(54) Title: LOW OXYGEN AFFINITY MUTANT HEMOGLOBINS

(57) Abstract: Non-naturally occurring mutant hemoglobins rHb (bN108Q) and rHb (bL105W) are provided that have a lower oxygen affinity than that of native hemoglobin, but high cooperativity in oxygen binding. rHb (bN108Q) also exhibits enhanced stability against autoxidation. The mutant hemoglobins are preferably produced by recombinant DNA techniques. Such mutant hemoglobins may be used as a component of a blood substitute and hemoglobin therapeutics.
LOW OXYGEN AFFINITY MUTANT HEMOGLOBINS

ACKNOWLEDGMENT

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FIELD OF THE INVENTION

This invention relates generally to novel mutant hemoglobins and more particularly relates to recombinant mutant hemoglobins "rHb (βN108Q)" (alternative designation "rHb (β108Asn→Gln)") and "rHb (βL105W)" (alternative designation "rHb (β105Leu→Trp)") that possess low oxygen affinity, and high cooperativity in oxygen binding. In particular, rHb (βN108Q) exhibits increased resistance to autoxidation as compared to other known low oxygen affinity mutants. This invention further relates to the preparation of mutant hemoglobins using recombinant DNA technology that are useful as substitutes for red blood cells and for hemoglobin-based therapeutics.

BACKGROUND OF THE INVENTION

The prevalence of infectious agents such as HIV and hepatitis in red blood cells of human blood products coupled with blood shortages from lack of suitable donors has led to great interest in the development of red blood cell substitutes, particularly human hemoglobin ("Hb") and its derivatives.

Hemoglobin is the oxygen-carrying component of blood, circulated through the blood stream inside erythrocytes (red blood cells). Human normal adult hemoglobin ("Hb A") is a tetrameric protein with a molecular weight of about 64,500 containing two identical α chains having 141 amino acid residues each and two identical β chains having 146 amino acid residues each, with each also bearing prosthetic groups known as hemes. The erythrocytes help maintain hemoglobin in its reduced, functional form. The heme-iron atom is susceptible to oxidation, but may be reduced again by one of two systems within the erythrocyte, the cytochrome bs, and glutathione reduction systems. For a review on hemoglobin, see, Dickerson, R.E., et al. *Hemoglobin: Structure, Function, Evolution, and Pathology*, Benjamin/Cummings, Menlo Park, CA (1983) (hereinafter "Dickerson, et al. (1983)"), the disclosure of which is incorporated herein by reference.

The oxygenation process of Hb A is cooperative, i.e., the binding of the first oxygen molecule enhances the binding of the second, third, and fourth
oxygen molecules. The oxygenation process is also regulated by interactions between individual amino acid residues and various solutes, known as heterotropic allosteric effectors. These effectors include ions or molecules such as hydrogen ion, chloride, carbon dioxide, inorganic phosphate, and organic polyanions, such as 2,3-bisphosphoglycerate ("2,3-BPG") and inositol hexaphosphate ("IHP").

Hemoglobin is able to alter its oxygen affinity, thereby increasing the efficiency of oxygen transport in the body, due to its dependence on the allosteric effector 2,3-BPG. 2,3-BPG is present within erythrocytes at a concentration that allows hemoglobin to release bound oxygen to tissues. In the absence of 2,3-BPG, hemoglobin binds oxygen very tightly and does not readily release its bound oxygen. The Hb A molecule alone, were it to be introduced into a subject, would not be able to properly allow oxygen to be delivered to tissues in the body due to a lack of 2,3-BPG, which lowers the oxygen affinity of Hb, in the blood plasma.

See, Winslow, et al. (1992). Any Hbs designed to be functional as Hb-based oxygen carriers or hemoglobin therapeutics should be able to deliver oxygen efficiently, i.e., they should load and unload cooperatively as Hb A does inside red blood cells.

The use of cell-free solutions of hemoglobin as a potential oxygen-carrying red cell substitute has been investigated for a long time. See, for
example, Mulder, A.G., et al., *J. Cell Comp. Physiol.* 5:383 (1934), the disclosure of which is incorporated herein by reference. However, the use of unmodified cell-free human hemoglobin purified from red blood cells suffers from several limitations in addition to contamination and supply limitations noted above, namely, an increase in oxygen affinity due to loss of allosteric effectors, such as 2,3-BPG, and dissociation of Hb tetramers into $\alpha\beta$ dimers which are cleared by renal filtration and which can cause long-term kidney damage. See, for example, Bunn, H.F., et al. *J. Exp. Med.* 129:909 (1969), the disclosure of which is incorporated herein by reference.

many respects, the \textit{E. coli} system is the best choice for such purposes because of its high expression efficiency and the ease of performing site-directed mutagenesis.

The natural N-terminal valine residues of Hb A are known to play important roles in regulating oxygen affinity, the Bohr effect, and interactions with allosteric effectors and anions as reported by Bunn, H.F., et al. eds. \textit{Hemoglobin: Molecular, Genetic and Clinical Aspects} (W.B. Saunders, Co., Philadelphia, PA) pp. 37-60 (1986) (hereinafter "Bunn, et al. (1986)"), the disclosure of which is incorporated herein by reference. The extra methionine can alter the N-terminal conformation of the Hb molecule as reported by Kavanaugh, J.S., et al. \textit{Biochemistry} 31:8640 (1992), the disclosure of which is incorporated herein by reference. Hence, the oxygenation properties of Hb depend on the integrity of the N-terminal residue thereby mandating the removal of the extra methionine residues from the N-termini of both the \(\alpha\)- and \(\beta\)-globins of the expressed Hb before the \textit{E. coli} system can be used effectively for the production of desired unmodified and mutant Hbs.

The cooperative oxygenation of Hb, as measured by the Hill coefficient ("\(n_{\text{max}}\)"), is a convenient measure of its oxygenation properties. See, Dickerson, et al. (1983). Hb A has an \(n_{\text{max}}\) value of approximately 3 in its binding with \(O_2\) under usual experimental conditions. Human abnormal Hbs with amino acid substitutions in the \(\alpha_1\beta_2\) (or \(\alpha_2\beta_1\)) subunit interface generally result in high
oxygen affinity and reduced cooperativity in O$_2$ binding compared to Hb A. See, for example, Dickerson, et al. (1983); Bunn, et al (1986) and Perutz, M.F., et al. *Mechanisms of Cooperativity and Allosteric Regulation in Proteins* Cambridge University Press (1990), the disclosure of which is incorporated herein by reference.

Hb A in its oxy form (Hb A with oxygen molecules) has a characteristic hydrogen bond between $\alpha$94Asp and $\beta$102Asn in the $\alpha_1$$\beta_2$ subunit interface as reported by Shaanan, B., et al. *J. Mol. Biol.* 171:31 (1983), the disclosure of which is incorporated herein by reference (hereinafter "Shaanan, et al. (1983)"). Human Hbs with an amino acid substitution at either the $\alpha$94Asp position such as Hb Titusville ($\alpha$94Asp$\rightarrow$Asn) (Schneider, R. G., et al. *Biochim. Biophys. Acta*. 400:365 (1975), the disclosure of which is incorporated herein by reference) or the $\beta$102Asn position such as Hb Kansas ($\beta$102Asn$\rightarrow$Thr) (Bonaventura, J., et al. *J. Biol. Chem.* 243:980 (1968), the disclosure of which is incorporated herein by reference), as well as others with mutations in the $\alpha_1$$\beta_2$ subunit interface, exhibit very low oxygen affinity. However, all these Hb mutants which directly disrupt the hydrogen bond between $\alpha$94Asp and $\beta$102Asn in the oxy form of Hb show greatly reduced cooperativity in the binding of oxygen and additionally dissociate easily into dimers when in the ligated state.
It has also been shown that during the transition from the deoxy-to the oxy-state, the $\alpha_2\beta_2$ subunit of Hb A undergoes a sliding movement, while the $\alpha_1\beta_1$ subunit interface remains nearly unchanged (See, Perutz, M.F. Nature 228: 726 (1970); Baldwin, J.M., et al. J. Mol. Biol. 129: 175 (1979); Baldwin, J.M., J. Mol. Biol. 136: 103 (1980); Shaanan, et al. (1983); and Fermi, G., et al. J. Mol. Biol. 175: 159 (1984), "Fermi, et al., (1984)", the disclosures of which are incorporated herein by reference. There are specific hydrogen bonds, salt bridges, and non-covalent interactions that characterize both subunit interfaces. The Hb molecule also has a lower oxygen affinity in the deoxy quaternary structure (T-structure) than in the oxy quaternary structure (R-structure) See, Dickerson, et al. (1983).

Low oxygen affinity human mutant Hbs which do not involve either $\alpha$94Asp or $\beta$102Asn also exist. For example, Hb Presbyterian ($\beta$108Asn→Lys) (Moo-Penn, W.F., et al. FEBS Lett. 92:53 (1978) and O'Donnell, J.K., et al. J. Biol. Chem. 269:27692 (1994) (hereinafter "O'Donnell, et al. (1994)")); Hb Yoshizuka ($\beta$108Asn→Asp), O'Donnell, et al. (1994) and recombinant Hb Mequon ($\beta$41Phe→Tyr) (Baudin, V., et al. Biochim. Biophys. Acta. 1159:223 (1992), the disclosures of which are incorporated herein by reference, all exhibit low oxygen affinity compared to Hb A, but they all exhibit a variable amount of cooperativity as measured by the Hill coefficient, with n varying from 1.8 to 2.9. Tsai, C.-H., et
(α96Val→Trp, β108Asn→Lys) which has low oxygen affinity and a greater
tendency to switch to the T quaternary structure. Jeong, S.T., et al., Biochemistry
38:13433 (1999) (hereinafter, "Jeong, et al. (1999)") report that Hb (α29Leu→Phe,
α96Val→Trp, β108Asn→Lys) exhibits low oxygen affinity and high cooperativity
combined with resistance to autoxidation.

Shen, et al. (1993) and U.S. Patent No. 5,753,465, the disclosures of
which are incorporated herein by reference, describe an E. coli expression plasmid
(pHE2) in which synthetic human α- and β-globin genes are coexpressed with the
E. coli methionine aminopeptidase gene under the control of separate tac
promoters. E. coli cells transformed with this plasmid express recombinant Hb A
(hereinafter "rHb A") from which the N-terminal methionines have been
effectively cleaved by the coexpressed methionine aminopeptidase. The resulting
rHb A which lacks an N-terminal methionine is identical to the native Hb A in a
number of structural and functional properties.

(hereinafter "Kim, et al. (1994)"), and U.S. Patent No. 5,843,888, the disclosures of
which are incorporated herein by reference, describe a non-naturally occurring
mutant hemoglobin (rHb (α96Val→Trp) (alternative designation "rHb (αV96W)"))
that has a lower oxygen affinity than that of native hemoglobin, but high cooperativity in oxygen binding.

There remains a need, however, for additional mutant hemoglobin species that can be used as a component of a hemoglobin-based blood substitute or therapeutic agent. Of particular interest is a mutant hemoglobin that possesses low oxygen affinity, high cooperativity in oxygen binding, and increased stability against autoxidation. There is a further need for such a hemoglobin produced by recombinant methods and an efficient expression system for producing such a mutant hemoglobin in high yield, especially for use in a blood substitute product or hemoglobin therapeutics.

**SUMMARY OF THE INVENTION**

Accordingly, it is a primary object of the present invention to provide mutant human hemoglobins with low oxygen affinity and high cooperativity in oxygen binding.

Another object of the present invention is to provide mutant hemoglobins with low oxygen affinity, high cooperativity in oxygen binding, and increased stability against autoxidation.

Another object of the present invention is to provide non-naturally occurring mutant human hemoglobins with low oxygen affinity and high cooperativity in oxygen binding.
Another object of the present invention is to provide non-naturally occurring mutant human hemoglobins with low oxygen affinity, high cooperativity in oxygen binding, and increased stability against autoxidation.

Another object of the present invention is to provide non-naturally occurring mutant human hemoglobins with low oxygen affinity, high cooperativity in oxygen binding, and preferably with stability against autoxidation that are produced artificially, preferably by recombinant means, and that have the correct heme conformation.

Another object of the present invention is to provide mutant hemoglobins that in a cell-free environment have similar oxygen binding properties as those of human normal adult hemoglobin in red blood cells.

Yet another object of the present invention is to provide mutant hemoglobins with low oxygen affinity and high cooperativity in oxygen binding in which the T-structure is stabilized while the R-structure is undisturbed.

Still another object of the present invention is to provide artificial hemoglobins for use as a hemoglobin-based oxygen carrier/red blood substitute or therapeutic agent.

These and other objects of the present invention are achieved by one or more of the following embodiments.
In one aspect, the invention features a non-naturally occurring mutant human hemoglobin wherein the asparagine residue at position 108 of the β-chains is replaced by a glutamine residue.

In a preferred embodiment, the hemoglobin possesses low oxygen affinity as compared to human normal adult hemoglobin, high cooperativity in oxygen binding, increased stability against autoxidation, and is produced recombinantly.

In another aspect, the invention features an artificial mutant hemoglobin which in a cell-free environment has oxygen binding properties comparable to those of human normal adult hemoglobin in red blood cells wherein said hemoglobin contains a mutation such that the asparagine residue at position 108 of the β-chains is glutamine.

A non-naturally occurring low oxygen affinity mutant hemoglobin with increased stability against autoxidation that has oxygen binding properties comparable to those of human normal adult hemoglobin in the presence of the allosteric effector 2,3-bisphosphoglycerate, wherein the asparagine residue at position 108 of each of the β-chains is replaced by a glutamine residue.

In yet another aspect, the invention features a non-naturally occurring mutant human hemoglobin wherein the leucine residue at position 105 of the β-chains is replaced by a tryptophan residue.
In a preferred embodiment, the hemoglobin possesses low oxygen affinity as compared to human normal adult hemoglobin, high cooperativity in oxygen binding, and is produced recombinantly.

In another aspect, the invention features an artificial mutant hemoglobin which in a cell-free environment has oxygen binding properties comparable to those of human normal adult hemoglobin in red blood cells wherein said hemoglobin contains a mutation such that the leucine residue at position 105 of the β-chains is tryptophan.

A non-naturally occurring low oxygen affinity mutant hemoglobin that has oxygen binding properties comparable to those of human normal adult hemoglobin in the presence of the allosteric effector 2,3-bisphosphoglycerate, wherein the leucine residue at position 105 of each of the β-chains is replaced by a tryptophan residue.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiment, and from the claims.

DESCRIPTION OF THE DRAWINGS

Figure 1A is a cDNA sequence (SEQ ID NO: 5) for the alpha- and beta-globin genes for rHb (βN108Q) derived from plasmid pHE7009.

Figure 1B is a cDNA sequence (SEQ ID NO: 7) for alpha- and beta-globin genes for rHb (βL105W) derived from plasmid pHE7004.
Figures 2A and 2B show the FPLC profiles of rHb (βN108Q) (peak b) (Figure 2A) and rHb (βL105W) (peak b) (Figure 2B).

Figures 3A and 3B show the pH dependence of the oxygen affinity ($P_{50}$) and the Hill coefficient ($n_{max}$), respectively, of rHb (αL29F) ( ]); rHb (βN108Q) (Δ); rHb (αL29F, βN108Q) (●); rHb (βL105W) (▲); and Hb A (O) in 0.1 M sodium phosphate buffer at 29°C. Oxygen dissociation data were obtained with 0.1 mM Hb.

Figure 4 shows oxygen-binding curves of rHb (βN108Q); rHb (αL29F, βN108Q); rHb (βL105W); and Hb A with and without the presence of allosteric effector, 5 mM 2, 3- BPG, in 0.1 M phosphate buffer at pH 7.4 and 29°C. Protein concentration was 0.1 mM heme.

Figure 5 shows the autoxidation of Hb A (O); rHb (βN108Q) (▲); rHb (βL105W) (Δ); rHb (αV96W) (V); rHb (αV96W, βN108K) (▼); rHb (αL29F, βN108Q) (◇); and rHb (αL29F, αV96W, βN108K) (□) in PlasmaLyte buffer in the presence of 5 mM EDTA and 5% D$_2$O at pH 7.4 and 37°C. The autoxidation process was measured by monitoring the rate of disappearance of the oxy-marker at -2.34 ppm upfield from DSS by 300-MHz $^1$H-NMR.

Figures 6A and 6B are 500-MHz $^1$H-NMR spectra showing exchangeable proton resonances (Figure 6A) and ring-current shifted proton
resonances (Figure 6B), respectively, of Hb A; rHb (βN108Q), and rHb (αL29W,
βL108Q), all in the CO form in 0.1 M sodium phosphate buffer at pH 7.0 and 29°C.

Figures 7A and 7B are 300-MHz 1H-NMR spectra showing ferrous hyperfine-shifted N$_2$H resonances of proximal histidines and hyperfine-shifted and exchangeable proton resonances, respectively, of rHb A; rHb (αL29F); rHb (βN108Q); and rHb (αL29W, βN108Q), all in the CO form, in 0.1 M sodium phosphate buffer at pH 7.0 and 29°C.

Figures 8A and 8B are 500-MHz spectra showing the exchangeable proton resonances of rHb (βN108Q) in the CO form in 0.1 M sodium phosphate buffer at pH 7.0 at 500 MHz at various temperatures (7°C, 11°C, 17°C, 23°C, 29°C), without an allostERIC effector (Figure 8A) and with 4 mM inositol hexaphosphate ("IHP") (Figure 8B).

Figures 9A and 9B are 500-MHz spectra showing the exchangeable proton resonances of rHb (αL29F, βN108Q) in the CO form in 0.1 M sodium phosphate buffer at pH 7.0 at various temperatures (7°C, 11°C, 17°C, 23°C, 29°C), without (Figure 9A) and with (Figure 9B) 4 mM IHP.

Figures 10A and 10B are 600-MHz 1H-NMR spectra showing exchangeable proton resonances (Figure 10A) and ring-current shifted proton resonances (Figure 10B) of 3-6% solutions of Hb A; rHb (βL05W); rHb (αD94A,
βL105W); and rHb (αD94A) in the CO form in 0.1 M sodium phosphate at pH 7.0 and 29°C.

Figures 11A and 11B are 600-MHz 2D heteronuclear multiple-quantum coherence ("HMQC") spectra of 5-8% solutions of $^{15}$N-labeled rHb (βL105W) (Figure 11A) and Hb A (Figure 11B) in the CO form in 90% H$_2$O/10% D$_2$O in 0.1 M sodium phosphate at pH 7.0 and 29°C.

Figures 12A-12D are 600-MHz 2D NOESY-HMQC ("NOESY"-nuclear Overhauser enhancement spectroscopy) spectra of a 5% solution of $^{15}$N-labeled rHb (βL105W) in the CO form in 90% H$_2$O/10% D$_2$O in 0.1 M sodium phosphate at pH 7.0 and 29°C recorded at various mixing times: 15 ms (Figure 12A); 30 ms (Figure 12B); 60 ms (Figure 12C); and 100 ms (Figure 12D).

Figures 13A-13C are 300-MHz 1H-NMR spectra of 3-6% solutions of Hb A; rHb (βL105W); rHb (αD94A, βL105W); and rHb (rHb (αD94A) in the deoxy form in 0.1 M sodium phosphate at pH 7.0 and 29°C. Figure 13A shows hyperfine-shifted N$_6$H resonances of proximal histidines acquired at 300-MHz; Figure 13B shows hyperfine shifted and exchangeable proton resonances acquired at 300-MHz; and Figure 13C shows exchangeable proton resonances acquired at 300-MHz. Since rHb (αD94A, βL105W) and rHb (αD94A) easily form met-Hb during the oxygenization process, a small amount of sodium dithionite was added to these NMR samples to diminish the formation of met-Hb.
Figure 14 is a 600-MHz 2D HMQC spectrum of 5-8% solutions of 
$^{15}$N-labeled rHb (βL105W) in the deoxy form in 90% H$_2$O/10% D$_2$O in 0.1 M 
sodium phosphate at pH 7.0 and 29°C.

Figures 15A-15D are 600-MHz 2D NOESY-HMQC spectra of 5% 
solution of $^{15}$N-labeled rHb (βL105W) in the deoxy form in 90% H$_2$O/10% D$_2$O in 
0.1 M sodium phosphate at pH 7.0 and 29°C recorded at various mixing times: 15 
ms (Figure 15A); 30 ms (Figure 15B); 60 ms (Figure 15C); and 100 ms (Figure 15D). 
The solid line between two cross peaks indicates the inter-residue NOE effect 
between the $^1$H$_{a1}$ of one residue and the $^1$H$_{a1}$ and $^1$H$_2$ of the other residue for 
$\beta$37Trp and $\beta$105Trp.

Figures 16A-16B show exchangeable proton resonances in 600-MHz 
$^1$H-NMR spectra of 3-6% solutions of Hb A; rHb (βL105W); rHb (αD94A, 
βL105W); and rHb (αD94A) in the CO form in 0.1 M sodium phosphate at pH 7.0 
and at 11°, 20°, and 29°C in the absence (Figure 16A) and presence (Figure 16B) of 
2 mM IHP.

Figure 17A-17D are 600-MHz 2D heteronuclear single-quantum 
coherence ("HSQC") spectra of 5-8% solutions of $^{15}$N-labeled rHb (βL105W) in the 
CO form in 90% H$_2$O/10% D$_2$O in 0.1 M sodium phosphate at pH 7.0 and various 
temperatures in the absence and presence of 2 mM IHP. Figure 17A - 29°C in the 
absence of IHP; Figure 17B - at 29°C in the presence of 2 mM IHP; Figure 17C - at
20°C in the presence of 2 mM IHP; and Figure 19D - 11°C in the presence of 2 mM IHP.

Figures 18A-18B show ring-current shifted proton resonances in 600-MHz ¹H-NMR spectra of 3-6% solutions of Hb A; rHb (βL105W); rHb (αD94A, βL105W); and rHb (αD94A) in the CO form in 0.1 M sodium phosphate at pH 7.0 and various temperatures in the absence (Figure 18A) and presence (Figure 18B) of 2 mM IHP.

**DETAILED DESCRIPTION OF THE INVENTION**

I. **DEFINITIONS**

As used herein, "Hb A" or "native Hb A" means human normal adult hemoglobin as obtained from human subjects.

"Recombinant human normal adult hemoglobin," "rHb A," and "unmodified rHb A" mean human normal adult hemoglobin produced through recombinant DNA technology and having essentially the same structure and function as native Hb A as described by Shen, et al. (1993), and U.S. Patent No. 5,753,465.

"rHb (βL105W)" refers to a recombinant mutant human hemoglobin in which the leucine residue at position 105 of each of the β-chains of the Hb molecule has been replaced by a tryptophan residue. This hemoglobin possesses low oxygen affinity and high cooperativity in oxygen binding compared to Hb A.
rHb (βL105W) is designed to form a hydrogen bond between α94Asp and β105Trp in the α₁β₂ subunit interface in order to lower the oxygen affinity by stabilizing its deoxy quaternary structure.

"rHb (βN108Q)" refers to a mutant human hemoglobin produced through recombinant DNA technology in which the asparagine residue at position 108 of the β-chains located in the α₁β₁ interface and in the central cavity of the Hb molecule, have been replaced by glutamine residues. This hemoglobin possesses low oxygen affinity, and high cooperativity in oxygen binding, and also increased resistance to autoxidation as compared to other known recombinant low oxygen affinity mutant hemoglobins, such as, rHb (αV96W) and rHb (αV96W, βN108K).

"Autoxidation" refers to the turning or conversion of oxyhemoglobin ("HbO₂" or "oxy-Hb") into methemoglobin ("met-Hb"). In HbO₂ the heme-iron atoms are in the reduced ferrous (Fe²⁺) state, however, in met-Hb, the heme-iron atoms are in the oxidized ferric (Fe³⁺) state.

"Deoxy" and "oxy" refer to the oxygenation state of the heme-iron atom in Hb A and rHbs. Oxyhemoglobin ("oxy-Hb" or "HbO₂") has four oxygen molecules bound to the heme groups; deoxyhemoglobin ("deoxy-Hb") contains no oxygen molecules. In normal arterial blood, normal adult hemoglobin A ("Hb A") is in the oxy form ("Hb O₂ A" or "oxy-Hb A"). In venous blood, a portion of Hb A is in the deoxy form ("deoxy-Hb A").
"Carbonmonox-Hb," "HbCO A," "rHbCO," and "CO form" all refer to hemoglobin bound to carbon monoxide molecules rather than oxygen molecules.

"Ferri-hemoglobin," "ferri-Hb," "ferric form," "methemoglobin," "meth-Hb," and "Fe⁺³ state" all refer to hemoglobin with their respective heme-iron atoms oxidized to the ferric (Fe⁺³) state. Ferri-Hb does not bind oxygen.

"Methionine aminopeptidase" refers to the enzyme methionine aminopeptidase which specifically cleaves the amino-(N) terminal methionine residue from a peptide sequence.

"Oxygen affinity" means the strength of binding of oxygen to a hemoglobin molecule. High oxygen affinity means hemoglobin does not readily release its bound oxygen molecules. The P50 is a measure of oxygen affinity.

"Cooperativity" refers to the binding of oxygen by the four subunits of the hemoglobin molecule and is measured by the Hill coefficient (nₘₐₓ). For Hb A in 0.1 M sodium phosphate at pH 7.4 and 29°C, nₘₐₓ is about 3.2.

The two classical quaternary structures are the T (tense) quaternary D structure for the low affinity deoxy-Hb and the R (relax) quaternary structure for the high affinity oxy-Hb. "R-type" or "R-like," and similar terms refer to those hemoglobins which exhibit characteristic quaternary structural markers, such as the proton resonance at 10.7 ppm from DSS on ¹H-NMR spectra. "T-type" or "T-
like" and similar terms refer to those hemoglobins which exhibit characteristic T quaternary structures, such as the proton resonance at ~14.0 ppm from DSS on $^1$H-NMR spectra.

II. METHODS AND RESULTS

Using the *Escherichia coli* expression system described by Shen, et al. (1993); U.S. Patent No. 5,753,465; and Kim, et al. (1995); U.S. Patent No. 5,843,888, new non-naturally occurring artificial recombinant hemoglobins ("rHbs") have been constructed, having low oxygen affinity while maintaining high cooperativity in oxygen binding. One of the rHbs, rHb (βN108Q) also exhibits increased resistance to autoxidation as compared to certain other known low oxygen affinity mutants. More particularly, the present invention is directed to: a recombinantly produced mutant of Hb A, denoted herein as rHb (βN108Q), in which the asparagine residues at position 108 of each of the β-chains, located in the $\alpha_1$$\beta_1$ subunit interface and in the central cavity of the Hb molecule, have been replaced by glutamine residue; and a recombinantly produced mutant of Hb A, denoted herein as rHb (βL105W) in which the leucine residues at position 105 of each of the β chains have been replaced by tryptophan and in this molecule a new hydrogen bond is formed from β105Trp to β94Asp in the $\alpha_1$$\beta_2$ subunit interface in order to lower the oxygen binding affinity by stabilizing its deoxy quaternary structure.
These new artificial hemoglobins, i.e., derived entirely from sources other than blood, possess a low oxygen affinity and high cooperativity in oxygen binding. Additionally, rHb (βN108Q) exhibits increased resistance to autoxidation as compared to other known low oxygen affinity mutants, such as rHb (αV96W) and rHb (αV96W, βN108K). Further, these new artificial hemoglobins exhibit no unused subunit disassociation when ligated. In a cell-free environment the rHbs of the present invention have similar or lower oxygen binding properties to those of Hb A in red blood cells. Such rHbs therefore are of value as hemoglobin-based oxygen carriers, i.e., potential blood substitutes, or hemoglobin therapeutics.

It is also within the scope of the present invention to prepare and use other low oxygen affinity hemoglobins with other appropriate mutations. In particular, the methods of the present invention may be used to produce other mutant hemoglobins with additional advantageous properties. Methods for evaluating the suitability of other useful mutants possessing the properties of such low oxygen affinity, high cooperativity, and increased resistance to autoxidation for use in a blood substitute or therapy are described herein below. The preferred materials and methods for obtaining rHb (βL105W) and rHb (βN108Q) are given in the following reference examples. While the rHbs of the present invention are preferably produced recombinantly, it is understood that non-recombinant methods may also be used.
The preferred mutant rHbs of the present invention, rHb (βL105W) and rHb (βN108Q), can switch from the R quaternary structure to the T quaternary structure in their ligated state upon the addition of an allosteric effector, IHP, and/or by lowering the temperature. The recombinant hemoglobins of the present invention can therefore be used to gain new insight regarding the nature of subunit interactions in the α1β2 and α1β1 interfaces and the molecular basis for the allosteric mechanism of hemoglobin.

As will be shown below, rHb (βN108Q) of the present invention shows a low oxygen affinity, an enhanced Bohr effect, but a similar cooperativity as that of Hb A, and also exhibits slower autoxidation to methemoglobin ("met-Hb") as compared to other known low oxygen affinity recombinant hemoglobins such as, for example, rHb (α96Val→Trp) and rHb (α96Val→Trp, β108Asn→Lys) (Kim, H.-W., et al. Biochemistry 35:6620-6627 (1996) (hereinafter "Kim, et al. (1996)"); Ho, C., et al. Blood Substitutes: Present and Future Perspectives of Blood Substitutes (Tsuchida, E., Ed.), Elsevier Science SA, Lausanne, Switzerland, pp. 281-296 (1998) (hereinafter "Ho, et al. (1998)"); Jeong, et al. (1999); and Tsai, et al. (1999), the disclosures of which are incorporated herein by reference), oxidize much faster. Therefore, rHb (βN108Q) can be useful for hemoglobin-based oxygen carriers and hemoglobin therapeutics.
Proton nuclear magnetic resonance ("^{1}H-NMR") studies show that rHb (βN108Q) has similar tertiary structure around the heme pockets and quaternary structure in the α₁β₁ and α₁β₂ subunit interfaces as compared to those of Hb A. ^{1}H-NMR studies also demonstrate that rHb (βN108Q) can switch from the R quaternary structure to the T quaternary structure without changing its ligation state upon the addition of an allosteric effector, IHP, and/or by lowering the temperature. This suggests that the T quaternary structure of rHb (βN108Q) is more stable than that of Hb A. This is consistent with the molecular mechanism of low-oxygen affinity found in rHb (αV96W) (Kim, H.-W., et al., J. Mol. Biol. 248:867 (1995) (hereinafter "Kin, et al. (1995)"); U.S. Patent No. 5,843,888 and rHb (αV96W, βN108Q) (Ho, et al. (1998); Tsai, et al. (1999)).

It has been reported by Carver, T.E., et al. J. Biol. Chem. 267: 14443 (1992); Brantley, R.E. Jr., et al. J. Biol. Chem. 268: 6995 (1993) (hereinafter "Brantley, et al. (1993)"; and Eich, R.F., et al. Biochemistry 35: 6976 (1996), the disclosures of which are incorporated herein by reference, that substitution of the Leu residue for phenylalanine at the B10 position can inhibit autoxidation in myoglobin and that at the B10 position of the α-chain can lower NO reaction with deoxy- and oxy-Hb A. Reduction of the NO reaction with oxy-Hb A by appropriate mutations, i.e., αL29F, in the distal heme pocket has been associated with reduction of the hypertensive effect recorded in vivo (Doherty, D.H., et al.
Nature Biotech. 16: 672 (1998), the disclosure of which is incorporated herein by reference). Hence, as detailed below, such mutation was further introduced into βN108Q to produce a double mutant, rHb (αL29F, βN108Q). It was found that this double mutant is more stable against autoxidation as compared to rHb (βN108Q), but exhibits comparable oxygen binding properties to those of Hb A in the presence of allosteric effector, 2 mM 2,3-BPG.

Mutant rHb (βL105W) was designed to form a new hydrogen bond from β105Trp to α94Asp in the α1β2 subunit interface in order to lower the oxygen binding affinity by stabilizing its deoxy quaternary structure. It was found that rHb (βL105W) possesses a very low oxygen affinity and maintains high cooperativity (P50 = 28.2 mm Hg, n_max = 2.6 in 0.1 M sodium phosphate at pH 7.4 and 29°C) as compared to Hb A (P50 = 9.9 mm Hg, n_max = 3.2 in 0.1 M sodium phosphate at pH 7.4 and 29°C). Mutant rHb (αD94A, βL105W) and rHb (αD94A) were designed to provide evidence that rHb (βL105W) forms a new hydrogen bond from β105Trp to α94Asp in the α1β2 subunit interface of the deoxy quaternary structure. The multinuclear, multidimensional nuclear magnetic resonance ("NMR") studies performed in accordance with the present invention in 15N-labeled rHb (βL105W) have identified the resonance of the indole nitrogen-attached proton of β105Trp for rHb (βL105W). 1H-NMR studies on Hb A and mutant rHbs were used to investigate the structural basis for the low oxygen
affinity of rHb (βL105W). NMR results show that rHb (βL105W) forms a new hydrogen bond from β105Trp to α94Asp in the α1β2 subunit of the deoxy quaternary structure. It is believed that the low oxygen affinity of rHb (βL105W) is due to the formation of a new hydrogen bond between β105Trp and α94Asp in the deoxy quaternary structure.

Proton nuclear magnetic resonance ("NMR") spectroscopy was used to study the tertiary and quaternary structures of Hbs in solution (Ho, et al. (1992)). Several exchangeable proton resonances at ~15 to ~9 ppm from the methyl proton resonance of 2,2-dimethyl-2-silapentane-5-sulfonate ("DSS") have been characterized as intersubunit H-bonds in the α1β1 and α1β2 subunit interfaces in both oxy and deoxy states of Hb A. These H-bonded protons observed by NMR can be used as structural markers in functional studies. In particular, the resonance at ~14 ppm from DSS has been identified as the inter-subunit H-bond between α42Tyr and β99Asp in the α1β2 interface of deoxy-Hb A, a characteristic feature of the T-structure of Hb A (Fung, L.W.-M., et al. Biochemistry 14:2526 (1975) (hereinafter "Fung, et al. (1975)"); 1975; Russu, I.M., et al. Biochem. Biophys. Acta 914:40 (1987) (hereinafter "Russu, et al. (1987)"). By observing this T-structure marker in both the deoxy and the CO forms of Hbs under various conditions, the stability of the T-conformation can be assessed and the transition from the T- to the R-structure can be monitored.
In the present invention, the strategy for designing rHbs with low oxygen affinity and high cooperativity was to stabilize the T-structure while not perturbing the R-structure. (See, Ho, et al. (1998); Tsai, et al. (1999)). This strategy has been demonstrated in the design of rHb (αV96W), which has low oxygen affinity and normal cooperativity (Kim et al. (1995); U.S. Patent No. 5,843,888). This designed mutation is located at the α₁β₂ subunit interface and in the central cavity of the Hb molecule. According to ¹H-NMR studies, rHbCO (αV96W) can switch from the R-structure to the T-structure without changing its ligation state when the temperature is lowered and/or when IHP, an allosteric effector, is added. The crystal structure of rHb (αV96W) in its T-state has shown a novel water-mediated H-bond between α96Trp Nε₁ and β101Glu Oε₂ in the α₁β₂ subunit interface (Puius, T.A., et al. Biochemistry. 37: 9258 (1998) (hereinafter "Puius", et al. (1998)"). Both ¹H-NMR studies and the crystal structure indicate that the T-structure of this rHb is stabilized. In the present invention, the NMR studies have also shown that rHbCO (βN108Q) and rHbCO (βN105W) can switch to the T quaternary structure even when they are still ligated. These results suggest that the T structure of these two rHbs are more stable than that of Hb A.

As stated above, the methods of the present invention may also be used to produce other mutant artificial hemoglobins with different properties as well as hemoglobins with mutations that compensate for mutants that are
naturally occurring. The preferred materials and methods for obtaining rHb (βN108Q) and rHb (βL105W) are given in the following reference example. Non-recombinant methods may also be used.

REFERENCE EXAMPLE

Construction of Expression Plasmids for rHb (βN108Q) and rHb (βL105W)

The E. coli Hb A expression plasmids pHE2 and pHE7, which respectively contain human α- and β-globin genes and cDNAs, were used as the starting plasmids for expressing the mutant hemoglobins of the present invention. The construction of plasmids pHE2 and pHE7 and properties of the rHb A produced thereby are fully described in Shen, et al. (1993), U.S. Patent No. 5,753,465, and Shen, T.-J., et al. Protein Eng. 10: 1085 (1997) (hereinafter "Shen, et al. (1997)"); Kim, et al. (1994), and U.S. Patent No. 5,843,888), the disclosure of which is incorporated herein by reference.

The construction of plasmid pHE2009 for expression of mutant r Hb (βN108Q) using synthetic globin genes was carried out as follows. The plasmid pHE2 was used as the starting plasmid and an oligonucleotide of sequence 5'-CGTCTGCTGGTTCAGGTACTAGTTTGC-3' (SEQ ID NO: 1) (mutated codon is underlined) was purchased from DNA International, Inc. (Lake Oswego, Oregon) and used as a primer to introduce the mutation βN108Q into pHE2.

Techniques for oligonucleotide synthesis are well known and this invention is not
limited to any particular technique. The site-directed mutagenesis procedure followed the protocol of an "Altered Sites II In-Vitro Mutagenesis System" kit (Promega Corporation, Madison, WI) and the resultant plasmid pHE2009 contained the expected mutation βN108Q.

The construction of plasmids pHE2020 (mutant rHb (αD94A) and pHE2004 (mutant rHb βL105W)) using synthetic globin genes was similar to that of pHE2009, except the mutation oligonucleotide 5'-CTGCCTGTT
GCTCGCTCATTCAATTCAACTG-3' (SEQ ID NO: 2, mutated codon αD94A is underlined) and 5'-GGAAAACTTCCGA TGGCTGGGTAACGTAC-3' (SEQ ID NO: 3, mutated codon βL105W is underlined) were used. Both oligonucleotides were purchased from DNA International, Inc. (Lake Oswego, Oregon).

The construction of plasmid pHE2017 (mutant rHb (αD94A, βL105W)) was accomplished by ligating the 0.51-kb SmaI-PstI fragment of pHE2020 with the 6.34-kb kb PstI-SmaI fragment of pHE2004. The construction of plasmid pHE2018 for expression of mutant rHb (αL29F, βN108Q) was accomplished by ligating the 6.06-kb PstI-BamHI fragment of pHE2009 with the 0.79-kb BamHI-PstI fragment of pHE284. The construction of plasmid pHE284 containing the mutation αL29F from plasmid pHE2 was reported previously by Jeong, et al. (1999).
The construction of plasmid pHE7009 for expression of mutant rHb (βN108Q) using the human globin cDNAs was carried out as follows. The coding sequences of human α- and β-globin cDNAs in plasmid pHE7 were inserted into pTZ18U (Bio-Rad Laboratories, Hercules, CA) by methods well known in the art.

Site-directed mutagenesis was performed as described by Kunkel, T.M. et al., Proc. Natl. Acad. Sci. USA 82:488 (1985) the disclosures of which are incorporated herein by reference, and Shen, et al. (1993). An oligonucleotide of sequence 5'-ACAGACCAG TACCTGTCC CAGGAGCCT-3' (SEQ ID NO: 4) (mutated codon Asn→Gln is underlined) was purchased from DNA International, Inc. (Lake Oswego, Oregon), and used as the mutation primer. The human normal β-globin cDNA in plasmid pHE7 was then replaced with the mutated cDNA to produce plasmid pHE7009. The DNA sequences for the α- and β-globin cDNAs in pHE7009 are shown in Figure 1A (SEQ ID NO: 5). Plasmid pHE7009 in host cell E. coli JM109 and designated pHE7009/JM109 was deposited with the American Type Culture Collection of Manassas, VA on April 27, 2000 under number PTA-1768.

The construction of plasmid pHE7004 for expression of mutant rHb (βL105W) using the human globin cDNAs was carried out in the similar way as that of plasmid pHE7009, except an oligonucleotide of sequence 5'-

CCTGAGAACTTC AGGTGG CTAGGCAACGTGCTGGTC-3' (SEQ ID NO: 6),
mutated codon Leu→Trp is underlined) was purchased from DNA International, Inc. (Lake Oswego, Oregon) and used as the mutation primer. The DNA sequences of the α- and β-globin cDNAs in pHE7004 are shown in Figure 1B (SEQ ID NO: 7). Plasmid pHE7004 in host cell *E. coli* JM109 and designated pHE7004/JM109 was deposited with the American Type Culture Collection of Manassas, VA on April 27, 2000 under number PTA-1769.

**Growth of Cells**

Plasmids pHE7009, and pHE7004 were individually transformed in *E. coli* strain JM109 (Promega, Madison, WI) by methods well known in the art. *E. coli* cells were grown in Terrific Broth ("TB") medium plus 100 μg/mL ampicillin in a 10-liter Microferm fermenter (New Brunswick Scientific, Model BioFlo 3000) at 32°C until the optical density at 600 nm reached 10. TB medium contains 1.2% bactotryptone, 2.4% bactoyeast extract, 0.17M KH₂PO₄, 0.072M K₂HPO₄, and 1% glucose solution. Expression of rHbs was induced by adding isopropyl β-thiogalactopyranoside (Sigma, St. Louis, MO) to a concentration of 0.1-0.4 mM. The culture was then supplemented with hemin (20-50 mg/liter) (Sigma) and the growth was continued for at least another 4 hr. The cells were harvested by centrifugation and stored frozen at -80°C until needed for purification. For details, refer to Shen, et al. (1993), and Shen, et al. (1997).
Although *E. coli* cells are presently preferred for expressing and producing the recombinant mutant hemoglobin of the present invention, the invention is not limited to *E. coli* cells. Other appropriate expression systems such as yeast, insect cells and transgenic animals such as pigs, sheep, and cows may also advantageously be used to express mutant hemoglobins. Plasmids pHE7009 and pHE7004 have been optimized for *E. coli* cells, but other expression systems may be advantageously used. The plasmids can also be constructed with human genes.

**Isolation and Purification of rHbs**

The recombinant hemoglobins obtained from cells transformed with plasmids pHE7009 and pHE7004 were purified as essentially described by Shen, et al. (1993), and Shen, et al. (1997). The frozen stored cell paste was put into lysis buffer (40 mM trisbase/1 mM benzamidine) (Sigma) at 3 ml/gm of cell paste. The cell lysis procedure was used to pass the cell paste through a high-pressure homogenizer (Model Emulsiflex-C5, Avestin) 3 times. The lysate was then centrifuged at 4°C for 2.5 hours at 13,000 rpm in a Beckman centrifuge (Beckman JA14 rotor). The supernatant from the lysate was saturated with CO gas and left at 30°C overnight as described by Tsai, et al. (1999). The supernatant was then put through a Millipore Minitan Acrylic Ultrafiltration system to concentrate the protein. Polyethyleneimine (Sigma) was added to a final concentration of 0.5% to
precipitate nucleic acids. After centrifugation, the sample was dialyzed against 20 mM Tris-HCl/0.5 mM triethylenetetraamine ("TETA") (Sigma) at pH 8.3 overnight with one or two changes of buffer. Throughout the above procedures, the sample was kept at 4°C and maintained in a CO atmosphere. Following the procedures of Shen, et al. (1993) and Shen, et al. (1997), the rHb fraction collected after passage through a Q-Sepharose Fast-Flow column (Pharmacia Anion Exchanger) Pharmacia was oxidized and reduced, and converted to the CO form. This Hb solution was then purified by eluting through a fast protein liquid chromatography ("FPLC") Mono-S column (Pharmacia Cation Exchanger, HR 16/10) with a gradient of 10 mM sodium phosphate in 0.1-0.5 mM ethylenediaminetetraacetic acid ("EDTA") at pH 6.8 (eluent A) and 20 mM sodium phosphate in 0.1-0.5 mM EDTA at pH 8.3 (eluent B).

rHb (βN108Q) was eluted individually in two major peaks. Figure 2A shows peak a and peak b for rHb (βN108Q). Figure 2B shows rHb (βL105W) was eluted individually in three major peaks, peaks a, b, and c. rHbs collected from peak b in both cases contained less than 2% methionine at the amino-termini and with the correct molecular weight.

Mass Spectrometry

Hb samples subjected to mass spectrometry were dialyzed extensively against distilled H₂O and then lyophilized. Immediately before
analysis, the samples were dissolved in water to a concentration of 125 pmol of Hb per µl of H$_2$O (7.8 mg/ml). Aliquots of these solutions were then diluted to give a final concentration of 10 pmol/µl of 50:50 water/acetonitrile containing 0.2% formic acid. Aliquots (10 µl) of these final solutions were introduced into the electrospray ion source at 5 µl/minute.

The electrospray ionization analyses were performed on a VG Quattro-BQ (Fisons Instruments, VG Biotech, Altrincham, U.K.), as described by Shen, et al. (1993). Automated cycles of Edman degradation were performed on an Applied Biosystems gas/liquid-phase sequencer (Model 470/900A) equipped with an on-line phenylthiohydantoin amino acid analyzer (Model 120A). These two analytical procedures were used to assess the quality of the rHbs. All rHbs used in this study had the correct molecular weights and contained less than 2% of methionine at the amino termini.

**Oxygen-Binding Properties of rHbs**

Oxygen dissociation curves of rHbs were measured by a Hemox-Analyzer (TCS Medical Products, Huntington Valley, PA) at 29°C as a function of pH. The concentration of Hb used was approximately 0.1 mM per heme. The methemoglobin ("met-Hb") reductase system described by Hayashi, A., et al. *Biochem. Biophys. Acta* 310:309 (1973), the disclosure of which is incorporated herein by reference, was used if needed to reduce the amount of met-Hb in the
sample. A visible absorption spectrum of each sample was recorded immediately after oxygen equilibrium measurement, and the met-Hb content was estimated by using the extinction coefficients of Hb reported by Antonini, E., Physiol. Rev. 45:123 (1965), the disclosure of which is incorporated herein by reference. Oxygen equilibrium parameters were derived by fitting Adair equations to each equilibrium oxygen-binding curve by a nonlinear least-squares procedures. $P_{50}$, a measure of oxygen affinity, was obtained at 50% saturation. The Hill coefficient ($n_{max}$), a measurement of cooperativity, was determined from the maximum slope of the Hill plot by linear regression. $n_{max}$ was derived between 60% and 65% oxygen saturation. The accuracy of $P_{50}$ measurements in mm Hg is ± 5% and that for $n_{max}$ is ± 7%.

$^1$H-NMR Spectroscopy Measurements of rHbs

$^1$H-NMR spectra of rHbs were obtained on Bruker AVANCE DRX-300, AVANCE DRX-500, and AVANCE DRX-600 NMR spectrometers that were operated at 300, 500, and 600 MHz, respectively, and at temperatures ranging from 10° C-36° C. All of the Hb samples were placed in 0.1 M sodium phosphate buffer (in 100% H$_2$O) at pH 7.0. The Hb concentration range was approximately 5% (~3 mM in terms of heme). The water signal was suppressed by using the "jump-and-return" pulse sequence as reported by Plateau, P., et al. J. Am. Chem. Soc. 104:7310 (1982) (hereinafter "Plateau, et al. (1982)"), the disclosure of which is
incorporated herein by reference. Proton chemical shifts are referenced to the methyl proton resonance of the sodium salt of 2,2-dimethyl-2-silapentane-5 sulfonate ("DSS") indirectly by using the water signal, which signal occurs at 4.76 ppm downfield from that of DSS at 29°C, as the internal reference.

5 Autoxidation of rHbs

The autoxidation of rHbs was recorded by monitoring the disappearing rate of the oxy-marker (-2.34 ppm from DSS) from Bruker AVANCE DRX-300 1H-NMR spectra. The autoxidation reaction was carried out in PlasmaLyte buffer (Baxter) (5% D2O) with 5 mM EDTA at pH 7.4 and at 37°C. The HbO2 concentration was 5% (~3 mM in terms of heme).

FUNCTIONAL STUDIES

Oxygen-binding Properties of rHbs

Figures 3A and 3B show the oxygen-binding measurements of rHb (αL29F), rHb (βN108Q), rHb (α29F, βN108Q), rHb (βLI05W), and Hb A in 0.1 M sodium phosphate buffer as a function of pH at 29°C. rHb (βN108Q) exhibits a significantly lower oxygen affinity as compared to that of Hb A over the pH range from pH 6.79 to pH 8.09. The oxygenation process of rHb (βN108Q) is very cooperative with an nmax value of about 2.7 to 3.1 depending on the pH, compared to about 3.2 for Hb A (Figure 3B). On the other hand, the mutation at the α-chain B10 position, i.e., αL29F, increases the oxygen affinity and decreases the
cooperativity. rHb (αL29F, βN108Q) shows slightly higher P$_{50}$ values as compared to those of Hb A at pH < 7.4, suggesting that the effect of the mutations on the oxygen affinity is additive. rHb (αL29F, βN108Q) preserves cooperativity in binding of oxygen with an $n_{ \text{max}}$ value of 2.4 to 2.8 (Figure 3B). rHb (βL105W) exhibits very low oxygen affinity (about 2-3 times lower) and maintains normal cooperativity from pH 7.0 to 8.0 as compared to Hb A.

Figure 4 shows that the oxygen affinities of rHb (βN108Q) and also rHb (βL105W) measured in the absence of 2,3-BPG are lower than that of Hb A in the presence of 5 mM 2,3-BPG, making them potential candidates for an oxygen carrier in a blood substitute system. Figures 3A and 3B also show that the alkaline Bohr effect (which, in Hb A, results in a decrease in oxygen affinity with a lowering of the pH) is enhanced in rHb (βN108Q) and rHb (αL29F, βN108Q) compared to Hb A.

Table 1 below compares the number of Bohr protons released upon oxygenation per heme calculated from the linkage equation $\Delta H^* = -\partial \log P_{50}/\partial pH$. (Wyman, J., Adv. Protein Chem. 4:407 (1948) and Adv. Protein Chem. 19:233 (1964), (hereinafter "Wyman, J. (1948) and (1964)") the disclosures of which are incorporated herein by reference. Both rHb (βN108Q) and rHb (αL29F, βN108Q) release more Bohr protons than Hb A.
Table 1

Bohr effect of Hb A, rHb (βN108Q), rHb (αL29F, βN108Q), and rHb (βL105W) in 0.1 M sodium phosphate buffer at 29°C

<table>
<thead>
<tr>
<th>Hemoglobin</th>
<th>( \frac{\partial \log P_{50}}{\partial \text{pH}} ) in 0.1 phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb A</td>
<td>0.48 (pH 6.79-8.00)</td>
</tr>
<tr>
<td>rHb (βN108Q)</td>
<td>0.56 (pH 6.79-8.09)</td>
</tr>
<tr>
<td>rHb (αL29F, βN108Q)</td>
<td>0.67 PpH 6.79-7.97</td>
</tr>
<tr>
<td>rHb (βL105W)</td>
<td>0.55 (pH 7.00-8.00)</td>
</tr>
</tbody>
</table>

---

Autoxidation.

The autoxidation process was monitored for oxy-Hb A, oxy-rHb (βN108Q), oxy-rHb (αL29F, βN108Q), oxy-rHb (βL105W) and three other known low-oxygen affinity mutants, oxy-rHb (αV96W), oxy-rHb (αV96W, βN108K) and oxy-rHb (αL29F, αV96W, βN108K), by a 300-MHz NMR spectrometer. The resonance at -2.34 ppm upfield from DSS has been assigned to the \( \gamma_2 \)-CH₃ of E11Val of Hb A in the oxy form of Hb A (Dalvit, C., et al., Biochemistry 24:3398 (1985), the disclosure of which is incorporated herein by reference). Monitoring the rate of disappearance of the oxy-marker (-2.34 ppm from DSS) as a function of time allows for the determination of the autoxidation rate of the Hb samples. The results are shown in Figure 5. The percentage of ferrous-Hb varies with time (t)
mono-exponentially and the autoxidation rate constant can be obtained from:

\[ [\text{ferrous-Hb}]_t = [\text{ferrous-b}]_{t=0} \times \exp (-K_{\text{auto}} \times t), \]

where \( K_{\text{auto}} \) is the autoxidation rate constant. The autoxidation rate constants of Hb A and r Hbs are listed in Table 2 below. At pH 7.4 and 37°C in PlasmaLyte buffer, rHb (βN108Q), rHb (βL105W), rHb (αV96W), and rHb (αV96W, βN108K) autoxidized 2.8-, 8-, 4.4-, and 8-times faster than Hb A. rHb (βN108Q) is shown to be more stable against autoxidation than other known low-oxygen affinity mutants developed in the laboratory; i.e., rHb (αV96W), rHb (βL105W), and rHb (αV96W, βN108K). The autoxidation rate is slowed down by introducing the mutation αL29F into rHb (βN108Q) and rHb (αV96W, βN108K). rHb (αL29F, βN108Q) and rHb (αL29F, αV96W, βN108K) autoxidized 2.5- and 2.8-times slower than rHb (βN108Q) and rHb (αV96W, βN108K), respectively. Thus, the mutation αL29F is very effective in slowing down the autoxidation process as suggested by the results on myoglobin (Brantley, et al., (1993), the disclosure of which is incorporated herein by reference). Hemichrome-like spectra are observed in the autoxidation process of only rHb (αL29F, αV96W, βN108K) among all the low oxygen affinity rHbs studied. Hemichrome forms when methemoglobin (met-Hb) converts from the ferric high-spin form to the ferric low-spin form in which the distal imidazole displaces the H₂O ligand (Levy et al., Biochemistry 29: 9311 (1990); Levy, et al., Biophys. J. 61: 750 (1992); Blumberg, et al., Adv. Chem. Series 100: 271 (1991)).
This is in accordance with the results from Jeong et al. (1999) in which the oxidized form of rHb ($\alpha$L29F, $\alpha$V96W, $\beta$N108K) exhibits of hemichrome-like spectra, making it undesirable to be considered as a candidate for an oxygen carrier.

Table 2

<table>
<thead>
<tr>
<th>Hemoglobin</th>
<th>$k_{auto}$ (hr$^{-1}$)$^a$</th>
<th>$P_{50}$ (mm Hg)$^b$</th>
<th>$n_{max}$$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb A</td>
<td>0.0158 ± 0.0002</td>
<td>9.64</td>
<td>3.28</td>
</tr>
<tr>
<td>rHb(βN108Q)</td>
<td>0.0449 ± 0.0007</td>
<td>17.46</td>
<td>3.10</td>
</tr>
<tr>
<td>rHb ($\alpha$L29F, βN108Q)</td>
<td>0.0181 ± 0.0006</td>
<td>12.06</td>
<td>2.77</td>
</tr>
<tr>
<td>rHb (βL105W)</td>
<td>0.123 ± 0.0048</td>
<td>28.2</td>
<td>2.60</td>
</tr>
<tr>
<td>rHb ($\alpha$V96W)</td>
<td>0.0689 ± 0.0008</td>
<td>16.38</td>
<td>2.94</td>
</tr>
<tr>
<td>rHb ($\alpha$V96W, βN108K)</td>
<td>0.125 ± 0.0051</td>
<td>50.65</td>
<td>2.36</td>
</tr>
<tr>
<td>rHb ($\alpha$L29F, $\alpha$V96W, βN108K)</td>
<td>0.0449 ± 0.0014</td>
<td>21.97$^c$</td>
<td>1.81$^c$</td>
</tr>
</tbody>
</table>

$^a$ Rate constants for the autoxidation ($k_{auto}$) of Hb A and r Hbs are obtained at [oxy-Hb] 3 mM heme in PlasmaLyte buffer at pH 7.4 and 37°C.

$^b$ Oxygen affinity and cooperativity were obtained in 0.1 M sodium phosphate buffer at 29°C and pH 7.4. Protein concentrations were 0.1 mM heme.

$^c$ From Jeong, et al. (1999).
STRUCTURAL STUDIES OF rHb (βN108Q) AND rHb (αL29F, βN108K)

\(^1\)H-NMR Investigations

\(^1\)H-NMR spectroscopy is an excellent tool for monitoring changes in the tertiary and quaternary structures of Hb A and its variants (see, e.g., Shen, et al. (1993); Kim, et al. (1994); Kim, et al. (1995); Kim, et al. (1996); and Barrick, D., et al. *Nat. Struct. Biol.* 4:78 (1997), the disclosures of which are incorporated herein by reference). Figure 6A shows the exchangeable proton resonances and Figure 6B shows the ring-current-shifted resonances of Hb A, rHb (βN108Q), and rHb (αL29F, βN108Q) in the CO form measured at 500 MHz. The ring-current-shifted resonances are sensitive to the orientation and/or conformation of the heme group relative to the amino acid residues in the heme pockets, i.e., the tertiary structure of the Hb molecule (see, Ho, C., *Adv. Protein Chem.* 43:153 (1992), (hereinafter "Ho (1992)"), the disclosure of which is incorporated herein by reference). The resonances at \(\approx -1.8\) and \(\approx -1.7\) ppm have been assigned to the \(\gamma_2\)-CH\(_3\) of the E11Val of the \(\beta\)-chain and \(\alpha\)-chain of HbCO A, respectively (Lindstrom et al. (1972); Dalvit et al. (1985)). These two resonances are not changed in rHbCO (βN108Q). However, the resonance assigned to the \(\gamma_2\)-CH\(_3\) of the \(\alpha\)-E11Val of rHbCO (αL29F, βN108Q) is shifted upfield to \(\approx -2.0\) ppm, suggesting that the \(\gamma_2\)-CH\(_3\) group of the \(\alpha\)-E11 valine residues in rHbCO (αL29F, βN108Q) is located closer to the normal of the heme than in HbCO A. \(\alpha\)29L is in close proximity to E11Val, hence, the amino acid substitution αL29F is expected to
alter the conformation of the distal heme pocket site of the \( \alpha \)-chain. There are some other changes in the ring-current-shifted resonances among these rHbs. The experience has been that minor differences in the intensity and positions of ring-current-shifted resonances are common features in many rHb mutants. (See, for example, Shen, et al. (1993); Kim, et al. (1994); Kim, et al. (1995); and Kim, et al. (1996); Ho, et al. (1998); Sun, D.P., et al. Biochemistry 36:6663 (1997) (hereinafter "Sun, et al. (1997)"), the disclosure of which is incorporated herein by reference; and Tsai, et al. (1999)). These changes reflect slight adjustments of the conformation of the hemes and/or the amino acid residues in the heme pockets as the result of the mutation.

The exchangeable proton resonances of the Hb molecule arise from the exchangeable protons in the subunit interfaces. Of special interest to the present invention are the exchangeable proton resonances at 14.2, 12.9, 12.1, 11.2, and 10.7 ppm from DSS, which have been characterized as the inter-subunit H-bonds in the \( \alpha_1 \beta_1 \) and \( \alpha_1 \beta_2 \) subunit interfaces in both deoxy (I) and/or oxy (R) states of Hb A (Russu, et al (1987); Fung, et al. (1975)); and Ho (1992), the disclosures of which are incorporated herein by reference). The resonances at 12.9 ppm and 12.1 ppm from DSS have been assigned to the H-bonds between \( \alpha 122 \)His and \( \beta 35 \)Tyr, and \( \alpha 103 \)His and \( \beta 131 \)Gln, respectively (see Russu, et al. (1987) and Simplaceanu, et al. Biophys. J. (in press) (2000) (hereinafter "Simplaceanu, et al.
In the spectra of rHbCO (βN108Q) and rHbCO (αL29F, βN108Q) (as seen in Figure 6A), three resonances instead of one occur corresponding to the chemical shift of HbCO A at 12.1 ppm. The main peak occurs at 12.0 ppm, with a shoulder at 11.8 ppm and an extra resonance at 12.3 ppm. The intensities of the resonances at 12.3 and 11.8 ppm are not even 1/10 of the ones at 12.0 ppm and at 12.9 ppm, indicating that these two extra resonances are unlikely to be contributed by additional protons. The sum of the integrated areas of the resonances at 11.8, 12.0, and 12.3 ppm is about the same as the area of the single resonance at 12.9 ppm, suggesting the coexistence of three conformers of rHb (βN108Q) in CO form.

Figure 7A shows the hyperfine-shifted and Figure 7B shows the exchangeable proton resonances of rHbs and Hb A in the deoxy form in 0.1 M phosphate at pH 7.0 and 29°C. The resonance at 63 ppm from DDS has been assigned to the hyperfine-shifted N3H-exchangeable proton of the proximal histidine residue (α87His) of the α-chain of deoxy-Hb A and the one at 77 ppm from DSS has been assigned to the corresponding residue of the β-chain (β92His) of deoxy-Hb A (Takahashi, S., et al. Biochemistry 19:5196 (1980) and La Mar, G.N., et al. Biochem. Biophys. Res. Commun. 96:1172 (1980), the disclosures of which are incorporated herein by reference). The chemical shift positions of these two proximal histidyl resonances in rHb (βN108Q) are exactly the same as those of Hb A, indicating no perturbations around the proximal histidine residues of this rHb.
However, the resonance at 63 ppm from DSS of rHb (αL29F) and rHb (αL29F, βN108Q) is shifted 4 ppm downfield to 67 ppm, reflecting a change in the environment of the proximal heme pocket of the α-chain as a result of the mutation at αL29F.

The spectral region from 10-25 ppm arises from the hyperfine-shifted resonances of the porphyrin ring and the amino acid residues situated in the proximity of the heme pockets and the exchangeable proton resonances. There are no noticeable differences seen in the resonances from 10-25 ppm between deoxy-Hb A and deoxy-rHb (βN108Q). However, there are spectral changes in rHb (αL29F) and rHb (αL29F, βN108Q) over the region from 16-20 ppm, reflecting changes in the environment of the heme pockets of both the α- and the β-chains. The resonance at 14.2 ppm has been identified as the inter-subunit H-bond between α42Tyr and β99Asp in the α1β2 interface in deoxy-Hb A (Fung, et al. (1975)), a characteristic feature of the deoxy (T) quaternary structure of Hb A (Perutz, (1970)). This resonance of rHb (αL29F) and rHb (αL29F, βN108Q) is shifted 0.5 ppm upfield to 13.7 ppm, indicating that this α1β2 interface H-bond in the deoxy form is perturbed by the mutation at αL29F.

A unique feature of the rHbs of the present invention with low oxygen affinity and high cooperativity is the appearance of the T-marker at 14.2 ppm on lowering the temperature and/or adding IHP to these rHbs in the CO
form (see Kim, et al. (1995); Ho, et al. (1998); Tsai, et al. (1999)). Studies on the
temperature dependence of exchangeable proton resonances of rHbs in the CO
form can be used to assess the structural effect on oxygen affinity. Figures 8A and
8B and Figures 9A and 9B show the exchangeable proton resonances of rHb
(βN108Q) and rHb (αL29F, βN108Q) in the CO form in the absence (Figs. 8A, 9A)
and presence (Figs. 8B, 9B) of 4 mM IHP in 0.1 M sodium phosphate buffer as a
function of temperature. The resonance at 14.2 ppm of rHb (βN108Q) is
observable starting at 23°C in 0.1 M phosphate at pH 7.0 in the presence of 4 mM
IHP (Figure 8B). The appearance of the T-marker in the presence of 4 mM IHP
and at low temperature in the spectra of CO-ligated rHb (βN108Q) and rHb
(αL29F, βN108Q) indicates that the T-states of rHb (βN108Q) and rHb (αL29F,
βN108Q) are more stable than that of Hb A. However, this resonance in the
spectra of Hb (αL29F, βN108Q) has a much smaller intensity than that in the
spectra of rHb (βN108Q) at low temperature, i.e., 11°C and in the presence of 4
mM IHP.

STRUCTURAL STUDIES OF rHb (βL105W)

rHb (βL105W) was designed to form a new hydrogen bond with
α94Asp in the α1β2 subunit interface in order to lower the oxygen binding affinity
by stabilizing its deoxy quaternary structure. rHb (αD94A, βL105W) and rHb
(αD94A) were constructed to provide evidence that β105Trp of rHb (βL105W)
does form a new hydrogen bond with α94Asp in the α₁β₂ subunit interface of the
deoxy quaternary structure. Multinuclear, multidimensional nuclear magnetic
resonance (NMR) studies on ¹⁵N-labeled rHb (βL105W) have identified the indole
nitrogen-attached proton resonance of β105Trp for rHb (βL105W). ¹H-NMR
studies were used to investigate the structural basis for the low oxygen affinity of
rHb (βL105W).

¹H-NMR Studies of the Structures of rHbs in the CO Form.

Figure 10A shows the exchangeable proton resonances of Hb A, rHb
(βL105W), rHb (αD94A, βL105W), and rHb (αD94A) in the CO form. The
exchangeable proton resonances arise from the exchangeable protons in the
subunit interfaces. Recent multinuclear, multidimensional NMR studies on the
¹⁵N-labeled rHb A have assigned the resonances at 10.6, 10.4 and 10.1 ppm to
β37Trp, α14TIP and β15Trp, respectively (Simplaceanu, et al. (2000)). The crystal
structure of Hb A in the oxy form (Shaanan, (1983)) suggested the likely candidate
to form an H-bond with β37Trp in the α₁β₂ subunit interface is α94Asp. The
spectrum of rHb (βL105W) in the CO form shows an additional proton resonance
in the region of exchangeable proton resonances (Figure 10A). Since residues β37
and β105 are both located in the α₁β₂ interface and are close in the R-quaternary
structure (Shaanan, (1983)), the replacement of Leu by Trp at β105 position may
cause the proton resonance of β37Trp to shift away from its original chemical
shift. It is suspected that the extra resonance (at either 11.0 ppm or 10.8 ppm) originates from β105Trp. Heteronuclear, two-dimensional ("2D") NMR studies on the 15N-labeled rHb (βLI05W) were, therefore, carried out to assign these resonances in the spectrum of rHb (βL105W). The spectrum of rHb (αD94A) in the CO form shows that the resonance at 10.6 ppm (assigned to β37Trp in Hb A) is missing and a new resonance shows up at 9.7 ppm compared to the spectrum of Hb A (Figure 10A). This result suggests that the shift of the resonance of β37Trp at 10.6 ppm to 9.7 ppm (closer to the water resonance) is due to the lack of an H-bond between α94 and β37 in rHb (αD94A) in the CO form. This result also confirms the assignment of the resonance at 10.6 ppm to the inter-subunit H-bond between α94Asp and β37Trp. The spectrum of rHb (αD94A, βL105W) in the CO form shows that one extra proton resonance appeared at 10.8 ppm compared to the spectrum of rHb (αD94A). The resonance at 10.8 ppm was assigned to the indole NH of β105Trp of rHb (αD94A, βL105W) and rHb (βL105W).

Figure 10B shows the ring-current-shifted proton resonances of Hb A, rHb (βLI05W), rHb (αD94A, βL105W), and rHb (αD94A) in the CO form. The ring-current-shifted resonances are very sensitive to the heme conformation and the tertiary structure of the heme pockets (Ho, (1992)). The spectrum for the ring-current-shifted proton resonances of rHb (βL105W) in the CO form differs only slightly from that of Hb A, while the spectra of rHb (αD94A, βL105W) and rHb
(αD94A) are very different from that of Hb A. These differences imply that some adjustments of the heme conformation and/or the amino acid residues in the heme pockets occurred due to the mutation αD94A. Previous studies have shown that minor differences in the ring-current-shifted resonances are common features in many mutant rHbs. (Kim, et al. (1994); Kim, et al. (1995); Kim, et al. (1996); Sun, et al. (1997)).

Heteronuclear 2D NMR Studies on $^{15}$N-labeled rHb (βL105W) in the CO Form.

In order to assign the proton resonances at 11.0 ppm and 10.8 ppm in the $^1$H-NMR spectrum of rHb (βL105W), heteronuclear 2D NMR studies on $^{15}$N-labeled rHb (βL105W) in the CO form were performed. The results are shown in Figures 11A and 11B and Figures 12A-12D. Figures 11A and 11B show the 600-MHz HMQC spectra of $^{15}$N-labeled rHb (βL105W) and rHb A in the CO form. A doublet is observed at the ($^1$H$_{61}$, $^{15}$N$_{61}$) chemical shift coordinates for Trp residues because this spectrum was acquired without $^{15}$N decoupling. In general, the $^1$H$_{61}$ resonances of Trp residues usually appear at ~ 9 to ~ 12 ppm (Cavanagh, et al. (1996); BioMagResBank (1999) (www.bmrbr.wisc.edu/ref_info/statsel.htm) in the proton dimension, and their $^{15}$N$_{61}$ resonances usually appear at ~ 121 to ~ 133 ppm (BioMagResBank) in the $^{15}$N dimension. The $^{15}$N chemical shifts for the proton resonances at 11.0 ppm and 10.8 ppm in the $^1$H-NMR spectrum of rHb (βL105W) are at 134 ppm and 129 ppm, respectively, suggesting that these
resonances originate from a Trp residue. Since the chemical shift of a proton is much easier to be affected than that of nitrogen by its environment, the assignment of (11.0, 134) ppm to β37Trp and (10.8, 129) ppm to β105Trp was made (Figures 11A and 11B). This also agrees with what is shown in Figure 10A. The HMQC spectrum also correlates the Trp 15Nε1 chemical shifts with the carbon-bound proton 1Hδ1. As shown in Figures 11A and 11B, the 1Hδ1 cross-peaks at (7.3, 129) and (7.1, 127) ppm are observed for α14Trp and β15Trp, respectively, in the spectra of both 15N-labeled rHb (βLI105W) and rHb A in the CO form. Also observed are 1Hδ1 cross-peaks (through two-bond coupling) at (7.4, 134) ppm with much weaker intensity for β37Trp in the spectrum of 15N-labeled rHb A in the CO form (results not shown in Figure 11B). Since the 1Hδ1 cross-peaks for β37Trp and β105Trp cannot be seen in the spectrum of 15N-labeled rHb (βLI105W) in the CO form (Figure 11A), NOESY-HMQC experiments have been performed at different mixing times to provide more evidence for the present Trp assignments. As shown in Figures 12A-12D, the 1Hδ1 and 1Hζ2 cross-peaks of all four Trp residues can be seen even at short mixing times. The intensities of these cross-peaks become weaker when the mixing time was 60 or 100 ms (Figs. 12C, 12D). Figures 12A-12D also show that the chemical shifts of 1Hδ1 and 1Hζ2 cross-peaks are very close to each other for β37Trp and β105Trp. All these results confirm the assignments for the Trp residues.
\(^{1}\)H-NMR Studies of the Structures of rHbs in the Deoxy Form.

Figure 13A shows the hyperfine-shifted \(N_2H\) resonances of proximal histidines in the 300-MHz \(^{1}\)H-NMR spectra of Hb A, rHb (\(\beta L105W\)), rHb (\(\alpha D94A,\) \(\beta L105W\)), and rHb (\(\alpha D94A\)) in the deoxy form. The spectrum for the hyperfine-shifted \(N_2H\) resonances of proximal histidines of mutant rHbs in the deoxy form is very similar to that of Hb A. Figure 13B shows the hyperfine-shifted and exchangeable proton resonances in the 300-MHz \(^{1}\)H-NMR spectra of Hb A, rHb (\(\beta L105W\)), rHb (\(\alpha D94A, \beta L105W\)), and rHb (\(\alpha D94A\)) in the deoxy form. The hyperfine-shifted proton resonances arise from the protons on the heme groups and their nearby amino acid residues due to the hyperfine, interactions between these protons and unpaired electrons of Fe(II) in the heme pocket (Ho (1992)). The hyperfine-shifted proton resonances of rHb (\(\beta L105W\)) in the region +24 to +16 ppm are very similar to those of Hb A, but those for rHb (\(\alpha D94A, \beta L105W\)) and rHb (\(\alpha D94A\)) are somewhat different from these for Hb A. Figure 13C shows the exchangeable proton resonances in the 300-MHz \(^{1}\)H-NMR spectra of Hb A, rHb (\(\beta L105W\)), rHb (\(\alpha D94A, \beta L105W\)), and rHb (\(\alpha D94A\)) in the deoxy form. The \(^{1}\)H resonance at \(\sim 14\) ppm has been identified as the intersubunit H-bond between \(\alpha 42\)Tyr and \(\beta 99\)Asp in the \(\alpha_1\beta_2\) interface in deoxy-Hb A, a characteristic feature of the deoxy (T)-quaternary structure of Hb A (Fung, et al. (1975)). The resonance at \(\sim 12.2\) ppm has been assigned to the H-bond between \(\alpha 103\)His and \(\beta 131\)Asp at the
α₁β₂ interface (unpublished results). The resonance at ~ 11.1 ppm has been tentatively assigned to the H-bond between α₉₄Asp and β₃₇Trp at the α₁β₂ interface (Fung, et al. (1975); Ishimori, et al. (1992)). Recent heteronuclear, multidimensional NMR studies on the ¹⁵N-labeled rHb A have assigned the resonance at ~ 13.0 ppm to α₁₂₂His, and confirmed the assignment of the resonance at ~ 11.1 ppm to β₃₇Trp (unpublished results). The spectrum of rHb (β₃₁₀₅W) in the deoxy form shows an additional proton resonance appearing in the region of the exchangeable proton resonances (Figure 13C). This suggests that the extra resonance at 12.7 ppm originates from β₁₀₅Trp. Due to the lack of an H-bond between residues α₉₄ and β₃₇ in rHb (αD₉₄A) in the deoxy form, the resonance for β₃₇Trp should shift away from its original chemical shift and closer to the water resonance (similar to what was observed in its CO form). The spectrum of rHb (αD₉₄A) in the deoxy form shows that the resonance at ~ 11.1 ppm (assigned to β₃₇Trp in Hb A) is missing (Figure 13C). However, it is not clear what is the new chemical shift for β₃₇Trp in rHb (αD₉₄A) in the deoxy form. The spectrum of rHb (αD₉₄A, β₃₁₀₅W) in the deoxy form shows an extra proton resonance appearing at 11.1 ppm compared to that of rHb (αD₉₄A). It appears that this resonance originates from β₁₀₅Trp of rHb (αD₉₄A, β₃₁₀₅W). The chemical shift for the NH resonance of β₁₀₅Trp in rHb (β₃₁₀₅W) shifts upfield 1.7 ppm and closer to the water resonance when α₉₄Asp is replaced by Ala in rHb.
(αD94A, βL105W) (Figure 13C). These results indicate that a new H-bond forms between β105Trp and α94Asp in rHb (βL105W) in the deoxy form.

Heteronuclear 2D NMR Studies on 15N-labeled rHb (βL105W) in the Deoxy Form.

In order to confirm the assignment of resonance at 12.7 ppm to β105Trp in the 1H-NMR spectrum of rHb (βL105W) in the deoxy form, heteronuclear 2D NMR experiments on 15N-labeled rHb (βL105W) in the deoxy form were performed (Figures 14 and 15A-15D). Figure 14 shows the 600-MHz HMQC spectrum of 15N-labeled rHb (βL105W) in the deoxy form. The 15N chemical shift for the proton resonance at 12.7 ppm in the 1H-NMR spectrum of rHb (βL105W) is at 134 ppm, suggesting that this resonance originates from a tryptophan residue. Also observed are the 1Hδ1 cross-peaks of Trp 15Nε1 at (7.8, 134), (7.6, 135), (7.1, 129), and (7.0, 127) ppm for β105Trp, β37Trp, α14Trp and β15Trp, respectively, in the HMQC spectrum of 15N-labeled rHb (βL105W) in the deoxy form. Also observed are the 1Hε1 and 1Hζ2 cross-peaks of His 15Nε2 for α103His at (8.3, 163) and (7.1, 163) ppm, respectively, and for α122His at (7.6, 167) and (7.0, 167) ppm, respectively. The NOESY-HMQC experiments were also performed at different mixing times to provide more evidence for the present assignments and to investigate the micro-environment for β105Trp in rHb (βL105W) in the deoxy form. For β105Trp (at 12.7 ppm), its 1Hδ1 and 1Hζ2 cross-peaks at 7.8 and 8.2 ppm, respectively, can be observed at all four mixing times.
(Figures 15A-15D). For β37Trp (at 11.2 ppm), its $^1$Hδ₁ and $^1$Hζ₂ cross-peaks at 7.6 and 7.3 ppm, respectively, also can be observed at all four mixing times (Figures 15A-15D). In addition, also observed is the NOE effect between residues of β105Trp and β37Trp in the NOESY-HMQC spectra of $^{15}$N-labeled rHb (βL105 W) in the deoxy form as shown in Figures 15A-15D.

The Effects of IHP and Temperature on the Spectra of Hbs in the CO Form

Figures 16A and 16B show the exchangeable protons resonances of Hb A, rHb (βL105W), rHb (αD94A, βL105W), and rHb (αD94A) in the CO form in the absence (Fig. 16A) and presence (Fig. 16B) of IHP at 11, 20, and 29°C. In the absence of IHP, the T marker can be observed only in the spectra of rHb (αD94A, βL105 W) at the lower temperature. In the presence of IHP, the T marker can be observed in the spectra of all three mutant rHbs. These results have shown that these mutant rHbs can switch from the R-structure to the T-structure without changing their ligation state when the temperature is lowered and/or when IHP is added. Besides the appearance of the T marker, the spectra of rHb (βL105W) in the CO form in the presence of IHP also show several differences compared to those in the absence of IHP. In the presence of IHP, new peaks at 13.1 and 11.2 ppm appear to gradually grow from the nearby resonances at 12.9 and 11.0 (or 10.8) ppm, respectively, when the temperature is lowered (Figure 16B).
In order to monitor these changes, more detailed HSQC experiments have been performed for $^{15}$N-labeled rHb (βL105W) in the absence and the presence of IHP. In the presence of IHP, the HSQC experiments have also been performed at lower temperatures (Figures 17A-17D). In the presence of IHP at 29°C, the (1H εL, $^{15}$N εL) cross-peak at (11.0, 134) ppm for β37Trp disappears. The (1H εL, $^{15}$N εL) cross-peak at (10.8, 129) ppm for β105Trp is much weaker in the presence of IHP at 29°C compared to that in the absence of IHP (Figures 17A and 17B). When the temperature is lowered in the presence of IHP, the cross-peak at (11.0, 134) ppm reappears and two new cross-peaks appear at (11.0, 131) and (10.9, 130) ppm (Figures 17C and 17D). It appears that these two new cross peaks originate from β37Trp and β105Trp.

Figures 18A and 18B show the ring-current-shifted proton resonances of Hb A, rHb (βL105W), rHb (αD94A, βL105W), and rHb (αD94A) in the CO form in the absence and presence of IHP at 11, 20, and 29°C. The ring-current-shifted proton resonances of rHb (βL105W) in the CO form differ only slightly from those of Hb A in the absence of IHP, while they are very different from those of Hb A in the presence of IHP. The ring-current-shifted proton resonances of rHb (αD94A, βL105W) and rHb (αD94A) in the CO form are very different from those of Hb A in both the absence and presence of IHP, but they are very similar to each other in the presence of IHP. In addition, the ring-current-
shifted proton resonances of rHb (βL105W) in the CO form in the presence of IHP turn out to be very similar to those of rHb (αD94A/βL105W) and rHb (αD94A) when the temperature is lowered (Figure 18B). It is believed that the spectra for the ring-current-shifted proton resonances of rHb (αD94A, βL105W) and rHb (αD94A) in the CO form in the presence IHP represent one type of spectrum for rHbs in the CO form with a stable T-structure. Therefore, the differences in heme pocket conformations between mutant rHbs and Hb A also suggest that these mutant rHbs are much easier to switch from the R-structure to T-structure in light of the T-marker from the exchangeable proton resonances. The resonances at -1.8 and -1.9 ppm have been assigned to the heme pocket distal valine (E11) of α- and β-chains of Hb A, respectively (Dalvit, C., et al., *Biochemistry* 24: 3398 (1985) and Craescu, C.T., et al., *Eur. J. Biochem* 181: 87 (1989), the disclosures of which are incorporated herein by reference). Compared to the spectra of Hb A, the resonance of distal valine (E11) of β-chain seems to be affected more in the spectra of mutant rHbs, especially in the presence of IHP, than that of α-chain (Figures 18A and 18B). These results imply that the structural switching from the R- to the T-structure induced by IHP, temperature, or the mutations described herein might occur mainly in the β-chain.

Appropriately cross-linked rHb (βN108Q) and/or rHb (βL105W) can be incorporated into a hemoglobin-based blood substitute or therapeutic
hemoglobin that is physiologically acceptable for use in clinical or veterinary medicine according to methods know in the art. See, for example, R.M. Winslow, et al. Eds. Blood Substitutes Physiological Basis of Efficacy (Birkhauser, Boston, Mass.) (1995), the disclosure of which is incorporated herein by reference. The hemoglobin of the present invention may also be advantageously used as a treatment for conditions such as septic shock, prevention of anaphylactic shock during dialysis.

Although the invention has been described in detail for the purposes of illustration, it is to be understood that such detail is solely for that purpose and that variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention except as it may be limited by the claims.
We Claim:

1. A non-naturally occurring mutant human hemoglobin wherein the asparagine residue at position 108 of the β-chains (SEQ ID NO: 5) is replaced by a glutamine residue.

2. The hemoglobin of Claim 1 possessing low oxygen affinity as compared to normal human adult hemoglobin.

3. The hemoglobin of Claim 2 further possessing high cooperativity in oxygen binding comparable to normal human adult hemoglobin.

4. The hemoglobin of Claim 3 further possessing increased stability against autoxidation.

5. The hemoglobin of Claim 1 which is produced recombinantly.

6. rHb (βN108Q) (SEQ ID NO: 5).

7. An artificial mutant hemoglobin which in a cell-free environment has oxygen binding properties comparable to those of human normal adult hemoglobin in red blood cells wherein said hemoglobin contains a mutation such that the asparagine residue at position 108 of the β-chains is glutamine (SEQ ID NO: 5).

8. The hemoglobin of Claim 7 which is produced recombinantly.

9. A non-toxic pharmaceutical composition comprising a non-naturally occurring mutant hemoglobin wherein the asparagine residue at
position 108 of the β-chains is replaced by a glutamine residue (SEQ ID NO: 5) in a pharmaceutically acceptable carrier.

10. The composition of Claim 9 wherein said hemoglobin in a cell-free environment has oxygen binding properties lower than those of human normal adult hemoglobin.

11. The composition of Claim 10 wherein said hemoglobin is rHb (βN108Q) (SEQ ID NO: 5).


13. A non-naturally occurring low oxygen affinity mutant hemoglobin with increased stability against autoxidation that has oxygen binding properties comparable to those of human normal adult hemoglobin in the presence of the allosteric effector 2,3-bisphosphoglycerate, wherein the asparagine residue at position 108 of each of the β-chains is replaced by a glutamine residue (SEQ ID NO:5).

14. A non-naturally occurring mutant human hemoglobin wherein said hemoglobin contains a mutation of the asparagine residue at position 108 of the β-chains (SEQ ID NO: 5) possessing oxygen-binding properties of oxygen affinity as measured by $P_{50}$ and cooperativity as measured by the Hill coefficient ($n_{max}$) and similar to those of Hb A in the presence of the allosteric
effector 2,3-bisphosphoglycerate as follows: $P_{50}$ about 17.4 mm Hg, $n_{\text{max}}$ about 3.1 in 0.1 M sodium phosphate at pH 7.4 and 29°C.

15. A method of producing artificial hemoglobin, comprising:
introducing an expression plasmid which contains a DNA coding sequence for human hemoglobin wherein the asparagine residue at position 108 of the $\beta$-chains is replaced by glutamine residue (SEQ ID NO:5) into a suitable host other than an erythrocyte and growing the transformed cells;
expressing said DNA to produce said artificial hemoglobin;
and
recovering and purifying said hemoglobin.

16. The method of Claim 15, wherein said host cells are E. coli.

17. The method of Claim 16, wherein said expression plasmid is pHE7009

18. A method of treating a human subject, comprising
administering to said subject a nontoxic composition comprising an artificial mutant hemoglobin, wherein said artificial mutant hemoglobin is rHb ($\beta$N108Q) (SEQ ID NO: 5).

19. rHb ($\beta$N108Q) (SEQ ID NO: 5) derived from cells transformed with pHE7009.
20. A non-naturally occurring mutant human hemoglobin wherein the leucine residue at position 105 of the β-chains (SEQ ID NO: 7) is replaced by a tryptophan residue.

21. The hemoglobin of Claim 20 possessing low oxygen affinity as compared to normal human adult hemoglobin.

22. The hemoglobin of Claim 21 further possessing high cooperativity in oxygen binding comparable to normal human adult hemoglobin.

23. The hemoglobin of Claim 20 which is produced recombinantly.

24. rHb (βL105W) (SEQ ID NO:7).

25. An artificial mutant hemoglobin which in a cell-free environment has oxygen binding properties comparable to those of human normal adult hemoglobin in red blood cells wherein said hemoglobin contains a mutation such that the leucine residue at position 105 of the β-chains is tryptophan (SEQ ID NO: 7).

26. The hemoglobin of Claim 25 which is produced recombinantly.

27. A non-toxic pharmaceutical composition comprising a non-naturally occurring mutant hemoglobin wherein the leucine residue at position
105 of the β-chains is replaced by a tryptophan residue (SEQ ID NO:7) in a pharmaceutically acceptable carrier.

28. The composition of Claim 27 wherein said hemoglobin in a cell-free environment has oxygen binding properties lower than those of human normal adult hemoglobin.

29. The composition of Claim 28 wherein said hemoglobin is rHb (βL105W) (SEQ ID NO: 7).

30. Plasmid pHET004.

31. A non-naturally occurring low oxygen affinity mutant hemoglobin that has oxygen binding properties comparable to those of human normal adult hemoglobin in the presence of the allosteric effector 2,3-bisphosphoglycerate, wherein the leucine residue at position 105 of each of the β-chains is replaced by a tryptophan residue (SEQ ID NO: 7).

32. A non-naturally occurring mutant human hemoglobin wherein said hemoglobin contains a mutation of the leucine residue at position 105 of the β-chains (SEQ ID NO: 7) possessing oxygen-binding properties of oxygen affinity as measured by $P_{50}$ and cooperativity as measured by the Hill coefficient ($n_{max}$) and similar to those of Hb A in the presence of the allosteric effector 2,3-bisphosphoglycerate as follows: $P_{50}$ about 28.2 mm Hg, $n_{max}$ about 2.6 in 0.1 M sodium phosphate at pH 7.4 and 29°C.
33. A method of producing artificial hemoglobin, comprising:
   introducing an expression plasmid which contains a DNA
   coding sequence for human hemoglobin wherein the leucine residue at position
   105 of the $\beta$-chains is replaced by tryptophan residue (SEQ ID NO: 7) into a
   suitable host other than an erythrocyte and growing the transformed cells;
   expressing said DNA to produce said artificial hemoglobin;
   and
   recovering and purifying said hemoglobin.

34. The method of Claim 33, wherein said host cells are $E. coli$.

35. The method of Claim 34, wherein said expression plasmid is
   pHE7004.

36. A method of treating a human subject, comprising
   administering to said subject a nontoxic composition comprising an artificial
   mutant hemoglobin, wherein said artificial mutant hemoglobin is rHb ($\beta$L105W)
   (SEQ ID NO: 7).

37. rHb ($\beta$L105W) derived from cells transformed with pHE7004.
FIG. 1A
p: tac
AAATGAGCTG TTAGACATTA ATCATGCSCT CGTATAATTGT GTGGAATTGT GAGCAGGATAA
EcoRI SacI KpnISmaI
CAATTTCCACA CAGGAAAACAG AATTCCGACT CGGTAACCAGAG GTTACATGGA GATTAACTCA
RBS
|-> α-globin
ATCTAGAGGG TATAATAAT GTATCGCTTA AATAGAAGGG AATAACATAT GGTGCTGTCT
CCTGCCGACA AGACCAACGT CAAGGCAGCC TGGGTGAAGG TGGCGCGCAG CGCTGCGGAG
TATGGTGCGG AGGCCCTGGA GAGGATGTTC CTGTCCCTCC CCACACCCCA GACCTACTTC
CCGCACCTCG ATCTGACCCA CGGCTCTGCC CAGGTTAAGG GCCACGCGCA GAGGTGGGCC
GGCGCGCTGA CCAACCGCGT GGGCAGCTTG GACGACATGC CCAACCGGCT GTCGCGGCTG
AGCGACCTGC AGCGCGCAACA GCTTGGTGTG GACGCCGCTCA ACTTCAAGCT CTAAGCCAC
TGGCTGCTGG TGACCTCTGSC CGGCCACCTC CCCGCCGAGT TCACCCCTGC GGTGCAAGCC
|->
TCCCTGAGCA AGTTCCTGSC TTCTGTGAGC ACCGTGCTGA CCTCAAATA CGTATAACT
RBS
|-> β-globin
AGAGGGTATT AATAATGATG CGGTTGAATA AGGGAGAATA ACATATGTTG CACCTGACTC
CTGAGGAGAA GTCTGCGGGT ACTGCCTCTGT GGGCAAAGGT GAACGTTGAT GAAAGTGTTG
GTGAGGGCTT GGGCAAGCTG CTGGTGCTCT ACCCTTGGAC CCGAGGTCT TTTGAGTCTT
TTGAGGATCT GTCCACTCTT GATGCTGTAA TGGGCAACCC TAAAGTTGAG GCTGTAGGCCA
AGAAAGTGGCT CAGTGCGCTCT AGTGATGCAC TGGCTCACCT GACAAACCTC AGGGCCACCT
TTGCGACACT GAGTGAGCGT CACTGTGACA AGCTGACAGT GGATCCTGAGA AACTTCAGGT
β105Leu->Trp
GCTGAGGCA CGTGTGCTGTC TGTGTTGCTGG CCGATCACTT TGGCAAAGAA TACCCCCAC
CAGTGCAGCC TGCCCTACAG AAAGTGTTGG CTGGTGCGGC TATAGCCCTT GCCCACAAGT
->| SphI
rzB (5S, T₁, T₂)
ATCAGTAAAGC ATGGCCTCTGT TTTGCGCGAT TAGAGAGGAT TTTGACCTTG ATACAGATTA
NsiI

FIG. 1B
FIG. 2B
FIG. 4
FIG. 10A  FIG. 10B
FIG. 11A

FIG. 11B

δN
110
120
130

δH (PPM from DSS)

ppm

δ15W
δ105W
δ37W

α14W
FIG. 13A  FIG. 13B  FIG. 13C
FIG. 14
SEQUENCE LISTING

Carnegie Mellon University
Low Oxygen Affinity Mutant Hemoglobins
00-40120-WO
Not Yet Assigned
2001-06-21
09/598,218
2000-06-21
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PatentIn version 3.1
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Artificial Sequence
Primer to introduce betaN108Q mutation into plasmid pHE2
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Primer to introduce betaN108Q mutation into plasmid pHE7

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Primer to introduce beta105W mutation into plasmid pHE7

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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C07K 14/80; C07H 17/00; C12P 21/06
US CL : 580/385; 580/23.1; 485/69.1; 520.1; 514/6
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 550/385; 580/23.1; 485/69.1; 520.1; 514/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>US 5,028,588 A (HOFFMAN et al.) 02 July 1991, Table 2.</td>
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Further documents are listed in the continuation of Box C. See patent family annex.

Date of the actual completion of the international search
20 AUGUST 2001

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 505-3280

Date of mailing of the international search report
18 OCT 2001

Authorized officer
KAREN COCHRANE CARLSON, PH.D.
Telephone No. (703) 505-0196

Form PCT/ISA/210 (second sheet) (July 1998)