A Ubiquitously Expressed Human Hexacoordinate Hemoglobin*

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We have identified a new human hemoglobin that we call histoglobin because it is expressed in a wide array of tissues. Histoglobin shares less than 30% identity with the other human hemoglobins, and the gene contains an intron in an unprecedented location. Spectroscopic and kinetic experiments with recombinant human histoglobin indicate that it is a hexacoordinate hemoglobin with significantly different ligand binding characteristics than the other human hexacoordinate hemoglobins, neuroglobin. In contrast to the very high oxygen affinities displayed by most hexacoordinate hemoglobins, the biophysical characteristics of histoglobin indicate that it could facilitate oxygen transport. The discovery of histoglobin demonstrates that humans, like plants, differentially express multiple hexacoordinate hemoglobins.

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Hemoglobins (Hbs) are a class of proteins long associated with functions in respiration and the storage and transport of oxygen. Recent research has also focused upon a potential physiological role of Hb relating to its interactions with nitric oxide (NO) during conditions of oxidative stress. Several examples of physiologically important reactions involving Hb and NO now exist: 1) Bacterial and yeast flavohemoglobins scavenge NO resulting from oxidative bursts, protecting these cells from the host immune response to infection (1–3). 2) A critical hindrance to cell-free Hb-based blood substitutes is the severe hypertension resulting from their scavenging of NO (4). 3) NO is scavenged by myoglobin (Mb) in cardiac tissue (5, 6). 4) The Hb from the parasitic worm Ascaris is reported to function as an NO-activated dioxygenase (7). 5) Mycobacterium tuberculosis contains a truncated Hb that is reported to be an NO scavenger (8). 6) The binding of NO to human Hb thiols has been implicated in vascular control (9). In addition, there is mounting evidence that Hbs are involved in cell survival during hypoxia. Human neuroglobin (NGb) has recently been linked to tissue protection during hypoxic ischemia injury (10), and similar low oxygen conditions induce the expression of a ubiquitous nonsymbiotic plant Hb (11, 12).

Both NGb and nonsymbiotic plant Hbs are members of a newly discovered class of “hexacoordinate” Hbs (hxHbs) that have been found in animals (13, 14), protists (15), cyanobacteria (16–18), and all plants (19, 20). In addition to potentially new physiological functions, hxHbs use an alternative mechanism to regulate ligand binding which involves reversible intramolecular coordination of the heme iron; prior to the discovery of this mechanism, an open coordination site was believed necessary for reversible ligand binding in Hbs (Fig. 1). Despite this apparent hindrance to entering ligands, hxHbs are capable of reversibly binding oxygen and other heme ligands with unusually high affinities (21). The molecular details of the hexacoordination mechanism are not known with certainty. Biophysical examination of the details of this reaction is necessary to distinguish whether hexacoordination is a mechanism for regulating ligand affinity or a requirement for a novel biochemical reaction.

Despite the prevalence of hxHbs, the physiological function(s) of these proteins is unknown. However, there is growing evidence linking hxHbs with NO scavenging and a protective role during hypoxia (10, 21). If hxHbs serve to protect cells from damage during the generation of NO or other reactive oxygen species, their expression in a wide range of tissues could be expected. Plants are known to contain two or three different hxHbs expressed in a variety of tissues (11, 12), but the human hxB Ngb is essentially expressed only in the brain (14). When coupled with a link to hypoxia in plant hxHbs and Ngb, this suggests that humans may harbor more than one hxHb. Our inquiry into this possibility has led to the discovery of histoglobin (HGb), a hxHb expressed ubiquitously in human tissues with behavior unique compared with the other human Hbs.

EXPERIMENTAL PROCEDURES

Identification, Cloning, and Sequence Analysis—The HGb gene was identified utilizing ALLGNE (22) to mine the publicly available expressed sequence tag (EST) and genomic sequencing data on Mus musculus and Homo sapiens for predicted genes harboring a globin domain. The resulting data were reduced by selection for genes with a GenBank identified EST clone. Of the remaining candidate genes, all coding for proteins containing more than 250 residues were culled because currently identified hxHbs are composed of ~200 or fewer amino acids. The final candidates were then evaluated based on sequence homology with the vertebrate Hbs to eliminate those likely originating from splicing errors of the known globin genes. This process identified the putative gene DT.40262016 (ALLGNE identification number) as a potential novel mammalian Hb. The IMAGE EST clone R87866 corresponding to DT.40262016 was purchased from Incyte Genomics. The human HGb cDNA sequence was found through sequencing of R87866. Intron determination and chromosome localization were performed using the public human genome data base. The complete cDNA sequence has been posted by the NCBI annotation project (accession no. XM058818). Oligonucleotide primers were designed to incorporate NdeI and EcoRI restriction sites at the 5’- and 3’-ends of the gene, respectively. The HGb cDNA was synthesized by PCR using these primers and then cloned into the Novagen expression vectors pET29a (no-tag) and pET28a (His6-tagged) to mine the publicly available expressed sequence alignments of the human Hbs.

Recombinant Protein Generation and Spectroscopy—Human Hgb was expressed in Z-competent (Zymo Research) Escherichia coli BL21(DE3)-CodonPlus-RP cells (Stratagene), using both a previously described fermentation apparatus (23) and 2-liter culture flasks. Recombinant expression cultures were grown at 37 °C for a period of

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† The abbreviations used are: Hb(s), hemoglobin(s); EST, expressed sequence tag; HGb, histoglobin; hxHb, hexacoordinate Hb; Mb, myoglobin; Ngb, neuroglobin; NO, nitric oxide; sulfHb, sulfhemoglobin.
16–16 h postinoculation in 2× YT nutrient medium supplemented with 50 μg/ml kanamycin. These cultures were harvested by centrifugation, and the cells were lysed with two passages through an Avestin EmulsiFlex-C5 homogenizer at 25,000 p.s.i.

The nontagged HgB was purified from lysate using a four-stage process composed of ammonium sulfate fractionation (40 and 70%), phenyl-Sepharose column chromatography (1.5 mM (NH₄)₂SO₄ binding, 0.75 mM (NH₄)₂SO₄ elution), DEAEE-cellulose column chromatography (0 mM NaCl binding, 50 mM NaCl elution), and a final size exclusion chromatography step (Sephacyr S-200). The protein solution was dialyzed into 20 mM Tris, pH 8.0, after the phenyl-Sepharose separation and before the DEAEE-cellulose purification step. The His₆-tagged protein was purified using an affinity column (nickel-nitrilotriacetic acid) followed by a desalting column (G-25). Purification was at 4 °C.

All protein experiments were initially conducted in quadruplicate, utilizing His₆-tagged and nontagged green and red HgB. All species of HgB exhibited functionally identical behavior, excluding the obvious spectral difference between the green and red protein samples. In light of this, further replications were conducted using the red versions of the species.

RNA Hybridization Assay—A human adult normal tissue RNA Dot-Blot I (BioChain Institute) was screened using 32P-labeled probes generated with random hexamer primers and HgB or Ngb cDNA. Hybridization buffer consisted of PerfectHyb Plus (Sigma) with 0.1 mg/ml sheared, denatured salmon testis DNA as a blocking reagent. The membrane was prehybridized for 1 h at 60 °C, and hybridization was allowed to proceed for 12 h at 60 °C. The membrane was washed once (2× SSC, 0.1% SDS) for 5 min at 25 °C, followed by two washes (0.5× SSC, 0.1% SDS) for 20 min at 60 °C and a final wash (0.1× SSC, 0.1% SDS) for 10 min at 60 °C. The membrane was then sealed in saran wrap and exposed to a PhosphorImager for 30 h. The membrane was first probed with HgB, then stripped, and the identical procedure outlined above was repeated using the probe generated from Ngb. The Ngb hybridized membrane required a 48-h exposure to the PhosphorImager.

Oxygen Sensitivity/Heme Exchange Experiments—The data illustrated in Fig. 4B were obtained from 1-liter cultures grown in 2× YT medium at 37 °C for 12 h. Each culture was inoculated simultaneously then differentially aerated by varying agitation speeds (rpm). After 12 h these cultures were harvested, and the HgB protein was purified. Protein samples were reduced with sodium dithionite, and a visible spectrum was taken. The 0% saturation data illustrated in Fig. 4C was obtained using a sealed 1-liter 2× YT culture where the media and flask were sparged with N₂ both before and immediately after inoculation. The 100% saturation data illustrated in Fig. 4C were obtained using a 1-liter 2× YT culture aerated with a 1-liter/min flow of pure O₂. Both cultures were agitated at 150 rpm. The heme cofactor was removed from samples of both red and green HgB using the methyl ethyl ketone method to produce the corresponding “green” or “red” apoprotein (24).

Titrating the apoprotein sample with hemin chloride solubilized in 0.1 M NaOH generated reconstituted holoproteins.

Kinetic Measurements—All kinetic experiments were performed at 20 °C, and protein samples were buffered in 100 mM potassium phosphate at pH 7.0, unless otherwise specified. Rapid mixing experiments were conducted using methods described previously (25, 26). Oxygen dissociation rate constants were measured using both the ligand displacement reaction (mixing oxygenated samples with carbon monoxide) and direct observation of oxygen dissociation rate constants (mixing oxygenated samples with solutions of carbon monoxide and sodium dithionite). The flash photolysis apparatus and the methods used to measure the hexacoordination and bimolecular association rate constants have been described previously (18, 27, 28). The program Igor

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**Fig. 1. Heme coordination states.** A, pentacoordinate heme; B, hexacoordinate heme. Two His side chains coordinate the heme iron in hzHbs.

| A            | Histogoblin | Neuroglobin | Myoglobin | AlphaGlobin | BetaGlobin | GammaGlobin |
|--------------|-------------|-------------|-----------|-------------|------------|-------------|
| CD1          |             |             |           |             |            |             |
| Histogoblin  | 1           | 1           | 1         | 1           | 1          | 1           |
| Neuroglobin  |       1     |             |           |             |            |             |
| Myoglobin    | 61          | 43          | 45        | 45          | 44         | 44          |
| AlphaGlobin  |             |             |           |             |            |             |
| BetaGlobin   |             |             |           |             |            |             |
| GammaGlobin  |             |             |           |             |            |             |
| Histogoblin  | 120         | 102         | 101       | 95          | 100        | 100         |
| Neuroglobin  |             |             |           |             |            |             |
| Myoglobin    |             |             |           |             |            |             |
| AlphaGlobin  |             |             |           |             |            |             |
| BetaGlobin   |             |             |           |             |            |             |
| GammaGlobin  |             |             |           |             |            |             |
| Histogoblin  | 180         |             |           |             |            |             |
| Neuroglobin  |             |             |           |             |            |             |
| Myoglobin    |             |             |           |             |            |             |
| AlphaGlobin  |             |             |           |             |            |             |
| BetaGlobin   |             |             |           |             |            |             |
| GammaGlobin  |             |             |           |             |            |             |

**Fig. 2. Sequence alignment of the major human Hbs and the Hgb gene structure.** A, primary sequence alignment of the five principal adult human Hbs. Residues sharing identity are highlighted in black, and those of high homology are shaded in gray. Sequence positions E7, F8, and CD1 (in reference to Mb) are annotated. B, schematic of the gene structure of Hgb.
was grown under conditions that varied the level of oxygen, and the relative color of the resulting proteins was assessed by spectroscopy. The data from these growths are plotted in Fig. 4, B and C. The data in Fig. 4C demonstrate that it is oxygen concentration and not some other aspect of culture agitation speed which influences the generation of green HGb. A correlation between the abundance of oxygen during recombinant protein expression and the amount of green protein generated is evident.

To assess whether the difference in color originates in the heme cofactor or the globin, apoHGb was generated by removing heme from the red and green proteins. Upon removal, heme from the green protein was green and that from the red protein, red. Reconstitution of the apoprotein generated from green HGb with heme b resulted in holoprotein having a visible spectrum identical to red HGb, as is shown in Fig. 4A. This indicates that the oxygen level-dependent modification giving rise to the green color is associated with the heme cofactor. Additional support of this is the identical size measured for both red and green proteins using SDS-PAGE (data not shown). However, this modification of the heme cofactor had no observable impact on either protein purification or kinetic characteristics of the red and green proteins.

Hexacoordination and Flash Photolysis Kinetics—Fig. 5 presents an overlay of the absorbance spectra of reduced, deoxygenated sperm whale Mb, human NGb, and HGb. The split peak in the visible region is characteristic of a hexacoordinate heme iron and a signature of the hxHb class of proteins (29, 30). The HGb absorbance peaks are nearly identical to those of NGb, suggesting bis-histidyl coordination in the ferrous deoxygynated form.

Time courses for bimolecular ligand recombination with pentacoordinate HbHs after flash photolysis are described by a single exponential decay. However, the time courses for ligand binding to hxHbs such as NGb are not monoeponential because intramolecular coordination competes with rebinding of the exogenous ligand as described by Reaction 1. In this reaction, the subscripts H, P, and L refer to the hexacoordinate, pentacoordinate, and ligand-bound forms of the Hb, respectively.

\[
\begin{align*}
Hb_H \overset{k_H}{\longrightarrow} Hb_P \overset{k_L [L]}{\longrightarrow} Hb_L
\end{align*}
\]

**REACTION 1**

Analysis of ligand binding under these circumstances is possible by using a procedure described in detail previously (27, 28). Time courses for oxygen and carbon monoxide binding to HGb after flash photolysis were initially analyzed using this method. Yet, unlike the obviously biexponential behavior exhibited by NGb (28), the ligand binding time courses for HGb were well described by a single exponential decay. Figs. 6A and 7A are the overlaid residuals from single and biexponential fits to the CO and O₂ rebinding time courses shown in Figs. 6B and 7B. These residuals are indistinguishable from one another.
and indicate that fitting these data to a biexponential decay is not warranted. To assess the bimolecular association rate constant for each ligand, rate constants extracted from single exponential fits were plotted against concentration as shown in Fig. 6C (CO) and Fig. 7C (O₂). The slopes of the linear fitted curves to these data are reported as the Hgb bimolecular rate constants in Table II.

The intercept of these linear fits should be zero if the data reflect either a simple bimolecular binding reaction (with a slow dissociation rate constant), or a reaction in which the bimolecular rate constant is substantially greater than all other binding events (k₁[L] > k₄[H] + k⁻¹[L]) (27). The linear fit to the oxygen binding data has an intercept within error of zero, indicating that the reaction measured reflects only the oxygen rebounding event. However, the linear fit to the CO data in Fig. 6C has a non-zero intercept at 440 s⁻¹. This suggests that the time courses giving rise to these data measure a reaction more complex than simple bimolecular rebinding of CO. As spectroscopic data indicate Hgb is hexacoordinate at equilibrium, the rate constants associated with the hexacoordination reaction are a likely source of the additional complexity observed.

**Stopped Flow Rapid Mixing Kinetics**—To investigate the possibility that Hgb possesses a hexacoordination dissociation rate constant (k⁻¹[H]) too slow to be measured with the flash photolysis method, CO binding was examined using rapid mixing to initiate the reaction. The time courses for CO binding to the ferrous, deoxygcnated protein at several different concentrations of CO are shown in Fig. 8A. Although [CO] ranges between 25 and 500 μM, the appearance of the ligand binding time courses does not show a 20-fold change in reaction half-time. This phenomenon has been described previously in hx-Hbs and is the typical behavior of these proteins (23). These time courses require fitting to a three-exponential decay curve to be described accurately. The fastest of the rate components was assigned as the hexacoordination dissociation rate constant (k⁻¹[H]) in accordance with the mechanism described in Reaction 1. The slower rate components of these reactions have been discussed previously in the context of a model for ligand binding to hxHbs (23). The fastest rate component extracted from the CO binding time courses is plotted against [CO] in Fig. 8B. As these values reach an asymptote at ~5 s⁻¹, this value is reported for k⁻¹[H] in Table II. This interpretation of the data indicates that the non-zero intercept in Fig. 6C arises from time courses with a hexacoordination contribution composed predominantly of the association reaction, and this is reflected in the reported value of 430 s⁻¹ for k⁻¹[H] in Table II.

The Hgb O₂ and CO dissociation rate constants reported in Table II were measured with ligand replacement reactions initiated by stopped-flow rapid mixing (25, 31). Reactions using 1,000 μM [CO] as the displacing ligand and protein samples saturated under different concentrations of oxygen (262 and 1,250 μM O₂) provide corroborating values of 0.35 s⁻¹ for k₀[H]. CO dissociation experiments used 2,000 μM NO as the displacing ligand and protein samples in less than 50 μM CO. Under these conditions, kᵦᵦ is equivalent to k₀[H] and was measured to be 0.003 s⁻¹ for Hgb.

**DISCUSSION**

Human Hb and Mb are proteins whose physiological roles in oxygen transport and respiration are among the most clearly defined and well understood. Yet, hxHbs have thus far demonstrated oxygen affinities that preclude their functioning within these roles (12, 15, 18, 21, 29). From its biophysical behavior to the tissues within which it is expressed, Hgb exhibits fundamental differences from the other human Hbs. The discussion that follows examines these differences in the context of potential physiological significance.

**Primary Structure Comparison**—Comparison of the nucleic acid and protein sequences of Hgb with the other human Hbs highlights both the similarities and differences that character-
Heme pocket and CO binding affinity

The amino acids comprising the heme pocket in human hemoglobin (Hb) are nearly identical to those of NGb, and these spectra illustrate the differences in the coordination states of these Hb molecules. The Soret peak wavelengths of HGb are in excellent agreement with those of NGb (Fig. 5) and indicate that there is a slight decrease in the heme pocket primary structure between Mb and HGb. The proximal His in NGb contains a polar residue, as opposed to the Ala residue in Mb. The F9 position adjacent to the heme pocket in HGb is occupied by a Lys in contrast to the Asp residue in Mb. For example, the E6 residue in the distal heme pocket of HGb and Mb is a Lys, in contrast to the Asp residue in Mb. The similarities in the heme pocket primary structure between Mb and HGb are intriguing, particularly in the context of the nearly identical O2 and CO equilibrium affinity constants for Mb and pentacoordinate Mb.

As is the case with NGb, there are putative HGb genes in both the rat and mouse which are highly homologous to the human version. The putative mouse HGb gene arises from an EST sequence (accession no. AK019410), and the rat homolog was noted in a very recent proteomic study where it was called Stellate Cell Activation-associated Protein (STAP) (34). Considering the numerous genes already bearing this acronym (35–37), the characterization of HGb as a hxHb and its expression in many tissues besides hepatic stellate cells, it seemed logical to continue our reference to this gene as HGb. The human HGb primary structure shares more than 90% identity with its homologs in the rat and mouse. This high degree of conservation implies that the function of these proteins is rigidly dependent upon the specific functional properties conveyed by these particular structures.

**Hexacoordination and Ligand Binding**—A key element in learning the physiological role(s) held by HGb, as well as other hxHbs, is biophysical study of the attributes that define the behavior of these proteins. The spectroscopic analysis of reduced, deoxygenated HGb (Fig. 5) clearly distinguishes it from pentacoordinate Mb. The Soret peak wavelengths of HGb are nearly identical to those of NGb, and these spectra illustrate the difference between the coordination states of these hxHbs and pentacoordinate Mb.

In addition to the equilibrium spectral signature, another manifestation of hexacoordinate character is biphasic time courses for ligand rebinding following flash photolysis (27, 28). However, the appearance of these biphasic time courses depends upon the relationship between the rate constants of the reaction.
hexacoordination and bimolecular ligand binding. If hexacoordination is outcompeted by bimolecular ligand rebinding, then single exponential time courses will be observed (27). The time courses illustrated in Figs. 6B and 7B exhibit this single exponential form and indicate that the rates associated with hexacoordination in HGb must be considerably slower than those of Ngb (28). However, the non-zero intercept in Fig. 6C suggests that CO rebinding rate constants at the lowest concentrations of CO were of an order similar to that of the HGb rate constants for hexacoordination. A more quantitative assessment of these values in HGb is possible if this intercept could be correlated with data from an independent method of measuring hexacoordination rate constants. These data were obtained using stopped-flow rapid mixing to ascertain the magnitude of the hexacoordination dissociation rate constant (Fig. 8).

The intercept from Fig. 6C \((-k_{11})\) and the approximate asymptote value from Fig. 8B \((-k_{-11})\) can be correlated with Equation 1, which describes the expected rate constant for ligand binding initiated by rapid mixing according to the mechanism described in Reaction 1 (23).

\[
 k_{\text{obs, H}} = \frac{k_{-11} k'_L [L]}{k_{-11} + k'_{1} [L] + k''_{L} [L]} 
\]  

(Eq. 1)

A simulation of the expected rate constants was created using Equation 1 and the ligand binding and hexacoordination rate constants reported for HGb in Table II, then overlaid on the observed data in Fig. 8B. The correspondence of this simulated curve with the observed values supports assignment of \(k_{-11}\) to the fastest phase of ligand binding observed after rapid mixing.

The rate constants associated with hexacoordination in HGb are considerably smaller than those observed thus far in other hxHbs (18, 27, 28). The effect of hexacoordination on equilibrium affinity constants can be calculated using the following equation, where \(K_{L,\text{Pent}}\) is the equilibrium constant for ligand association to the pentacoordinate form of the protein \((k'/k_{1})\), and \(K_{H}\) is the equilibrium constant for hexacoordination \((k''/k_{-11})\) (28).

\[
 K_{\text{L, effective}} = \frac{K_{L,\text{Pent}}}{1 + K_{H}} 
\]  

(Eq. 2)

The \(K_{H}\) reported for HGb in Table II is the largest hexacoordination equilibrium constant yet observed in a hxB, and it differs dramatically from the \(K_{H}\) of Ngb. With the contribution of hexacoordination factored in, the equilibrium affinity constants for HGb are very similar to those of Mb (Table II).

**Formation of Green HGb**—Green heme proteins are not unprecedented, and their color can arise through several different mechanisms. 1) Myeloperoxidase contains a conventional heme prosthetic group, and its color can arise through several different mechanisms. 2) Biliverdin and verdoheme are green heme derivatives produced by heme oxygenase during the degradation of heme (39). 3) Sulphemoglobin (sulfHb), a green protein associated with certain blood pathologies, owes its color to the incorporation of sulfur into the porphyrin ring, forming sulfohemin (40).

The green color in HGb is caused by a heme modification, because the addition of iron protoporphyrin IX to (previously green) apoHGb results in red protein. Additionally, red HGb is stable and does not degrade to the green protein. This suggests that green HGb is not a result of mechanisms 1) and 2) described above. However, it is possible that green HGb is a sulfHb. In support of this view is the fact that formation of sulfHbs is known to be dependent on the availability of oxygen (40), as we have shown to be the case with formation of green HGb. And like sulfHb, the absorbance spectrum of green HGb is very similar to that of the red protein (41), containing only the additional peak around 630 nm. However, sulfmyoglobin is

| Protein | \(k'_{CO}\) | \(k''_{CO}\) | \(k''_{L,\text{Pent}}\) | \(k'_{CO}\) | \(k''_{CO}\) | \(k'_{-11}\) | \(k''_{-11}\) | \(K_{H}\) |
|---------|-------------|-------------|-----------------|-------------|-------------|-------------|-------------|----------|
| Histoglobin | 30 | 0.35 | 1.0 | 5.6 | 0.003 | 21.7 | ~5 | 430 | 86.0 |
| Neuroglobin | 130 | 0.3 | 200 | 38 | 0.007 | 2,500 | 8,200 | 9,800 | 1.2 |
| Hb \(\alpha\)-chain R-state | 19 | 15 | 1.3 | 2.2 | 0.009 | 245 | |
| Hb \(\beta\)-chain R-state | 74 | 47 | 1.6 | 5.9 | 0.01 | 590 | |
| Myoglobin | 17 | 15 | 1.1 | 0.51 | 0.02 | 25.5 | |

\(a\) Hexacoordinate hemoglobins are in bold.
\(b\) These values were calculated using Equation 2.
\(c\) Ref. 23.
\(d\) Ref. 59.
\(e\) Ref. 60.

**TABLE II**

**Rate constants associated with ligand binding to the human hemoglobins**

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**Fig. 8.** Rapid mixing ligand binding kinetics. A, time courses for CO binding to the ferrous, deoxygenated form of HGb after rapid mixing are shown for several different concentrations of CO. The time scale for these reactions indicates that they measure a component of the binding reaction other than just the bimolecular event. B, plot of the fastest rate components extracted from three-exponential fits to the time courses shown in A versus CO concentration. A curve simulating \(k_{\text{obs, H}}\) generated using Equation 1 with the ligand binding and hexacoordination rate constants reported for HGb in Table II is overlaid on the observed values in B. This plot demonstrates that the reported hexacoordination rate constants are consistent with observed ligand binding data.

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**Human Histoglobin**

19543
associated with 2,500- and 10-fold reductions in O2 and CO binding, respectively (42, 43). In marked contrast to this, green HGB appears to differ little from red HGB in ligand binding behavior. It is possible that the reaction time courses we observed for green HGB are attributable to the presence of a fraction of the red protein. Yet, if this were the case, smaller absorbance change amplitudes would be expected for the CO and O2 ligand binding reactions with the green protein compared with the red, and this difference was not observed (data not shown).

The mechanism for generation of sulfHbs involves a ferryl heme iron and sulfide (41). Given the conditions under which it is generated, if green HGB is a sulfHb it must either have a more stable ferryl oxidation state compared with other Hbs or harbor a readily accessible sulfur atom. In support of the latter is a cysteine located in the distal heme pocket (position E9) which might facilitate sulfheme formation. With regard to the former, a ferryl heme iron is a component of peroxidase compound I, and HGB has been attributed peroxidase activity (34). However, the level of activity is very low compared with known peroxidases (44). In fact, the level of peroxidase activity attributed to HGB is similar to the “pseudo” peroxidase activity of other Hbs including Mb and soybean leghemoglobin (45, 46). This suggests that the primary role of HGB is not that of a peroxidase.

**Physiological Significance**—The transport and facilitated diffusion of oxygen and other ligands by Hbs have been subjects of investigation since the 1960s (47, 48). The relationship between Hb kinetic rate constants and the environment in which the protein functions is fairly well established for these physiological roles (49–51). Although other hxHbs characterized thus far possess oxygen affinities that are too high for these functions, the oxygen affinity of HGB is of the same order as Mb and should allow it to serve in a similar role. It is therefore possible that HGB supports the facilitated diffusion of oxygen in those tissues that do not express Mb. In this scenario, hexa-coordination would serve simply to decrease oxygen affinity, thereby allowing transport to a higher affinity oxidase. Another hypothesis is that hxHbs (including HGB) are involved in a general mechanism for scavenging NO and/or other reactive oxygen species in both plants and animals. Plant hx-Hbs and Ngb are both up-regulated by hypoxia (10, 19, 20). Expression of plant hxHbs is also stimulated by conditions that activate the plant disease resistance pathway (52–54), which generates NO and other reactive oxygen species (55, 56). Reperfusion injury following ischemia in animals has been associated with NO (57); it is intriguing that both plants and animals express biochemically similar hxHbs in response to this type of challenge.

The expression of HGB has not yet been linked with hypoxia. Nevertheless, this possibility is interesting in the context of the oxygen-dependent modification of the heme cofactor. Although this modification may be an artifact of recombinant expression, it has not been observed during the generation of other recombinant Hbs in this laboratory. It has been proposed previously that hxHbs may play a role in sensing gaseous ligands (29, 58). If HGB serves this function, the heme modification may be a component of the sensory mechanism.

**Conclusions**—The study of HGB presented here identifies a new human gene that encodes a member of a biophysically defined class of proteins called hexacoordinate Hbs. These proteins are found in most organisms and possess a regulatory ligand binding mechanism that differs fundamentally from traditional pentacoordinate Hbs. In HGB this mechanism results in exogenous ligand equilibrium affinity constants that are very similar to those of Mb. And although considerable uncertainty remains as to the physiological role(s) served by HGB or the other hxHbs, mounting evidence suggests a potential protective function during conditions of oxidative stress.

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**Note Added in Proof**—While this manuscript was in press, another article describing this gene was published (Burmester, T., Ebner, B., Weich, B., and Hankeln, T. (2002) Mol. Biol. Evol. 19, 416–421).
