Familial secondary erythrocytosis due to increased oxygen affinity is caused by destabilization of the T state of hemoglobin Brigham ($\alpha_2\beta_2^{\text{Pro100Leu}}$)

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Abstract: Hemoglobin Brigham (β Pro100 to Leu) was first reported in a patient with familial erythrocytosis. Erythrocytes of an affected individual from the same family contain both HbA and Hb Brigham and exhibit elevated O₂ affinity compared with normal cells (P_{50} = 23 mm Hg vs. 31 mmHg at pH 7.4 at 37°C). O₂ affinities measured for hemolysates were sensitive to changes in pH or chloride concentrations, indicating little change in the Bohr and Chloride effects. Hb Brigham was separated from normal HbA by nondenaturing cation exchange liquid chromatography, and the amino acid substitution was verified by mass spectrometry. The properties of Hb Brigham isolated from the patient's blood were then compared with those of recombinant Hb Brigham expressed in *Escherichia coli*. Kinetic experiments suggest that the rate constants for ligand binding and release in the high (R) and low (T) affinity quaternary states of Hb Brigham are similar to those of native hemoglobin. However, the Brigham mutation decreases the T to R equilibrium constant (L) which accelerates the switch to the R state during ligand binding to deoxy-Hb, increasing the rate of association by approximately twofold, and decelerates the switch during ligand dissociation from HbO₂, decreasing the rate approximately twofold. These kinetic data help explain the high O₂ affinity characteristics of Hb Brigham and provide further evidence for the importance of the contribution of Pro100 to intersubunit contacts and stabilization of the T quaternary structure.

Keywords: hemoglobin; allostery; kinetics; ligand binding

Abbreviations: ACN, acetonitrile; CO, carbon monoxide; DT, sodium dithionite; DTT, dithiothreitol; HbA, hemoglobin; HPLC, high-performance liquid chromatography; Met, ferric iron oxidation state; O₂, oxygen; OEC, oxygen equilibrium curve; rHb, recombinant hemoglobin; TFA, trifluoroacetic acid.

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Introduction

Normal adult human Hemoglobin A (HbA) is a highly conserved oxygen (O₂) transport protein that is found in the erythrocytes of all vertebrates.^{1–3} This tetramer consists of two alpha (α) and two beta (β) subunits, each bearing a single ferroporphyrin IX (heme) prosthetic group which is responsible for reversible O₂ binding. There are over a thousand known mutant hemoglobins (Hbs) that are caused by single point mutations.⁴ Some of these mutants are associated with hematological disorders such as anemia or erythrocytosis, but others are asymptomatic. Over the years, investigations of these abnormal Hbs and their functional differences have provided considerable insight into our current understanding of structure-function relationships in the Hb molecule and O₂ transport in the microcirculation.^{2,3,5}

Hb Brigham was discovered as a variant in which one or both β subunit proline 100 (G2) residues are mutated to leucines (βPro100Leu; βP100L) in a patient with mild erythrocytosis (hematocrit ~55%) and lowered red cell *P*₅₀ values.⁶ In individuals affected with this hemoglobinopathy, the presence of large amounts of mutant Hb suggest that the βP100L replacement is not significantly destabilizing.⁶ Moreover, hemolysates containing mixtures of HbA and Brigham Hb show relatively normal Bohr effect properties and sensitivity to the allosteric effector 2,3-bisphosphoglycerate (BPG).⁶

Several similar Hb variants involving proline to leucine substitutions have previously been identified, including Hb Manawatu (αP37L (C2)), Milledgeville (αP44L (CE2)), Georgia (αP95L (G2)), and Nouakchott (αP114L (GH2)).^{7–11} Like Hb Brigham, these mutations have not been associated with severe hematological phenotypes.^{7–11} These studies suggest that proline to leucine substitutions in Hb tend to result in variant tetramers that have physical properties similar to wild-type HbA and are, in some cases, indistinguishable by electrophoresis.^{7–11} This identity in charge impedes clinical detection and complicates chromatographic separation of the variant tetramers. When they are identified, these mutants are often only characterized in hemolysates containing native HbA, making it difficult to determine the effects of the proline replacements on overall function.

In the present study, we describe a novel method for chromatographically separating intact Hb Brigham from wild-type HbA using hemolysates obtained from an affected heterozygous individual. We also constructed the genes for recombinant Hb Brigham (α(wt)β(P100L)), expressed them in *Escherichia coli*, purified the rHb mutant tetramer, and showed its properties were identical to those of the protein obtained from the patient's blood, further verifying the purification procedure. Consistent with the earlier work of Lokich *et al.*,⁶ we find that Hb Brigham exhibits an elevated O₂ affinity. Rapid mixing and flash photolysis studies on separated frac-

tions suggest that this effect is due to destabilization of the low affinity (T) quaternary state, which favors formation of the high-affinity (R) state and leads to impaired O₂ off-loading in peripheral tissues. The probable mechanism for this behavior is disruption of interactions between the native β P100, βT34, and other nearby residues by the mutant L100 side chain. The resultant decrease in efficiency of O₂ transport per Hb molecule is compensated by increased production of red cells and erythrocytosis.

Results

Case report

The subject of this study was a 39-year-old female referred to the National Institutes of Health for evaluation of mild erythrocytosis, recent deep vein thrombosis, and a pulmonary embolism, which were thought to have been precipitated by air travel and her underlying mild polycythemia. Her Hb was elevated (16.3 g/dL, reference range 11.2–15.7 g/dL), but the erythropoietin level was normal (10.6 mIU/mL, reference range 3.7–31.5 mIU/mL). Venous blood was analyzed and showed a pH of 7.38, a pO₂ of 19 mmHg, a percent saturation of 50.7%, and a pCO₂ of 44 mmHg. An initial algorithm^{12,13} was used to estimate the O₂ partial pressures that give rise to 50% Hb saturation (*P*₅₀). Preliminary data suggested a *P*₅₀ value of 18.4 mmHg, consistent with a high O₂ affinity Hb variant.

Oxygen affinity and Bohr properties for intact red cells and hemolysates

We measured *P*₅₀ values and O₂ binding cooperativity (Hill coefficient; *n*₅₀) for intact erythrocytes from the patient using established methods.¹⁴ Representative data are depicted in Figure 1(A). Consistent with the report of Lokich *et al.*,⁶ we find that erythrocytes containing Hb Brigham and HbA mixtures exhibit lower *P*₅₀ values than control erythrocytes, 23.6 mmHg as compared with 31.1 mmHg, respectively. We also observed that red cells containing Hb Brigham exhibit less cooperativity for O₂ binding than normal erythrocytes [*n*₅₀ = 2.2 vs. *n*₅₀ = 2.7 for control erythrocytes; Fig. 1(B)]. We also measured the effect of pH on O₂ affinity (Bohr Effect) and the effect of chloride ion (Cl[−]) concentration on the O₂ affinity of BPG-depleted hemolysates. Both the Bohr Effect and Cl[−] coefficients were evaluated from the maximal slopes of the plots of *P*₅₀ as a function of pH and [Cl[−]], respectively. These data are presented in Table I and indicate that the heterozygotic red cells containing Hb Brigham possess normal Bohr and Cl[−] effects.^{16,17}

To confirm these findings, we expressed and purified recombinant Hb Brigham using established techniques¹⁸ and conducted oxygen equilibrium measurements using pure mutant protein, which is completely free of wild-type HbA. As the plots in

Figure 1. Oxygen binding equilibria of intact erythrocytes in whole blood. A. Oxygen equilibrium curves of intact red cells. B. Hill plots. C. Oxygen equilibrium curves of hemolysates and recombinant Hb Brigham. Experiments were conducted in 30 mM *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid, 135 mM NaCl, 5 mM KCl, pH 7.4 at 37°C. Samples contained approximately 1 mg/mL bovine serum albumin and 4 μ L/mL of polydimethylsiloxane to mitigate foaming (AFA-25; TCS Scientific, New Hope, PA). Measurements were made within 6 h of venipuncture, and samples were stored on ice before use. Whole blood was used for all measurements, diluted to an Hb concentration of approximately 75 μ M in heme in volumes of approximately 3 mL. Data were collected in 5 mmHg increments of O₂ partial pressures between 0 and 150 mmHg. Lines in B were generated using the least squares method and Excel (Microsoft Corporation, Redmond, WA). Y, fractional O₂ saturation as estimated by the instrument.

Figure 1(C) show, recombinant Hb Brigham possesses a much higher O₂ affinity than wild-type HbA. The P_{50} values are 3.8 and 15.6 mmHg for mutant recombinant and native HbA, respectively, under these *in vitro* conditions.

Separation of Hb Brigham from wild-type HbA

Erythrocyte lysates from our patient were subjected to hydrophobic interaction and ion exchange chro-

matography using various salts, ionic strengths, pH values, and chromatography media. We found that it was possible to resolve HbA from Brigham Hb in patient hemolysates using a 5 mm $\times$ 100 mm Protein Pak SP 8HR prepac column (1000 Å, 8 μ m particle size) with a 50 mM sodium phosphate buffer, pH 5.8 at 22°C, and isocratic elution. This elution resulted in several chromatographic peaks. We analyzed all fractions obtained from these separations using the methods described below and compared the observed peaks with those observed from control hemolysates. We found that one peak present in Hb Brigham hemolysates differed from the peaks observed for the control hemolysates. Representative portions of these chromatograms are shown in Figure 2(A,B). As shown, samples from homozygotic HbA erythrocytes elute as a single fraction in this region, whereas samples from heterozygotic cells containing Hb Brigham elute in two fractions. We refer to these fractions as F_I and F_{II}.

Verification of chromatographic fractions

Samples of wild-type HbA, F_I, and F_{II} were subjected to reversed-phase chromatography to investigate their composition. Figure 2(C) shows the elution profiles of these samples superimposed for comparison. As has been established in previous work,^{19,20} β - and α -globin chains elute at approximately 26 and 28 min postinjection, respectively, and the heme group elutes at 17 min. Upon comparison of the observed chromatograms, it is clear that the heme, α -globin, and β -globin peaks from F_I are similar to wild-type HbA. However, the peak at approximately 26 min corresponding to native β -globin is decreased significantly and replaced by a peak at approximately 32 min in F_{II}. This delayed retention time indicates the presence of a mutated amino acid with increased hydrophobicity, and strongly suggests that F_{II} consists of a mixture of wild-type β subunits and β P100L subunits. This delayed retention is consistent with other studies involving Pro to Leu Hb point mutations.^{9,21–25}

Based on our initial HPLC data, we hypothesized that F_I contains wild-type HbA and F_{II} contains Hb Brigham consisting of tetramers with two mutated β subunits. To investigate further, we

Table I. Bohr Effect and the Effect of Chloride on O₂ Binding Properties of Hb Brigham

Sample	$\Delta\text{Log}(P_{50})/\Delta\text{pH}$	$-\text{H}^+/4\text{O}_2$	$\Delta\text{Log}(P_{50})/\Delta\text{log}[\text{Cl}^-]$	$[\text{Cl}^-]/4\text{O}_2$
Control Hb	-0.31	1.2	0.25	1.0
Brigham Hb	-0.30	1.2	0.29	1.1

Acidic and alkaline Bohr effects were measured in hemolysates from Brigham patient and control using 0.1M Bis-Tris buffer in the pH range of 5.5 to 7.0, at 37°C, and 0.1M Tris buffer in the pH range of 7.5 to 9.0, at 37°C. Samples were depleted of BPG by passage through a column containing Sephadex G-25 media before use. All preparative steps were done at 4°C to reduce oxidative damage, denaturation, and precipitation. Chloride binding was measured using 0.1M HEPES buffer, pH 7.4 at 37°C, and the same buffer containing 1.0M NaCl. Samples contained approximately 4 μ L/mL of polydimethylsiloxane as an anti-foaming agent (AFA-25; TCS Scientific, New Hope, PA), as well as approximately 5 μ L/mL of hexamethylphosphoramide. The Hayashi reduction system¹⁵ was used to control autoxidation during these measurements.

Figure 2. Chromatography of wild-type HbA and Hb Brigham. A. Strong cation exchange chromatography of wild-type HbA. B. Strong cation exchange chromatography of wild-type HbA and Hb Brigham. C. Reversed-phase chromatography of HbA, Fraction I (F_I), and Fraction II (F_{II}). Separation conditions are described in the Materials and Methods section. Protein elution was monitored by optical absorbance at 280 nm.

utilized both LC-ESI-MS (for intact mass measurement) and LC/MS/MS (tandem mass spectrometry) analysis to qualitatively assess the subunit content represented in Brigham patient hemolysate and F_I and F_{II} fractions, respectively. We measured the intact masses of all detectable species present in the Brigham patient's hemolysates using a single quadrupole Agilent HP 1100 MSD series mass spectrometer. In these samples, we detected the presence of only wild-type α , wild-type β , and $\beta P100L$ subunits, with all subunits exhibiting near complete N-terminal methionine cleavage. No other variant globins were detected. Data from these experiments are set forth in Figure 3. The deconvoluted spectra of our wild-type control HbA show α and β subunits at $\sim 15,127$ Da and $\sim 15,868$ Da, respectively, which agrees with the literature values of $15,126.7 \pm 1.4$ Da for α and $15,867.4 \pm 0.7$ Da for β [Fig. 3(B)].²⁶ By contrast, Hb Brigham hemolysates show three peaks at $\sim 15,127$, $\sim 15,868$, and $\sim 15,884$ Da, corresponding to α , $\beta 100P$, and $\beta P100L$ subunits, respectively [Fig. 3(A,C)].

Patient-derived F_I and F_{II} were investigated by tandem mass spectrometry following trypsin digestion. The data from these fractions were subjected to MASCOT searches followed by MassSieve analysis as described in the Materials and Methods section for a semiquantitative assessment of the HPLC data. Interpretation of these analyses suggests that F_{II} contains a higher abundance of the Hb Brigham

mutant as signified by a majority of spectra matched to peptides that contain the leucine substitution at $\beta P100$ (11 spectra matching $\beta(83-104)$). Two spectra matching $\beta(83-104)$ contain proline at $\beta P100$ were also obtained suggesting minor contamination of HbA. On the other hand, the F_I analysis resulted in seven spectra matching $\beta(83-104)$ attributed to HbA. No spectra could be matched to $\beta(83-104)$ containing the leucine substitution at $\beta P100$ suggesting that F_I consisted either solely or primarily of HbA. The MS/MS spectra, showing the fragmentation pattern of the b and y ions, corresponding to the MH_2^+ ion of F_I and F_{II} $\beta(83-104)$ peptides are shown in Figure 3(D,E), respectively. Taken together the results from both intact mass data and LC/MS/MS experiments correlate with and further support the identification of F_{II} as Hb Brigham containing the $\beta P100L$ mutation.

Autoxidation studies

Autoxidation of samples of wild-type HbA, F_I , and F_{II} were measured in air-equilibrated phosphate buffer at pH 7.4, 37°C. All three samples exhibited nearly identical autoxidation rates and spectra. Representative spectra for the autoxidation of F_{II} are shown in Figure 4(A), and time courses for the decreases in absorbance at 576 nm due to autoxidation of both fractions and an HbA control are shown in Figure 4(B) along with the fitted rate time courses. Before the start of each reaction, spectra were recorded to confirm that less than 5% of the starting materials contained metHb.²⁷

The time courses show that the $\beta P100L$ mutation does not appreciably affect the rate of autoxidation, nor does it appear to affect Hb stability as estimated by the lack of solution turbidity and precipitation during and after the autoxidation process. The observed rates for autoxidation of the native controls were somewhat larger than literature values for tetrameric HbA.²⁸⁻³⁴ However, our studies were conducted using 30 μM Hb, and at these concentrations a significant fraction of HbO_2 is present as dimers, which are known to autoxidize more rapidly than tetramers.^{28,32}

Hb Brigham ligand binding and dissociation kinetics

To further investigate the ligand binding differences between HbA and Hb Brigham, we measured the rates of ligand binding to and dissociation from HbA, isolated Hb Brigham F_I and F_{II} , and recombinant Hb Brigham. We observed significant differences between native HbA and Hb Brigham when CO binding to the deoxy-Hb T-state forms was measured in rapid mixing experiments [Fig. 5(A-C) and Table II]. Both deoxy-HbA and F_I show identical accelerating time courses with an apparent $k'_{CO} \approx 0.20 \mu M^{-1}s^{-1}$. In contrast, F_{II} shows a more rapid CO

Figure 3. Mass spectrometry of Brigham hemolysate and fractions I and II. A. LC-ESI mass spectrum of Hb Brigham hemolysate denoting the charge states corresponding to α and β globin chains. The inset shows the ions corresponding to the $\alpha(+18)$ and two $\beta(+19)$ charge states. B. Deconvoluted masses corresponding to our wild-type HbA control (α : 15,127 Da; β : 15,868 Da). C. Deconvoluted masses corresponding to Brigham hemolysate (α : 15,127 Da; β : 15,868 Da; β : 15,884 Da). In these data, an additional peak with a +16 Da increase was observed that corresponds to the β P100L mutation. D. MSMS spectra of the MH_2^+ ion at m/z 1293.65 corresponding to $\beta(83-104)$ peptide from F_I. E. MH_2^+ ion at m/z 1301.97 corresponding to $\beta(83-104)$ peptide from F_{II}. The cysteines identified in each peptide in panels D and E are carboxaminomethylated cysteines. The fragmentation pattern showing the b and y ions are indicated (ions marked with * indicates loss of NH_3 or H_2O). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

binding, both in the presence and absence of IHP [Fig. 5(A,B)], this result was verified with recombinant Hb Brigham [Fig. 5(C)]. This finding suggests that the β P100L mutation exerts its effects through destabilization of the T-state of Hb as successive ligands bind, resulting in an approximately twofold higher net rate of ligand uptake.

We also examined the overall rate of O_2 dissociation by mixing HbO_2 complexes with anaerobic buffer containing high concentrations of sodium dithionite to scavenge all free O_2 [Fig. 6(A,B)]. This experiment mimics O_2 off loading in capillaries adjacent to rapidly respiring tissues where the O_2 tension is low. The fitted apparent overall rates of O_2

Figure 4. Autoxidation rates and accompanying spectral changes for HbA, F_I , and F_{II} fractions. A. F_{II} autoxidation. B. Absorbance changes at 576 nm. The time courses in B were fit to a first-order exponential decay expression using Excel (Microsoft Corporation, Redmond, WA). Wild-type HbA and F_I exhibited similar spectra to the data shown in A.

dissociation (Table II) from recombinant Hb Brigham and F_{II} from the Hb Brigham patient are approximately twofold smaller than those of HbA, as would be expected if the mutant were stabilized in the high affinity (R) quaternary conformation and prevented switching to the T-state until two ligands had been off-loaded. The presence of IHP had a smaller effect on O_2 dissociation from Hb Brigham (F_{II}) than from HbA (F_I) [Fig. 6(A)]. IHP decreases O_2 affinity by stabilization of the T-state, which has a 100-fold higher rate of O_2 dissociation.^{35,36} Thus,

Figure 5. Time courses for CO binding to Hb. A. CO binding to HbA, F_I , F_{II} , and patient-derived hemolysate. B. Effects of IHP binding on CO binding rate. C. CO binding to recombinant Hb Brigham. Concentrations of Hb, CO, and IHP are 4, 23, and 60 μM postmixing and in heme equivalents where applicable. Absorbance changes were followed at 430 nm and experiments were conducted using 50 mM Bis-Tris, pH 7.0 at 23°C. 5 mg/mL sodium dithionite was included in both syringes to reduce the protein and to eliminate ambient O_2 contamination.

Table II. CO Binding (k'_{CO}) and O_2 Dissociation (k_{O_2}) Rate Constants for HbA, F_I , F_{II} , and Recombinant Hb Brigham Determined by Rapid Mixing Stopped-Flow Spectrophotometry

Sample	$k'_{CO, \text{ apparent}}$ ($\mu M^{-1} s^{-1}$)	k_{O_2} (s^{-1})
Wild-type HbA	0.19	39.6
Wild-type HbA (+IHP)	0.08	51.0
F_I	0.17	38.4
F_I (IHP)	0.09	69.2
F_{II}	0.27	33.9
F_{II} (+IHP)	0.12	34.7
Recombinant Brigham	0.27 (0.15)	21 (36)
Recombinant Brigham (+IHP)	0.11 (0.06)	33 (54)

To measure CO binding, anaerobic solutions of protein in 0.05M Bis-Tris, 5 mg/mL sodium dithionite, pH 7.0 at 22°C, were rapidly mixed with solutions of the identical buffer plus CO gas. Postmixing Hb and CO concentrations were 4 μM and 23 μM , respectively. Absorbance changes at 430 nm were recorded. Because these reactions are either accelerating, as in the case of HbA, or slight biphasic, as in the case of the mutant proteins, the apparent association rate constants, k'_{CO} , were computed from the half-times of the reactions and the ligand concentration. These values are similar to those obtained by single exponential fitting. Oxygen dissociation measurements were done using 0.05M Bis-Tris buffer, pH 7.4 at 22°C. Solutions of Hb were rapidly mixed with solutions containing 1.5 mg/mL sodium dithionite. Postmixing heme concentrations were 30.0 μM , and absorbance changes were followed at 437.5 nm. The parenthetical values listed in the recombinant protein rows correspond to wild-type HbA data collected on the same day under identical conditions.

the smaller effect on F_{II} and recombinant Hb Brigham supports the conclusion that the P100L mutation increases O_2 affinity by destabilizing the T-state conformation in the mutant protein.

Figure 6. Oxygen dissociation following rapid mixing of HbO₂ with excess sodium dithionite. A. Effects of inositol hexaphosphate for HbA and F_{II} . B. Representative time courses for HbA and rHb Brigham. In A, solutions of 15 μM Hb in 0.1M Tris buffer, pH 7.4 at 25°C, were rapidly mixed with solutions of anaerobic buffer containing 1.5 mg/mL sodium dithionite. Where indicated, Hb samples were preincubated with the indicated postmixing concentrations of inositol hexaphosphate (IHP). Absorbance changes at 437.5 nm were recorded and the observed rates for HbA (○) and Hb Brigham, F_{II} (●) have been plotted to show the effects of IHP on O_2 dissociation. In B, premixing concentrations of 10 μM in heme were and 4 mg/mL sodium dithionite in 50 mM Bis-Tris, pH 7.0 at 23°C, were mixed and absorbance changes were recorded at 430 nm. IHP was approximately 60 μM postmixing where indicated.

Figure 7. R-state rates of O_2 binding to and dissociation from Hb. A. Rebinding of O_2 to HbA and F_{II} following partial dissociation by laser flash photolysis. B. Replacement of O_2 by CO bound to HbA and F_{II} following rapid mixing in a stopped-flow apparatus. In A, a 1-mm sealed cuvette containing 50 μM of either HbA or F_{II} in 100 mM Bis-Tris, 1.25 mM O_2 , pH 7.4 at 22°C, was flashed with a 300 ns laser pulse using a 0.32 neutral density optical filter to produce 10% photolysis. Following this pulse, O_2 rebinding to R-state Hb was monitored by optical absorbance changes at 430 nm. In B, a solution containing 4 μM HbA or F_{II} in 100 mM Bis-Tris, 0.625 mM O_2 , pH 7.0, 20°C was rapidly mixed with a solution containing 0.5 mM CO. Absorbance changes at 424 nm report on the displacement reaction of O_2 by CO. All concentrations are listed as postmixing values and in heme equivalents where applicable. Experiments with recombinant Hb Brigham yielded equivalent findings (not shown).

We also examined O_2 and CO binding parameters for the R state conformation. As shown in Figure 7, there are no differences between the time courses for O_2 rebinding to native HbA and F_{II} after partial photolysis of the Hb O_2 complexes. Similarly, there are no differences between the time courses for the displacement of O_2 from the fully saturated Hb O_2 complexes by high concentrations of CO. Identical time courses for these reactions were also observed for recombinant Hb Brigham and HbA, and all three proteins showed superimposable time

courses for after partial photolysis of HbCO samples. Thus, the R state k'_{O_2} (80–40 $\mu M^{-1}s^{-1}$), k_{O_2} (15–30 s^{-1}), and k'_{CO} ($\sim 5 \mu M^{-1}s^{-1}$) values for HbA and Hb Brigham are experimentally the same and unaffected by the $\beta P100L$ mutation. This conclusion is supported by laser full photolysis experiments for HbA, F_{II}, and rHb Brigham CO and O_2 bound samples (not shown). In these experiments, both fast (R) and slow (T) phases are observed because the rate of switching from the R to T state competes with CO and O_2 rebinding at high ligand concentrations. The observed rate constants are the same for all three hemoglobins, but the fraction of slow, T-state phase is significantly smaller for F_{II} and rHb Brigham, presumably because the $\beta P100L$ replacement slows switching from the R to T state.

Discussion

One known cause of familial erythrocytosis is the presence of abnormal Hb with increased O_2 affinity. There are nearly 100 known Hb variants with high O_2 affinities that can be grouped into several categories depending on the site and location of the mutation. Most commonly reported changes are located at the $\alpha_1\beta_2$ interface or the C-terminus of the β chain.³⁷ Most of the structural changes that occur in these regions lead to destabilization of the T-state. Other mechanisms that are known to contribute to erythrocytosis include changes to those amino acids located in and around the 2,3-BPG and heme pockets.³⁸ We initially suspected that the $\beta P100L$ mutation might result in altered Hb dimerization. However, size exclusion chromatography and other experiments based on the methods of Nagel and Gibson suggested that this was not the case.³⁹ For example, time courses for the reaction of 1 μM recombinant Hb Brigham with 1 μM human haptoglobin 1-1 were almost identical to those for the

Figure 8. Structure of the $\beta P100L$ mutation. Images in both panels were generated from an X-ray crystal structure of deoxy HbA (2DN1).⁴⁰ A. Wild-type Hb. B. Hypothetical structure for Hb Brigham. Heme groups are shown as red sticks and the color schemes for ribbon structures are: α , gray, and β , yellow. Pro100 and Leu100 are shown in green, and Thr38 and Asp94 are shown in cyan. Corey-Pauling-Koltun colors are otherwise used throughout. Distances were measured and the theoretical model was generated using the PyMol Molecular Graphics System distance measuring utility and site-directed mutagenesis wizard, respectively.

reaction of HbA with Hp 1-1 (data not shown), implying similar extents of dimerization and tetramer to dimer dissociation rate constants.³⁹

Since Hb Brigham was originally discovered in 1973,⁶ no biochemical characterization of the molecular basis of this high O₂ affinity Hb has been reported. Hb Brigham behaves normally using standard electrophoretic techniques,⁶ but familial erythrocytosis was detected in the original case report.⁶ Oxygen equilibrium studies carried out with fresh blood and hemolysates confirmed the high O₂ affinity binding characteristics of the patient's blood. Brigham Hb exhibited a normal Bohr effect and a measurable effect when 2,3-BPG concentration was increased. Also, preliminary ligand binding kinetic studies using unfractionated hemolysates revealed no large abnormalities compared with HbA.

It was argued that definitive functional studies on Hb Brigham and the true contribution of the β P100L mutation to the increased overall O₂ affinity could not be determined until the mutant was separated from wild-type HbA.⁶ Using nondenaturing cation exchange liquid chromatography we were able to separate Hb Brigham from HbA even though the β P100L mutation causes no change in charge or polarity. We then verified the functional properties of this fraction with rHb Brigham generated by recombinant DNA technology and expression in *E. coli* in the absence HbA.

Proline is a nonpolar amino acid with a cyclic side chain found in turns. The conformational rigidity of this residue disrupts helices. All 12 of the reported Leu→Pro variant Hb subunits display significant structural instability, probably due to disruption of helical secondary structure. In contrast, Pro→Leu mutants are not known to cause significant changes in globin stability. β Pro100 is among one of 20 amino acids in the β subunit that maintains critical allosteric contacts in the $\alpha_1\beta_2$ interface and appears to interact with α Thr38 and α Asp94 (Fig. 8). Disruptions and alterations of these contacts have been predicted to perturb the T quaternary state.⁴¹ Thus, the β P100L side chain modification in Hb Brigham is thought to favor the R state and increase O₂ affinity without altering the active site of the heme pocket (Fig. 8).^{41,42}

Our O₂ equilibrium measurements in the acid and alkaline regions as well as experiments carried out in a chloride-free HEPES buffer showed no impairment of either the Bohr and chloride responses as a result of the Brigham mutation. Conformational changes in the vicinity of this mutation did not seem to propagate to other regions of the molecule, including those amino acids that are involved in H⁺ and Cl⁻ binding.

Similarly, the rates of spontaneous oxidation of HbO₂ Brigham and native HbA are almost identical

(Fig. 4). As shown in Figure 7, the intrinsic R state rates of O₂ binding and release are unaffected, and the same is true of the bimolecular CO association rate constant for the high affinity R state (partial photolysis experiments, not shown). Thus, our data indicate that the β P100L mutation affects only the rate and equilibrium constants for the R to T conformational transition and not the intrinsic reactivity of the β heme iron atom.

The effects of the Hb Brigham replacement are similar to those of other mutations in this region which also only effect the relative stabilities of the R and T states.^{17,37} Similar mutations associated with erythrocytosis in heterozygotes, such as Hbs Chesapeake, Yakima, and Kempsey, interfere with the normal point of contact between the subunits in the $\alpha_1\beta_2$ interface and destabilize the tetramer in the deoxy or T-state conformation of the mutants.⁴³ Hb Brigham shares other properties with these more well-characterized variants including a normal Bohr effect but lower *n* values derived from the Hill plots.

Clinical relevance

The causes of polycythemia can be grouped mechanistically into primary causes involving autonomous proliferation of erythrocyte precursors from mutations of the Jak-Stat pathway (polycythemia vera) or secondary causes mediated by the overproduction of erythropoietin, the key regulator of erythrocyte production. Secondary polycythemia is characterized by excess production of erythropoietin, and can be due to shunting of blood from the right to the left heart chambers, carbon monoxide poisoning, Hb variants with high O₂ affinity, and erythropoietin secreting tumors. It is critical to distinguish between primary and secondary polycythemia for proper clinical management because successful treatment depends on the underlying cause.

High O₂ affinity Hb variants in heterozygotes can be difficult to identify in clinical laboratories unless they demonstrate altered mobility on electrophoresis or altered retention times on HPLC. In the case of Hb Brigham, there is no difference in electrophoretic mobility under alkaline or acidic conditions with respect to HbA, but HPLC of the denatured protein or proteolysis fragments was able to resolve Brigham Hb from HbA in a more sophisticated clinical laboratory setting. The ultimate identification of the underlying mutation depends on mass spectrometry or DNA sequencing, which are labor intensive, time consuming, and not readily available even in large clinical laboratories. Functional characterization of the variant can be achieved by either purifying the protein from mixtures within the patient's red cells or, once the substitution is known, constructing, expressing, and producing the rHb mutant

in *E. coli*. We verified the use of both approaches in this work.

The detection of an abnormal Hb variant is important not only for management of the polycythemic patient, but also for genetic counseling or testing of children of the patient. In the case of Hb Brigham, a low P_{50} was estimated by venous blood analysis using a relatively simple clinical algorithm which is described elsewhere.^{12,13} An abnormally low P_{50} is the definition of a high O₂ affinity Hb, and if this phenotype is accompanied by polycythemia, the time and expense involved in determining the underlying mutation is justified, particularly when electrophoresis and/or HPLC methods allow detection of the presence of an Hb variant. In the event that a similar, previously described variant has been found, the published literature can serve as a guide in clinical management. If a new variant is discovered, there may be novel insights into hemoglobin biology derived from detailed mechanistic studies with either the isolated, naturally occurring variant or recombinant Hb construct, as in this report.

Materials and Methods

Materials

Unless otherwise indicated, all chemicals, solvents, and reagents were purchased from either Sigma Aldrich (Saint Louis, MO) or Fisher Scientific (Pittsburgh, PA). Trypsin was purchased from Roche Diagnostics (Indianapolis, IN). Gases were purchased from Roberts Oxygen Company, Inc. (Rockville, MD). Control and Brigham blood samples were collected with informed consent in accordance with IRB-approved protocols at the National Institutes of Health (Bethesda, MD).

Hemoglobin isolation

HbA was purified following osmotic lysis and centrifugation within 1 day of collection according to established methods.⁴⁴ The resulting materials were then stored at -80°C until used. Catalase was removed from wild-type Hb using an XK 26/100 column containing Superdex-200 chromatography media (GE Healthcare Biosciences, Piscataway, NJ). Brigham Hb purification steps are described in greater detail below. Recombinant hemoglobin Brigham was generated by site-directed mutagenesis of the pHE2 construct that was kindly provided by C. Ho and T.-J. Shen (Carnegie Mellon University, Pittsburgh, PA).¹⁸ Protein was then expressed in *E. coli* and purified using established methods.¹⁸

Oxygen equilibrium measurements

Oxygen equilibrium binding studies were carried out using a Hemox Analyzer (TCS Scientific, New Hope, PA). Sample deoxygenation and reoxygenation were

performed using pure nitrogen gas and air, respectively. Oxygen tensions were measured using a Clark Model 5331 O₂ Probe (Yellow Springs Instrument Company, Yellow Springs, OH). Oxygen equilibrium curves (OECs) were recorded for whole blood suspensions, whole hemolysates, BPG-stripped hemolysates, purified Hb fractions, and recombinant Hb Brigham. Oxygen fractional saturation was determined by optical absorbance spectroscopy using a dual-wavelength spectrophotometer at varied O₂ partial pressures. A typical experiment was conducted using 3 mL sample volumes of either erythrocytes or hemolysates. Buffers for each experiment are identified in the corresponding figure legends. Data obtained from these studies were fit by nonlinear least-squares analysis using Adair equations built-in to the Hemox Analyzer (P50 Plus, Version 1.2).

Liquid chromatography

Hb Brigham was separated from HbA using patient hemolysates by strong cation exchange chromatography. Whole blood containing Hb Brigham was centrifuged for 10 min at 7155g to pellet the red blood cells and the supernatant was discarded. Red blood cells were resuspended in ice cold water using a volume equaling three times that of the original sample. This suspension was incubated overnight at 4°C with gentle agitation. The next day, the sample was centrifuged several times for 10 min at 10,000g to remove cell debris, and was concentrated to approximately 3 mM in heme equivalents.

Hb Brigham fractionation was accomplished with a 5 mm $\times$ 100 mm Protein Pak SP 8HR pre-packed column (1000 Å, 8 μm particle size; Waters Corporation, Milford, MA) driven by a Waters HPLC system equipped with a 626 pump, 2487 dual-wavelength detector, and 600 s controller, all controlled by Millennium32 software (Waters Corporation, Milford, MA). Before injection, the column was pre-equilibrated with 50 mM sodium phosphate buffer, pH 5.8 at 22°C . Samples were injected in 100 μL injection volumes (750 μM in heme equivalents), and were subjected to isocratic elution. A 1 L solution of buffer was prepared by mixing 35 mL of 200 mM sodium phosphate monobasic with 215 mL of 200 mM sodium phosphate dibasic, adjusting the pH to 5.8 at 22°C using 1N HCl, and raising the buffer to a final volume of 1 L using water. Between re-equilibration and subsequent injections, the column was washed for 10 min using an identical buffer containing 0.5M NaCl.

Fractionation of Hb Brigham from wild-type Hb was verified by analytical reversed-phase HPLC using a 250 mm $\times$ 4.6 mm Zorbax Stable Bond 300 C3 column connected with a guard column (Agilent Technologies, Palo Alto, CA) using the previously described Waters HPLC.^{19,20,45,46} Running Solvents A and B were prepared with 0.1% trifluoroacetic (TFA)

acid in H₂O or 0.1% TFA in acetonitrile (ACN), respectively. The mobile phase gradient program was based on existing methods.^{19,20,45,46} Briefly, the column was equilibrated with 35% Solvent B, followed by sample injection and an increase to 37% Solvent B over 5 min, then to 40% Solvent B over 5 min, and then to 43% Solvent B over 2 min. The concentration of Solvent B was then maintained at 43% for 23 min, and then increased to 80% over 5 min. Sample volumes of 10 μ L were injected at a flow rate of 1 mL per minute. This separation was done at 23°C, and the optical absorbance of the eluate was monitored at 280 nm and 405 nm throughout each run.

Mass spectrometry

HbA and Brigham Hb fractions were digested with trypsin and analyzed by one-dimensional liquid chromatography tandem mass spectrometry (1D LC/MS/MS) experiments using a ThermoElectron Surveyor HPLC system coupled online to a ThermoFinnigan LTQ linear ion trap mass spectrometer equipped with a nanoflow electrospray source as previously described (Thermo Fisher Scientific, Incorporated, Waltham, MA).⁴⁷ These experiments were operated such that spectra were acquired for 140 min in the data dependent mode with dynamic exclusion enabled. The top seven peaks in the 350 to 2000 *m/z* range of every MS survey scan were fragmented.

MS data files were searched against the Swiss-Prot Human database containing a sequence corresponding to Hb Brigham, porcine trypsin, and reverse translated sequences of all entries using the Mascot search engine (Matrix Sciences, London, UK). Search parameters were as follows: trypsin specificity, 1 missed cleavages, carboxaminomethylation of cysteine as a static modification, methionine oxidation (+16 Da) as a variable modification, and +1 through +4 charge states. The precursor ion mass tolerance was ± 1.0 Da and the fragment ion mass tolerance was ± 0.8 Da. Mascot output files were analyzed using the software MassSieve.⁴⁸ MassSieve filters were adjusted to only include peptide identifications with Mascot Ion Scores equal to or exceeding their identity scores (corresponds to $\geq 95\%$ confidence). This resulted in calculated false-positive discovery rates (FDRs) to be 1.89% for the *F_{II}* dataset and 3.88% for the *F_I* dataset, respectively. A minimum of two peptide identifications including the peptide with the amino acid substitution were required for HbA and/or Hb Brigham to be considered identified. Additionally, spectra matched to peptides were used as a semiquantitative metric to access the abundance levels of HbA and Hb Brigham in the fractions. The MassSieve data representing these experiments are available as Supporting Information tables.

LC-ESI MS data were obtained using a single quadrupole MS (Agilent HP 1100 MSD series). Hun-

dred microliters of the sample was injected through a C₃ column and similar gradient conditions were used as reported elsewhere.⁴⁹ Deconvolution of the multiply charged species was performed using HP Chemstation software on the peaks integrated between 35 and 45 min by specifying a molecular mass range of 10 to 30 kDa, 5% abundance cutoff and three minimum peaks.

Autoxidation

Hb autoxidation experiments were performed using 50 mM potassium phosphate buffer, 20% glycerol, pH 7.4 at 37°C. Optical absorbance spectra between 400 nm and 800 nm were recorded every 20 min for 48 h using 1 nm intervals and an averaging time of 0.5 s. Hb concentrations were fixed at 30 μ M in heme equivalents. Optical absorbance spectroscopy measurements were made using an Agilent 8453 UV-Visible Spectrophotometer (Agilent Technologies, Incorporated; Santa Clara, CA).

Stopped-flow and laser flash photolysis

Stopped-flow measurements were made using an Applied Photophysics SX-18 microvolume stopped-flow spectrophotometer (Leatherhead, Surrey, UK). The pathlength and slit width configurations for each experiment are specified in the individual descriptions. Glass syringes were used to mitigate contamination by ambient gases (Cadence Science, Lincoln, RI). Laser flash photolysis was performed using a Phase-R model 2100B dye laser as previously described (Rice University, Houston, TX).⁵⁰ The rate of dissociation of O₂ from HbO₂ and the rate of CO binding to fully deoxygenated Hb were measured by stopped-flow rapid mixing spectrophotometry.^{51,52} The rate constant for bimolecular association of CO with deoxygenated Hb was determined by rapid mixing, measuring the dependence for the pseudo-first-order rate of association of [CO].^{53,54} These rate constants were confirmed in complete laser flash photolysis experiments.⁵⁵ The rate of O₂ and CO binding to R-state tetramers was investigated both by partial laser flash photolysis and ligand replacement reactions using previously developed rapid mixing and photolysis methods.^{52,55,56}

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