that the chloride-independent effect is very weak. In human Hb, transition to the R state squeezes Tyr β145 out of the pocket it occupies in the T state, so the C-terminal residues of the β subunit have much more conformational freedom in oxy Hb and the terminal histidine adopts it natural p*K*. A similar situation is found in the goose oxy Hb structure, but it is interesting to note that His β146 of bar-headed goose Hb is found close to His β139 and has a relatively low temperature factor. The importance of this interaction on the Bohr effect is unclear as the p*K* of the imidazole ring may be lowered by the proximity of His β139 (which is Asn in human Hb). The chloride-independent Bohr effect will be weakened if the p*K* of His β146 is lowered more in the T state than the R state since it arises from a higher p*K* of this group in the T state.

A recent study on Hb from the emperor penguin *Aptenodytes forsteri* shows that it too has a very weak chloride-independent Bohr effect (Tamburrini *et al*., 1994). The authors suggest that the salt-bridge between His β146 and Asp β94 either does not exist in the T state or does not break in the R state. From the structure described in this paper however it is impossible to determine whether His β146 bonds to Asp β94 in the T state as in human Hb. Given the similarity between the Hbs it seems likely that His β146 contributes as little to the Bohr effect in geese Hbs as it does in the penguin protein. A weak and almost completely chloride-dependent Bohr may be a general feature of avian haemoglobins rather than an adaptation to prolonged diving or the habit of penguins to live on fat reserves for long periods as Tamburrini *et al*. have suggested. Almost all bird Hb sequences known do not have a histidine at position α89. This histidine residue has been mutated to glutamine in human Hb and the Bohr effect of the engineered protein was found to be reduced by about 25% in 100 mM chloride (Imai *et al*., 1989).

IPP binding

Vertebrate haemoglobins bind a variety of allosteric effectors in the T state to reduce their oxygen affinity inside the red cell. Human haemoglobin binds to DPG and fish haemoglobins use ATP and GTP. All of these effector molecules bind to the same site on the haemoglobin, at the interface between the two β subunits (Arnone, 1972; Perutz, 1983). The IPP binding site of bird haemoglobins is unaltered between bar-headed and greylag geese and cannot therefore be involved in adaptation to high altitudes. The level of IPP is the same in red cells from bar-headed and greylag geese, and the two bird haemoglobins both show the same affinity for IPP (Rollema & Bauer, 1979). A very similar pattern of amino acid changes is found around the site in all sequenced bird haemoglobins, so it appears that the mode and strength of IPP binding is common to all these proteins. DPG and IPP are asymmetric molecules which bind to haemoglobin on the 2-fold axis of the protein. By growing crystals of DPG-bound human haemoglobin under low salt conditions in which the molecular symmetry is broken by crystal contacts (the β subunits are found in slightly different environments) Richard *et al*. (1993) managed to determine precisely the interactions formed between the protein and DPG. The precise mode of binding of IPP can only be found however from the crystal structure of the bird Hb in the deoxy state with IPP bound. Val β1, His β2, Lys β82, Arg β104, Arg β143, Arg β135, His β139, Lys β144 and His β146 form a pocket lined with positive charge between the β chains. Four His residues (His β139 and His β146) and two Arg residues (Arg β135) form the bottom of the pocket and the Arg β143 form the wall (Figure 6). This positively charged region is undoubtedly the IPP binding site, although some of the charged groups around the site may not interact with IPP directly, but simply serve to provide positive charge. Both bar-headed and greylag goose Hbs bind IPP very tightly. At pH 7.2 and 25°C the association constants are 3×10^5 M and 4×10^9 M for the oxy and deoxy proteins, respectively (Rollema & Bauer, 1979). Under similar conditions the IPP binding constants of human oxy and deoxy Hb are 2.5×10^3 M and 1×10^6 M. The R state bird Hbs therefore bind IPP only about three times less tightly than T state human Hb.

Discussion

The model of bar-headed goose Hb shows that the molecule adopts a structure very similar to that of human Hb. This is to be expected since the protein sequences are 69% identical, and fish Hbs which have many more amino acid differences also adopt very similar folds (Camardella *et al*., 1992; J. Tame, unpublished results). Perutz has previously shown that the altered properties of a number of animal Hbs can be ascribed to one

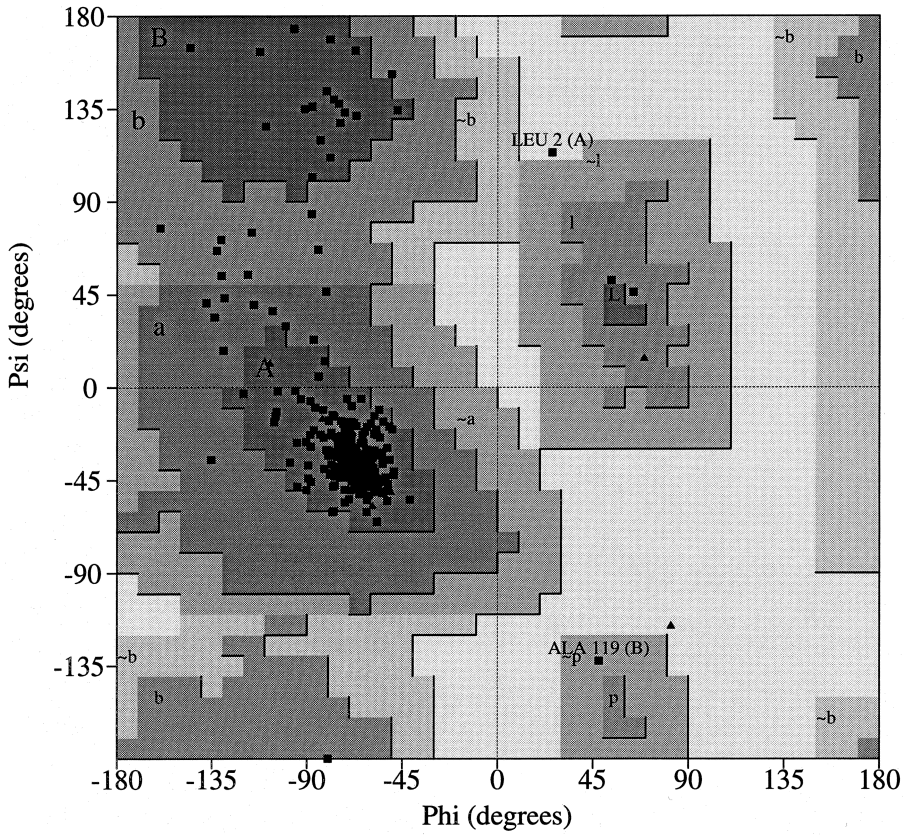

Figure 5. Ramachandran plot for bar-headed goose Hb, drawn with PROCHECK (Laskowski *et al*., 1993). Glycine residues are shown as triangles. Two residues have unusual $\phi\psi$ angles, Ala β119 and Leu α2. The N-terminal region of the α chain could not be readily fitted into the density and the temperature factors of atoms in the first five residues are high, indicating some disorder. In contrast, Ala β119 is well ordered and the C^{β} and carbonyl oxygen atoms can be clearly observed in the electron density map. This residue adopts a similar conformation to the glycine found in most vertebrate haemoglobins at this position at the GH corner.

or only a few mutations (Perutz, 1983). Their effects may be dramatic or subtle, but the basic bodyplan of the molecule seems unaffected. Several properties of animal Hb have been transferred to human Hb by mutagenesis, including altered Bohr effects and carbon dioxide binding (Luisi & Nagai, 1986; Komiyama *et al*., 1995). These generally involve a small number of residues, consistent with the

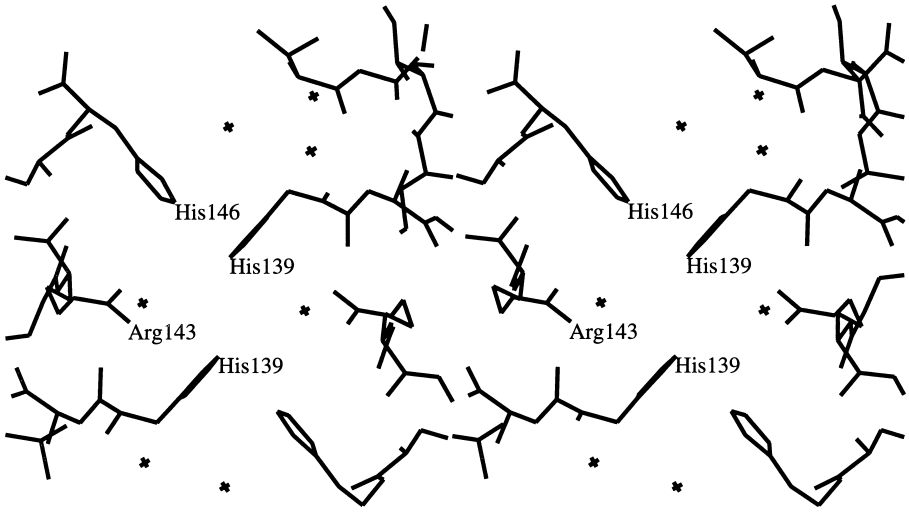

Figure 6. The IPP binding site of bar-headed goose Hb. A total of eight positively charged residues (four from each β subunit) line the site, which is found at the equivalent position to the DPG binding site of human Hb. Lys β82 is not shown.

neutral theory of protein evolution (Kimura, 1979). Bar-headed goose Hb is a further example where a functional property of the molecule can be modified by minimal changes in the amino acid sequence, in this case by a single mutation. This is because the allosteric equilibrium of Hb is so finely balanced, and strong selective pressure maintains this balance despite the large sequence variation which occurs between different Hbs. For a high flying bird or burrowing mammal the optimal oxygen affinity of the blood will be different to that of a human being, but only very slight changes are necessary to bring about the required shift in the relative stability of the R and T states of haemoglobin. When first putting forward his two-state model of haemoglobin cooperativity, Perutz (1970a) did not consider the $\alpha_1\beta_1$ contacts to be of great functional significance, but it has since emerged that mutations here can have a large effect on the oxygen affinity. Any weakening of this subunit contact tends to allow the subunits to adopt a structure, and hence ligand affinity, closer to that of the monomeric form (Amiconi *et al*., 1989). Although very unusual, the proline to alanine mutation found in bar-headed geese appears to cause no significant change in the protein structure. Of the four mutations found between bar-headed and greylag geese Hbs, Gly α18 to Ser, Ala α63 to Val, Glu β125 to Asp and Pro α119 to Ala, only the latter need be introduced into human Hb to cause a comparable increase in oxygen affinity (Weber *et al*., 1993). The structure of the protein gives no indication that the other three amino acid changes will have a functional effect. The interaction between proline α119 and methionine β55 in deoxy human Hb appears very weak, yet its loss causes considerable physiological effects. The Andean goose, another bird adapted to high altitudes, has evolved a Hb in which β55 has mutated to serine; this has the same effect as the α119 mutation when introduced into human Hb (Weber *et al*., 1993). The fact that two species of high flying bird have independently evolved Hbs in which this same contact is not formed indicates how finely tuned to the environment Hb can become. This is not the only means of a adaptation to high altitude however. Vultures of the falconiform family fly to exceptional altitudes and have developed high affinity Hbs by a totally different set of mutations in the α subunits (Weber *et al*., 1988). Hb appears extremely robust to mutation (hundreds of human Hb mutants have been detected clinically, most with no apparent effect on the patient) which has allowed a variety of mutations to arise to adapt to high altitudes.

Experimental

Red blood cells from a bar-headed goose were washed in saline to remove plasma proteins and then lysed with distilled water. Cell membrane and other insoluble material were removed by saturating the solution with salt and spinning in an ultra-centrifuge at 10,000 rpm for an hour. Crystals were grown by the hanging drop method using 10% PEG 6000, 50 mM potassium phosphate (pH 6.8) and a protein solution of 25 mg/ml. Oxidation of the protein to metHb was minimised by growing the crystals at 4°C in the presence of 1 mM EDTA. The crystals are in space-group $P422_12$ with unit cell dimensions $a = b = 81.59$ Å $c = 107.28$ Å. This suggests that the asymmetric unit is occupied by an αβ dimer and the molecular 2-fold axis coincides with a crystallographic axis. Data were collected at the Photon Factory, Tsukuba, Japan, using the Weissenberg camera and radiation of 1.0 Å wavelength (Sakabe, 1983). A total of 92,268 observations of 29,297 independent reflections were collected. The data were processed with WEISS to give an R_{merge} of 5.12%. The crystals diffract to 1.8 Å but data beyond 2.0 Å resolution were not used since the data are very weak and incomplete beyond this limit.

Structure determination

An initial model for refinement was obtained by molecular replacement using the coordinates of the human oxy-haemoglobin $\alpha_1\beta_1$ dimer (Shaanan, 1983). Bar-headed goose haemoglobin has 69% sequence identity with human haemoglobin, so the structures were expected to show a high degree of structural similarity. In common with human Hb, bar-headed and greylag geese Hbs have 141 residues in the α subunit and 146 in the β subunit. The rotation and translation searches were carried out with AUTOMR (Matsuura, 1991) using 10 to 4 Å resolution data. The human oxyHb dimer was taken from the Brookhaven Protein Data Bank and rotated by (30, 50, 40) degrees in the polar angle system and then used as search model. A Patterson radius of 25 Å was used in the rotation function calculation. The two highest peaks were located at position $\psi = 60.382$ $\phi = 175.728$ $\kappa = 260.752$ and $\psi = 44.988$ $\phi = 265.492$ $\kappa = 135.351$. The translation function was calculated using data between 12 and 10 Å resolution. The two rotation peaks gave the same translation vector $X = 40.80$ Å, $Y = 0.00$ Å $Z = 32.19$ Å showing that these two rotation peaks are equivalent. The result also shows that the bar-headed goose oxy Hb molecule is located on the 42 axis in the *ac* plane. The molecular dyad axis which correlates the $\alpha_1\beta_1$ dimer and $\alpha_2\beta_2$ dimer coincides with the crystal 42 axis, while the two pseudo molecular dyad axes which correlate the α and β subunits are in the direction of $a + b$ and $a - b$ of the crystal. The molecular replacement solution gave an R value of 46.7% calculated with 10 to 4 Å resolution data. The goose Hb model was built with FRODO (Jones, 1982). Refinement was carried out with simulated annealing by XPLOR (Brunger, 1992) and subsequently PROLSQ (Konnert & Hendrickson, 1980; CCP4, 1994). Manual adjustment of the model to improve agreement with the calculated electron density map was done using FRODO and later O (Jones & Kjeldgaard, 1991). Water molecules were added using the program ARP (Lamzin & Wilson, 1993); there are 108 in the final model, which has an R factor of 19.8% calculated with all data between 10.0 and 2.0 Å. The rms deviations in bond distances and angles are 0.020 Å and 0.055 Å, respectively. The Ramachandran plot (Figure 5) shows that almost all residues in the structure are found in a helical conformation, and only two have unfavourable positions.

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References

Amiconi, G., Ascoli, F., Barra, D., Bertollini, A., Matarese, R. M., Verzili, D. & Brunori, M. (1989). Selective oxidation of methionine β55D6 at the $\alpha_1\beta_1$ interface in hemoglobin completely destabilizes the T state. *J. Biol. Chem.* **264**, 17745–17749.

Arnone, A. (1972). X-ray diffraction study of binding of 2,3 diphosphoglycerate to human haemoglobin. *Nature*, **237**, 146–149.

Baldwin, J. & Chothia, C. (1979). Haemoglobin: the structural changes related to ligand binding and its allosteric mechanism. *J. Mol. Biol.* **129**, 175–220.

Brunger, A. T. (1992). *XPLOR version 3.1*, Yale University, CT, USA.

Camardella, L., Caruso, C., D'Avino, R., di Prisco, G., Rutigliano, B., Tamburrini, M., Fermi, G. & Perutz, M. F. (1992). Haemoglobin of the Antarctic fish *Pagothenia bernachii*: amino acid sequence, oxygen equilibria and crystal structure of its carbonmonoxy derivative. *J. Mol. Biol.* **224**, 449–460.

CCP4. (1994). The CCP4 Suite: programs for protein crystallography. *Acta Crystallog. sect D*, **50**, 760–763.

Demma, L. S. & Salhany, J. M. (1979). Subunit inequivalence in super-oxide anion formation during photooxidation of human oxyhemoglobin. *J. Biol. Chem.* **254**, 4532–4535.

Emsley, P. E. (1991). D.Phil thesis. University of York.

Gelin, B. R. & Karplus, M. (1977). Mechanism of tertiary structure change in hemoglobin. *Proc. Natl Acad. Sci. USA*, **74**, 801–805.

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.

Imai, K., Shih, D. T.-B., Tame, J., Nagai, K. & Miyazaki, G. (1989). Structural and functional consequences of amino acid substitutions in hemoglobin as manifested in natural and artificial mutants. *Protein Seq. Data Anal.* **2**, 81–86.

Jessen, T.-H., Weber, R. E., Fermi, G., Tame, J. & Braunitzer, G. (1991). Adaptation of bird hemoglobins to high altitudes: demonstration of molecular mechanism by protein engineering. *Proc. Natl Acad. Sci. USA*, **88**, 6519–6522.

Jones, T. A. (1982). In *Computational Crystallography*, pp. 3303–3317, Clarendon Press, Oxford.

Jones, T. A. & Kjeldgaard, M. (1991). *O—The Manual*. Uppsala University, Sweden.

Kimura, M. (1979). The neutral theory of molecular evolution. *Sci. Amer.* **241**, 94–104.

Kleinschmidt, T. & Sgouros, J. G. (1987). Hemoglobin sequences. *Biol. Chem. Hoppe-Seyler* **368**, 579–615.

Komiyama, N. H., Miyazaki, G., Tame, J. & Nagai, K. (1995). Transplanting a unique allosteric effect from crocodile into human haemoglobin. *Nature*, **373**, 244–246.

Konnert, J. K. & Hendrickson, W. A. (1980). A restrained parameter thermal-factor refinement procedure. *Acta Crystallog. sect. A*, **36**, 344–349.

Lamzin, V. S. & Wilson, K. S. (1993). Automated refinement of protein models. *Acta Crystallog. sect. D*, **49**, 129–147.

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.

Luisi, B. F. & Nagai, K. (1986). Crystallographic analysis of mutant haemoglobins made in *Escherichia coli*. *Nature*, **320**, 555–556.

Mansouri, A. & Winterhalter, K. H. (1973). Nonequivalence of chains in hemoglobin oxidation. *Biochemistry*, **12**, 4946–4949.

Matsuura, Y. (1991). AUTOMR: an automatic processing program system for the molecular replacement method. *J. Appl. Crystallog.* **24**, 1063–1066.

Obertheur, W., Braunitzer, G. & Wuerdunger, I. (1982). Das Haemoglobin des Streifengans (Anser indicus). Primaerstruktur und Physiologie det Atmung, Stematik und Evolution. *Hoppe-Seyler's Z. Physiol. Chem.* **363**, 581–590.

Olson, J. S., Mathews, A. J., Rohlfs, R. J., Springer, B. A., Edeberg, K. D., Tame, J., Renaud, J. P. & Nagai, K. (1988). The role of the distal histidine in myoglobin and haemoglobin. *Nature*, **336**, 265–266.

Perutz, M. F. (1970a) Stereochemistry of cooperative effects in haemoglobin. *Nature*, **228**, 726–734.

Perutz, M. F. (1970b). The Bohr effect and combination with organic phosphates. *Nature*, **228**, 734–739.

Perutz, M. F. (1983). Species adaptation in a protein molecule. *Mol. Biol. Evol.* **1**, 1–28.

Perutz, M. F., Fermi, G., Luisi, B. F., Shaanan, B. & Liddington, R. C. (1987). Stereochemistry of cooperative effects in haemoglobin. *Acc. Chem. Res.* **20**, 309–321.

Petschow, D., Wuerdinger, I., Baumann, R., Duhm, J., Braunitzer, G. & Bauer, C. (1977). Causes of high blood oxygen affinity of animals living at high altitude. *J. Appl. Physiol.* **42**, 139–143.

Richard, V., Dodson, G. G. & Mauguen, Y. (1993). Human deoxyhemoglobin-2,3 diphosphoglycerate complex low salt structure at 2.5 Å resolution. *J. Mol. Biol.* **233**, 270–274.

Rollema, H. S. & Bauer, C. (1979). The interaction of inositol pentaphosphate with the hemoglobins of highland and lowland geese. *J. Biol. Chem.* **254**, 12038–12043.

Sakabe, N. (1983). A focusing Weissenberg camera with multi-layer-line screens for macromolecular crystallography. *J. Appl. Crystallog.* **16**, 542–547.

Shaanan, B. (1983). Structure of human oxyhemoglobin at 2.1 Å resolution. *J. Mol. Biol.* **171**, 31–59.

Shih, D. T., Luisi, B. F., Miyazaki, G., Perutz, M. F. & Nagai, K. (1993). A mutagenic study of the allosteric linkage of His(HC3)146β in haemoglobin. *J. Mol. Biol.* **230**, 1291–1296.

Swan, L. W. (1970). Goose of the Himalayas, *Nat. Hist.* **79**, 68–75.

Tamburrini, M., Condo, S. G., di Prisco, G. & Giardina, B. (1994). Adaptation to extreme environments: structure-function relationship in emperor penguin haemoglobin. *J. Mol. Biol.* **237**, 615–621.

Weber, R. E., Hiebl, I. & Braunitzer, G. (1988). High altitude and hemoglobin function in the vultures *Gyps rueppelli* and *Aegypius monachus*. *Biol. Chem. Hoppe-Seyler*, **369**, 233–240.

Weber, R. E., Jessen, T.-H., Malte, H. & Tame, J. (1993). Mutant hemoglobins (α^{119}-Ala and β^{55}-Ser): functions related to high-altitude respiration in geese. *J. Appl. Physiol.* **75**, 2646–2655.

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