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Hemoglobin Variants: Biochemical Properties and Clinical Correlates

Christopher S. Thom[1,2], Claire F. Dickson[3], David A. Gell[3], and Mitchell J. Weiss[2]

[1]Cell and Molecular Biology Graduate Group, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

[2]Hematology Department, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104

[3]Menzies Research Institute, University of Tasmania, Hobart, Australia

Correspondence: weissmi@email.chop.edu

Diseases affecting hemoglobin synthesis and function are extremely common worldwide. More than 1000 naturally occurring human hemoglobin variants with single amino acid substitutions throughout the molecule have been discovered, mainly through their clinical and/or laboratory manifestations. These variants alter hemoglobin structure and biochemical properties with physiological effects ranging from insignificant to severe. Studies of these mutations in patients and in the laboratory have produced a wealth of information on hemoglobin biochemistry and biology with significant implications for hematology practice. More generally, landmark studies of hemoglobin performed over the past 60 years have established important paradigms for the disciplines of structural biology, genetics, biochemistry, and medicine. Here we review the major classes of hemoglobin variants, emphasizing general concepts and illustrative examples.

Globin gene mutations affecting hemoglobin (Hb), the major blood oxygen (O_2) carrier, are common, affecting an estimated 7% of the world's population (Weatherall and Clegg 2001; Kohne 2011). These mutations are broadly subdivided into those that impair globin protein subunit production (thalassemias) and those that produce structurally abnormal globin proteins (Hb variants). The latter class is mainly composed of missense mutations that cause single amino acid substitutions in the globin protein, resulting in an abnormal, or "variant" Hb tetramer. Less commonly, Hb variants are associated with deletions, multiple amino acid substitutions, antitermination mutations, and altered posttranslational processing (Table 1).

Naturally occurring Hb mutations cause a range of biochemical abnormalities, some of which produce clinically significant symptoms. The most common and medically important Hb variants include HbS (Cao and Kan 2012; Lettre 2012; Schechter and Elion 2012; Serjeant and Rodgers 2012; Williams and Weatherall 2012), HbC (Cao and Kan 2012; Lettre 2012; Schechter and Elion 2012; Serjeant and Rodgers 2012; Williams and Weatherall 2012), HbE (see the sections on Selected Variants that Illustrate Important Aspects of Hemoglobin Biology and

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Table 1. Hb variants that are discussed in this article

Name	Globin site (fold)	Amino acid substitution	Molecular mechanism	Clinical phenotype	Other biochemical and laboratory findings
			Unstable Mutants		
Brockton	β138 (H16)	Ala > Pro	Altered secondary structure	Hemolytic anemia, reticulocytosis	
Philly	β35 (C1)	Tyr > Phe	Altered α1β1 interface	Hemolytic anemia, reticulocytosis	Decreased cooperativity, increased oxygen affinity
Peterborough	β111 (G13)	Val > Phe	Altered α1β1 interface	Hemolytic anemia, reticulocytosis	Decreased oxygen affinity
Stanmore	β111 (G13)	Val > Ala	Altered α1β1 interface	Hemolytic anemia	Decreased oxygen affinity
J-Guantanamo	β128 (H6)	Ala > Asp	Altered α1β1 interface	Hemolytic anemia	Target cells
Khartoum	β124 (H2)	Pro > Arg	Altered α1β1 interface	Normal	
Prato	α1 or α2 31 (B12)	Arg > Ser	Altered α1β1 interface	Anisocytosis, hypochromia	Mildly unstable in isopropanol
Lombard	α2 103 (G10)	His > Tyr	Altered α1β1 interface	Anemia	
Contaldo	α1 or α2 103 (G10)	His > Arg	Altered α1β1 interface	Hemolytic anemia	
Foggia	α2 117 (GH5)	Phe > Ser	Altered α1β1 interface	Microcytosis	Rapidly degraded α chains
Groene Hart	α1 119 (H2)	Pro > Ser	Altered α1β1 interface, disrupted AHSP binding	Hemolytic anemia, microcytosis	
Turriff	α1 or α2 99 (G6)	Lys > Glu	Altered α1β1 interface, disrupted AHSP binding	Normal	Comigrates with HbA_{1C}, rapidly degraded α chains
Beziers	α1 99 (G6)	Lys > Asn	Altered α1β1 interface, disrupted AHSP binding	Normal	Comigrates with HbA_{1C}
Hirosaki	α2 43 (CE1)	Phe > Leu	Altered heme pocket	Heinz body hemolytic anemia	Hyperunstable
Terre Haute	β106 (G8)	Leu > Arg	Altered heme pocket	Heinz body hemolytic anemia, dominant inclusion body thalassemia	Hyperunstable
			High Affinity Variants		
Kempsey	β99 (G1)	Asp > Asn	Unstable T state	Erythrocytosis	Decreased cooperativity
Hiroshima	β146 (HC3)	His > Asp	Mutated Bohr proton donor	Erythrocytosis	Decreased cooperativity, decreased Bohr effect
York	β146 (HC3)	His > Pro	Mutated Bohr proton donor	Erythrocytosis	Decreased cooperativity, decreased Bohr effect
Cowtown	β146 (HC3)	His > Leu	Mutated Bohr proton donor	Erythrocytosis	Decreased Bohr effect

Continued

Table 1. *Continued*

Name	Globin site (fold)	Amino acid substitution	Molecular mechanism	Clinical phenotype	Other biochemical and laboratory findings
Rahere	β82 (EF6)	Lys > Thr	Altered 2,3DPG binding site	Erythrocytosis	
Providence	β82 (EF6)	Lys > Asn	Altered 2,3DPG binding site	Erythrocytosis	Low oxygen affinity
Helsinki	β82 (EF6)	Lys > Met	Altered 2,3DPG binding site	Erythrocytosis	Decreased Bohr effect
			Low Affinity Variants		
Kansas	β102 (G4)	Asn > Thr	Unstable R state	Cyanosis	Decreased cooperativity
Beth Israel	β102 (G4)	Asn > Ser	Unstable R state	Cyanosis	Decreased cooperativity
St. Mandé	β102 (G4)	Asn > Tyr	Unstable R state	Cyanosis	
			Methemoglobin Variants		
M-Iwate	α1 or α2 (F8)	His > Tyr	Oxidized heme	Pseudocyanosis (Methemoglobinemia)	Abnormal visible spectrum
M-Saskatoon	β63 (E7)	His > Tyr	Oxidized heme	Pseudocyanosis (Methemoglobinemia)	Abnormal visible spectrum
			Globin Chain Elongation Variants		
Constant Spring	α2 142 (HC3)	Stop > Gln	Antitermination mutant	Microcytosis	Decreased mRNA stability
Cranston	β145 (HC3)	+CT	Frameshift, elongated globin	Hemolytic anemia	Increased oxygen affinity, decreased cooperativity
			Variants with Multiple Effects		
HbE	β26 (B8)	Glu > Lys	Unstable, reduced synthesis	Microcytosis	
Bruxelles	β41 (C7) or β42 (CD1)	Phe > 0	Altered heme pocket	Hemolytic anemia, cyanosis, splenomegaly, reticulocytosis	Heinz bodies, decreased cooperativity
Warsaw	β42 (CD1)	Phe > Val	Altered heme pocket	Hemolytic anemia, cyanosis	Heinz bodies, decreased cooperativity
Hammersmith	β42 (CD1)	Phe > Ser	Altered heme pocket	Hemolytic anemia, cyanosis	Heinz bodies, decreased cooperativity
Buccuresti-Louisville	β42 (CD1)	Phe > Leu	Altered heme pocket	Hemolytic anemia, cyanosis	Heinz bodies, decreased cooperativity
Zurich	β63 (E7)	His > Arg	Altered heme pocket	Normal, but hypersensitive to oxidative stress	Decreased cooperativity, increased oxygen affinity, increased CO affinity
Jamaica Plain	β6 (A3) and β68 (E12)	Glu > Val and Leu > Phe	Altered secondary structure	Hemolytic anemia, cyanosis, splenomegaly, splenic sequestration	Unstable, Heinz bodies, sickle cell phenotype, decreased oxygen affinity

Continued

Table 1. *Continued*

Name	Globin site (fold)	Amino acid substitution	Molecular mechanism	Clinical phenotype	Other biochemical and laboratory findings
Quebec-Chori	β87 (F3)	Thr > Ile	Altered interaction with HbS polymer	Normal	Promotes HbS polymerization
D-Ibadan	β87 (F3)	Thr > Lys	Altered interaction with HbS polymer	Normal	Inhibits HbS polymerization
Bristol-Alesha	β67 (E11)	Val > Met	Altered heme pocket	Hemolytic anemia, reticulocytosis	Heinz bodies, decreased cooperativity, decreased Bohr effect, decreased oxygen affinity
Toms River	γ67 (E11)	Val > Met	Altered heme pocket	Anemia, cyanosis	Unstable, low oxygen affinity

For a full listing of hemoglobin variants, see The Globin Gene Server (http://globin.bx.psu.edu; Hardison et al. 2002; Giardine et al. 2011).

Variants that Affect Multiple Hemoglobin Functions; see also Musallam et al. 2012), and some thalassemias (e.g., "thalassemic hemoglobinopathies"), all of which are under positive genetic selection because they confer survival advantages in areas where malaria is endemic (Weatherall and Clegg 2001). In addition to these prevalent mutant proteins, there are also >1000 other known naturally occurring Hb variants, which are rare individually but common collectively. Most Hb mutants are cataloged on the Globin Gene database (HbVar, http://globin.bx.psu.edu; Hardison et al. 2002; Giardine et al. 2011). By convention, these variants are named after the geographic origin of the affected individual. Although many Hb variants are clinically silent, some produce clinical manifestations of varying severity. Analyses of these variants, which can be considered to be "experiments of nature" (Garrod 1928), have generated valuable insights into structure–function relationships within the Hb molecule, with interesting and important clinical consequences.

The goal of this work is to provide a succinct conceptual framework for understanding the biology and clinical implications of Hb variants. We explore major concepts of Hb biology followed by a discussion of selected Hb variants that reinforce basic principles. For more extensive reviews of this topic, see Nathan and Oski's *Hematology of Infancy and Childhood* (Nathan et al. 2009); *Hemoglobin: Molecular, Genetic, and Clinical Effects* (Bunn and Forget 1986); *Disorders of Hemoglobin: Genetics, Pathophysiology, and Clinical Management* (Steinberg et al. 2001); and the Globin Gene database (Hardison et al. 2002; Giardine et al. 2011).

BASIC PRINCIPLES

Hemoglobin Synthesis, Structure, and Function

Hemoglobin is a heterotetramer composed of α-like and β-like globin subunits, each bound to a heme prosthetic group. The major functions of Hb are to transport oxygen (O_2) from the lungs to peripheral tissues and carbon dioxide (CO_2) from the tissues to the lungs. The kinetics of Hb-O_2 binding and release are fine-tuned for this purpose and adaptable according to developmental ontogeny and metabolic perturbations. Moreover, the Hb molecule must limit potential problems caused by its associated iron and free O_2, reactive molecules capable of inflicting damage through the production of reactive oxygen species. Efforts to understand how Hb structure imparts these critical functions have

been ongoing for more than 50 years. Hemoglobin was one of the first proteins to be sequenced (Konigsberg et al. 1961; Schroeder et al. 1961; Watson and Kendrew 1961) and the globin genes were among the earliest to be cloned (Rabbitts 1976). In the late 1950s, Perutz and colleagues determined the three-dimensional structure of Hb through X-ray crystallography (Perutz 1960; Perutz et al. 1960). More recent studies refined this structure to high resolution (Paoli et al. 1996; Park et al. 2006). In addition, O_2 and other ligand-binding properties have been measured in detail for native Hb and many naturally occurring mutants. All of this work provides a substantial framework for defining the O_2 delivery properties of Hb, as well as the molecular consequences of variant mutations (see, for example, Lehmann 1957; Konigsberg and Lehmann 1965; Shimizu et al. 1965; Perutz and Lehmann 1968; Perutz 1970; Bunn and Forget 1986).

Globin polypeptides are synthesized from separate α-like and β-like globin gene clusters located on human chromosomes 16 and 11, respectively. Nascent globin chains rapidly incorporate heme, which stabilizes their native folding into Hb subunits composed of seven or eight α helices named A–H, which fold together into a globular structure (Fig. 1A). Hb subunits bind O_2 and other ligands via the heme iron buried within an evolutionarily conserved hydrophobic pocket that faces the outside of HbA tetramers (Fig. 1B). Heme iron is axially coordinated to globin proteins by an invariant histidine residue in helix F8, termed the "proximal" histidine (Fig. 1C). The opposite axial position binds O_2, which is stabilized by interaction with the conserved "distal" histidine in helix E7 (Fig. 1C). Multiple additional amino acids within the globin proteins stabilize heme binding through noncovalent interactions (Fig. 1C). Iron must be in its reduced (ferrous, Fe^{2+}) state for Hb to bind O_2. Oxidized or "met" Hb (ferric, Fe^{3+}) cannot bind O_2 and is relatively unstable, tending to lose hemin and denature. Thus, red blood cells have evolved elaborate mechanisms to maintain Hb in its reduced state (Bunn and Forget 1986; Ganz and Nemeth 2012; Schechter 2012). For example, the methemoglobin reductase system converts methemoglobin (metHb) to its reduced form. Not surprisingly, globin mutations that alter amino acids within the ligand pocket frequently produce strong functional effects, including destabilization, altered affinity for O_2, and increased rates of metHb formation and heme loss (see the section on Selected Variants that Illustrate Important Aspects of Hemoglobin Biology). Variants promoting autoxidation are termed "M-Hbs" (see the sections on Selected Variants that Illustrate Important Aspects of Hemoglobin Biology and Methemoglobin ("M-Type") Variants). Because Hb gains its distinctive color from the heme group, alterations that affect the environment of the heme iron, including changes in the surrounding amino acids, different gas ligands, or redox state, produce characteristic changes in visible light absorption. These color changes are used clinically to assess Hb-O_2 saturation, metHb formation and the effects of Hb variants.

Within the Hb tetramer, each globin subunit binds the unlike chain through two distinct interfaces, termed α1β1 and α1β2 (Fig. 1D). Individual globin subunits form dimers through the extremely high affinity α1β1 interaction. Globin chain monomers are relatively unstable compared with dimers, with a tendency to form intracellular precipitates that damage erythrocytes, causing hemolytic anemia. Thus, mutations that impair the α1β1 interaction can cause erythrotoxicity by favoring the accumulation of monomeric subunits (Fig. 2C). The α1β2 interaction, which is lower affinity, mediates tetramerization. Oxygen binding destabilizes the α1β2 interaction, resulting in a transition of the quaternary structure from the "T" (tense, low affinity, deoxygenated) to "R" (relaxed, high affinity, oxygenated) state, which facilitates O_2 binding to additional subunits (Fig. 2A). This process causes cooperative O_2 binding, which is illustrated by the characteristic sigmoidal shape of the Hb-O_2 equilibrium curve (Fig. 2B). Cooperativity allows maximal O_2 release over relatively small drops in O_2 tension. Mutations within the α1β2 interaction region can alter the functional properties of Hb, mainly by perturbing O_2-binding characteristics (Fig. 2C, cyan spheres; Table 1).

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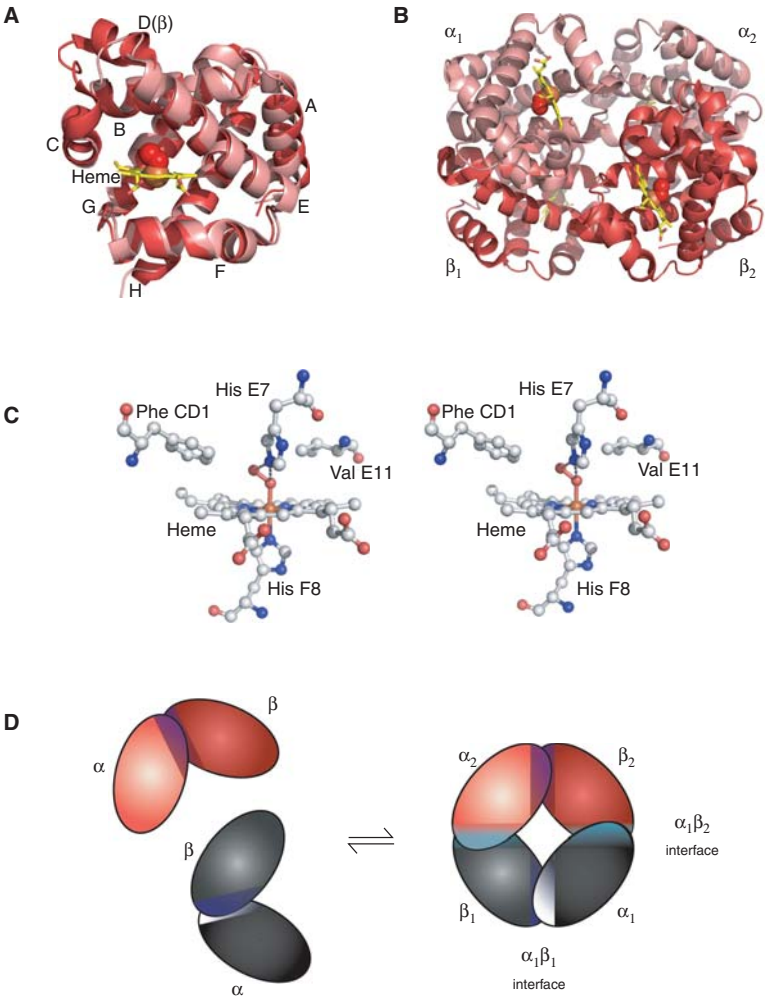

Figure 1. The structure of Hb. (*A*) The α (pink) and β (red) Hb subunits have conserved α-helical folds. Helices are labeled A–H from the amino terminus. The α subunit lacks helix D. (*B*) The high O_2 affinity R state quaternary structure of Hb with O_2 (red spheres) bound at all four heme sites (protoporphyrin-IX as yellow sticks, with central iron atom as orange sphere). (*C*) Stereo (wall-eye) diagram of the heme pocket of β showing the proximal (F8) and distal (E7) histidines and selected residues in the distal heme pocket that influence ligand binding and autoxidation. (*D*) Hb tetramer is assembled from two identical αβ dimers (shown in red and gray for clarity). In the tetramer, each subunit makes contact with the unlike chain through a high affinity dimerization α1β1 interface and a lower affinity α1β2 dimer–tetramer interface (cyan).

Cooperativity represents a general phenomenon termed allosteric regulation, in which effector molecules control the properties of enzymes or other proteins by binding to regions that are distinct from the active sites. Several allosteric regulators, in addition to O_2, influence the properties of Hb. For example, H^+ binds Hb to promote O_2 release in a process termed the Bohr effect (Perutz et al. 1984). In peripheral tissues, abundant CO_2 is taken up by red blood cells and metabolized by carbonic anhydrase to carbonic acid, resulting in the production of H^+ and consequent O_2 release. In contrast, relatively low CO_2 and high pH in the lung favors O_2 binding to Hb. Additionally, CO_2 forms carbamino adducts with the amino termini of both α and β

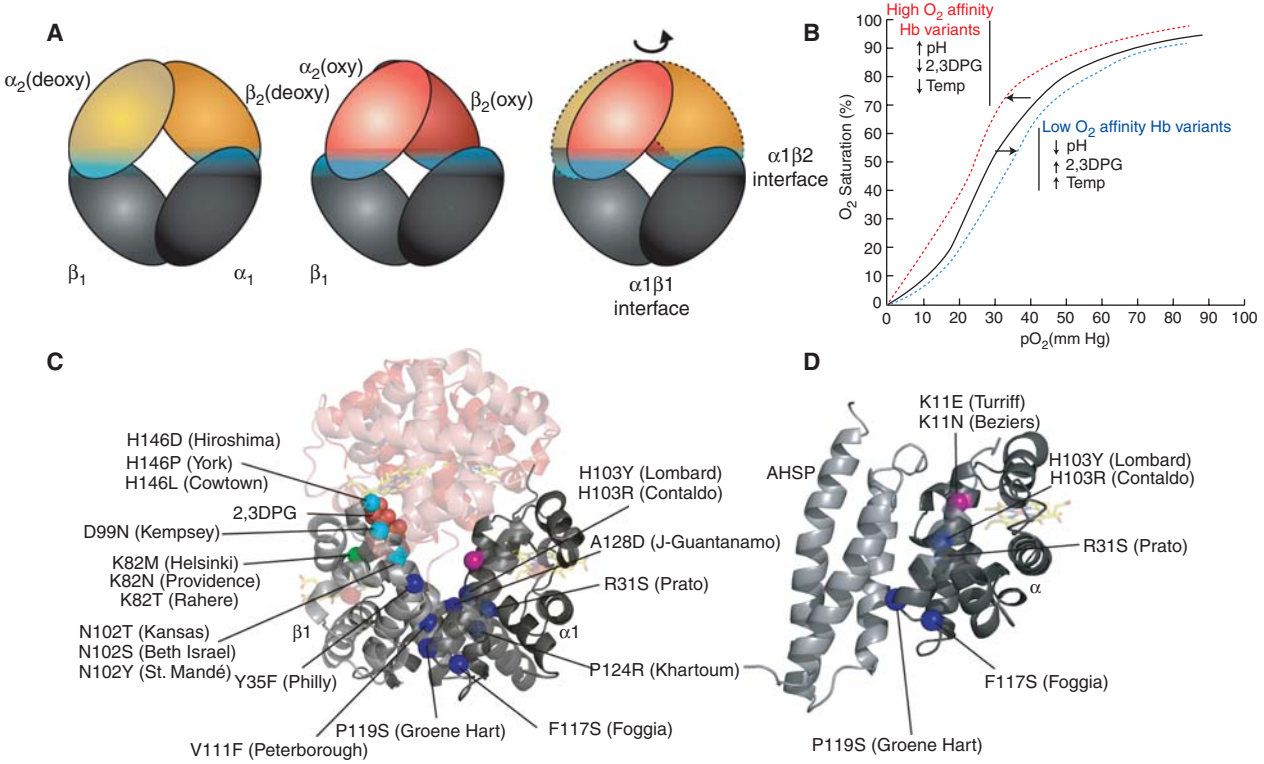

Figure 2. Hb variants with altered subunit interactions. (*A*) Conversion from the low O_2 affinity (deoxy, T state) to high O_2 affinity (oxy, R state) involves a relative rotation of the α1β1 and α2β2 dimers, with changes in contacts across the α1β2 (and α2β1) interface (cyan). In this cartoon, the α1β1 dimer is held stationary to reveal the relative motion of the α2β2 dimer in going from the deoxy (orange) to the oxy (red) states. (*B*) The sigmoidal shape of the Hb-O_2 saturation curve shows allosteric regulation by changes in pH, temperature, and 2,3DPG. These regulators, as well as Hb variants, influence the shape of the curve. High oxygen affinity variants, high pH, low 2,3DPG, or low temperature induce a "left shift" in the saturation curve (red line). Conversely, low oxygen affinity variants, low pH, high 2,3DPG, or high temperature induce a "right shift" (blue line). (*C*) Hb sequence variants at the allosteric α1β2 interface (cyan spheres) show an impaired response to O_2 binding. Some sequence variants disrupt binding to other allosteric regulators, e.g., substitutions at βK82 (green) disrupt interactions with 2,3DPG that normally stabilize the low O_2 affinity T state. Mutations that disrupt α1β1 (and α2β2) dimerization (blue spheres) increase the concentration of free monomers, which are unstable. (*D*) Some α mutations that disrupt binding to β may also disturb binding to the chaperone, AHSP. Other α variants, such as Turriff and Beziers (pink sphere) may inhibit only AHSP binding.

globins in vivo. These interactions produce minor effects on whole blood O_2 affinity compared to the impact of CO_2 on pH (Bunn and Forget 1986). The compound 2,3-diphosphoglycerate (2,3DPG), formed as a by-product of glycolysis and present at relatively high concentrations in red blood cells, is another important allosteric regulator of Hb. 2,3DPG binds and stabilizes T state Hb to facilitate O_2 release. Hemoglobin binding sites for H^+ and 2,3DPG have been identified (Perutz et al. 1969, 1984; Perutz 1970; Arnone 1972). Together, these interactions allow Hb to sense metabolic activity and modulate O_2 binding accordingly. This environmental sensory function can be altered by mutations that affect the affinity of Hb for its allosteric regulators (Fig. 2C, green sphere; Table 1).

Identification of Hb Variants and Their Clinical Implications

Many Hb variants are readily ascertained through physical examination and/or routine laboratory testing, which explains why so many have been discovered. Uncommonly, some variants are identified through evaluation of ill patients with severe anemia or clinically significant cyanosis. Many amino acid substitutions alter surface charge and are thus detected by electrophoresis or chromatography, techniques which are routinely performed on neonates in North America and Europe. Interestingly, a few Hb variants migrate similarly to HbA_{1c}, a glycosylated form of Hb that reflects long-term control of blood glucose levels in diabetic patients (Table 1) (reviewed in Little and Roberts 2009). In this way, specific Hb amino acid substitutions can artificially elevate HbA_{1c} measurement and interfere with diabetic management. Other Hb variants are clinically benign but produce obvious changes in skin color. For example, mutations that increase Hb-O_2 affinity typically stimulate erythropoietic drive by inhibiting O_2 tissue delivery, causing erythrocytosis associated with a ruddy complexion (Nathan et al. 2009). Mutations that reduce O_2 affinity produce a bluish hue to the skin (cyanosis) caused by abnormally high levels of deoxyHb. Mutations that favor oxidation of Hb iron to the met form (M-Hbs), also cause blue-tinged skin, whereas the blood itself appears brown. Studies of one family with congenital cyanosis led Horlein and Weber to describe the first known hemoglobinopathy, caused by the variant Hb-M Saskatoon (Horlein and Weber 1948). The M-Hb variant Iwate, which causes "black blood" or "hereditary nigremia," was first described more than 200 years ago in Japan (Shibata et al. 1960).

It is important to note that many Hb variants affecting skin color are not clinically damaging beyond their cosmetic effects. However, these conditions can mimic more life-threatening problems such as cardiopulmonary and myeloproliferative disorders, which must be excluded. Thus, patients with cyanotic or polycythemic Hb variants may mistakenly undergo unnecessary and potentially dangerous medical procedures. Historical examples include cyanotic patients undergoing surgery or catheterization for presumed heart defects and polycythemic patients receiving radioactive ^{32}P for presumed polycythemia vera (Steinberg et al. 2001; Nathan et al. 2009). As stated, "the primary reason for establishing the diagnosis [of M Hbs] is to prevent iatrogenic misadventures that might arise under the mistaken impression that the patient has a cardiac or pulmonary disorder" (Bunn and Forget 1986). Most Hb variants with altered O_2 affinity are relatively simple to diagnose through history, physical examination, and laboratory testing (Wajcman et al. 2001; Wajcman and Moradkhani 2011).

Globin Gene Synthesis Is Developmentally Regulated

Developmental regulation of the α-like and β-like globin gene families is of great medical significance (Sankaran and Orkin 2012). Hemoglobin F ($\alpha_2\gamma_2$) is the most highly expressed form during late fetal gestation. After birth, expression gradually switches to HbA ($\alpha_2\beta_2$) over several months. Thus, symptomatic mutations affecting α or γ globins are present prenatally or at birth, whereas the manifestations of β-globin mutations are typically delayed until a few months after birth. Interestingly, γ-globin gene mutations that are apparent at birth, most

typically reflected by cyanosis or hemolytic anemia (e.g., Hb-F Toms River, sections on Selected Variants that Illustrate Important Aspects of Hemoglobin Biology and Variants that Affect Multiple Hemoglobin Functions, and Table 1), fade over a few weeks as Hb production switches from F ($\alpha_2\gamma_2$) to A ($\alpha_2\beta_2$).

Laboratory Testing for Hb Variants

In many countries, routine testing of all newborns is performed to identify common hemoglobinopathies such as some thalassemias and HbS. Isoelectric focusing or high-performance liquid chromatography (HPLC), the most commonly used tests, identify most structurally abnormal Hbs (Wajcman et al. 2001). In this way, many benign Hb variants are discovered incidentally. Clinical indications for laboratory testing to investigate potential Hb variants are listed in Table 2.

Specific laboratory tests to investigate Hb variants include:

a. *Physical methods of Hb separation*. These include electrophoretic and chromatographic techniques that examine the physical properties of $\alpha 1\beta 1$ dimers or individual globin subunits. Specific standards, such as HbA, HbS, HbC, HbF, and HbA_2, are typically examined as controls. Hb variants may show altered migration in these assays. Historically, cellulose acetate and citrate agar electrophoresis were most commonly used to detect variant Hbs. Current clinical testing more typically uses isoelectric focusing and HPLC, which are more sensitive.

Table 2. Clinical indications for laboratory testing to diagnose Hb variants

Indications for hemoglobin testing
Routine newborn testing for common hemoglobinopathies (i.e., HbS, HbC, thalassemias)
Cyanosis with adequate arterial oxygenation and no apparent cardiopulmonary disease
Erythrocytosis with normal or elevated erythropoietin levels
Unexplained hemolytic anemia
Unexplained thalassemia phenotype
Family history consistent with an Hb variant

b. *Hb-O_2 binding curve*. This test, performed on whole red blood cells or hemolysate, indicates the percent (%) oxygenated Hb at a given O_2 partial pressure (Fig. 2B). Hemoglobin variants with an abnormally high O_2 affinity (see sections on Selected Variants that Illustrate Important Aspects of Hemoglobin Biology and High Oxygen Affinity Variants) will become saturated at lower O_2 pressures producing a "left-shifted" O_2 equilibrium curve, whereas mutations that reduce O_2 affinity (see sections on Selected Variants that Illustrate Important Aspects of Hemoglobin Biology and Low Oxygen Affinity Variants) will cause the opposite "right shift." Determination of Hb-O_2 affinity responses to allosteric regulators, particularly 2,3DPG or H^+ (pH changes), can provide insight into the structural causes of the observed phenotypes. Unfortunately, few clinical laboratories currently offer this assay.

c. *Visible wavelength spectroscopy*. Hemoglobin variants with amino acid substitutions in the heme pocket affect visible light absorbance. For example, M-type Hbs show characteristic spectra that can distinguish them from methemoglobinemia caused by an enzyme deficiency in the metHb reductase system (Bunn and Forget 1986; Dailey and Meissner 2012; Ganz and Nemeth 2012; Schechter 2012). Pulse oximetry is a noninvasive spectrophotometric test that measures absorbance ratios at specific wavelengths for oxygenated (660 nm) and deoxygenated (940 nm) blood (Tremper and Barker 1989). This can produce confusing and sometimes misleading results in patients with variant Hbs that show unique light absorbance properties (Verhovsek et al. 2010). In these cases, analysis of arterial blood O_2 concentration may be required to rule out hypoxia. Analyzing these variant Hbs using light absorbance throughout the full visible wavelength spectrum can provide useful diagnostic information.

d. *Hemoglobin stability testing.* Typically, Hb stability is impaired in variants that are associated with hemolytic anemia. Hemoglobin stability tests measure the propensity for Hb to denature on exposure to various stresses including heat (Carrell and Kay 1972), isopropanol (Bender et al. 1981), mechanical agitation (Asakura et al. 1975), and zinc acetate (Roth et al. 1976). The Heinz body test uses supravital stains, such as methylene blue or crystal violet, to detect aggregated globins within erythrocytes (Eisinger et al. 1985).

e. *Specialized testing.* Mass spectrometry analysis of patient hemolysate and DNA sequencing of globin genes are specialized confirmatory tests to identify amino acid and nucleotide alterations associated with suspected Hb variants (Wajcman et al. 2001; Wajcman and Moradkhani 2011). DNA sequencing may readily elucidate the presence of an Hb variant. However, additional biochemical and structural studies, including those discussed in this section, are required to determine how the variant affects Hb function. Crystallographic analysis is the highest resolution approach to determine the effects of globin amino acid substitutions on molecular structure. Crystallography has been used historically as a research tool to assess the effects of some interesting Hb variants (see, for example, Pulsinelli et al. 1973; Perutz et al. 1984).

SELECTED VARIANTS THAT ILLUSTRATE IMPORTANT ASPECTS OF HEMOGLOBIN BIOLOGY

Unstable Variants

Unstable variants frequently cause congenital Heinz body hemolytic anemia detected by laboratory screening and clinical symptoms (see sections on Basic Principles and Laboratory Testing for Hb Variants and Table 2). Mutations that alter any step in globin processing, including subunit folding, heme interaction, dimerization, or tetramerization, can destabilize Hb. Bunn and Forget note five general mechanisms that destabilize Hbs: amino acid substitutions within the heme pocket, disruption of secondary structure, substitution in the hydrophobic interior of the subunit, amino acid deletions, and elongation of the subunit (Bunn and Forget 1986).

More than 75% of Hb is α helical (Perutz et al. 1960; Park et al. 2006). This structure is particularly susceptible to disruptions by proline substitutions (Levitt 1981). For example, in Hb Brockton (β138 [H16] Ala > Pro) the substituted proline disrupts intermolecular hydrogen bonding between β138Ala and β134Val in helix H (Russu and Ho 1986; Moo-Penn et al. 1988). This produces an unstable variant with a propensity to precipitate and aggregate, thereby damaging erythrocytes and predisposing to hemolysis. Hb Brockton does not show altered O_2 binding affinity or electrophoretic mobility shifts. This variant was identified by HPLC analysis of patient globin chains and its altered X-ray crystallography diffraction pattern shows local disruption of helix H (Moo-Penn et al. 1988).

Mutations at the α1β1 interface can cause hemolytic anemia by inhibiting heterodimer formation, favoring the accumulation of free globin subunits, which themselves are unstable, particularly α chains (Fig. 2C, blue spheres). Examples include Hb Philly (β35 [C1] Tyr > Phe) (Rieder et al. 1969), Hb Peterborough (β111 [G13] Val > Phe) (King et al. 1972), Hb Stanmore (β111 [G13] Val > Ala) (Como et al. 1991), and Hb J-Guantanamo (β128 [H6] Ala > Asp) (Martínez et al. 1977). Hb Khartoum (β124 [H2] Pro > Arg) contains a substitution at the α1β1 interface that is mildly destabilizing in vitro, but does not cause clinical symptoms (Clegg et al. 1969; Argos et al. 1979).

Interestingly, some α-globin (*HBA*) gene mutations affecting the α1β1 interface may also destabilize free α chains by inhibiting their binding to α-hemoglobin stabilizing protein (AHSP), an erythroid molecular chaperone that facilitates Hb assembly (Fig. 2D, blue spheres) (reviewed in Weiss et al. 2005; Favero and Costa 2011). These α-globin variants include Hbs Prato (α1 or α2 31 [B12] Arg > Ser) (Marinucci et al. 1979), Lombard (α2 103 [G10] His > Tyr) (Hoyer et al. 2002), Contaldo

(α1 or α2 103 [G10] His > Arg) (Sciarratta et al. 1984), Foggia (α2 117 [GH5] Phe > Ser) (Lacerra et al. 2008), Groene Hart (α1 119 [H2] Pro > Ser) (Harteveld et al. 2002; Vasseur-Godbillon et al. 2006; Giordano et al. 2007; Vasseur et al. 2009), and others (Wajcman et al. 2008; Yu et al. 2009). Naturally occurring α-globin variants with amino acid substitutions at position 99 including Hb Turriff (α1 or α2 99 [G6] Lys > Glu) (Langdown et al. 1992) and Hb Beziers (α1 99 [G6] Lys > Asn) (Lacan et al. 2004) bind β globin normally but show impaired interaction with AHSP and may be mildly destabilizing (Fig. 2D, violet spheres) (see also Yu et al. 2009; Khandros et al. 2012; Mollan et al. 2012). Antitermination mutations can also destabilize α globin in part by impairing its binding to AHSP (Turbpaiboon et al. 2006).

Hyperunstable Hb variants precipitate shortly after synthesis and are not incorporated into Hb tetramers. These ephemeral proteins are difficult to isolate. In this case, electrophoresis may be falsely negative due to the rapid turnover of these variants, making DNA sequencing a critical diagnostic test. Affected patients show a dominantly inherited "inclusion body thalassemia" resulting from both the precipitated variant globin and consequent chain imbalance with accumulation of the unaffected globin, which is unstable in its free form (Stamatoyannopoulos et al. 1974). Patient erythrocytes typically display abnormal morphology with microcytosis, hypochromia, moderate to severe anisopoikilocytosis, basophilic stippling, and inclusions that may be become particularly prominent following splenectomy (Steinberg et al. 2001). Weatherall, Thein, and colleagues characterized several hyperunstable mutations in exon 3 of the β-globin gene (Thein et al. 1990). All of the mutations were frameshifts or nonsense codons that produced relatively long (>120 amino acid) proteins with carboxy-terminal truncations. The investigators proposed that truncated globins causing dominantly inherited thalassemia are long enough to bind heme posttranslationally, which rendered them relatively resistant to proteolytic degradation, allowing for subsequent precipitation of heme-containing aggregates detected as Heinz bodies. Missense mutations also cause hyperunstable Hb variants. Hb Hirosaki (α2 43 [CE1] Phe > Leu) was discovered in a family with hemolytic anemia (Ohba et al. 1975; Tanaka et al. 2005). After several tests failed to identify a soluble variant Hb within erythrocytes, DNA sequencing was used to characterize the mutation. Hb Terre Haute (β106 [G8] Leu > Arg) is another hyperunstable variant associated with a severe Heinz body hemolytic anemia and globin chain imbalance (Coleman et al. 1991). In initial studies of patient erythroid cells, performed in 1979, abnormal Hb tetramers were not detected and peptide mapping of radiolabeled nascent globins identified a β112 (G14) Cys > Arg substitution, originally termed Hb Indianapolis (Adams et al. 1979). However, the β112 Cys > Arg mutation was subsequently identified in unrelated individuals with much less severe disease. In 1991, reevaluation of the original pedigree by DNA analysis discovered a β106 Leu > Arg mutation, which was renamed Hb Terre Haute (Coleman et al. 1991). Most likely, incomplete tryptic cleavage of the abnormal β-globin peptide in the earlier studies led to misidentification of the causative mutation. This work reflects the interesting historical point that many Hb variants were identified through laborious and technically challenging protein studies performed some time ago, before DNA sequence analysis of patient globin genes was feasible. Reevaluation of these mutations through genetic testing has yielded some interesting surprises (see also the discussion on Hb Bristol-Alesha and the section on Variants that Affect Multiple Hemoglobin Functions).

High Oxygen Affinity Variants

Hemoglobin variants with increased O_2 affinity cause erythrocytosis by stimulating erythropoietic drive (see Hebbel et al. 1978 for a description of the associated physiology). This commonly results from amino acid substitutions that stabilize the R (high O_2 affinity) state relative to the T (low O_2 affinity) state and/or inhibit responses to environmental allosteric regulators that stimulate O_2 release, including H^+ (Bohr effect) or 2,3DPG (see the sections on

Basic Principles and Hemoglobin Synthesis, Structure, and Function). Because T to R state transitions are mediated largely through α1β2 interactions, high affinity variants frequently result from substitutions that alter this interface (Fig. 2C, cyan spheres). For example, the amino acid replacement in Hb Kempsey (β99 [G1] Asp > Asn) perturbs α1β2 interactions by preventing the formation of a hydrogen bond between β99 Asp and α42 Tyr, which normally stabilizes the deoxygenated low O_2 affinity T state (Fig. 3) (Reed et al. 1968; Lindstrom et al. 1973; Bunn et al. 1974). This structural change shifts quaternary equilibrium toward the oxygenated R form, which impairs O_2 release to peripheral tissues and thus increases erythropoietic drive. The carboxyl termini of globin chains are also involved in α1β2 interactions that stabilize the low O_2 affinity T state and numerous substitutions within these regions cause high O_2 affinity variants. In addition, β146 His at the carboxyl terminus contributes significantly to the Bohr effect by forming a salt bridge with β94 Asp (Perutz et al. 1984). This interaction is disrupted in several high O_2 affinity variants with β146 substitutions: Hb Hiroshima (β146 [HC3] His > Asp) (Hamilton et al. 1969; Perutz et al. 1971; Imai et al. 1972; Olson et al. 1972), Hb York (β146 [HC3] His > Pro) (Bare et al. 1976), and Hb Cowtown (β146 [HC3] His > Leu) (Fig. 2C) (Schneider et al. 1979; Perutz et al. 1984). These variants show reduced Bohr effect with impaired release of O_2 under acidic conditions.

Several high affinity Hb variants are caused by substitutions that inhibit interaction with 2,3DPG, which normally binds globin chains to stimulate O_2 release (Fig. 2C). For example, Hb Rahere (β82 [EF6] Lys > Thr) replaces an

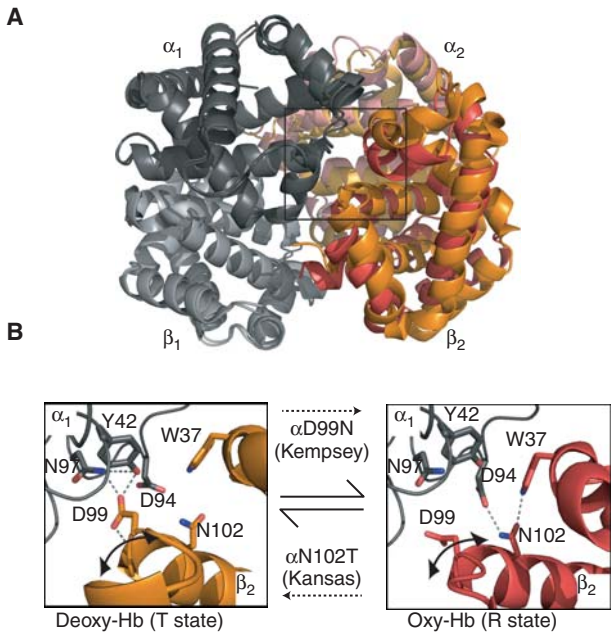

Figure 3. Hb variants that affect allosteric regulation. (*A*) The α2 and β2 subunits indicated on the *right* side of the figure show an overlay of the R-state (red/pink) and T-state (orange/light orange) quaternary structures of Hb. The allosteric $\alpha_1\beta_2$ interface is boxed. (*B*) Detail of the $\alpha_1\beta_2$ interface in the deoxy T state (β_2 chain in orange, PDB 2DN2) and oxy R state (β_2 chain in red, PDB 2DN1) showing selected H-bonding interactions. Note that the two H-bonding networks use nonoverlapping sets of side chains, hence mutations in these residues affect only one state. Mutation of Asp99 to Asn (Hb Kempsey) compromises electrostatic interactions in the deoxygenated state, thereby favoring the R state and causing impaired O_2 release. Mutation of Asn102 to Thr (Hb Kansas) abrogates interactions with Asp94, favoring the T state and O_2 binding inhibition.

invariant lysine in the 2,3DPG binding site of β globin, thereby reducing the affinity for this allosteric regulator (Lorkin et al. 1975; Sugihara et al. 1985). Consequently, Hb Rahere shows blunted O_2 release in response to added 2,3DPG in vitro. In vivo, O_2 release in peripheral tissues is inhibited, resulting in elevated blood Hb levels. Similarly, Hb Providence (β82 [EF6] Lys > Asn) (Bonaventura et al. 1976; Moo-Penn et al. 1976) and Hb Helsinki (β82 [EF6] Lys > Met) (Ikkala et al. 1976; Charache et al. 1977) are high O_2 variants caused by different amino acid substitutions at the 2,3DPG binding site on β82.

Low Oxygen Affinity Variants

Low O_2 affinity Hb variants typically present with cyanosis. In general, these variants are caused by globin amino acid substitutions that tip the quaternary equilibrium of Hb tetramers from the high affinity oxygenated R state to the low affinity deoxygenated T state; more or less the opposite of what occurs for high O_2 affinity variants (see the sections on Selected Variants that Illustrate Important Aspects of Hemoglobin Biology and High Oxygen Affinity Variants). This does not inhibit Hb-O_2 release in tissue capillaries, but rather, interferes with Hb-O_2 uptake if the P_{50} has increased to $\geq$50 mm Hg. Paradoxically, low O_2 affinity Hb variants can be associated with mild anemia thought to be caused by increased O_2 tissue delivery with reduced erythropoietic drive (Stamatoyannopoulos et al. 1969). In addition, some low O_2 affinity mutants are unstable and therefore associated with not only cyanosis but also Heinz body hemolytic anemia (see the sections on Selected Variants that Illustrate Important Aspects of Hemoglobin Biology and Unstable Variants).

Several low affinity variants involve replacements at the α1β2 interface, which plays an important role in Hb cooperativity. Hb Kansas (β102 [G4] Asn > Thr) is a well-studied low O_2 affinity variant (Fig. 3B) (Reissmann et al. 1961; Bonaventura and Riggs 1968; Gibson et al. 1973; Riggs and Gibson 1973). Affected individuals are markedly cyanotic, although clinically well. Replacement of Asn102 at the α1β2 interface inhibits the formation of a hydrogen bond with Asp94 that normally stabilizes the oxygenated R structure. A similar mechanism causes low O_2 affinity in two other Hb variants through different substitutions of the same amino acid residue (B102 [G4] Asn) in Hb Beth Israel (β102 [G4] Asn > Ser) (Nagel et al. 1976) and Hb St. Mandé (β102 [G4] Asn > Tyr) (Arous et al. 1981; Poyart et al. 1990).

Methemoglobin ("M-Type") Variants

Hemoglobin iron must be in its reduced (Fe^{2+}, ferrous) state to bind O_2. Moreover, oxidized (Fe^{3+}, ferric, met) Hb is intrinsically unstable with a tendency to release heme. Hemoglobin reduction is maintained through intrinsic features of the Hb protein and extrinsic antioxidant pathways within red blood cells (see the sections on Basic Principles and Hemoglobin Synthesis, Structure, and Function). Exposure to oxidant drugs or toxins, genetic alterations in erythroid metHb reductase enzyme systems (Ganz and Nemeth 2012; Schechter 2012), or globin chain variants can predispose to methemoglobinemia. These disorders present as "pseudocyanosis," (i.e., low Hb-O_2 saturation), despite adequate arterial oxygenation. Detailed in vitro analyses of the red cell and isolated Hb samples can usually distinguish wild-type metHb resulting from toxins or defective reductase systems and M-type Hb variants that are predisposed to spontaneous oxidation (Bunn and Forget 1986; Steinberg et al. 2001; Nathan et al. 2009). For example, various M-Hbs show characteristic visible absorbance spectra.

Globin variants associated with metHb formation are typically caused by amino acid substitutions within the heme pocket. For example, four different M-Hbs occur when tyrosine replaces the α- or β-globin proximal or distal histidine residues that interact with heme (Fig. 1C) (reviewed in Adachi et al. 2011). In Hb M-Iwate (α1 or α2 87 [F8] His > Tyr), the proximal histidine is replaced by tyrosine (Fig. 4A), which deprotonates and coordinates to the heme iron (Fig. 4B) (Konigsberg and Lehmann 1965; Shimizu et al. 1965). Ferric heme, bound through the native His F8, is readily reduced by metHb

reductase (Fig. 4C). Tyrosine (F8) coordination stabilizes the oxidized ferric state and decreases reactivity with metHb reductases. This interaction also distorts the position of heme and helix F within the altered α subunits. In native Hb, movement of the proximal His F8 and F helix away from the heme group stabilizes the deoxygenated T state and reduces the O_2 affinity of the native β subunit partners. Hence, substitution of the normal His F8 side chain for a longer Tyr F8 (Fig. 4A) also stabilizes the deoxygenated T state and reduces O_2 affinity of the native β subunit in Hb M-Iwate (Nagai et al. 2000; Jin et al. 2004). These biochemical and structural alterations underlie the lack of cooperativity and severe cyanosis in patients with Hb M-Iwate, in which metHb levels can exceed 20% (normal <2%) (Ameri et al. 1999). In contrast, Hb M-Saskatoon (β63 [E7] His > Tyr) replaces the distal His with Tyr (Fig. 4D) (Horlein and Weber 1948; Hayashi et al. 1966). In this variant, the protonated form of mutant Tyr can bind ferric heme iron to generate a hexacoordinate structure that is relatively easily reduced by cellular metHb reductases (Fig. 4E). Consequently, patients with Hb M-Saskatoon have lower levels of circulating oxidized Hb compared with those with Hb M-Iwate. Comparative studies of these patients and their variant M-Hbs have contributed greatly to understanding the biochemical properties of the heme iron, including its interactions with various ligands and nearby amino acids such as the proximal and distal histidines.

Globin Chain Elongation Mutants

Antitermination and frameshift mutations that add irrelevant amino acids to the carboxyl terminus of globin proteins produce interesting variants that can damage erythrocytes (Nathan et al. 2009). The most clinically significant

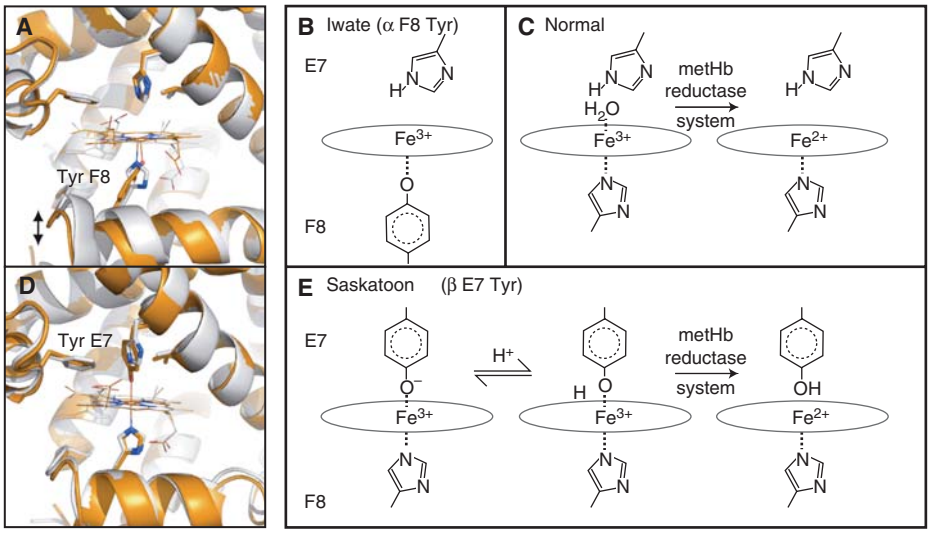

Figure 4. Examples of M-type hemoglobins. (*A*) The heme pocket of wild-type Mb (gray) and the F8 His > Tyr mutation (orange, PDB 1HRM), which serves as a model for Hb M-Iwate. Effects on the protein fold are to increase the distance from the heme to the F helix, recapitulating features of the deoxy-α (T state) structure. (*B*) In Hb M-Iwate, α 87 Tyr F8 is deprotonated and favors Fe^{3+} oxidation state, resulting in rapid autoxidation. This ferric form is not reduced by met-Hb reductase. (*C*) With the normal His F8 present, ferric heme is readily reduced. (*D*) Substituting the distal His E7 side chain in Mb for a larger Tyr E7 (orange, PDB 1MGN), as also occurs in Hb Saskatoon, brings the Tyr hydroxyl group within binding distance of the iron, forming a hexacoordinate iron site. (*E*) In Hb Saskatoon the hexacoordinate ferric iron in the effected β chains *can* be reduced by met-Hb reductase, possibly owing to a transient protonation of Tyr E7. Note that Mb is used as a model for Hb subunits in *A* and *D*, whereas *B* and *E* are based on Hb spectroscopy.

example is Hb Constant Spring (α2 142 [HC3] Stop > Gln), caused by an antitermination mutation at the α2 stop codon (Clegg et al. 1971; Efremov et al. 1971; Milner et al. 1971; Clegg and Weatherall 1974). This elongates the protein by 31 amino acids, generating an unstable protein that is relatively underrepresented in hemolysates, but can be detected by physical methods (see sections on Basic Principles, Laboratory Testing for Hb Variants, and *Physical methods of Hb separation*). In addition, Hb Constant Spring mRNA is rapidly degraded in developing erythrocytes, owing to ribosomal entry into the 3′UTR, causing displacement of RNA-bound stabilizing proteins with a resultant thalassemia syndrome (Hunt et al. 1982; Derry et al. 1984; Weiss and Liebhaber 1994; Morales et al. 1997).

Hb Constant Spring contributes to α-thalassemia syndromes, particularly when combined with two α-globin deletional alleles ($-/\alpha^{CS}\alpha$), which produces a severe form of HbH disease (Viprakasit and Tanphaichitr 2002). Isolated Hb Constant Spring, in its heterozygous ($\alpha\alpha/\alpha^{CS}\alpha$) or homozygous ($\alpha^{CS}\alpha/\alpha^{CS}\alpha$) forms, results in more severe anemia than occurs when the same α alleles are deleted ($\alpha\alpha/-\alpha$) or ($-\alpha/-\alpha$) (Schrier et al. 1997). This is due to the cytotoxic effects of the unstable Constant Spring protein. Although most common in Southeast Asia, Hb Constant Spring is increasingly identified in other geographic regions, largely through global migration (Lal et al. 2011). In fact, it was first discovered in a Chinese family living in Constant Spring, Jamaica.

One example of a β-globin chain elongation mutant is Hb Cranston (β 145 [HC3] +CT) (Bunn et al. 1975). This mutation introduces a frameshift at the normal stop codon to generate a β chain that is extended by 11 amino acids. This results in an unstable Hb tetramer with high O_2 affinity and diminished cooperativity (McDonald et al. 1980; Shaeffer et al. 1980). Affected patients show compensated hemolytic anemia with the variants accounting for 30% of total Hb in the hemolysate. Interestingly, the structure of Hb Cranston was investigated simultaneously with studies to determine the β-globin mRNA 3′ untranslated sequence (Forget et al. 1975). Cross-comparison of the protein and mRNA sequencing data allowed Bunn, Forget, and colleagues to more rapidly define normal β-globin gene structures and ascertain that the Hb Cranston mutation likely arose by nonhomologous crossover of two normal β-globin genes.

Variants that Affect Multiple Hemoglobin Functions

Not surprisingly, amino acid substitutions within critical regions of globin proteins can produce multiple effects. For example, HbE (β26 [B8] Glu > Lys), a common variant in Southeast Asia, contains an amino acid substitution that renders β chains mildly unstable in vitro with minimal clinical significance (Frischer and Bowman 1975; Huisman 1997; Rees et al. 1998; see also Musallam et al. 2012). However, this mutation also creates an alternate splice site in the β-globin mRNA, leading to reduced synthesis of productive transcripts with resultant thalassemia (Orkin et al. 1982). HbE is particularly deleterious when coinherited with a more severe β-thalassemic allele, which happens commonly in Southeast Asia.

Mutations that alter the heme pocket commonly produce multiple biochemical effects. For example, deletion or substitution of the conserved Phe residue at the CD1 helical region in the heme pocket markedly destabilizes the affected globin and also alters its O_2 affinity. Thus, Hb Bruxelles (β42 [CD1] Phe > 0) (Blouquit et al. 1989; Griffon et al. 1996), Hb Warsaw (β42 [CD1] Phe > Val) (Honig et al. 1990), Hb Hammersmith (β42 [CD1] Phe > Ser) (Dacie et al. 1967), and Hb Buccuresti-Louisville (β42 [CD1] Phe > Leu) (Bratu et al. 1971; Keeling et al. 1971) cause both congenital Heinz body hemolytic anemia and cyanosis. These combined effects arise from severely reduced cooperativity, rapid rates of autoxidation and hemin loss, and unfolding of these unstable globin variants (Griffon et al. 1996).

Another interesting heme pocket variant is Hb Zurich (β63 [E7] His > Arg) in which the distal His is replaced by Arg (Huisman et al. 1961; Bachmann and Martihr 1962; Frick et al.

1962; Tucker et al. 1978; Phillips et al. 1981; Springer et al. 1989). The large, highly polar variant His side chain swings out of the distal heme pocket, and the positively charged guanidino group forms a salt bridge with a heme propionate (Fig. 5A). This results in an enlarged ligand-binding pocket, destabilizing O_2 binding and causing iron autoxidation via exposure to water. Affected individuals show increased sensitivity to oxidant agents, including sulfa drugs, which more easily enter the expanded heme pocket. Loss of the distal histidine markedly decreases O_2 affinity but has little effect on carbon monoxide (CO) binding. As a result, individuals with Hb Zurich tend to have supranormal levels of CO-Hb, which ironically protects the heme iron from oxidation and the globin from denaturation. Affected subjects who are cigarette smokers accumulate even higher levels of CO-Hb, which tends to protect against hemolysis. Thus, "the pathology of a mutant protein is ameliorated by a normally toxic pollutant" (Bunn and Forget 1986).

Two other recently identified Hb variants illustrate how multiple biochemical defects produce unique phenotypes. Hb Jamaica Plain

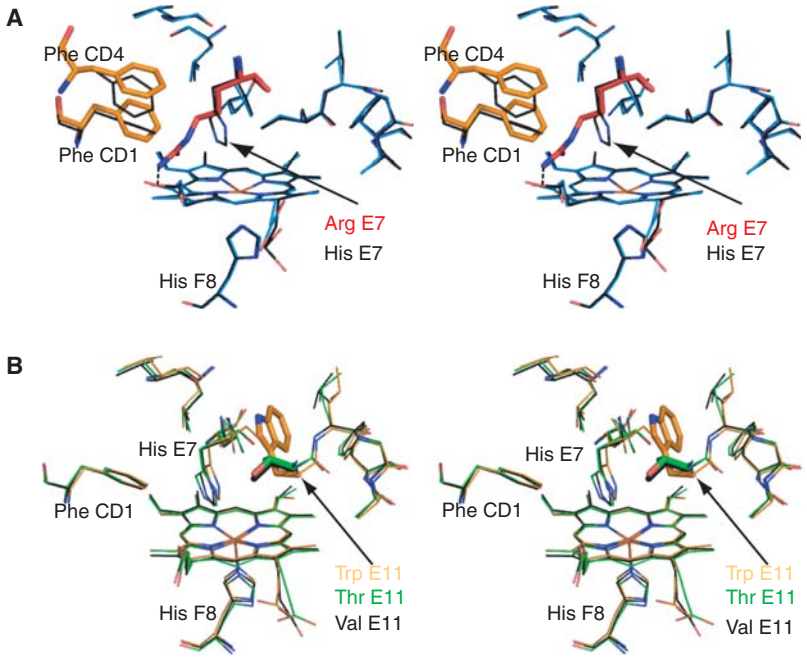

Figure 5. Hb variants with amino acid changes in the heme pocket. (*A*) Stereo diagram of a model of the deoxy Hb Zurich β heme pocket (blue) overlaid with the wild-type β heme pocket (black, PDB 2DN2). This model of Hb Zurich is based on the structures solved by Phillips et al. (1981) and Tucker et al. (1978). It was generated with the macromolecular modeling program Coot (Emsley et al. 2010) by mutating the distal His of deoxy-β (PDB 2DN2) to Arg. The distal Arg E7 (red) is oriented toward the CD corner, disturbing the position of Phe CD1 and Phe CD4 (orange). The heme pocket entrance is much wider allowing increased access to ligands. However, unlike normal His E7, mutant Arg is unable to stabilize bound O_2 via hydrogen bonding. (*B*) Stereo diagram showing the structural changes associated with substitution of β Val E11. The wild-type structure carrying the branched hydrophobic side chain Val (black bonds, PDB 2DN2) is overlaid with structures carrying the largest aromatic side chain Trp E11 (orange, PDB 101K) or a polar side chain Thr E11 (green PDB, 1HDB). It is clear that no major backbone or side chain repacking occurs. Thus, mutations in this position are likely to have specific effects in changing the volume of the heme pocket accessible to solvent or diatomic ligands and the electrostatic properties of the pocket. These changes will manifest as differences in O_2 binding, ligand selectivity, autoxidation, and heme loss.

(β6 [A3] Glu > Val and β68 [E12] Leu > Phe) contains two defects in the same β chain, a β6 Glu to Val substitution that causes sickle cell anemia (Serjeant and Rodgers 2012) and a β68 amino acid substitution that reduces O_2 affinity, probably by destabilizing the oxygenated conformation through steric effects introduced into helix E (Geva et al. 2004). The affected patient, who was heterozygous for the mutant allele, showed symptoms of sickle cell anemia that were precipitated by infection or airplane travel. Thus, an amino acid substitution that reduces O_2 affinity exacerbates the effects of a sickling mutation in the same globin chain.

Several other Hb variants modulate the severity of sickle cell anemia (see also Cao and Kan 2012; Schechter and Elion 2012; Serjeant and Rodgers 2012). For example, γ globin inhibits polymerization of HbS (Nagel et al. 1979). This effect is attributable to differences in several amino acid residues compared with the corresponding β chain, including γ80 and γ87 (Adachi and Asakura 1979; Nagel et al. 1979; Adachi et al. 1996). Hb D-Ibadan (β87 [F3] Thr > Lys), which introduces a lysine residue at the β87 position, is presumed to have decreased interaction with the mutant Val residue at HbS β6 (Watson-Williams et al. 1965; Nagel et al. 1979). Thus, Hb D-Ibadan inhibits HbS polymerization. In contrast, Hb Quebec-Chori (β87 [F3] Thr > Ile) was identified in a compound heterozygous patient with mild to moderately severe sickle cell anemia (Witkowska et al. 1991). The introduction of an isoleucine at β87 causes Hb Quebec-Chori to promote deoxygenated HbS polymerization.

Hb Bristol-Alesha (β67 [E11] Val > Met) (Molchanova et al. 1993; Rees et al. 1996) and Hb Toms River (γ67 [E11] Val > Met) (Crowley et al. 2011), which contain analogous amino acid substitutions in β and γ chains, respectively, represent interesting globin variants with multiple biochemical defects. Hb Bristol-Alesha was initially observed in patients with moderately severe hemolytic anemia. Studies of the mutant protein in patient erythrocytes revealed a β67 Val > Asp substitution, predicted to render the protein unstable by introducing a highly charged polar residue into the hydrophobic heme pocket. However, subsequent DNA analysis of the same patient identified a Val > Met codon substitution (Rees et al. 1996). The investigators concluded that the mutant Met residue was converted to Asp posttranslationally, probably through oxidative mechanisms. More recently, the analogous variant was identified in fetal (γ) globin (Hb Toms River) (Crowley et al. 2011). The affected patient was a newborn presenting with both cyanosis and anemia. DNA testing revealed the codon change (Val > Met at E11). Mass spectrometry of patient hemolysate indicated a mixture of variant γ globins containing either Met or Asp at position E11. Although no structural studies have been performed with Hb chains carrying Met or Asp E11, structures with polar (Thr) or large aromatic (Trp) substitutions are available. These indicate that a range of amino acids can be accommodated without gross changes in the heme pocket structure (Fig. 5B). Instead, altered steric and electrostatic interactions with the distal His and diatomic ligands entering the heme pocket are likely to be functionally significant. Biochemical studies indicated that the Hb Toms River Met substitution produced a stable, low O_2 affinity variant γ globin, causing cyanosis. Its gradual posttranslational conversion to Asp destabilized the molecule, causing hemolytic anemia. This provides an example of how posttranslational modifications of variant globins can modify phenotypes. The reason that Hb Bristol-Alesha causes predominantly hemolytic anemia whereas Hb Toms River causes mainly cyanosis probably reflects different rates of Met conversion to Asp in the variant globin chains.

CONCLUDING REMARKS

More than 1000 Hb variants are known to exist (Globin Gene Server; Hardison et al. 2002; Giardine et al. 2011). These are mainly missense mutations that destabilize Hb, alter Hb-O_2 affinity, or most commonly, alter Hb function minimally. Moreover, variants that do alter Hb biochemistry are rarely life threatening or health compromising. Nonetheless, studies of these variants have been of great benefit to science and medicine for two main reasons. First,

identification of Hb gene mutations as a cause for cyanosis, erythrocytosis, or mild hemolysis in otherwise healthy patients provides reassurance and minimizes additional diagnostic procedures, sparing expense and risk. Second, efforts to understand how Hb variants produce their structural, biochemical, and clinical effects has generated important insights into red blood cell function and also created general paradigms for the study of protein biology. Despite hundreds of studies over more than 50 years, new Hb variants continue to emerge, yielding new insights into this important molecule.

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