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Evolution

Adaptation of bird hemoglobins to high altitudes: Demonstration of molecular mechanism by protein engineering

(physiology/$\alpha_1\beta_1$ subunit contact/x-ray structure)

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ABSTRACT **Of two closely related species of geese, one, the greylag goose, lives in the Indian plains all year round, while the other, the bar-headed goose, lives at the Tibetan lakes and migrates across the Himalayas to winter in India. Another species, the Andean goose, lives in the High Andes all year round. Possession of a Hb with high oxygen affinity helps to adapt bar-headed and Andean geese to high altitudes. The Hb amino acid sequences of the bar-headed and the greylag geese differ by four substitutions, of which only one is unique among bird sequences: Pro-119α(H2) → Ala. Perutz proposed that the two-carbon gap left by this substitution at the $\alpha_1\beta_1$ contact raises the oxygen affinity, because it relaxes the tension in the deoxy or T structure [Perutz, M. F. (1983) *Mol. Biol. Evol.* 1, 1–28]. It was later found that the Hb of the Andean goose has a gap in the same position, due to the complementary substitution Leu-55β(D6) → Ser. We have tested Perutz's hypothesis by introducing each of these substitutions into human globin synthesized in *Escherichia coli*. The reconstituted Hbs combine cooperatively with oxygen. Their oxygen affinities exceed that of normal human Hb by an even larger factor than that found between the high-flying geese and the greylag goose. The mutant Hb Met-55β(D6) → Ser was crystallized. Its structure is the same as that of HbA, except in the immediate environment of the gap left by the substitution of the serine for the methionine side chain, which evidently causes the increased oxygen affinity of this Hb.**

Many of Konrad Lorenz's classic studies concerned the behavior of geese (1). Some species of geese are adapted to life and flight at very high altitudes (2). Like high-altitude mammals and amphibians, they have Hbs with high oxygen affinity. Perutz (3) has argued that such adaptation arises from one or a few amino acid substitutions in key positions, while the majority of substitutions between species is functionally neutral. DNA technology has allowed us to test this idea by introducing single amino acid substitutions into human Hb in order to try and "evolve" an increase in oxygen affinity similar to that found in the Hbs of high-flying birds.

The bar-headed goose (*Anser indicus*) is one of the most remarkable species in the goose family; these birds live and hatch their young at the Tibetan lakes (altitude, 4000–6000 m), but at the end of autumn they migrate to the plains of northwest India (4). Flocks have been observed flying over the summit of Mt. Everest (5). Using the principle of minimal genetic distance, the Hb sequence of the bar-headed goose (6) was compared with that of the greylag goose (7), the Canada goose (8), and the mute swan (8), which are its closest lowland relatives. The alignment of the protein sequences of the bar-headed goose and the greylag goose showed only four mutations, the smallest number of replacements found (amino acids of greylag goose Hb given first): Gly-18α(A16) → Ser, Ala-63α(E12) → Val, Pro-119α(H2) → Ala, and Glu-125β(H3) → Asp. The substitution at Gly-18α(A16) affects an external residue, Ala-63α(E12) lies in a surface crevice, and Glu-125β(H3) is placed so that a hydrophilic side chain would protrude into the surrounding water (9). None of these substitutions is likely to affect function. Pro-119α(H2), on the other hand, touches leucine β D6 at the $\alpha_1\beta_1$ contact. Its replacement by alanine leaves a two-carbon gap. Experience with abnormal human Hbs has shown that any gap in the deoxy or T (tense) structure is liable to raise its oxygen affinity by relaxing its tension, while such gaps leave the oxygen affinity of the oxy or R (relaxed) structure unchanged, because it is already relaxed (9). For this reason, Perutz (3) suggested that the substitution Pro-119α(H2) → Ala alone is responsible for the high oxygen affinity of the bar-headed goose Hb.

The Andean goose (*Chloephaga melanoptera*) lives in South America at altitudes of 5000–6000 m. Its Hb also shows a high oxygen affinity. Andean goose blood has a half-saturation oxygen tension (P_{50}) of 33.9 mmHg (t = 40°C; pH 7.1) (10), comparable to the value found for bar-headed goose blood (29.7 mmHg; t = 37°C; pH 7.4) (11), but much lower than the P_{50} of greylag goose blood, which is 39.5 mmHg (t = 37°C; pH 7.4) (11). The globin of the Andean goose differs from that of the greylag goose by nine replacements in the α chain and seven in the β chain (12). Nevertheless, we postulated that its high oxygen affinity is caused by the single replacement Leu-55β(D6) → Ser, which leaves a two-carbon gap at the contact with Pro-119α(H2) at the $\alpha_1\beta_1$ interface, the same proline that is replaced by alanine in the bar-headed goose.

To test these hypotheses, we synthesized two human Hb mutants, Pro-119α(H2) → Ala (HbPα119A) and Met-55β(D6) → Ser (HbMβ55S), compared their oxygen affinities with that of native human Hb, and crystallized one of the mutants (HbMβ55S) to determine its structure by x-ray analysis.

MATERIALS AND METHODS

The human globin genes were mutated by site-directed mutagenesis in phage M13 by the gapped duplex method (13). The mutated genes were cloned into the expression vector pLcIIFX$\beta\alpha$ and used to transform *Escherichia coli* QY13 (14, 15). Inclusion bodies of the fusion proteins were produced by heat induction of the cells. The purified fusion proteins were cleaved with blood coagulation factor Xa to liberate the mutated globin chains, which were reconstituted into Hb tetramers by refolding in the presence of hemin and the appropriate native Hb subunit. We followed essentially the reconstitution procedure described by Nagai and Thøgersen

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(15) and modified by Tame *et al.* (16). The purity of the resulting HbPα119A and HbMβ55S was confirmed by absorption spectra, cellulose acetate electrophoresis, fast protein liquid chromatography, and protein sequencing (17).

The oxygen equilibrium curves of the native and the mutant Hbs were measured in a modified diffusion chamber according to Weber (18). All measurements were performed at 25°C. According to spectroscopic measurements, the oxidation of the samples was <4%.

Hb crystals of the HbMβ55S mutant were grown by the method of Perutz (19). The crystals were isomorphous with those of native deoxy-HbA in space group $P2_1$. Data were collected on a FAST/MADNESS area detector to 2.5-Å resolution and corrected for Lorentz and polarization effects, and batch scale and temperature factors were applied (batches of 5° in rotation angle). The data were merged to yield 15,582 unique reflections, or 83% of the total in the 10- to 2.5-Å range, with a merging R factor of 7.5% on intensity. The reduced structure amplitudes were scaled to the 1.74-Å native HbA data set of Fermi *et al.* (20) with an R factor of 12.7%, and a difference electron density map was calculated with the native phases.

RESULTS

Both recombinant proteins could be synthesized in *E. coli* and reconstituted to fully functional Hbs in amounts sufficient to perform oxygen equilibrium measurements and crystallographic investigations. Fig. 1 shows the oxygen binding properties of the native and the mutant Hbs. Since the recombinant wild-type Hbs obtained by our method of reconstitution (15, 16) show the same P_{50} and cooperativity as native HbA (21), we used the latter as a standard for comparison with the mutants.

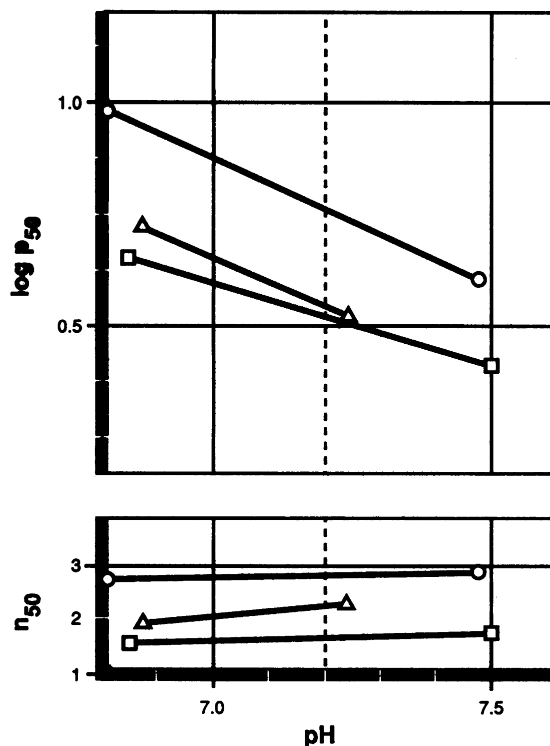

FIG. 1. Oxygen binding properties of native and mutant Hbs. ○, Native human HbA; □, HbPα119A; △, HbMβ55S. Experimental conditions were as follows: 100 mM NaHepes/100 mM KCl; 25°C. Heme concentration of HbA and HbPα119A was 148 μM, and heme concentration of HbMβ55S was 330 μM. P_{50} is the partial pressure of oxygen in mmHg (1 mmHg = 133 Pa) at 50% saturation of Hb, and n_{50} is the slope of the Hill plot at P_{50}.

Both mutant Hbs show an increased intrinsic oxygen affinity; the n_{50} and n_{max} values are decreased, as is often found in naturally occurring Hb mutants with high oxygen affinity, due to the bell-shaped curve relating oxygen affinity to cooperativity (22). Both mutations give rise to very similar increases in oxygen affinity. Table 1 compares our data with the known oxygen affinity values of geese Hbs. The increase in oxygen affinity (Δlog P_{50}) produced by either of the two complementary substitutions in human Hb is actually greater than the difference in oxygen affinity between the Hbs of the bar-headed and the greylag geese. This confirms that the gap left by these substitutions at the $\alpha_1\beta_1$ contact is a sufficient cause for the increase in oxygen affinity observed in nature.

Fig. 2 shows a difference Fourier map of the mutant HbMβ55S versus HbA. The region of positive density suggests the position of the serine OH, and negative density marks the replaced methionine side chain. The serine OH points away from 119α(H2). Superimposition of van-der-Waals shells (not shown here) shows no contact between residues α H2 and β D6. Weak negative density at the end of the D β-helix and the beginning of the E β-helix indicates slight shifts of these helices, widening the gap between the $\alpha_1\beta_1$ subunits. Positive density in the gap may represent a new water molecule. No other significant changes in density were observed.

DISCUSSION

In the allosteric transition of Hb, the $\alpha\beta$ dimers turn relative to each other as rigid units and the extensive $\alpha_1\beta_1$ contacts remain rigid. Amino acid replacements at this contact were therefore expected to leave the allosteric equilibrium unaltered. Contrary to this expectation, in Hb Philly the replacement Tyr-35β(C1) → Phe, which disrupts a hydrogen bond with Asp-126α(H9) at the $\alpha_1\beta_1$ contact, raised the oxygen affinity by the equivalent of 3 kcal/mol (1 cal = 4.184 J) (24). This discovery led to the recognition that disruption of any bond anywhere in the Hb molecule relaxes the tension on which the low oxygen affinity of the T state depends, while it hardly affects the oxygen affinity of the R structure, because this is about the same as that of fully relaxed $\alpha\beta$ dimers (24). The difference Fourier map presented here proves that the only significant disturbance introduced by the replacement Met-55β(D6) → Ser is the loss of contact of the methionine side chain with Pro-119α(H2). The increase in oxygen affinity produced by the replacement amounts to Δlog P_{50} = 0.23, which is equivalent to a difference in free energy per mol of $\alpha\beta$ dimer of 650 cal, or about one-quarter of an internal hydrogen bond. The energy equivalent of the difference in the oxygen affinity between the Hbs of the bar-headed and the greylag geese is even smaller; it is remarkable that a difference in bond energy of only 800 cal per tetramer should have given these geese a selective advantage that adapted them to flight and life at extreme altitudes.

The HbMβ55S mutant was crystallized in preference to the HbPα119A mutant, since the only function of Met-55β_1(D6)

Table 1. Oxygen affinities of human and goose Hbs

Hb	P_{50}, mmHg	log P_{50}	Δ log P_{50}
Human HbA	5.8	0.76	
Human HbPα119A	3.3	0.53	0.23
Human HbMβ55S	3.4	0.54	0.22
Greylag goose	2.8	0.45	
Bar-headed goose	2.0	0.30	0.15

Experimental conditions were as follows: pH 7.2, 0.1 M Cl^-, 25°C. The P_{50} values of human Hbs were calculated from Fig. 1. The P_{50} values of the greylag and bar-headed goose Hb were measured by Rollema and Bauer (23) in 100 mM [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane, the heme concentration was 120 μM.

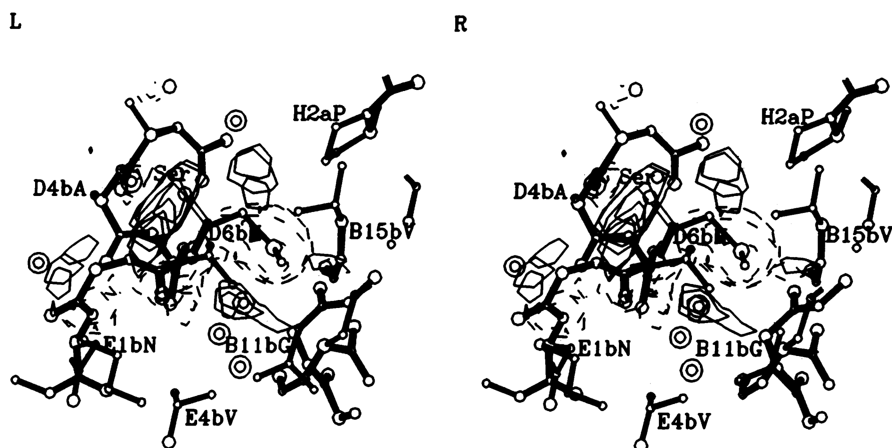

FIG. 2. Difference Fourier map at 2.5-Å resolution of HbMβ55S minus HbA, plotted on coordinates of deoxy-HbA. The $\alpha_1\beta_1$ contact Pro-119α(H2)–Met-55β(D6) (H2aP–D6bM) and its surroundings are shown. Solid contours (——), positive density; broken contours (- - -), negative density. The serine side chain is marked by an open bond. The map was averaged about the molecular dyad to improve accuracy, as there were no qualitative differences between symmetry-related regions. The contour level is ±0.12 electron per Å, or ≈3 times the rms value of the unaveraged map. D4bA, Ala-53β(D4); E1bN, Asn-57β(E1); E4bV, Val-60β(E4); D6bM, Met-55β(D6); H2aP, Pro-119α(H2); B15bV, Val-33β(B15); B11bG, Gly-29β(B11).

is the formation of a van-der-Waals contact to Pro-119α_1(H2) (9): on the other hand, this proline makes contacts with Arg-30β_1(B12) and Val-33β_1(B15) as well as with Met-55β_1(D6), which could complicate the interpretation of the results. The difference Fourier map of the HbMβ55S mutant shows that major disturbances in the structure are restricted to the immediate neighborhood of the amino acid substitution. This explains why this recombinant Hb is fully functional and proves that the increase of oxygen affinity is caused exclusively by the gap introduced at the $\alpha_1\beta_1$ contact.

Human Hb shows 70% amino acid identity with goose Hb; moreover, the residues at the heme and subunit contacts are nearly all conserved, which indicates that the structures are homologous (25). Proline α H2 has been conserved in the Hb of all mammals, birds, and fish (26), even though most other prolines have been exchanged for other residues. The sole exception among birds is the bar-headed goose, which has an alanine residue instead. In bird Hbs, the Met-55β(D6) of mammalian Hbs is usually replaced by the similar amino acid leucine, but in the Andean goose it is replaced by serine. Our results confirm that the introduction of a smaller amino acid in either residue at the H2–D6 $\alpha_1\beta_1$ contact leads to a higher oxygen affinity. There are, of course, additional anatomical and physiological factors that allow these geese to fly at altitudes up to 10,000 m (27–30), but a raised blood oxygen affinity has been proved to be advantageous for animals living at high altitudes (11, 31, 32), because it increases the oxygen saturation of the arterial blood (27, 28, 31). Some authors have calculated that the bar-headed goose might have oxygen reserves in the venous blood even at the summit of Mt. Everest (33), which is amazing when one considers that the P_{O_2} there is only one-third of that at sea level.

The increases in intrinsic Hb oxygen affinities associated with the mutations at the $\alpha_1$119–$\beta_1$55 contact appear to be an integral part of the adaptation of the bar-headed and the Andean geese to hypoxia at extreme altitudes and of their abilities to exploit ecological niches that other species cannot tolerate. It is remarkable that these vital mutations have occurred at the same subunit contact, but on different globin chains, in two different species of high-flying geese that evolved in widely separated parts of the world.

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