Allosteric Modulation by Tertiary Structure in Mammalian Hemoglobins

INTRODUCTION OF THE FUNCTIONAL CHARACTERISTICS OF BOVINE HEMOGLOBIN INTO HUMAN HEMOGLOBIN BY FIVE AMINO ACID SUBSTITUTIONS*

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Bovine erythrocytes do not contain 2,3-diphosphoglycerate, the principal allosteric effector of human hemoglobin. Bovine hemoglobin has a lower oxygen affinity than human hemoglobin and is regulated by physiological concentrations of chloride (Fronticelli, C., Bucci, E., and Razynska, A. (1988) J. Mol. Biol. 200, 343-348). It has been proposed that the chloride regulation in bovine hemoglobin is introduced by particular amino acid residues located in the amino-terminal region of the A helix and in the E helix of the β subunits (Fronticelli, C. (1990) Biochem. J. 37, 141-146). In accordance with this proposal we have constructed two mutant human hemoglobins, β(V1M+H2deleted+T4I+P5A) and β(V1M+H2deleted+T4I+P5A+A76K). These are the residues present at the proposed locations in bovine hemoglobin except for isoleucine at position 4. Oxygen binding studies demonstrate that these mutations have introduced into human hemoglobin the low oxygen affinity and chloride sensitivity of bovine hemoglobin and reveal the presence of a previously unrecognized allosteric mechanism of oxygen affinity regulation where all the interactions responsible for the lowered affinity and chloride binding appear to be confined to individual β subunits.

Hemoglobin (Hb) is present within the red cell at about 5 mM concentration and functions to transport oxygen from the lungs to the tissues (Antonini and Brunori, 1970). This protein is a tetramer composed of two pairs of identical α and β subunits. The four subunits have the same tertiary folding, and they each contain eight α helices, which are identified as A through H. The tetrameric molecule can assume two conformations that are in equilibrium with one another. The predominant conformation when no oxygen is bound is called T state, and the predominant conformation when the oxygen is coordinated to the iron is termed R state. The affinity of the protein for oxygen is regulated by position of the conformational equilibrium, i.e. the equilibrium between the R and T states. The position of the conformational equilibrium is also influenced by pH and the concentration of certain anions, termed effectors. In the absence of effector anions, the oxygen affinity of human Hb is too high for efficient release of oxygen at the oxygen partial pressure maintained in most tissues, approximately 40 torr (Vandegriff, 1992).

In human Hb, the effector regulating the conformational equilibrium in the red cells is 2,3-diphosphoglycerate (DPG) (Benesch et al., 1968). On the other hand, bovine Hb has an intrinsically lower oxygen affinity than human Hb, and the affinity is lowered further by interaction with physiological concentrations of chloride ions (100-150 mM) (Fronticelli et al., 1984, 1988; Perutz et al., 1993). At this concentration of chloride, the oxygen affinity of bovine Hb is insensitive to polyanions such as DPG and has an oxygen affinity similar to that of human whole blood (Bucci et al., 1988).

In the development of hemoglobins to be used clinically as a cell-free oxygen carrier, an understanding of the molecular mechanism that regulates the functional characteristics of bovine Hb could lead to the design of human Hb variants that are capable of efficient oxygen transport in cell-free solutions within the circulatory system at the physiological chloride concentrations.

Searching for structural differences between the human and bovine hemoglobins, we examined their hydropathy plots (Fronticelli, 1990). No differences were detected between the α chains of human and bovine Hb; however, in the β chains two regions of different hydrophobicities were observed. One comprised a portion of the A helix, and the other comprised a portion of the E helix. In order to correlate the different hydrophobicities with relevant differences in sequence, we compared sequences of several primates and ruminant β subunits. The results, reported in Table I, show the presence of consistently different sequences that in ruminant β subunits result in an increase in the hydrophobic index of the amino-terminal end of the protein and a decrease in the index of the E helix relative to human β subunits. Thus, we hypothesized that amino acid substitutions of the amino-terminal residues and of the A and E helices of bovine Hb were responsible for introducing in bovine Hb a different mechanism of oxygen affinity regulation (Fronticelli, 1990).

To test this hypothesis we have constructed mutant human hemoglobins containing some of the amino acid substitutions reported in Table I. Substitution of valine β1 with a methionine and deletion of histidine β2 produced a stabilization of the T state but was not sufficient to introduce the low oxygen affinity...
of bovine hemoglobin into human hemoglobin (Fronticelli, 1992). However, this mutation removed the Cl⁻ binding site between Val¹¹¹ and Lys¹⁴² as well as the Cl⁻-dependent Bohr effect and introduced at the amino-terminal of the human β subunit the conformation present in bovine β subunits (Fronticelli et al., 1994; Perutz et al., 1993). Additional amino acid substitutions have now been introduced, and in this paper we report the functional properties of two mutant hemoglobins, β(V1M + H2deleted + T4I + P5A) and β(V1M + H2deleted + T4I + P5A + A76K).

Our results confirm the original hypothesis that the functional characteristics of bovine hemoglobin are principally dependent on the presence of specific amino acid residues in the A and E helices. Most important, they also reveal a novel mechanism of oxygen affinity modulation within a single β subunit, regulated by tertiary conformational changes.

### MATERIALS AND METHODS

Protein Cloning, Expression, and Purification—The plasmid bearing the β globin mutations, M1V, H2 deleted, T4I, and P5A, was generated from p JK13, which contains the β globin cDNA fused to a Factor X recognition sequence and a truncated flu virus protein, NS1 (Fronticelli et al., 1991). pJ K05 has only two Bsu36I (MsII) sites, both within the globin gene. The first Bsu36I site is in the region of the Pvu I codon, 33 nucleotide residues downstream from the single Ncol site, which is at the juncture between NS1 gene and the Factor X recognition sequence. The second Bsu36I site is 159 nucleotide residues beyond the first. The vector was prepared by first digesting pJ K05 exhaustively with Ncol followed by a partial digestion with Bsu36I, and the DNA was gel purified. The recognition sequence of Bsu36I is 5'-CC ↓ TXAGG and is not the same in the two β globin sites; the first is CC ↓ TGAGG and the second is CC ↓ TAAGG. Therefore, in the vector, Bsu36I generates a 5'TGA↓ overhang at the first site and a STTA — overhang at the second site. Taking advantage of this difference, a double stranded oligonucleotide was designed that would anneal and ligate selectively to the first site. Accordingly, two oligonucleotides were synthesized that coded for the sequence changes indicated above, were complementary, and had a 5'-CATG (Ncol) overhang on the one end with a 3'-ACT-5'

Electrophoretic separation (Paragon system, Beckman) of human Hb (lane 1), bovine Hb (lane 2), α(bovine)-β(human), (lane 3), and α(human)-β(bovine), (lane 4). The dots represent the site of the samples deposition.

### RESULTS AND DISCUSSION

Hybrids of Human and Bovine Hemoglobin—We have constructed two human-bovine hemoglobin hybrids, α(bovine)-β(human), and α(human)-β(bovine) (Fronticelli, 1992). This method cannot be used with bovine hemoglobin, which does not have cysteine residues at the α1β1 interface, and the bovine α and β globins were separated by reverse phase chromatography (Fronticelli et al., 1990). The peak containing the β globin was dialyzed against water, the precipitate collected, solubilized in 0.1 M NaOH and dialyzed under nitrogen against 0.04 M borate buffer at pH 9.0 containing 1 mM dithioerythritol and 1 mM EDTA. The bovine β globin was reconstituted into tetrameric hemoglobin upon the addition of an equimolar amount of heme and human α subunits. The bovine α globin after dialysis against water was dissolved in 5 M acetic acid and dialyzed against 20 mM phosphate buffer at pH 5.5 and reconstituted with heme and human β subunits. The reconstituted hybrid proteins were purified on ion exchange chromatography (Fronticelli et al., 1991). The Paragon electrophoresis system (Beckman) was used to confirm the degree of purity.

Oxygen Equilibrium Curves—These data were obtained using the thin layer dilution technique (Dollman and Gill, 1978). The protein concentration was 20-30 mg/ml at 25°C. The buffer was 50 mM Hepes. Effectors were added as specified in the legends of the figures. The experimental data were fitted to the Adair equation (Adair, 1925) as shown by the continuous lines in the figures.

The rationale for the experiment was that if the entire molecule is necessary for expressing the decrease in oxygen affinity, the two hybrids would have similar oxygen affinities. Otherwise, it would be found only in the hybrid containing the enabling subunit.

The hybrid containing the bovine β chains has a 3-fold de-
crease in oxygen affinity (Fronticelli, 1992) with respect to the hybrid containing the human \(\beta\) chains (Fig. 2). This is the same oxygen affinity difference observed between bovine and human Hb (Fronticelli et al., 1984), supporting the proposition that the reduced oxygen affinity of bovine Hb is expressed via the \(\beta\) subunits.

**Pseudobovine Hemoglobins**—We have constructed two mutant hemoglobins, both with normal human \(\alpha\) subunits. In the first mutant, \(\beta(V1M^1H2del+T4I^1P5A^1)\), hereafter abbreviated \(\beta(PB4)\), valine \(\beta1\) was replaced by a methionine, histidine \(\beta2\) was deleted, proline \(\beta5\) was substituted by alanine, and, in order to further increase the hydrophobicity of the A helix, threonine \(\beta4\) was replaced by isoleucine. The second mutant, \(\beta(V1M^1H2del+T4I^1P5A^1A76K)\), abbreviated \(\beta(PB5)\), has the sequence modifications of \(\beta(PB4)\) plus the substitution of alanine \(\beta76\) to lysine. With the exception of the threonine \(\beta4\) to isoleucine substitution, all the residue changes are those found in the \(\beta\) subunits of bovine Hb.

**Peptide Analysis**—The amino acid substitutions in the recombinant \(\beta\) globin were confirmed by peptide analysis and sequencing. In Fig. 3 the comparison of the peptide maps shows that peptide 1 is eluted at 54 and 65 min in the \(\beta\) globins of human Hb and \(\beta(PB5)\), respectively. Also, peptide 9 is split into two peptides, 9a and 9b, in \(\beta(PB5)\), where a lysine is present at position 76. The rest of the peptide maps are identical in profile. Table II shows the sequencing of peptides 1 and 9 from human Hb and \(\beta(PB5)\).

**Functional Data**—In the absence of chlorides, \(\beta(PB4)\), \(\beta(PB5)\), and bovine Hb all have the same oxygen affinity, which is 3-fold lower than in human Hb (Fig. 4A). This result indicates that the amino acid substitutions present in \(\beta(PB4)\) produce a stabilization of the T state sufficient for introducing into human Hb the intrinsic low oxygen affinity of bovine Hb. X-ray crystallography of bovine Hb and of the double mutant \(\beta(V1M^1H2deleted)\) suggests that this effect is due to a conformational change in the A helix that mimics the effect of DPG in human Hb (Perutz et al., 1993; Fronticelli et al., 1994).

In the presence of 200 mM chloride, the oxygen affinity of human Hb is decreased; however, the oxygen affinity of \(\beta(PB4)\) remains nearly the same as that observed in the absence of chloride (Fig. 4B). This result demonstrates that although the bovine-like sequence of the amino-terminal region lowers the oxygen affinity, it also greatly decreases the sensitivity to chloride. This latter property is not unexpected because we have previously shown that the replacement of Val\(^{11}\) \(\rightarrow\) Met and the deletion of His\(^{92}\) destroys the anion binding site between Val\(^{11}\) and Lys\(^{182}\) as well as the chloride-dependent Bohr effect (Fronticelli et al., 1994). In contrast to \(\beta(PB4)\), the oxygen affinities of both bovine Hb and \(\beta(PB5)\) are decreased to the same extent in the presence of 200 mM Cl\(^{-}\) (Fig. 4B). Clearly, the additional substitution of Ala\(^{76}\) \(\rightarrow\) Lys in \(\beta(PB5)\) creates a new chloride-dependent regulatory site. A possible location of the chloride binding site is between Lys\(^{76}\), Lys\(^{78}\), and perhaps His\(^{77}\) of the same \(\beta\) subunit, as earlier proposed (Fronticelli, 1990). The same Cl\(^{-}\) binding site has been postulated to be present in pig hemoglobin, where these three residues are also present. (Con-

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**Figure 2**. Oxygen binding curves of hybrids of \(\alpha(\text{bovine})\)-\(\beta(\text{human})\), (circles) and \(\alpha(\text{human})\)-\(\beta(\text{bovine})\), (squares). Buffer, 100 mM Hepes with 100 mM Cl\(^{-}\) at pH 7.4. Temperature, 25°C.

**Figure 3**. Tryptic peptide maps of \(\beta\) chains of human Hb (a) and of \(\beta(PB5)\) (b).

**Table II**. Sequencing by Edman degradation of peptides 1 and 9 obtained by tryptic digestion of human hemoglobin and \(\beta(PB5)\)

| Fragment | Sequence        |
|----------|-----------------|
| HbA      | VHHTPKEEK       |
| \(\beta(PB5)\) | 1               |
| HbA      | M-LIAEEK        |
| \(\beta(PB5)\) | 9               |
| \(\beta(PB5)\) | 9b              |
| HbA      | VLGAFSDGLHDLNKL |
| \(\beta(PB5)\) | 9b              |
| HbA      | HLDNLK          |
do et al., 1992; Katz et al., 1994).

In the presence of 100 mM Cl\(^-\), the oxygen affinity of \(\beta(PB5)\) is 3-fold lower than the oxygen affinity of human Hb but similar to the oxygen affinity of human Hb in the presence of 2 mM DPG (Fig. 4C). Like bovine Hb in presence of 100 mM Cl\(^-\), the oxygen affinity of \(\beta(PB5)\) is virtually unaffected by the presence of 2 mM DPG (Fronticelli et al., 1988; Perutz et al., 1993). This indicates that the amino acid substitutions in \(\beta(PB5)\) have introduced a mechanism of oxygen affinity modulation similar to the one present in bovine Hb.

Fig. 5 presents the Cl\(^-\) titration of \(\beta(PB4)\), \(\beta(PB5)\), human Hb, and bovine Hb, in 50 mM Hepes buffer at pH 7.4. From the slope of the curve, it can be calculated (Wyman, 1964) that the Cl\(^-\) exchanged is 2.5/tetramer for human Hb, bovine Hb, and \(\beta(PB5)\). This indicates that these hemoglobins bind the same number of chloride ions. In \(\beta(PB4)\) the Cl\(^-\) exchanged is only 0.8/tetramer. This confirms that the replacement of Val\(^{\alpha 1}\) with Met and the deletion of His\(^{\beta 2}\) results in the loss of the Cl\(^-\) binding site present at Val\(^{\beta 1}\) in human Hb (Fronticelli et al., 1994) and indicates that a new chloride binding site is introduced in \(\beta(PB5)\) by the mutation Ala\(^{\beta 76}\) → Lys.

Conclusion—These results are consistent with the proposition that the mechanism of oxygen transport in bovine Hb has...
its molecular basis on the presence of particular amino acid residues in the A and E helices of the β subunits. (Fronticelli, 1990). The results indicate that the low oxygen affinity of bovine Hb compared with human Hb is due to the sequence of the amino-terminal end, which stabilizes a low affinity conformation independent of heterotropic effectors and that the Cl- sensitivity is due to the formation of an oxygen-linked Cl- binding site involving a lysine at position β76 in the E helix. Crystallographic data on the deoxy human mutant β(V1M+H2del) show that the presence of a methionine at position β1 and the deletion of histidine at position β2 places the amino terminus in close proximity to Aspβ79 (Fronticelli et al., 1994), thereby weakening the interaction present between Aspβ79 and Lysβ8. Thus, Lysβ8 acquires a greater conformational flexibility and can establish with Lysα76 a new Cl- binding site, which provides additional stability to the tertiary conformation that was imposed on the β subunits by the amino-terminal residues of the A helix. Upon oxygenation the interaction between Metβ11 and Aspβ79 would be disrupted (Fronticelli et al., 1994), the interaction between Lysβ8 and Aspβ79 would be re-established, and the Cl- would be released. The amino acid residues in the A and E helices of the β subunits. Thus, these data present evidence for the presence of an allosteric mechanism of oxygen affinity modulation regulated solely by tertiary conformational changes. Notably, although there are 24 amino acid differences between the human and bovine β globins, this mechanism is introduced into human Hb by the replacement of only five amino acid residues.

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