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Introduction of a new regulatory mechanism into human hemoglobin

Clara Fronticelli[a,b,*], Kevin M. Bobofchak[a], Michael Karavitis[b], Maria Teresa Sanna[b,1], William S. Brinigar[c]

[a]*Department of Anesthesiology, Johns Hopkins University School of Medicine, Baltimore MD 21287, USA*
[b]*Department of Biochemistry and Molecular Biology, University of Maryland Medical School, Baltimore, MD 21201, USA*
[c]*Department of Chemistry, Temple University, Philadelphia, PA 19122, USA*

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Abstract

Previous studies on bovine hemoglobin (HbBv) have suggested amino acid substitutions, which might introduce into human hemoglobin (HbA) functional characteristics of HbBv, namely a low intrinsic oxygen affinity regulated by Cl^-. Accordingly, we have constructed and characterized a multiple mutant, PB5, [β(V1M+H2Δ+T4I+P5A+A76K)] replacing four amino acid residues of HbA with those present at structurally analogous positions in HbBv, plus an additional substitution, βT4I, which does not occur in either HbBv or HbA. This 'pseudobovine' hemoglobin has oxygen binding properties very similar to those of HbBv: the P_{50} of HbA, PB5 and HbBv in the absence of Cl^- are 1.6, 4.6 and 4.8 torr, respectively, and in 100 mM Cl^- are 3.7, 10.5 and 12 torr, respectively. Moreover, PB5 has 3-fold slower autoxidation rate compared to HbA and HbBv. These are desirable characteristics for a human hemoglobin to be considered for use as a clinical artificial oxygen carrier. Although the functional properties of PB5 and HbBv are similar, van't Hoff plots indicate that the two hemoglobins interact differently with water, suggesting that factors regulating the R to T equilibrium are not the same in the two proteins. A further indication that PB5 is not a functional mimic of HbBv derives from $PB5_{control}$, a human hemoglobin with the same substitutions as PB5, except the βT4I replacement. $PB5_{control}$ has a high oxygen affinity ($P_{50}=2.3$ torr) in the absence of Cl^-, but retains the Cl^- effect of PB5. The Cl^- regulation of oxygen affinity in PB5 involves lysine residues at β8 and β76. PB4, which has the same substitutions as PB5 except βA76K, and PB6, which has all the substitutions of PB5 plus βK8Q, both have a low intrinsic oxygen affinity, like HbBv and PB5, but exhibit a decreased sensitivity to Cl^-. Since HbBv

Abbreviations: Hb: hemoglobin; HbA: human hemoglobin; HbBv: bovine hemoglobin; rβHbA: human hemoglobin with recombinant β-chains; PB2: hemoglobin with mutations β(V1M+H2); PB4: hemoglobin with mutations β(V1M+H2+T4I+P5A); PB5: hemoglobin with mutations β(V1M+H2+T4I+P5A+A76K); $PB5_{control}$: hemoglobin with mutations β (V1M+H2+P5A+A76K); PB6: hemoglobin with mutations β(V1M+H2+T4I+P5A+K8Q+A76K); apoMb: apomyoglobin; 2,3-DPG: 2,3 Diphosphoglycerate; IHP: inositol hexaphosphate

*Corresponding author. Department of Anesthesiology, Johns Hopkins University, School of Medicine, 600 N. Wolfe St. Baltimore MD 21287. Tel.: +410-614-9420; fax: +410-955-7185.

E-mail address: cfrontic@jhmi.edu (C. Fronticelli).

[1] Present address: Dipartimento di Science Applicate ai Biosistemi Universita di Cagliari 09042 Monserrato Cagliari, Italy.

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has lysine residues at both β8 and β76, these results imply that Cl^- regulation in HbBv likewise involves these two residues. The mechanism responsible for the low intrinsic oxygen affinity of HbBv remains unclear. It is suggested that residues peculiar to HbBv at the $\alpha_1\beta_1$ interface may play a role.

Keywords: Recombinant hemoglobin; Bovine hemoglobin; Autoxidation; Low intrinsic oxygen affinity; Heme transfer; Circular dichroism

1. Introduction

In the development of artificial oxygen carriers, the availability of hemoglobins with a range of oxygen affinities is desirable to accommodate various therapeutic applications. An approach in our laboratory has been to use molecular engineering to create human hemoglobins with lowered intrinsic oxygen affinity and a cooperativity similar to that of natural human Hb. These mutant human hemoglobins should also retain their conformational stability and be resistant to autoxidation. Modifications in the heme pocket have been described, which resulted in a decreased oxygen affinity; however, these mutations are usually accompanied by an increase in the autoxidation rate and protein instability [1–3].

Mammalian hemoglobins having a low oxygen affinity and a heme pocket structure essentially the same as human hemoglobin have been described [4]. Ruminant hemoglobins belong to this group. In these hemoglobins, the affinity for 2,3-DPG is lower than for human hemoglobin and oxygen affinity is modulated, in vivo, by the interaction with Cl^- within the erythrocytes. This is a favorable mechanism for hemoglobins that are to be used as artificial oxygen carriers, because 2,3 DPG modulation of oxygen affinity can be effective only when a mixture of hemoglobin and 2,3-DPG is encapsulated, whereas blood plasma is 100 mM in Cl^- and therefore would interact with hemoglobin free in circulation.

The low oxygen affinity of ruminant hemoglobins must reside in their amino acid sequence. Searching for structural differences between the high oxygen and 2,3-DPG affinities of primate hemoglobins vs. the low oxygen and 2,3-DPG affinities of ruminant hemoglobins, we have compared their hydropathy plots [5,6]. No differences were apparent between the α-chains, however, in the β-chains two regions of different hydrophobicity were observed, one comprising a portion of the A-helix and the other a portion of the E-helix. In these regions, consistent sequence differences were observed between primate and ruminant hemoglobins. Human and bovine Hbs were taken as prototypes of the primate and ruminant families. In order to investigate whether these amino acid differences were the basis for the low oxygen affinity of the ruminant hemoglobins, we constructed hybrids of human and bovine hemoglobins [7]. Oxygen equilibrium measurements indicated that the α-human β-bovine hybrid had a lower oxygen affinity than the α-bovine β-human hybrid, confirming the predominant role of the bovine beta chains in conferring a low oxygen affinity. It had been proposed that the intrinsic low oxygen affinity of HbBv is due to the hydrophobic character of the N-terminal residues of the β-chains which stabilizes the low affinity T-structure [8]. However, the oxygen affinity of a mutant HbA carrying the N-terminal residues present in HbBv, retains a high oxygen affinity [9]. To further explore the basis for the low affinity of ruminant hemoglobins, we engineered human hemoglobins carrying amino acid sequences present in either the A or the A and E helices of bovine β-chains [7]. Additionally, β4 threonine was replaced by isoleucine. This substitution is absent in HBv and was introduced for increasing the hydrophobicity of the A-helix. Of these mutants, the 'pseudobovine' mutant, PB4, β(V1M + H2Δ + T4I + P5A) has the low intrinsic oxygen affinity exhibited by HbBv. The 'pseudobovine' PB5, β(V1M + H2Δ + T4I + P5A + A76K) has additional functional characteristics remarkably similar to those of HbBv, namely low intrinsic oxygen affinity regulated by Cl^-. Here, we report further characterization of these and related vari-

ants that provide insight into how new mechanisms may be introduced into the hemoglobin molecule for manipulating its functional properties.

2. Materials and methods

2.1. Plasmid construction, protein expression and purification

Construction of plasmids expressing the mutant β-globins, PB4 and PB5 have been described previously [7]. The additional mutations reported here were constructed using the Promega (Madison, WI, USA) Altered Sites Mutagenesis System. The 934 bp fragment bearing the gene for the fusion protein, NS1-FX-β-globin between HindIII sites of PB4 and PB5 was inserted into pAlter, which had been linearized with HindIII. Orientation of the gene was established by hydrolysis with NcoI and EcoRV. The mutagenic oligonucleotides for the conversion of 4Ile to Thr was: 5′-CGTATGCTGACTGCAGAGGAGAAGTC-3′ and

the one for 8Lys to Gln was 5′-GCTGATTGCTGAAGAGCAGTCTGCGGTTAC-

3′. Residues changed are underlined. The deoxy oligonucleotides were synthesized by DNAgency (2545 Whitehorse Rd., Berwyn, PA 19132, USA). The gel purified 934 bp fragments from HindIII digestion of the mutated genes were inserted into the expression plasmid, pJKO5 [10], which had been digested with HindIII and gel purified. The sequences of the entire β-globin genes were verified by sequencing carried out by the DNA Sequencing Facility in the Department of Genetics, University of Pennsylvania. The mutant NS1-FX-β-globin fusion protein was expressed, cleaved with Factor X_a, and reconstituted with α-globin as previously described for pJKO5 [10].

2.2. Oxygen binding curves

Measurements of pH and Cl^- dependence were obtained using the thin layer dilution technique [11]. The protein concentration was 20–30 mg/ml at 25 °C. The buffer was 50 mM MOPS, HEPES or TAPS depending on the pH of the measurements. Effectors (Cl^-, 2,3-DPG and IHP) were added as specified in the text for the different experiments. The amount of methemoglobin formed during the measurements was estimated by spectral deconvolution of the initial and final spectrum. Measurements in which methemoglobin formation exceeded 10% were discarded. Van't Hoff plots were measured in 50 mM glycine at pH 9.0. The data were analyzed globally as described by Bucci et al. [12].

2.3. CD spectroscopy

The CD spectra were recorded at 15 °C in 20 mM phosphate buffer (pH 7.0) on An AVIV model 202 spectropolarimeter (Aviv Associates, Lakewood NJ, USA). Each spectrum represents the average of eight scans. Carbonmonoxy derivatives were obtained by treating the sample with dithionite and filtering through a Sephadex 25 column that was pre-equilibrated in the measuring buffer. Soret spectra were measured between 450 and 400 nm, scanning every 0.5 nm; Hb concentration was 0.10 mg/ml. Near UV spectra were recorded between 320 and 245 nm, with a step size of 0.1 nm, and a Hb concentration of 0.50 mg/ml.

2.4. Thermal denaturation

An AVIV model 202 spectropolarimeter with a Peltier temperature controller was used to determine the T_m values. The hemoglobin samples, in the carbonmonoxy form, were at a concentration of 0.1 mg/ml, in 10-mM Phosphate buffer at pH 7.0. The change in ellipticity was recorded at 222 nm, under continuous stirring in a 1-cm cuvette. The temperature was increased at a rate of 10 °C/min, from 25 to 100 °C, with a step size of 2 °C. Equilibration at each temperature step was continued for 30 s before data collection, which was averaged over an additional 30 s. The data presented are the averaged results.

2.5. Autoxidation measurements

These were carried out using a HP 8452A diode array spectrophotometer, as previously described [13]. Prior to the measurements, the hemoglobin was treated with dithionite and filtered trough a

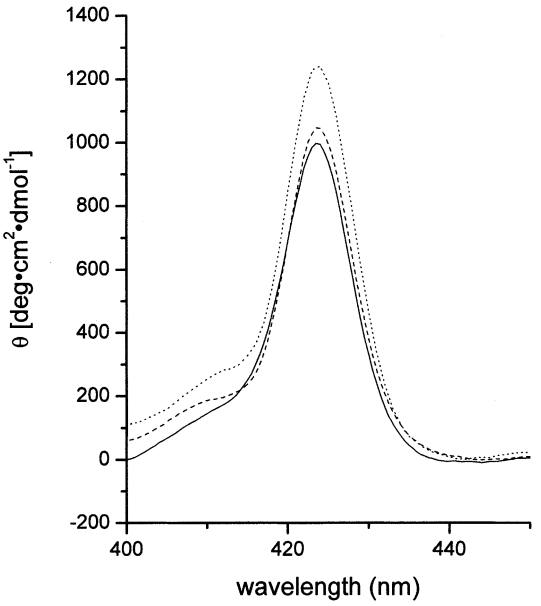

Fig. 1. CD Spectra of the Soret region for CO-liganded HbA (dotted line), rβHbA (dashed lined), and PB5 (solid line). The results are the average of eight scans, with a step size of 0.5 nm. Samples were at a concentration of 0.1 mg/ml in 20 mM phosphate, pH 7.0, and kept at 15 °C during the measurements.

G25 Sephadex column. The measurements were carried out at a protein concentration of 0.1 mg/ml, in a 3-ml cuvette with a screw cap, containing 0.1 M phosphate buffer at pH 7.0, with 1 mM EDTA and beef liver catalase (Boehringer Mannheim) at a molar ratio of 0.003 M heme^{-1}. All experiments were performed at 37 °C. Wavelength scans were taken from 400 to 700 nm at 2 nm intervals and were obtained every 30 min. The absorption spectra were decomposed linearly into a series of predeterminated standard spectra of oxyhemoglobin, deoxyhemoglobin and methemoglobin (at pH 7.0). The wavelength dependence of the baseline was estimated from the difference of the absorption spectra of solutions and after clearing. The fraction of methemoglobin was plotted as function of time and then fitted to a first or second order kinetic equation using Origin 6.1 (OriginLab, Northampton, MA, USA).

2.6. Dimer–tetramer association constant

These measurements were carried on the aquomet derivatives by Dr Maurizio Gattoni in the laboratory of Prof. Emilia Chiancone, using the method described by Gattoni et al. [14,15]. The conditions of the measurements were the same as those for the heme transfer experiments.

2.7. Heme transfer experiments

These experiments were performed and analyzed as described by Gattoni et al. [15,16]. A known amount of immobilized albumin (0.8 ml of packed gel corresponding to approximately 9×10^{-5} mmol) was mixed in a test tube with 2.0 ml of 0.1 M Tris buffer, pH 9.0, plus 0.1 M NaCl containing ferric hemoglobin at concentrations in the range 2.2×10^{-5}–7.2×10^{-5} M. The test tube was placed in a water bath thermostatted at 20 °C. At established times, the solid phase was separated from the supernatant by pressing a filter sampler (Porex Medical, Fairburn, GA, USA) gently into the test tube. The absorbance of the supernatant was measured between 650 and 400 nm, with a mod HP8452 Hewlett Packard spectrophotometer thermostatted at 20 °C. The loss of heme from hemoglobin could thus be followed without interference from the immobilized protein. Alternatively, the affinity of the proteins for heme was measured following the method of Hargrove et al. [17]. Spectra were recorded using an HP model 8452 spectrophotometer, with a water-controlled temperature cell holder. The samples were kept at 25 °C during the experiments. Hb concentration was 3 μM in heme and apoMb was 15 μM. Proteins were buffered in 0.15 M phosphate, 0.45 M sucrose, pH 7.0. Spectra were recorded every 5 min for a total of 600 min. The absorbance at 406 nm for each measurement was plotted and fitted with a second-order kinetics equation with amplitudes fixed at 50:50, using the program Origin 6.1 to obtain the rate constants.

3. Results

3.1. Conformational characteristics and stability to denaturation

The conformation of the heme pocket of natural HbA, rβHbA and PB5 was investigated by observing the CD spectra. In Fig. 1 the Soret CD spectra

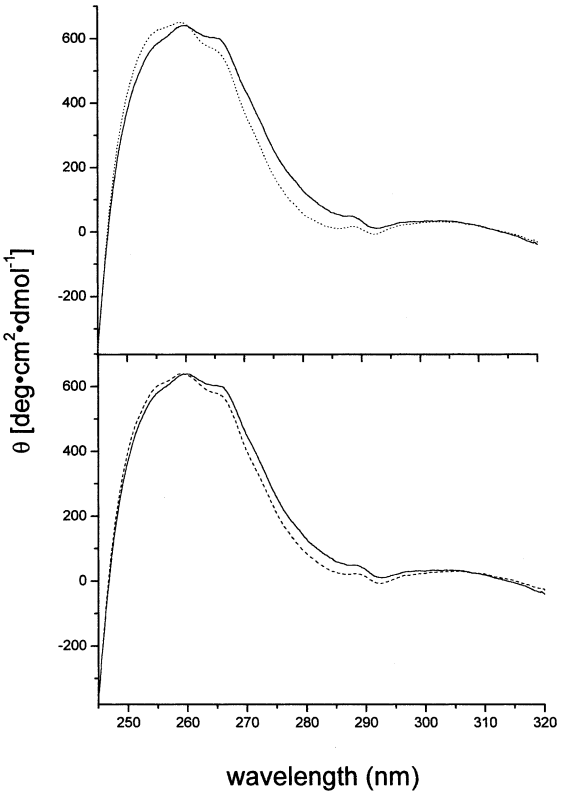

Fig. 2. CD Spectra of the Near-UV region for hemoglobins in the carbonmonoxy form. Top panel: PB5 (solid line), HbA (dotted line). Bottom panel: PB5 (solid line), rβHbA (dashed line). The results are the average of eight scans with a step size of 0.1 nm. Samples were at a concentration of 0.5 mg/ml in 20 mM phosphate, pH 7.0, and kept at 15 °C during the measurements.

indicate that the ellipticity present in HbA is decreased PB5. Fig. 2a,b shows the Near UV spectra of PB5 vs. that of HbA and rβHbA, respectively. Similar modifications in the CD of the PB5 are observed and are more evident in the comparison with HbA. CD spectra in the Far UV (not reported) indicate the secondary structure in these proteins was the same.

The stability toward denaturation of the ferric derivatives of HbA, rβHbA and of PB5 was investigated as a function of temperature. Fig. 3 shows the first derivative of the temperature scanning measurements. HbA and rβHbA have a similar stability, with $T_m = 76$ °C, whereas in PB5 a

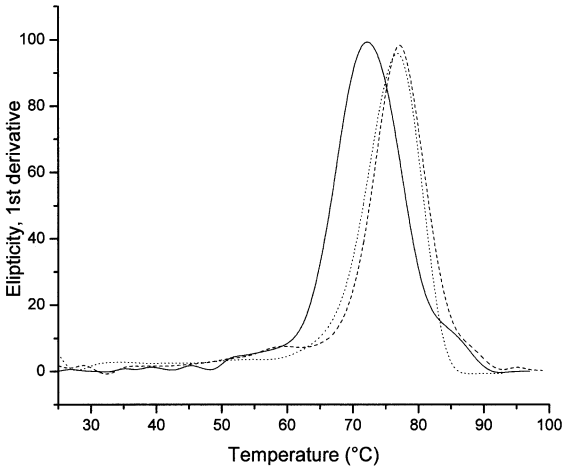

Fig. 3. Thermal Denaturation of CO-liganded HbA (dotted line), rβHbA (dashed line) and PB5 (solid line). The secondary structure unfolding was monitored using the CD signal at 222 nm. Protein concentration was 0.1 mg/ml in 10 mM phosphate, pH 7.0. HbA and βrHbA both have T_m values of 76 °C, while PB5 was a slightly lower 70 °C.

small decrease in stability is observed, with $T_m =$ 70 °C.

3.2. Dimer–tetramer association constants and rate of heme transfer

These measurements were performed on ferric derivatives and the results are listed in Table 1. The dimer–tetramer association constant, $K_{2,4,}$ was

Table 1
Dimer–tetramer association constants and rates of heme release of natural HbA, rβHbA and pseudobovine hemoglobins PB4 and PB5

Proteins	$K_{2,4}$ (M^{-1})	$k_H^F(h^{-1})$ Albumin transfer	$k_H^S(h^{-1})$ Albumin transfer	$k_H^F(h^{-1})$ ApoMb transfer	$k_H^S(h^{-1})$ ApoMb transfer
HbA[a]	8.0×10^5			1.7	0.15
rβHbA[a]	7.2×10^5	3.6	0.2	2.0	0.20
PB4	2.8×10^5	4.2	0.3		
PB5	2.1×10^5	12.0	1.0	6.2	0.3

[a] Taken from [15].

The standard error of the heme transfer experiments is rather high ~30%. Two different methods were used [14,15,17], the results were consistent in indicating a faster rate of heme release by PB5.

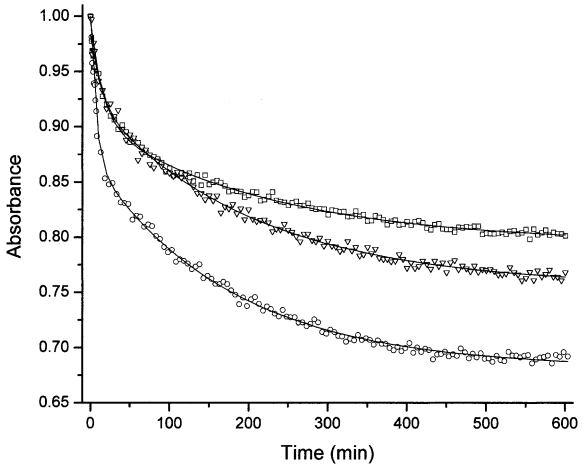

Fig. 4. Heme transfer experiments of HbA (□), rβHbA (▽), and PB5 (○) at 25 °C. Hb samples were in the ferric state, at a concentration of 3–4 μM, and apo-Mb concentration was 25–30 μM. A 1.0 cm path-length, 800 μl quartz cuvette was used for the experiment. The buffer conditions were 0.2 M phosphate, 0.45 M sucrose, pH 7.0. The OD_{406} results were normalized to 1.0 for each of the samples to afford comparison. Each data set was fitted with a 2nd-order exponential curve to obtain the rate constants, with amplitudes fixed at 50:50.

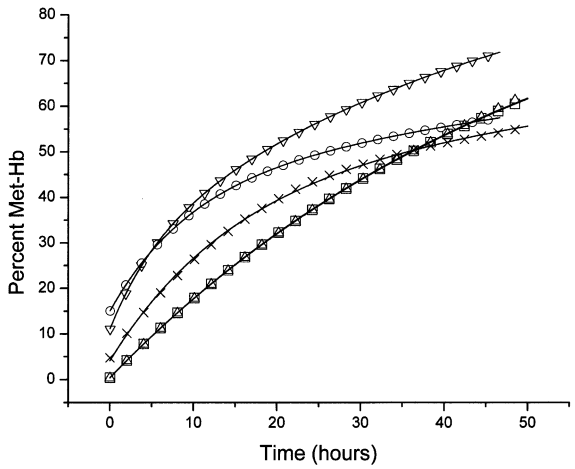

Fig. 5. The rates of autooxidation of HbA (□), HbBv (△), rβHbA (▽), PB4 (×) and PB5 (○) at 37 °C. Protein concentration was 0.1 mg/ml in 0.1 M phosphate, 1 mM EDTA and beef liver catalase in a molar ratio of 0.003 M heme^{-1}.

determined by means of differential gel filtration as previously described [14,15]. This method enables the detection of 1–2% differences in elution volume, giving highly reproducible values. Both PB4 and PB5 are more dissociated than the recombinant rβHbA. The rate of heme transfer was determined by two methods. In one, the transfer occurred to immobilized albumin [15,16], in the other, to apomyoglobin, ApoMb, in the solution [17]. Consistent results were obtained with the two methods. The data were described by a fast k^F and a slow k^S process, approximately one order of magnitude different. Similar rates were obtained for rβHbA and PB4, but the rate of heme loss by PB5 was clearly increased. Representative experiments are shown in (Fig. 4), where the symbols are the experimental data and the lines the fitted curves from which the rates were obtained.

3.3. Autoxidation

Representative first order plots for the autoxidation reaction of HbA, rβHbA and PB5, are shown in Fig. 5. The time courses of the autoxidation were analyzed and the results are listed in Table 2. The autoxidation rates of HbA and HbBv were described by a single exponential, with a rate of 0.019 h^{-1}. In the recombinant hemoglobins, the rate of autoxidation was biphasic, with 10–15-fold difference in the two rates. The slow rate, (80%) of rβHbA, was similar to that of HbA and HbBv, with $t_{1/2}$, ~35 h. In PB4 and PB5 the $t_{1/2}$ of the slow rate was increased 3–4 times to ~130 h, however, the relative amount of this fraction was decreased (~60%). The increased resistance to autoxidation is also reflected in the fast rate of

Table 2
Autoxidation rates of HbA, HbBv, rβHbA and PB5

Protein	k (h^{-1})	%	$t_{1/2}$, (h)
HbA	0.019	100	36.3
HbBv	0.019	100	36.3
rβHbA	0.180	20	3.8
	0.020	80	34.5
PB4	0.068	41	10.0
	0.005	59	138.0
PB5	0.100	34	6.9
	0.006	66	115.0

The error of the measurements was 10% or less. The measurements were carried out at 37 °C in 0.1 M phosphate, pH 7.0.

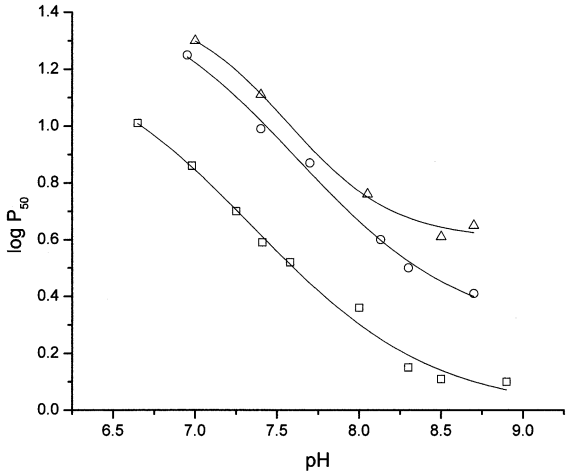

Fig. 6. Bohr effect of HbBv (△); PB5 (○); HbA (□) in 50 mM Mops, Hepes, Taps buffer + 100 mM NaCl. Protein concentration was 30 mg/ml and temperature was 25 °C.

autoxidation of PB4 and PB5, which is decreased with respect to the fast rate of rβHbA.

3.4. Bohr effect

In Fig. 6, the pH dependence of oxygen affinities for HbA, HbBv and PB5 are illustrated. All measurements were carried out in the presence of 100 mM Cl^-. The three hemoglobins have a similar Bohr effect and the oxygen affinity of PB5 with respect to HbA is decreased at all pH values. The number of protons exchanged at pH 7.4 is 0.55/heme. The cooperativity was good, with *n* values comprised between 2.3 and 3.0.

3.5. Sensitivity of oxygen affinity to the presence of effectors

The data listed in Table 3 demonstrate that in the absence of effectors, the oxygen affinity of PB5 and HbBv is approximately 3-fold lower than that of HbA. Upon addition of 100 mM Cl^-, the oxygen affinity of HbA, PB5 and HbBv is similarly decreased between 2- and 3-fold. When organic phosphates (2,3 DPG or IHP) are added in the presence of 100 mM Cl^-, the oxygen affinity of PB5 and HbBv is not modified by 2,3 DPG; a small decrease in affinity is observed in HbBv in the presence of IHP. Conversely, the oxygen affinity of HbA is decreased 2.5- and 9.5-fold in the presence of 2,3-DPG and IHP, respectively.

Table 3
Oxygen affinities of HbA, PB5 and HbBv, in 50 mM Hepes at pH 7.4 in the absence and in the presence of effectors (Cl^-, Cl^- + 2,3-DPH, Cl^- + IHP)

Effectors	HbA P_{50} torr	PB5 P_{50} torr	HbBv P_{50} torr
Absent	1.6	4.6	4.8
100 mM Cl^-	3.7	10.5	12.0
100 mM Cl^- + 5 mM 2,3DPG	10.4	11.0	13.0
100 mM Cl^- + 1mM IHP	35	11.0	16.0

3.6. Van't Hoff plots of HbA, HbBv and PB5

Fig. 7 shows the van't Hoff plot of HbA, PB5 and HbBv. These measurements were carried out in 50 mM glycine at pH 9.0 where the Bohr effect is absent, in order to eliminate the enthalpic contribution associated with protons release. The enthalpy of oxygenation of HbBv is strikingly

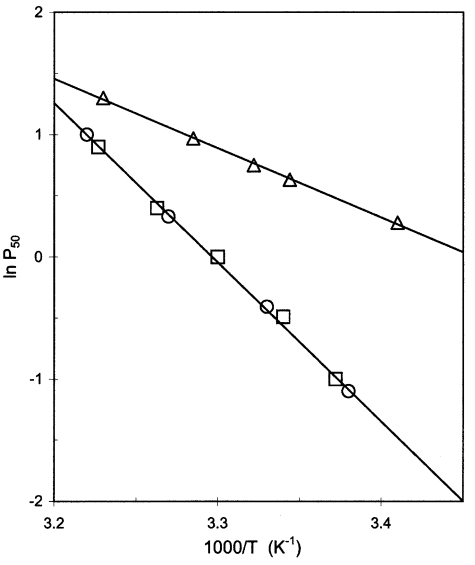

Fig. 7. Van't Hoff plot for the temperature dependence of the oxygen affinity of HbBv (△) HbA (□) and PB5 (○). Buffer was 50 mM glycine at pH 9.0. Protein concentration was 25 mg/ml.

Table 4
Pseudobovine hemoglobins constructed and characterized in our laboratory

Hemoglobin	Mutations introduced	Log $P_{50\ (torr)}$ in the absence of Cl^-	Number of oxygen linked Cl^- binding sites	References
PB2	V1M + H2Δ (deleted)	0.23	1.2	9
PB4	V1M + H2Δ + T4I[a] + P5A	0.60	1.2	7
PB5	V1M + H2Δ + T4I[a] + P5A + A76K	0.60	2.2	7
$PB5_{control}$	V1M + H2Δ + P5A + A76K	0.23	2.2	
PB6	V1M + H2Δ + T4I[a] + P5A + K8Q + A76K	0.60	1.2	
HbA		0.18	2.2	7
HbBv		0.62	2.2	7

[a] In both human and bovine hemoglobins this residue is a Thr.

different from that observed for HbA and PB5. Correction for the heat of solution of oxygen (−3.0 Kcal/mol) yields an enthalpy value of −7.0 kcal/mol for HbBv, while for HbA and PB5 the enthalpy value is of −20.0 Kcal/mol.

4. Discussion

The development of recombinant techniques offers the possibility of constructing mutant hemoglobins with functional and conformational characteristics, tailored to specific therapeutic needs. With the aim of obtaining a human hemoglobin with a decreased oxygen affinity and Cl^- acting as the principal effector, we have substituted certain residues present in HbA with those present at analogous positions in HbBv [6,7,9]. These pseudobovine hemoglobins are listed in Table 4, with their oxygen affinities and the number of oxygen linked Cl^- binding sites.

It has been proposed that the low oxygen affinity of HbBv with respect to HbA is due to the amino acid composition of the N-terminal end of the HbBv β-chains, which can move toward the central cavity of the molecule, stabilizing the low affinity T-structure [8]. Crystallographic and functional analyses on PB2 indicate that the substitution of the β-N-terminal Val with Met and the deletion of β2His, abolishes an anion binding site between the amino terminus and the EF corner and slightly decreases the oxygen affinity [9]. Two additional substitutions were introduced into PB2 to give PB4: β4Thr was replaced with Ile and β5Pro was replaced by Ala, the residue at that position in HbBv. Although Thr is the 4th residue in both HbA and HbBv, molecular modeling suggested that an Ile at this position would extend into the hydrophobic pocket formed by the side chains from β3Leu, β78 Leu, β81 Leu and β133 Val, providing additional stabilization of the T-state and consequently lowering the oxygen affinity. The Pro to Ala substitution should increase the flexibility of the β-N-terminal residues that might also increase stabilization of the T-state. Oxygen binding measurements showed that PB4 had a low intrinsic oxygen affinity similar to HbBv, but had a decreased Cl^- sensitivity [7]. PB5 has all the mutations present in PB4 plus β76Ala was replaced by Lys, one of the more prominent differences between the E helices of human and bovine β-chains. This pseudobovine Hb has the functional characteristics of HbBv, namely a low intrinsic oxygen affinity that is subject to Cl^- concentration. Based on these results, we proposed that the oxygen linked Cl^- binding site of PB5 involved β8Lys, β76Lys and possibly β77His, all within individual β-subunits [7]. The amino acid substitutions of PB4 and PB5 have minimal effects on the protein conformation and stability and they greatly increase their resistance to autoxidation. Thus, these mutants represent a significant advance in the development of hemoglobin-based oxygen carriers. The critical role of an isoleucine at position β4 for introducing the low intrinsic oxygen affinity in PB4 and PB5 is evident from the results obtained with $PB5_{control}$ (Table 4 and Fig. 9). In

Table 5
Amino acid differences at the $\alpha_1\beta_1$ interface between HbA and HbBv

HbA	α104C	α111A	α115A	α116E	β112C	β116H	β117H	β125P	β129A
HbBv	S	S	S	D	V	R	N	V	D

this mutant, β4Thr has not been replaced by isoleucine and the intrinsic oxygen affinity remains similar to that of PB2.

4.1. Conformational data

CD spectra in the Soret region are sensitive to the position of the heme in relation to the aromatic residues lining the heme pocket (Fig. 1). A decreased ellipticity of rβHbA and PB5 with respect to HbA is observed. The decrease is not associated with a spectral shift. It could be due to an increased amount of inverted heme in recombinant hemoglobins [18] or to a non-perfect folding of the heme pocket of the recombinant subunit [19]. In the Near UV, differences in the spectrum are observed between 260 and 300 nm (Fig. 2). The ellipticity in this region of the spectrum results from the aromatic residues [20,21]. The CD spectra indicate a difference in ellipticity at the level of tryptophan, (270–280 nm) and phenylalanine (~265 nm). Modifications at the level of tryptophan may be consistent with the increased dissociability of PB5, altering the exposure of β37 tryptophan at the $\alpha_1\beta_2$ interface. The possible relevance of the modification at the level of the phenylalanine is discussed later. PB5 has a decreased stability toward denaturation ($T_m = 70$ °C) with respect to HbA, rβHbA ($T_m = 76$ °C). PB5 is more dissociated than rβHbA; the decrease in T_m observed may be due, at least in part, to the increased fraction of dimers present in solution.

4.2. Functional data

A correlation was not always observed between the dimer–tertramer association constant, the rate of heme release and the rate of autoxidation. A similar observation has been reported studying a different group of mutant recombinant hemoglobins [15]. Both PB4 and PB5 are more dissociated than rβHbA, (Table 1). These hemoglobins have the same amino acid substitutions at the N-terminal end and the replacement β76Ala→Lys does not affect the dissocialbility of PB5. A relationship of the N-terminal residues of the β-chains and the conformational characteristics of the $\alpha_1\beta_2$ interface, also reflected in dimer formation, has been observed [14,22,23].

While the autoxidation of natural HbA and HbBv is described by a single rate, in the recombinant proteins a second rate approximately one order of magnitude faster is observed. The fraction of hemoglobin undergoing this faster rate is 20 and 34% for rβHbA and PB5, respectively. At the protein concentration used for these measurements the fraction of dimers present in HbA and rβHbA is similar (20–25%), however, only in rβHbA is a faster oxidation rate is observed. The molecular origin of this phenomenon is not clear at present. It may be due to the larger amount of inverted heme present in the recombinant proteins, although the two conformers have similar ligand affinity [24,25]. A non-perfect refolding of some of the heme pockets of the recombinant β-subunits could also be present [19]. Surprisingly, PB5 has an increased stability toward autoxidation. This is an unusual feature since a decrease in oxygen affinity is usually associated with an increase in the autoxidation rate. Also, the increased rate of heme transfer measured for PB5 is at variance with the higher resistance to autoxidation present in PB5. The only conformational evidence on which we can tentatively speculate, is the modified ellipticity observed for the aromatic residues in the Near UV circular dichroism measurements (Fig. 2). There are two phenylalanine residues in the heme pocket of the α and β chains, Phe $\alpha^{43}\beta^{42}$ (CD1) and Phe $\alpha^{42}\beta^{45}$(CD4); a different orientation of these residues in the heme pocket of PB5 could alter the geometry of the heme pocket and the processes there associated [26,27].

The functional characteristics of PB5 and HbBv are very similar, as indicated by the Bohr effect

(Fig. 6) and sensitivity to organic effectors (Table 3). The temperature dependence of oxygen affinity shown in Fig. 7 shows that HbA and PB5 have a similar van't Hoff with enthalpy value ($\Delta H = -20.0$ Kcal/heme). This indicates that the mutations introduced do not modify the overall cooperative mechanism present in the two hemoglobins. Conversely, the enthalpy value of HbBv ($\Delta H = -7.0$ Kcal/heme) suggests a different interaction of HbBv with the solvent and the possible modification of the mechanism that controls ligand affinity in this hemoglobin. Consistent with these data, recent crystallographic data of R-state HbBv indicate that this protein has a quaternary structure more compact than R-state HbA, and that the α and β subunit heme groups, are not fully relaxed in the R-state [28].

4.3. Molecular mechanisms regulating the functional characteristics of pseudobovine hemoglobins and HbBv

Two different aspects must be considered, the low intrinsic oxygen affinity, and the role of Cl^- as principal regulator of oxygen affinity in these hemoglobins.

Our data on PB4 and PB5 and $PB5_{control}$ (Table 4) clearly indicate that the amino acid sequence present at the βN-terminal end of HbBv is not sufficient to introduce a low intrinsic oxygen affinity in the Hb. This is obtained only when an isoleucine is present at position β4. This suggests a different molecular mechanism responsible for the low intrinsic oxygen affinity of HbBv and PB4 and PB5. Inspection of the sequence of HbA and HbBv indicates the presence at the $\alpha_1\beta_1$ interface of nine amino acid differences, listed in Table 5. These differences have been selected on the basis of residues within 6.0 Å or less from the partner subunit at the interface [29]. Fig. 8 shows that these substitutions span the entire interface. Future work will determine the importance of this observation. The relevance of the residues at the $\alpha_1\beta_1$ interface on the functional characteristics of hemoglobin has been recently demonstrated with recombinant human hemoglobin mutants [14,15,30,31]

In a previous paper, we postulated the presence in PB5 of an oxygen linked Cl^- binding site,

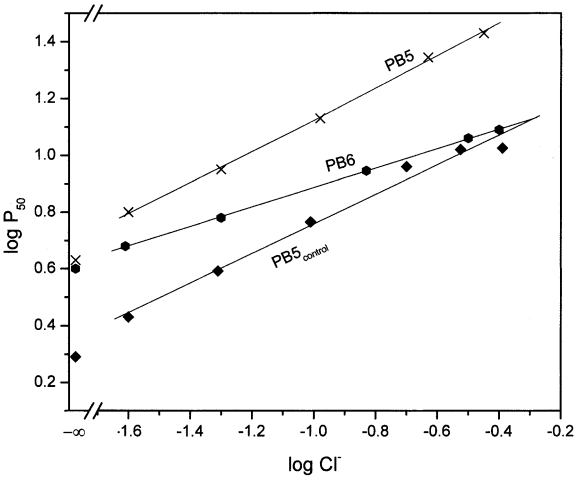

Fig. 8. Oxygen affinity of PB5 (×), PB6 (●), $PB5_{control}$ (◆) in 50 mM Hepes buffer at pH 7.4 at increasing Cl^- concentration. Protein concentration was 30 mg/ml and temperature was 25 °C.

comprised of β8Lys and β76Lys [7] and regulated by tertiary conformational changes at the N-terminals of the A-helix. In order to verify this proposition, we have constructed a mutant Hb, PB6, β(V1M + H2del + T4I + P5A + K8Q + A76K) (Table 4), in which the replacement of β8Lys, present in both PB5 and HbBv, by a Gln, results in loss of the Cl^- binding site. As shown in Fig. 9, this mutant behaves very similarly to PB4. It has a low intrinsic oxygen affinity, but has lost the Cl^- regulation of oxygen affinity. The amino acids establishing this Cl^- binding site in PB5 are also present in HbBv, suggesting that a similar Cl^- binding site may also be present in HbBv.

In conclusion, we have obtained a recombinant hemoglobin having a low oxygen affinity, regulated by Cl^-, and an increased resistance to autoxidation. From this study, we have also gained new insight into the regulatory mechanisms that can be introduced in hemoglobin. Mutations that increase the hydrophobic interactions between the A-helix and the hydrophobic core of the β-subunits decrease the oxygen affinity. The possibility of engineering a regulatory mechanism in which the Cl^- ions in the solution are the principal effectors has been demonstrated, by engineering oxygen

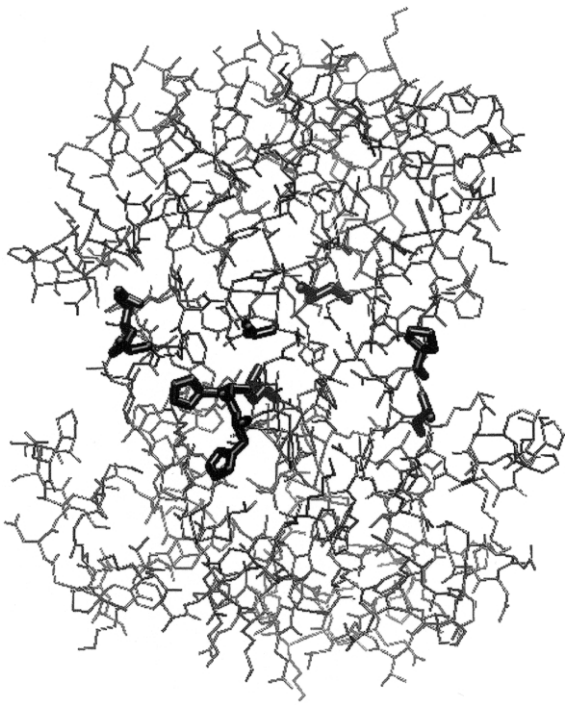

Fig. 9. Residue differences between HbA and HbBv in the α1β1 interface (α-chains blue, β-chains red). The structure is a dimer of HbA (PDB ID: 2hhb [32]). The nine amino acid differences are listed in Table 5.

linked Cl^- binding sites far removed from any interface.

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