

BRIEF REPORT

Hemoglobin Jamaica Plain — A Sickling Hemoglobin with Reduced Oxygen Affinity

Alon Geva, Jennifer J. Clark, M.D., Yuxun Zhang, Ph.D., Anthony Popowicz, Ph.D., James M. Manning, Ph.D., and Ellis J. Neufeld, M.D., Ph.D.

SUMMARY

A baby girl presented with symptomatic sickle cell disease exacerbated by mild hypoxemia, despite a newborn-screening diagnosis of sickle cell trait. DNA sequencing of the β globin gene revealed that her maternal β globin allele was normal. Her paternal allele had not only the expected sickle-trait mutation, β^{Glu6Val} , but also a second, charge-neutral mutation, β^{Leu68Phe} . Analysis of the patient's hemoglobin revealed that the double-mutant protein, which we called "hemoglobin Jamaica Plain," had severely reduced oxygen affinity. Structural modeling suggested destabilization of the oxy conformation as a molecular mechanism for sickling in a heterozygote at an ambient partial pressure of oxygen.

From the Division of Hematology–Oncology, Children's Hospital Boston (A.G., J.J.C., E.J.N.); the Dana–Farber Cancer Institute (J.J.C., E.J.N.); Harvard Medical School (J.J.C., E.J.N.); and the Department of Biology, Northeastern University (Y.Z., J.M.M.) — all in Boston; and Rockefeller University, New York (A.P.). Address reprint requests to Dr. Neufeld at the Division of Hematology–Oncology, Children's Hospital Boston, Karp 8210, 300 Longwood Ave., Boston, MA 02115, or at ellis.neufeld@childrens.harvard.edu.

N Engl J Med 2004;351:1532-8.
Copyright © 2004 Massachusetts Medical Society.

PATIENTS WHO ARE HOMOZYGOUS FOR THE MUTATION IN THE GENE ENCODING the β chain of hemoglobin (β globin) that results in the substitution of valine for glutamic acid at position 6 (β^{Glu6Val}) have hemoglobin S (HbS) and sickle cell anemia. Hemoglobin from these patients polymerizes in the deoxy conformation into long fibers composed of strands of HbS tetramers. The β^{Glu6Val} mutation in deoxy-HbS favors a hydrophobic interaction between each strand and its neighbor. Other residues on the β chain participate in the binding of adjacent tetramers within each strand and between strands. The interaction between the valine at position 6 with the phenylalanine at position 85 and the leucine at position 88 on the partner strand is stereochemically unavailable in oxyhemoglobin.¹ The fibers of polymerized deoxy-HbS are responsible for dehydration, rigidity, and lysis of red cells.

In persons who are heterozygous for the β^{Glu6Val} mutation, hemoglobin can polymerize if the non-HbS allele encodes a permissive mutant hemoglobin (such as HbC, HbD, or HbO Arab). In other words, patients with sickling disorders due to two heterozygous mutations in β globin tend to have compound heterozygosity, such as HbS/C or HbS/D.¹

Mutations in β globin that cause sickle hemoglobin polymerization in persons who have simple heterozygosity are rare. The HbS Antilles mutation ($\beta^{\text{Glu6Val,Val23Ile}}$) is an example, but its mechanism is not completely understood.² Here we report the case of an infant girl with symptomatic sickle cell disease that was exacerbated by intercurrent respiratory infection and airplane travel. Genetic and functional studies of her hemoglobin revealed reduced oxygen affinity, which was caused by heterozygosity for a double-mutant sickling hemoglobin ($\beta^{\text{Glu6Val,Leu68Phe}}$) that we termed hemoglobin Jamaica Plain (Hb JP).

CASE REPORT

A baby girl of Puerto Rican descent was identified on newborn screening to have a variant hemoglobin isoelectric focusing pattern, which indicated the presence of HbF, HbA, and a variant band running near HbS. Since the father was known to be heterozygous for the β^{Glu6Val} mutation, the result was reported as the hemoglobin S trait. Splenomegaly and normocytic anemia were noted when the child was admitted for dehydration with gastroenteritis at the age of five months and during episodes of bronchiolitis. At the age of six months, she was admitted with a respiratory infection; the arterial oxygen saturation, estimated by pulse oximetry, was less than 90 percent. Sick cells and basophilic stippling were noted on a peripheral-blood smear (data not shown). Whereas a stain for Heinz bodies was negative, an isopropanol stability test indicated the presence of an unstable hemoglobin. She had marked splenomegaly, which diminished after the transfusion of packed red cells. Laboratory test results are listed in Table 1. Over the course of the following year, she had recurrent splenomegaly and anemia, requiring transfusion every six to eight weeks, and occasional transient indirect hyperbilirubinemia.

At the age of 19 months, during her first airplane

trip, the child became acutely ill in flight, with her spleen reaching the pelvic brim (as reported by a physician on board). She was cyanotic and hypotensive. She received oxygen for the duration of the flight. After landing, she was hospitalized and found to have a hematocrit of 18 percent. Packed red cells were transfused; the hematocrit then rose to 28 percent with resolution of symptoms and a decrease in splenomegaly. Because of the apparent splenic sequestration crisis, splenectomy was performed when she was two years old. Since that time, she has been asymptomatic, and she has required no transfusions for the past 24 months. Serial laboratory findings are shown in Table 1.

METHODS

After informed consent had been obtained from the family, blood for DNA sequencing and hemoglobin analysis was drawn from the patient and her parents. The institutional review board at Children's Hospital Boston approved the protocol, Genetic Basis of Blood Diseases.

DNA SEQUENCING

To identify the variant hemoglobin, DNA was prepared from a specimen of peripheral blood from the patient with a Puregene DNA Purification Kit

Table 1. Laboratory Values during Respiratory Infection and before and after Splenectomy.*

Variable	Patient's Values			Normal or Expected Range
	Respiratory Infection (age, 6 mo)	Before Splenectomy (age, 22 mo)	After Splenectomy (age, 36 mo)	
Hemoglobin (g/dl)	8.2	7.4	8.0	10.9–13.0
Mean cell volume (μm^3)	73	88.8	80.4	70.0–86.0†
Reticulocyte count (%)	9	13.1	5.9	0.8–2.1
Results of hemoglobin electrophoresis (%)	58 A, 27 variant, 3.4 A ₂ , 11.5 F	ND	ND	25–40 S, 2.1–3.1 A ₂ , 2.7–13 F‡
Oxygen saturation by pulse oximetry in room air (%)	<90	95	97	>95
Heinz-body preparation	Negative	ND	ND	Negative
Result of isopropanol stability test	Positive	ND	ND	Negative
Lactate dehydrogenase (U/liter)	Normal	Normal	Normal	100–295
Bilirubin (mg/dl)§	Normal	1.3 (total) 0.3 (direct)	Normal	0.3–1.2 (total) 0–0.4 (direct)

* ND denotes not determined.

† For children two to six years of age, the normal range is 75.0 to 87.0 μm^3 .

‡ A range of 25 to 40 percent HbS is expected for the S trait.

§ To convert the values for bilirubin to micromoles per liter, multiply by 17.1.

(Genra Systems). DNA was amplified by the polymerase chain reaction (PCR) with the use of a primer set encompassing exons 1 and 2 and another encompassing exon 3 (exons 1 and 2 forward primer, 5'AGTCAGGGCAGAGCCATCTA3'; exons 1 and 2 reverse primer, 5'TCCCCTTCCTATGACATGAAC3'; exon 3 forward primer, 5'CAAGCTAGGCCCTTTTGCTA3'; and exon 3 reverse primer, 5'TTGGACTTAGGGAACAAAGGAA3'). The resulting fragments were purified with a QIAquick PCR Purification Kit (Qiagen). The fragments were initially sequenced directly with the use of standard, automated, fluorescent dye-terminator reactions and were compared with the published sequence (*HBB*, GenBank accession number NM_000518); nucleotide positions are given according to the official Human Genome Organisation genomic-DNA-based description. To confirm that both mutations occurred on the same allele, amplified, purified DNA was cloned, and 10 independent clones were sequenced. The parents' DNA was also isolated, amplified, and sequenced.

To confirm the presence of the putative β globin mutation, a cytosine-to-thymine transition at nucleotide 335 (β 335C→T), and to eliminate the possibility of a PCR artifact, we designed reverse PCR primers specific for the C and T alleles at this position, with 3' terminal nucleotides complementary either to the wild-type sequence or to the mutant sequence (wild type, 5'GTGAGCCAGGCCATCATAAAGGCACCGTG3'; mutant, 5'GTGAGCCAGGCCATCACTAAAGGCACCGTA3'). The penultimate nucleotide of each reverse primer was mismatched to increase specificity, according to Little's criteria for amplification-refractory mutation system (ARMS) analysis.³ A common forward primer was used for amplification (5'AGGAGACCAATAGAACTGGGCATGTGGAG3'). After PCR amplification, reaction products were analyzed by agarose-gel electrophoresis and detected by ethidium bromide staining. An ARMS assay was also performed on wild-type DNA to confirm the specificity of the assay.

HEMOGLOBIN ANALYSIS

Hemoglobin isoelectric focusing was performed with the use of the Resolve-Hb hemoglobin test kit, pH 6 to 8 (Perkin-Elmer Life and Analytical Sciences). Hemoglobin was purified from a hemolysate of the patient's blood by chromatography on a CM52 carboxymethylcellulose column. The purity of the resulting hemoglobin was assessed by fast protein liquid chromatography (FPLC) with cation

exchange to analyze the percentage of mutant as compared with normal hemoglobin.

Oxygen-binding studies were performed as previously described⁴ for dialyzed hemolysate prepared from the patient's blood and for purified Hb JP and HbS alone and in the presence of either sodium chloride or 2,3-diphosphoglycerate. Binding studies were performed with the use of 0.6 mM hemoglobin, and 150 mM sodium chloride or 3.0 mM 2,3-diphosphoglycerate was added as specified. In addition, the association constant for hemoglobin polymerization was determined for Hb JP both in the absence and in the presence of 2,3-diphosphoglycerate. Tetramer stability was assayed as previously described.⁵

RESULTS

The DNA sequences of 7 of 10 independent PCR clones from the patient's genomic DNA were entirely normal. Three clones revealed an adenine-to-thymine transversion at position 20 (β 20A→T) in exon 1 (corresponding to β ^{Glu6Val}, which is standard HbS). The same three clones also revealed a β 335C→T mutation in exon 2 (Fig. 1A). Exon 3, amplified separately, was normal in all clones (data not shown). *XmnI* digestion of amplified DNA containing exon 2 resulted in three fragments 594, 410, and 184 bp in size, findings consistent with heterozygosity for the β 335C→T mutation, which creates an *XmnI* recognition sequence (5'GAANNNTTC3', where the mutated nucleotide is underscored) that is absent from the wild-type sequence. ARMS analysis confirmed that the patient was heterozygous for β 335C→T (Fig. 1B). Wild-type DNA failed to amplify with the mutant primer (data not shown). The mother's β globin gene was wild-type according to DNA analysis, and the father's β globin gene contained only the β 20A→T (S trait) mutation, and not the β 335C→T mutation. This β 335C→T mutation, encoding β ^{Leu68Phe}, was previously reported as an isolated finding, Hb Rockford,⁶ also known as Hb Loves Park.⁷ The heterozygous mutation was also present in DNA from a buccal swab from the patient, confirming that it was a germ-line mutation. We interpret these results to mean that the patient acquired a new mutation on her paternal HbS allele and inherited a normal β globin allele from her mother and is thus heterozygous for a double-mutant hemoglobin, Hb JP (β ^{Glu6Val,Leu68Phe}).

The properties of the mutant hemoglobin in hemolysates of the patient's red cells and in purified

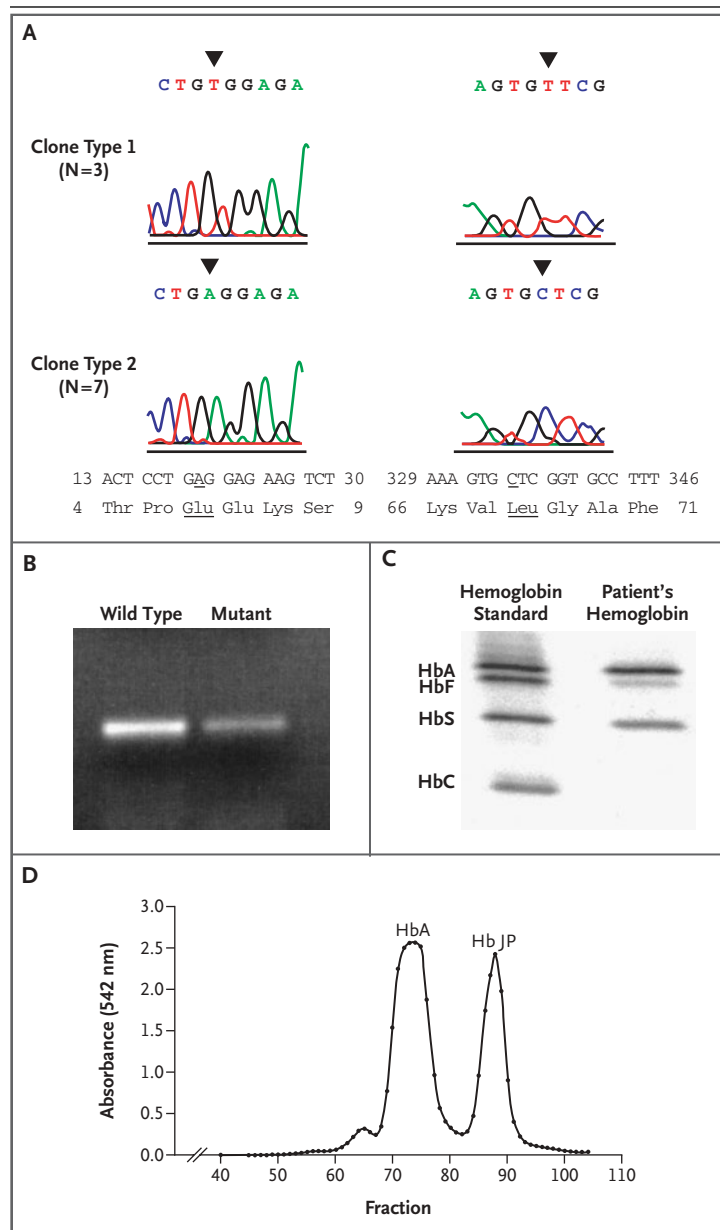
form were studied next. Isoelectric focusing of the hemolysate revealed HbA, HbF, and a band running near HbS that corresponded to the charge-neutral Hb JP double mutant (Fig. 1C). When the patient was 22 months of age, FPLC showed 62.6 percent HbA, 28.5 percent Hb JP, and 8.9 percent HbF. The isolated mutant protein was 93 percent pure on FPLC after separation by carboxymethylcellulose chromatography (Fig. 1D). Purified HbS and Hb JP could not be separated by FPLC or high-performance liquid chromatography. Dialyzed hemolysate from the patient had an oxygen-binding curve in which the partial pressure of oxygen at 50 percent dissociation was 48 mm Hg, a value substantially higher than (i.e., shifted far to the right of) that for HbA (Fig. 2A). This result demonstrated that reduced oxygen affinity is intrinsic to the mutant hemoglobin. The partial pressure of oxygen at 50 percent dissociation was only slightly higher for the purified mutant hemoglobin alone than for HbS (10.0 vs. 6.5 mm Hg), but the difference increased in the presence of 150 mM sodium chloride (22.2 vs. 13.0 mm Hg) and even more dramatically in the presence of 3.0 mM 2,3-diphosphoglycerate (43.0 vs. 24.0 mm Hg) (Fig. 2B). Cooperativity of oxygen binding to hemoglobin, as assessed by the Hill coefficient,⁸ did not significantly differ among Hb JP, HbS, and HbA (data not shown). The addition of

2,3-diphosphoglycerate or sodium chloride did not alter this result.

In anaerobic conditions, sickle hemoglobin precipitated readily as a polymer. The average (\pm range) solubility (C_{sat} , an index of the hemoglobin saturation) in dextran was 36.14 ± 1.5 mg per milliliter (average of two measurements) in the absence of 2,3-diphosphoglycerate and 22.9 ± 0.3 mg per milliliter (average of two measurements) in the presence of a threefold molar excess of 2,3-diphosphoglycerate. Hb JP was slightly less soluble under

Figure 1. Genetic Sequencing and Protein Analysis of the Patient's Hemoglobin.

Panel A shows sequencing chromatograms revealing two clone types derived from the patient's DNA. Clone type 1 contained the sickle mutation $\beta 20A \rightarrow T$ in codon 6 and the spontaneously arising mutation $\beta 335C \rightarrow T$ in codon 68, indicating that both mutations in the patient were on the same chromosome. Clone type 2 was wild type. Both positions are marked with arrowheads, and the encoded (wild type) amino acids are shown below the chromatograms, with the mutated nucleotide and amino acid underscored. Amplification-refractory mutation system analysis, shown in Panel B, confirmed the presence of both the wild-type ($\beta C335$) allele and the $\beta 335C \rightarrow T$ mutation, indicating that the patient was heterozygous for that mutation. Isoelectric focusing of the patient's hemolysate (Panel C) revealed HbA, HbF, and a band comigrating with HbS. Trace HbA₂ was also present on the original blot, although it is not visible in the figure. At left is a standard hemoglobin ladder. Hb JP was purified by chromatography on a CM52 carboxymethylcellulose column (Panel D). The points on the curve represent the absorbance of each collected fraction. The resulting hemoglobin was 93 percent pure on fast protein liquid chromatography (data not shown).



these conditions, with corresponding average values of 28.34 mg per milliliter and 21.72 mg per milliliter (both duplicate measurements). Also, tetramer stability in the mutant hemoglobin was within the normal range and was not significantly different from that of HbS (Fig. 2C). Hb JP synthesized in yeast by standard recombinant DNA techniques had properties identical to those described above for purified Hb JP, thus corroborating the observed results (data not shown).

DISCUSSION

We report a double-mutant hemoglobin, Hb JP, with clinical consequences of severe hemolytic anemia and sickling of erythrocytes, which in this case were exacerbated by respiratory infection and the low arterial oxygen saturation associated with high altitude. The patient presented with clinically severe vaso-occlusion due to erythrocyte sickling and splenomegaly during an airplane flight; she had a history of more minor episodes of vaso-occlusion and splenomegaly. Splenectomy improved the severe clinical findings at sea level; we propose that she was prone to intrasplenic sickling and splenomegaly because of the relative hypoxic environment and slow transit time of red cells in the spleen. Genetic analysis indicated that a spontaneous mutation, $\beta 335C \rightarrow T$, previously reported as a sole mutation encoding Hb Rockford (β^{Leu68Phe}),⁶ arose on an existing paternal β^{Glu6Val} allele. Hb Rockford is reported to be a low-affinity, stable hemoglobin that does not result in hemolysis. The double mutation, by contrast, resulted in a hemoglobin variant with substantially reduced oxygen affinity, especially in the presence of 2,3-diphosphoglycerate. As a physiologic adaptation, 2,3-diphosphoglycerate levels in erythrocytes would be elevated in chronic anemia and would play a key role in the hypoxic environment of the spleen at the low partial pressure of oxygen typical of a pressurized airplane cabin, where the ambient partial pressure of oxygen falls to 126 mm Hg⁹ and the arterial oxygen correspondingly falls to 56 mm Hg in healthy passengers.¹⁰

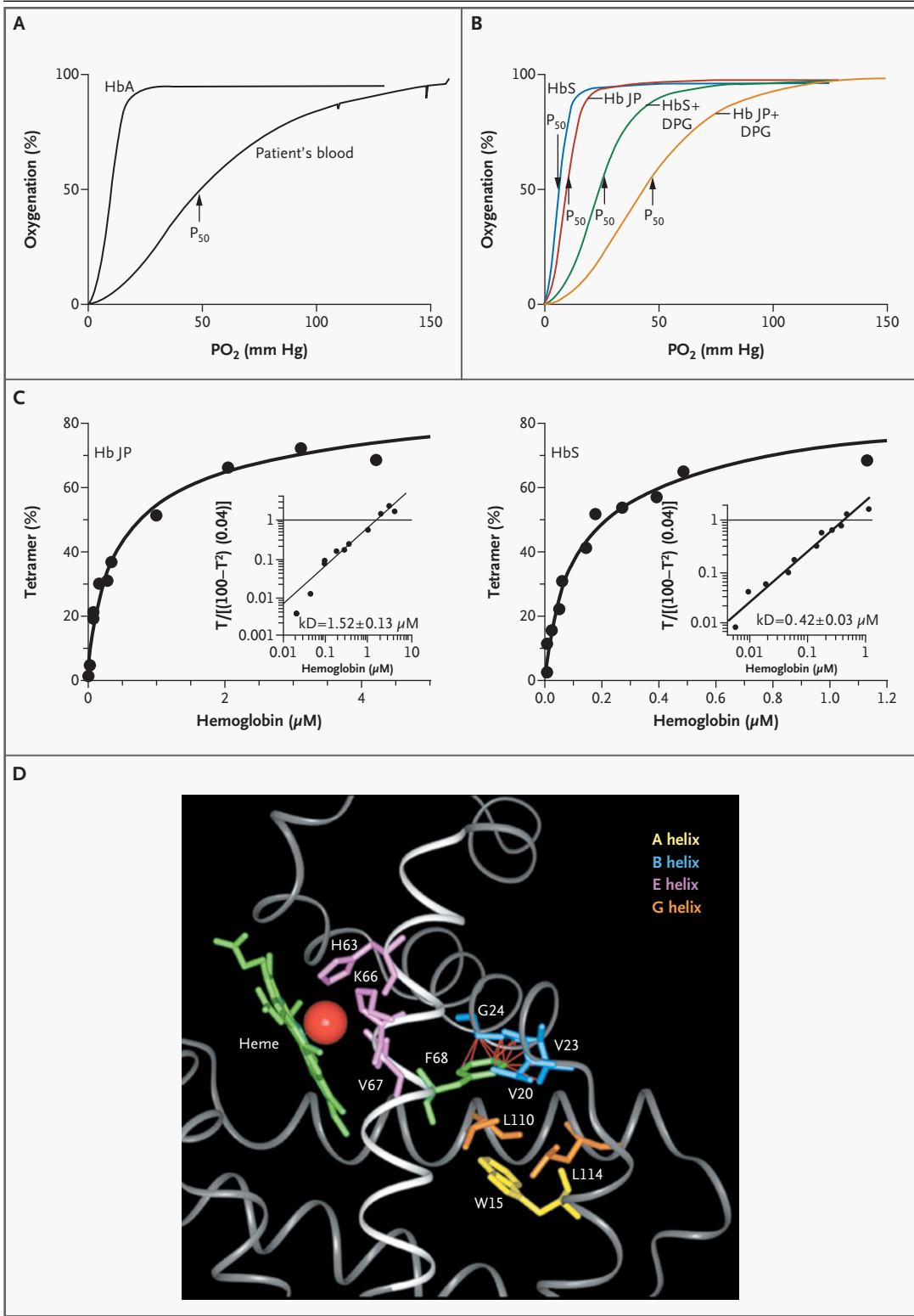
In normal hemoglobin, the leucine residue at position 68 is buried within the folded β globin chain.^{11,12} Modeling of the substituted phenylalanine at this position predicted unfavorable steric interactions in the oxy conformation, but not in the deoxy conformation, with amino acids that are nearby (Fig. 2D). The modeling predicted that the deoxy conformation is stabilized relative to the oxy con-

Figure 2 (facing page.) Functional Analysis of Hb JP.

Dialyzed hemolysate prepared from the patient's blood had a higher partial pressure of oxygen (PO_2) at 50 percent dissociation (P_{50}) than wild-type HbA (Panel A). Whereas the P_{50} of purified Hb JP was only slightly higher than that of HbS alone, the difference increased in the presence of 3.0 mM 2,3-diphosphoglycerate (DPG) (Panel B). Tetramer formation for Hb JP was within the normal range and not significantly different from that of HbS (Panel C). Insets show the data replotted to obtain the dissociation constant (kD). T denotes the extent of tetramer formation, expressed as a percentage. Panel D shows a proposed mechanism for the reduced oxygen affinity of Hb JP. The phenylalanine substitution at position 68 of the β chain was modeled according to the coordinates from normal HbA to assess how it would affect the binding of oxygen (red ball). The oxy-HbA backbone was not changed in this model, but the leucine residue at position 68 was replaced with phenylalanine. Side chains surrounding the heme moiety (histidine at position 63, lysine at 66, and valine at 67) are shown for reference (purple). Additional side chains shown for reference include tryptophan at position 15 (yellow) and leucine at positions 110 and 114 (orange). The solid red lines between the bulky mutant phenylalanine residue at position 68 (green) and nearby chains (valine at positions 20 and 23 and glycine at position 24 [blue]) represent highly unfavorable contacts. A rearrangement would probably occur in order to compensate for these adverse contacts. When the same analysis of phenylalanine substitution is performed with deoxy-HbA coordinates (not shown), the unfavorable interactions are much less severe. Hence, the deoxy structure would be preferred, suggesting that oxygen binding would be weaker (i.e., there would be a rightward shift of the hemoglobin dissociation curve). Amino acid residues are labeled with their single-letter codes. Colors denote the alpha helix (A, B, E, or G) of β globin in which the amino acids are located.

formation — a mechanism for decreased oxygen affinity similar to that of Hb Kansas.¹³ However, unlike Hb Kansas, Hb JP does not result in a weak hemoglobin tetramer. In addition, the cooperativity of globin subunits is unaffected, as indicated by Hill coefficients within the normal ranges. It is likely that helical movements to accommodate pressure on the heme from the phenylalanine residue at position 68 in Hb JP would affect some outside residues to increase polymerization and result in lower hemoglobin solubility. X-ray crystallography will be required to confirm the mechanisms proposed here and to specify further the nature of the perturbations resulting from the phenylalanine substitution that lead to the polymerization of deoxy-Hb JP.

The marked decrease in oxygen affinity of Hb JP — which is a greater decrease than that of HbS An-



tilles, a double-mutant hemoglobin that has been used in a murine model of sickling disorders¹⁴ — might make it useful for testing antisickling agents at ambient partial pressures of oxygen in heterozygous mice. This charge-neutral double-mutant hemoglobin is difficult to detect without DNA sequencing. Since it cannot be distinguished from HbS by isoelectric focusing, FPLC, or high-perfor-

mance liquid chromatography, Hb JP may account for sporadic cases of clinically severe vaso-occlusion in other cases of S variants or the presumed S trait. Sequencing of the β globin gene should be considered in such cases.

We are indebted to the patient and her family for their participation.

Supported in part by K24 grants (HL04184 to Dr. Neufeld and HL18819 to Dr. Manning) from the National Institutes of Health.

REFERENCES

1. Bunn HF, Forget BG. Hemoglobin: molecular, genetic, and clinical aspects. Philadelphia: W.B. Saunders, 1986:453-564.
2. Monplaisir N, Merault G, Poyart C, et al. Hemoglobin S Antilles: a variant with lower solubility than hemoglobin S and producing sickle cell disease in heterozygotes. *Proc Natl Acad Sci U S A* 1986;83:9363-7.
3. Little S. Amplification-refractory mutation system (ARMS) analysis of point mutations. In: Dracopoli NC, Haines JL, Korf BR, et al., eds. *Current protocols in human genetics*. New York: John Wiley, 2000:9.8.1-9.8.12.
4. Dumoulin A, Manning LR, Jenkins WT, Winslow RM, Manning JM. Exchange of subunit interfaces between recombinant adult and fetal hemoglobins: evidence for a functional inter-relationship among regions of the tetramer. *J Biol Chem* 1997;272:31326-32.
5. Manning LR, Jenkins WT, Hess JR, Vandegriff K, Winslow RM, Manning JM. Subunit dissociations in natural and recombinant hemoglobins. *Protein Sci* 1996;5:775-81.
6. Perrault J, Fairbanks VF, McCormick DJ, et al. Hemoglobin Rockford, β 68(E12)Leu \rightarrow Phe: a new HB variant associated with mild anemia. *Blood* 1997;90:Suppl 1:30b. abstract.
7. Hb Loves Park. In: Hardison RC, Chui DHK, Riemer C, et al. *Human hemoglobin variants and thalassemias*. (Accessed September 13, 2004, at http://globin.cse.psu.edu/cgi-bin/hbvar/query_vars?mode=output&display_format=page&i=375.)
8. Perutz MF. Mechanisms of cooperativity and allosteric regulation in proteins. *Q Rev Biophys* 1989;22:139-237.
9. Lee AP, Yamamoto LG, Relles NL. Commercial airline travel decreases oxygen saturation in children. *Pediatr Emerg Care* 2002;18:78-80.
10. Gendreau MA, DeJohn C. Responding to medical events during commercial airline flights. *N Engl J Med* 2002;346:1067-73.
11. Steinberg MH, Benz EJ Jr. Pathobiology of the human erythrocyte and its hemoglobins. In: Hoffman R, Benz EJ Jr, Shattil SJ, et al., eds. *Hematology: basic principles and practice*. 3rd ed. New York: Churchill Livingstone, 2000:356-67.
12. Bunn HF, Forget BG. Hemoglobin: molecular, genetic, and clinical aspects. Philadelphia: W.B. Saunders, 1986:13-35.
13. Bonaventura J, Riggs A. Hemoglobin Kansas, a human hemoglobin with a neutral amino acid substitution and an abnormal oxygen equilibrium. *J Biol Chem* 1968;243:980-91.
14. Popp RA, Popp DM, Shinpock SG, et al. A transgenic mouse model of hemoglobin S Antilles disease. *Blood* 1997;89:4204-12.

Copyright © 2004 Massachusetts Medical Society.

FULL TEXT OF ALL JOURNAL ARTICLES ON THE WORLD WIDE WEB

Access to the complete text of the *Journal* on the Internet is free to all subscribers. To use this Web site, subscribers should go to the *Journal's* home page (www.nejm.org) and register by entering their names and subscriber numbers as they appear on their mailing labels. After this one-time registration, subscribers can use their passwords to log on for electronic access to the entire *Journal* from any computer that is connected to the Internet. Features include a library of all issues since January 1993 and abstracts since January 1975, a full-text search capacity, and a personal archive for saving articles and search results of interest. All articles can be printed in a format that is virtually identical to that of the typeset pages. Beginning six months after publication, the full text of all Original Articles and Special Articles is available free to nonsubscribers who have completed a brief registration.