LOW AFFINITY MUTANT HEMOGLOBINS AND WHOLLY ARTIFICIAL HEMOGLOBINS AS BLOOD SUBSTITUTES.

BACKGROUND OF THE INVENTION

Field of Invention

This invention relates to novel hemoglobin compositions useful as substitutes for red blood cells, and to methods of preparing same. It further relates to the preparation, using recombinant DNA technology, of mutant hemoglobins for use in such compositions.

Information Disclosure Statement

It is not always practical to transfuse a patient with donated blood. In these situations, use of a red blood cell substitute is necessary. The product must effectively transport O_2, just as do red blood cells. ("Plasma expanders", such as dextran and albumin, do not transport oxygen.) The two types of substitutes that have been studied most extensively are hemoglobin solutions and fluorocarbon emulsions.

Hemoglobin (Hgb) is the oxygen-carrying component of blood. Hemoglobin circulates through the bloodstream inside small enucleate cells called erythrocytes (red blood cells). Hemoglobin is a protein constructed from four associated polypeptide chains, and bearing prosthetic groups known as hemes. The erythrocyte helps maintain hemoglobin in its reduced, functional form. The heme iron atom is liable to oxidation, but may be reduced again by one of two enzyme systems within the erythrocyte, the cytochrome b_5 and glutathione reduction systems.

Hemoglobin exhibits cooperative binding of oxygen by the four subunits of the hemoglobin molecule (two alpha-globins and two beta-globins in the case of Hgb A), and this cooperativity greatly facilitates efficient oxygen transport. Cooperativity, achieved by the so-called heme-heme interaction, allows hemoglobin to vary its affinity for oxygen. Hemoglobin reversibly binds up to four moles of oxygen per mole of Hgb. At high oxygen concentration, such as that found in the lungs, the oxygen affinity is high and hemoglobin is almost saturated with oxygen. At low oxygen concentration, such as that found in actively respiring tissue, the oxygen affinity is lowered and oxygen is unloaded.

Oxygen-carrying compounds are frequently compared by means of a device known as an oxygen dissociation curve. This curve is obtained when, for a given oxygen carrier, oxygen saturation is graphed against the partial pressure of oxygen. The percentage of saturation increases with partial pressure according to a sigmoid relationship. The P_{50} is the partial pressure at which the oxygen-carrying solution is half saturated with oxygen. It is thus a measure of oxygen-binding affinity; the higher the P_{50}, the more loosely the oxygen is held.

When the oxygen dissociation curve of a oxygen-carrying solution is such that the P_{50} is less than that for whole blood, it is said to be "left-shifted."

The oxygen affinity of hemoglobin is lowered by the presence of 2,3-diphosphoglycerate (2,3-DPG), chloride ions and hydrogen ions. Respiring tissue

releases carbon dioxide into the blood and lowers its pH (i.e. increases the hydrogen ion concentration), thereby causing oxygen to dissociate from hemoglobin and allowing it to diffuse into individual cells.

The ability of hemoglobin to alter its oxygen affinity, increasing the efficiency of oxygen transport around the body, is dependent on the presence of the metabolite 2,3-DPG. Inside the erythrocyte 2,3-DPG is present at a concentration nearly as great as that of hemoglobin itself. In the absence of 2,3-DPG "conventional" hemoglobin binds oxygen very tightly and would release little oxygen to respiring tissue.

Aging erythrocytes release small amounts of free hemoglobin into the blood plasma where it is rapidly bound by the scavenging protein haptoglobin. The hemoglobin-haptoglobin complex is removed from the blood and degraded by the spleen and liver.

It is clear from the above considerations that free native hemoglobin A, injected directly into the bloodstream, would not support efficient oxygen transport about the body. The essential allosteric regulator 2,3-DPG is not present in sufficient concentration in the plasma to allow hemoglobin to release much oxygen at venous oxygen tension, and free hemoglobin would be rapidly inactivated as an oxygen carrier by auto-oxidation of the heme iron.

Nonetheless, solutions of conventional hemoglobin have been used as RBC substitutes. The classic method of preparing hemoglobin solutions employs outdated blood. The red cells are lysed and cellular debris is

removed, leaving what is hopefully "stromal-free hemoglobin" (SFH).

Several basic problems have been observed with this approach. The solution must be freed of any toxic components of the red cell membrane without resorting to cumbersome and tedious procedures which would discourage large-scale production. DeVenuto, "Appraisal of Hemoglobin Solution as a Blood Substitute", Surgery, Gynecology and Obstetrics, 149: 417-436 (1979).

Second, as expected, such solutions are "left-shifted" (lower P_{50}) as compared to whole blood. Gould, et al., "The Development of Polymerized Pyridoxylated Hemoglobin Solution as a Red Cell Substitute", Ann. Emerg. Med. 15: 1416-1419 (Dec. 1986).

Third, SFH has a half-life in the circulatory system of only about 2-4 hours. This is because oxyHgb partially dissociates into a dimer that is small enough to be filtered by the kidney.

Finally, SFH has a high colloid osmotic pressure (COD). Thus, administration of SFH in a dose that would have the same oxygen-carrying capacity as a unit of packed red blood cells is inadvisable, since the high osmotic pressure (60mm Hg) would cause a massive influx of water from the cells into the bloodstream, thus dehydrating the patient's tissues. This consideration limits the dose of SFH to about 6-8 gm Hgb/dl.

In an effort to restore the desired P_{50}, researchers added 2,3-DPG to the hemoglobin solution. Unfortunately, 2,3-DPG was rapidly eliminated from the circulation. Scientists then turned to other organic phosphates, particularly pyridoxal phosphate. Like 2,3-DPG, these compounds stabilized the "T state" of the Hgb by forming a salt bridge between the N-termini of the two beta chains. The pyridoxylated hemoglobin had a P_{50} of 20-22 torr, as compared to 10 torr for SFH and 28 torr for whole blood. While this is an improvement over SFH, the pyridoxylated Hgb remains "high affinity" relative to whole blood.

Hemoglobin has been chemically modified (by intramolecular or intermolecular crosslinking) to increase intravascular retention and reduce osmotic pressure. Unfortunately, this polymerization also causes a "left shift" of the molecule's oxygen dissociation curve. Thus, for polymerized-pyridoxylated Hgb, the P_{50} is about 18 torr.

For chemical modifications of hemoglobin, See Iwashita, U.S. 4,412,989 and 4,301,144 (with polyalkylene glycol), Iwasaki, U.S. 4,670,417 (with polyalkylene oxide), (with a polysaccharide); Nicolau, U.S. 4,321,259 and U.S. 4,473,563 (with inositol phosphate); Wong, U.S. 4,710,488 and 4,650,786 (with inositol phosphate and a polysaccharide); Bonhard, U.S. 4,336,248 (with other proteins or gelatin derivatives); Walder, U.S. 4,598,064 and U.S. 4,600,531 (intramolecularly crosslinked hemoglobin) and Ajisaka, U.S. 4,377,512 (with inulin).

The human alpha- and beta-globin genes have both been cloned and sequenced. Liebhaber, et al., P.N.A.S.

(U.S.A.) 77: 7054-58 (1980)(alpha-globin genomic DNA); Marotta, et al., J. Biol. Chem., 252: 5040-53 (1977)(beta-globin cDNA).

Nagai and Thorgerson (Nature, 309: 810-812, 1984) expressed in E. coli a hybrid protein consisting of the 31 amino-terminal residues of the lambda cII protein, an Ile-Glu-Gly-Arg linker, and the complete human beta globin chain. They cleaved the hybrid at the single arginine with blood coagulation factor Xa, thus liberating the beta-globin chain.

Later, Nagai, et al., P.N.A.S. (U.S.A.), 82: 7252-55 (1985) took the rDNA-derived human beta globin, naturally derived human alpha globin, and a source of heme and succeeded in producing active human hemoglobin. Additionally, they produced two semi-artificial analogues of the naturally occurring mutant hemoglobins Hb Nympheas and Hb Daphne by site-directed mutagenesis of the cloned beta-globin gene, expression of the modified gene, and combination of the rDNA-derived beta chain with naturally occurring alpha chain and a source of heme. Like the naturally occurring mutants, these semiartificial analogues exhibited increased oxygen affinity as compared to "normal" hemoglobin. In subsequent studies, the structural basis for this change in oxygen binding was established. Luisi and Nagai, Nature, 320: 555-56 (1986); and cp. Nagai, et al., Nature, 329: 858-860 (Oct. 1987)(similar production of mutant hemoglobins with replacements of Val(67beta)E11).

Surprisingly, the expression of the human alpha globin gene in heterologous cells has not been reported. Indeed, merely substituting the alpha globin

gene for the beta globin gene in the E. coli expression system of Nagai, et al. results in negligible expression of the lambda cII protein/alpha-globin fusion. It appears that there is something about the native alpha globin mRNA sequence which interferes with efficient translation in a bacterial host.

SUMMARY OF THE INVENTION

We have discovered that the disadvantages of hemoglobin solutions as blood substitutes are overcome if a mutant hemoglobin species is selected which, in a typical cell-free blood substitute solution, would impart to the solution a P_{50} comparable to that of non-mutant hemoglobin in RBC-bound state. Naturally occurring hemoglobin mutants which, in the erythrocyte environment, would impart to the erythrocyte a P_{50} higher than the normal value for whole blood (28 torr) are of particular interest, both in their own right and for what they teach about the structural foundations of oxygen affinity. It is expected that many such "right-shifted" species, outside the erythrocyte environment (and thus the right-shifting influence of 2,3-DPG), will assume a P_{50} comparable to or greater than that of the normal P_{50} for whole blood.

For the purposes of this invention, the term "conventional hemoglobin A" refers to the species of Hgb A whose alpha and beta chains are of the amino acid sequence given in Figure 1. This is the species which is most frequently found in human erythrocytes and which imparts to such erythrocytes a P_{50} of about 28 torr. A "hemoglobin A mutant" is defined as any species of hemoglobin A whose alpha or beta chain is of an amino acid sequence different from that set forth in

Figure 1. A "low affinity" hemoglobin A mutant is one which has a P_{50} at least about 10% greater than that of "conventional hemoglobin A" in the same environment. It is particularly desirable that its P_{50} be at least that twice of conventional (wild type) hemoglobin A in the absence of 2,3-DPG. A "recombinant" hemoglobin is one composed of an alpha and beta globin at least one of which is obtained by expression of a globin gene carried by a recombinant DNA molecule, whether the hemoglobin is a conventional hemoglobin or a mutant species.

A large number of naturally occurring low affinity Hgb A mutants are known. (See Table I). The mutations may appear in either the alpha or the beta chains of the molecule (or both, of course). Thus, Hgb Hazebrouck is a beta mutant (38(C4):thr->pro) whose P_{50} is 36 (in whole blood), declining to 27-29 torr in vitro.

Clearly, one cannot depend on nature to provide an adequate supply of these low affinity mutants. Consequently, the mutant polypeptide chain will usually be prepared artificially, either by direct polypeptide synthesis, or, more preferably, by in vivo expression of the corresponding mutant gene in a suitable host cell. This gene may be obtained directly from the genome of the mutant erythrocyte precursor (a mature erythrocyte does not contain DNA), as a complementary DNA (cDNA) transcribed from the messenger RNA of the mutant erythrocyte precursor, by direct polynucleotide synthesis, or, preferably, by in vitro mutagenesis of the gene encoding conventional hemoglobin.

If one of the chains is identical to that of "conventional" hemoglobin, it may be obtained either naturally or synthetically. In addition, to create a functional hemoglobin molecule, it is necessary to provide the prosthetic heme groups and to couple the alpha and beta chains.

It is also within the contemplation of this invention to prepare and use non-naturally occurring low affinity mutants by appropriate modification and expression of the alpha or beta globin gene and subsequent assembly of a recombinant hemoglobin. Methods for selecting candidate sequences and evaluating their suitability for use in a blood substitute product are described herein.

We also have discovered that human alpha-globin may be obtained from a bacterial host by (1) constructing a fused gene which comprises not only the alpha-globin gene but also at least a portion of the beta globin gene, separated by spacer DNA encoding a selective protease cleavage site; (2) expressing the fused gene in the form of a fusion protein; and (3) cleaving the fusion protein at the aforementioned cleavage site in order to liberate the human alpha-globin.

As a result of this discovery, it is possible to prepare entirely artificial human hemoglobin, that is, hemoglobin in which both the alpha and beta globin chains are formed in cells other than human erythrocytes. Such fully artificial hemoglobin is ideal for use in blood substitute solutions. Of course, semi-artificial hemoglobins (one chain obtained from non-erythrocyte source) may still be used.

When extracted natural hemoglobin is used as a blood substitute, one must be concerned with the toxicity of red blood cell membrane components which might contaminate the product. It is known that erythrocyte stroma can cause dyspnea, bronchospasm, hypotension, arrythmia, disseminated intravascular coagulation, activation of complement, and renal, myocardial and hepatic changes associated with ischemia and acute inflammation. See Feola, Surgery, Gynecology & Obstetrics, 166: 211-222 (March 1988); MacDonald, et al., F.A.S.E.B. J., 2(6) Abstr. 8217 (1988); Stone, et al., Surgery, Gynecology and Obstetrics, 149: 874-876 (1979); Rabiner, et al, J. Exp. Med., 126: 1127-42 (1967). While purified preparations of natural hemoglobin are known (so called "stroma-free hemoglobin"), Feola comments, "a truly pure hemoglobin solution has not been produced."

Another concern with natural hemoglobin is contamination with infectious agents communicated by blood. Bove, Progr. Hematol., 14: 123-145 (1986) reported that hepatitis viruses, cytomegalovirus, Epstein-Barr virus, serum parvoviruses, syphilis, malaria, filariasis, trypanosomiasis, babesiosis, numerous pathogenic bacteria, and AIDS are all transmitted by blood transfusions. AIDS has even been transmitted by blood transfusions screened as negative for HIV antibody. Ward, et al., New Engl. J. Med., 318: 473-78 (1988).

The alpha-globin of the present invention may be identical in sequence to natural normal human alpha-globin or to a naturally occurring hemoglobin mutant,

or it may be an alpha globin mutant which is not known in nature.

According to one aspect of the invention there is provided the use of cell-free mutant (as defined above) hemoglobin in the manufacture of a non-toxic composition for administration to a patient so as to supplement the oxygen-carrying capacity of the patient's blood.

The above mutant hemoglobin may be a naturally-occurring or non-naturally-occurring hemoglobin.

It is particularly preferred for the mutant hemoglobin to be wholly artificial.

Preferably the cell-free mutant hemoglobin has a P_{50} of at least about 10% greater than that of cell-free conventional hemoglobin under the same conditions.

Preferably the mutant hemoglobin contains a mutation which affects the equilibrium between the R state and the T state of the hemoglobin molecule.

Examples of mutant hemoglobins include the following:

mutant hemoglobins containing a mutation of the beta Asn102 (G4) residue.

mutant hemoglobins containing a beta Asn102 (G4) -> Thr mutation.

mutant hemoglobins containing a His 63 Phe mutation.

Preferably the mutant hemoglobins contain a mutation of an amino acid residue within 4 angstroms, as determined on a nearest atom-to-nearest atom basis, of the heme moiety, most preferably a mutation of a first amino acid residue on a first chain which is within 4 angstroms of a second residue on a second and distinct chain, as determined on a nearest atom-to-nearest atom basis, wherein said first and second residues are a part of the alphalbeta2 or alpha2beta1 interfaces.

Examples include:

mutant hemoglobins containing a mutation of beta residue 45 (CD4).

mutant hemoglobins containing a mutation of beta residue 70 (E14).

mutant hemoglobins containing a mutation of beta 67 Val.

A specific example of the last-mentioned is the mutant hemoglobin contains a mutation of beta 67 Val -> Ile.

Other preferred mutant hemoglobins for use according to the invention contain a mutation wherein another amino acid residue is converted to cysteine, and wherein said cysteine is cross-linked to another cysteine residue.

The compositions used acording to the invention preferably further comprises an organic phosphate which stabilizes the T state of hemoglobin.

Nontoxic pharmaceutical composition comprising a non-naturally occurring mutant (as defined above) hemoglobin in a pharmaceutically acceptable carrier are novel and form a further aspect of the invention.

The invention further provides hemoglobin compositions comprising cell-free wholly artificial, biologically functional hemoglobin characterised in that both the α- and the β-globin subunits are derived by expressing recombinant DNA coding sequences.

Said compositions may further comprise sodium ions, potassium ions and chloride ions, an oncotic agent, and an antioxidant.

The α-globin subunit of the hemoglobin compositions may advantageously be derived by cleavage of a chimeric protein including at least a 10 amino acid segment of a β-globin.

Polypeptides comprising a covalent conjugate consisting of an α-globin moiety and at least a 10 amino acid segment of a β-globin are further provided according to the invention.

Recombinant DNA molecules for expressing polypeptides useful in producing mutant hemoglobins as described herein form further aspects of the present invention. These include recombinant DNA molecules comprising a fused gene, said fused gene comprising a first nucleotide sequence which codes on expression for an alpha globin, and a second nucleotide sequence which codes on expression for at least a 10 amino acid segment of a beta globin.

In such recombinant DNA molecules said first and second nucleotide sequences are separated by a first spacer DNA sequence which codes on expression for a sequence of amino acids which provide a selective cleavage site for a site-selective protease, e.g. Factor Xa.

The second nucleotide sequence is advantageously positioned so that its first codon is read prior to the reading of the first codon of the first nucleotide sequence in the course of the expression of the fused gene and preferably codes on expression for the N-terminal of a beta globin.

Methods of producing hemoglobin which comprises (a) producing alpha globin by (i) introducing a recombinant DNA molecule as defined above into a suitable host other than an erythrocyte, (ii) expressing alpha globin either in free form or as part of a fusion protein, (iii) if need be, cleaving said fusion protein at a selective cleavage site to liberate the alpha globin, and (iv) recovering and purifying said alpha globin; (b) obtaining beta globin from a source other than blood; and (c) preparing hemoglobin by combining said alpha and beta globin and a source of heme form further aspects of the invention.

Hemoglobin compositions comprising a solution of cell-free, mutant (as defined herein) hemoglobin in a pharmaceutically acceptable carrier, said solution having a P_{50} under standard physiological conditions of 24-32 torr are also provided according to the invention.

Mutant hemoglobins, whether of reduced or enhanced oxygen affinity, may be of value for altering O_2 concentrations in cell cultures, or for extracting O_2 from fluids.

The appended claims are hereby incorporated by reference into this specification as a statement of the preferred embodiments.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the amino acid sequence of the (a) alpha and (b) beta chains of conventional human hemoglobin A, and the nucleotide sequences encoding these chains.

Figure 2 shows selected DNA sequences and enzyme restriction maps of (a) M13 mp11 FX, (b) pLcIIFX beta, and pLcII beta, and (c) pLCIIFX-beta-FX-alpha. Note that pLCIIFX alpha lacks the codon encoding beta His 2 in Figure 2(b).

Figure 3 shows equilibrium oxygen binding curves for artificial conventional human hemoglobin and mutant human hemoglobin having the structure of Hgb Kansas.

DETAILED DESCRIPTION OF THE INVENTION

One aspect of this invention is the cloning and expression of an exogenous alpha globin gene in a suitable host. The host may be a procaryotic cell

(such as a E. coli) or a eucaryotic cell (such as a yeast cell or a mammalian cell). The alpha globin gene will usually code on expression for a polypeptide corresponding to a naturally occurring human alpha globin, normal or abnormal, but may correspond to a nonhuman alpha globin, or indeed be a non-naturally occurring analogue of known alpha globins. Preferably, a mutant alpha globin is prepared and assembled into a low affinity hemoglobin mutant for use in a blood substitute composition.

The alpha globin gene is expressed as part of a fused gene which also codes on expression for at least a portion of the beta globin gene. In a preferred embodiment, the alpha and beta globin sequences are separated by spacer DNA encoding a selective protease cleavage site, in particular, a site susceptible to specific cleavage by Factor Xa.

Preferably, the aforementioned fused gene includes a subsequence which codes on expression for the 20 amino acid N-terminal of beta globin.

As previously noted, the alpha globin of the present invention is advantageously combined with rDNA-derived beta globin and a source of heme to obtain fully artificial (derived entirely from sources other than blood) hemoglobin. Such hemoglobins, and especially low oxygen affinity hemoglobin mutants produced through use of selectively modified alpha and/or beta globin genes, are of value as blood substitutes. Semi-artificial hemoglobins, in which the only chain obtained from a non-erythrocyte source is a mutant sequence and the mutation imparts reduced oxygen affinity to the molecule, are also encompassed by the

present invention and may also be used as blood substitutes. Unless otherwise indicated, the term "artificial" embraces both wholly and semi-artificial forms.

In order to appreciate possible strategies for designing low affinity alpha or beta mutants of hemoglobin for use as blood substitutes, it is necessary to understand the structure of the hemoglobin molecule.

The structure of conventional hemoglobin is well known. We herewith incorporate by reference the entire text of Bunn and Forget, eds., Hemoglobin: Molecular, Genetic and Clinical Aspects (W.B. Saunders Co., Philadelphia, PA: 1986) and of Fermi and Perutz "Hemoglobin and Myoglobin," in Phillips and Richards, Atlas of Molecular Structures in Biology (Clarendon Press: 1981).

The primary structure of a polypeptide is defined by its amino acid sequence and by identification of any modifications of the side chains of the individual amino acids.

About 92% of the normal adult human hemolysate is Hgb A (designated alpha2 beta2, because it comprises two alpha and two beta chains). The alpha chain consists of 141 amino acids (See Figure 1). The iron atom of the heme (ferroprotoporphyrin IX) group is bound covalently to the imidazole of his 87 (the "proximal histidine"). The beta chain is 146 residues long (see Figure 1) and heme is bound to it at His 92.

Other recognized hemoglobin species are Hgb A_2, Hgb A_{1a}, Hgb A_{1b}, and Hgb A_{1c}, as well as the rare species Hgb F, Hgb F_1, Hgb Gower-1, Hgb Gower-2, Hgb Portland, Hgb H, and Hgb Bart. They are distinguished from Hgb A by a different selection of polypeptide chains.

Segments of polypeptide chains may be stabilized by folding into one of two common conformations, the alpha helix and the beta pleated sheet. In its native state, about 75% of the hemoglobin molecule is alpha-helical. Alpha-helical segments are separated by segments wherein the chain is less constrained. It is conventional to identify the alpha-helical segments of the each chain by letters, e.g., the proximal histidine of the alpha chain is F8 (residue 8 of helix F). The non-helical segments are identified by letter pairs, indicating which helical segments they connect. Thus, nonhelical segment BC lies between helix B and helix C. In comparing two variants of a particular hemoglobin chain, it may be enlightening to attempt to align the helical segments when seeking to find structural homologies. For the amino acid sequence and helical residue notation for conventional human hemoglobin A_o alpha and beta chains, see Table 4.

The tertiary structure of the hemoglobin molecule refers to the steric relationships of amino acid residues that are far apart in the linear sequence, while quaternary structure refers to the way in which the subunits (chains) are packed together. The tertiary and quaternary structure of the hemoglobin molecule have been discerned by X-ray diffraction analysis of hemoglobin crystals, which allows one to

calculate the three-dimensional positions of the very atoms of the molecule.

In its unoxygenated ("deoxy", or "T" for "tense") form, the subunits of hemoglobin (alpha1, alpha2, beta1, and beta2) form a tetrahedron having a twofold axis of symmetry. The axis runs down a water-filled "central cavity". The subunits interact with one another by means of Van der Waals forces and hydrogen bonds (and, in the case of deoxyhemoglobin, by "salt bridges"). The alpha1beta1 and alpha2beta2 interfaces remain relatively fixed during oxygenation. In contrast, there is considerable flux at the alpha1beta2 (and alpha2beta1) interface. In its oxygenated ("oxy", or "R" for "relaxed" form), the intersubunit distances are increased.

The deoxy conformation is stabilized by numerous interactions, including, for example, the hydrogen bond between Tyr42alpha and Asp99beta. In the oxygenated form, this bond is broken and a new one formed between Asp94alpha and Asn102beta.

Various different approaches to modification of hemoglobin may be adopted. In each case, a candidate mutant is selected which, on the basis of the available evidence, is believed to be likely to have a lower affinity for oxygen than conventional hemoglobin.

In making this selection, it is possible to consider not only the known effects of various mutations of human hemoglobin, but also the oxygen binding capacity of known forms of animal hemoglobins, and of related compounds such as carboxyhemoglobin, methemoglobin, myoglobin, etc.

Alpha Chain Mutants

Thanks to our success in overcoming the problems of expressing the alpha globin gene in a heterologous system, it is now possible to conveniently prepare alpha globin mutants.

Several low oxygen affinity hemoglobin alpha chain mutants are already known. Of these, Hb Titusville (alpha94Asp=>Asn), Hb Setif (alpha94Asp->Tyr), Hb Torino (alpha43Phe->Val), Hb Hirosaki (alpha43Phe->Leu) and Hb Moabit (alpha86Leu->Arg) are of special interest.

Alpha globins are more readily oxidized than beta globins because the His(F8) to O_2 bond on the alpha chain is slightly stronger than on beta, so that an electron is more readily lost by the oxygen. Alpha globins could be modified to make them less oxidizable, for example, by the change alpha63His->Gln or Val.

Chloride ion binds to the alpha chain by bridging between the N-terminal NH_3^+ and the hydroxyl of alpha131Ser. The effect of chloride binding is to increase the P_{50} slightly. It is believed that by changing alpha131 to Glu, Asp or Asn one could achieve the same effect without resorting to chloride. Alternatively, the pK_a of the N-terminal could be increased. The natural human N-terminal is valine, with a pK_a of9.72. This could be replaced with Ile (9.76), Pro (10.60) or Thr (10.43).

Beta globin mutants, and additional alpha globin mutants which are likely to impart reduced oxygen affinity, are discussed below.

Stabilizing the T state

It is not possible to raise the plasma concentration of 2,3-DPG sufficiently to maximize the oxygen carrying efficiency of free conventional hemoglobin in the blood. This problem can be overcome by stabilizing the T state with additional salt-bridges or hydrogen bonds introduced by protein engineering. The cross-linking of hemoglobin may itself stabilize the T structure to some extent.

Hydrogen bonds and ionic salt bridges are the predominant stabilizing forces on the surface of proteins. Hydrogen bonds are weak non-ionic bonds formed between electronegative atoms (eg. oxygen, nitrogen, sulfur) and protons that are covalently attached to other electronegative atoms. Individually, hydrogen bonds are weak (eg -1 kcal/mol), but in a protein there are hundreds to thousands of hydrogen bonds that collectively amount to a large stabilizing force. An example of an hydrogen bond that is important to hemoglobin structure is the hydrogen bond formed between alpha-asp^{94} and beta-asn^{102} in the oxy-state. When either of these residues is mutated to a residue that can no longer form this hydrogen bond the oxy state is destabilized and the molecule has a much lower O_2 affinity. Hg Kansas (beta thr^{102}), Hg Beth Israel (beta ser^{102}), Hg Richmond (beta lys^{102}), Hg St. Mande (beta tyr^{102}), Hg Titusville (alpha asn^{94}) and Hg Setif (alpha tyr^{102}) are all examples of the importance of this hydrogen bond. Other likely non-natural mutants that will achieve the same effect are beta asp^{102}, beta glu^{102}, beta arg^{102}, beta his^{102}, beta

gly^{102} and beta cys^{102}; alpha gln^{94}, alpha thr^{94}, alpha ser^{94}, alpha lys^{94}, alpha gly^{94} and alpha arg^{94}.

Ionic interactions are salt forms formed between juxtaposed residues of opposite charge. These interactions are of much greater strength mole for mole than hydrogen bonds. An example of attraction of unlike charges would be the interaction between a lys and asp residue; at physiologic pH both of these residues are charged (positive and negative, respectively). Repulsion of two juxtaposed positive charges or two juxtaposed negative charges may also occur; interactions such as these are destabilizing.

Stabilization of the deoxy state of Hg by 2,3-DPG is an example of ionic interaction. The 2,3-DPG molecule is highly charged at neutral pH (5 negative charges) and interacts with eight residues in the 2,3-DPG pocket that are positively charged. It is felt that by engineering more positive charge into this binding pocket that 2,3-DPG would bind more tightly to the engineered Hgb than to HgbA. Another example is the $alpha_1/beta_2$ interface where alpha asp^{94} hydrogen bonds to beta asn^{102}. Replacing beta asn^{102} with a negatively charged group such as asp or glu will interfere with oxy state stabilization by charge repulsion with the like charged alpha asp^{94}.

Thus, certain amino acid residue changes can facilitate the formation of the desired hydrogen bonds and salt bridges.

The T conformation can also be stabilized by the substitution of cysteine residues for other residues.

Cysteine residues may be cross-linked by disulfide bonds. Examination of the published x-ray data of methemoglobin suggests that the $alpha_1/beta_1$ interface would be a logical place to put the disulfide. Conveniently, there are two alpha ala residues, G17 and G18 (ala is sterically similar to cys) adjacent to beta G14 cys. Hence, at first glance it is thought that alpha G17 or G18 would be likely spots for cys residues to be added. Further strategy about where to place disulfides can be guided by Thornton,J.M. J. Mol. Biol. 151, 261-287, 1981. Oxidation of cysteines to disulfide (Cystines) bonds can be carried out by treatment with O_2 or catalyzed by thioredoxin (Pigiet, V Am. Biotech. Lab 6, 48-52, 1988).

Modifying residues near the oxygen binding site

Heme (haem) is the prosthetic group of hemoglobin, myoglobin, catalase, peroxidase, and cytochrome b. The heme is inserted in a cleft between the E and F helices. The heme iron is linked covalently to the imidazole nitrogen of the "proximal" F8 histidine. The "distal" E11 valine appears to guard the access of oxygen to the heme pocket.

Val-E11 and His-E7 are highly conserved residues which are in Van der Waals contact with the oxygen molecule liganded to the heme iron atoms of hemoglobin; by replacing these residues the intrinsic oxygen affinity of hemoglobin can be altered. Val-E11 has been replaced with Ile, Leu, Ala and Met. The oxygen affinity of the Ala-E11beta mutant was higher than that of HbA; that of the Ile-E11beta mutant was lower. X-ray crystallographic study of the latter mutant showed that the delta-methyl group of the Ile side chain must

be pushed to one side if oxygen is to bind to the iron atom.

Another alteration that we have made is beta His63->Phe. This mutant has an extraordinarily low oxygen affinity (See Table 3).

It has therefore been shown that the oxygen affinity of hemoglobin can be altered at will by replacing residues close to the oxygen binding site. By adjusting oxygen affinity in this way the efficiency of oxygen transport can be maximized in the absence of allosteric effectors such as 2,3-DPG.

The following residues of human deoxyhemoglobin are, on a nearest atom-to-nearest atom basis, within 4 angstroms of the heme moiety: Alpha B13(D)Met, C7(E)Tyr, CE1(D)Phe, CE3(E)His, CE4(D)Phe, E7(D)His, E10(D)Lys, E11(D)Val, E14(D)Ala, F4(P)Leu, F7(P)Leu, F8(P)His, FG3(P)Leu, FG5(P)Val, G4(P)Asn, G5(P)Phe, G8(D)Leu, H15(P)Val and H19(P)Leu; and Beta B13(D)Leu, C7(E)Phe, CD1(D)Phe, CD3(E)His, CD4(D)Phe, E7(D)His, E10(D)Lys, E11(D)Val, E14(D)Ala, F4(P)Leu, F7(P)Leu, F8(P)His, FG3(P)Leu, FG5(P)Val, G4(P)Asn, G5(P)Phe, G8(D)Leu, H15(P)Val, and H19(P)Leu. See Fermi, et al., J. Mol. Biol., 175: 159-174 (1984). (In the above list, "P" denotes "proximal", "D", "distal", and "E", "edge-on".)

These residues are therefore candidates for modification. Consideration should also be given to pairs contacted through bound water molecules. See Ladner, et.al., Mol. Biol., 114: 385-414 (1977).

Mutations of beta residues 42 (CD1), 45 (CD4) and 70 (E14) are of particular interest. Other beta residues of interest include 43 (CE1), 46 (CE4), 58 (E7), 61 (E10) and 62 (E11). Alpha residues of interest include 43 (CE1), 46 (CE4), 58 (E7), 61 (E10) and 62 (E11).

In general, mutations around the heme-O_2 binding site that interfere with O_2 binding are also desirable because of their low-affinity O_2 binding nature. Replacing residues that are adjacent to the face of heme that binds O_2 can result in lower affinity. A naturally occurring mutant Hg Bristol (beta^{67} val-->asp) has been described with low affinity. Other mutants that are desirable are the beta ile^{67} described herein, beta asp^{67} and beta glu^{67}. Other residues are also in the vicinity of the O_2 binding site. Histidine E7 (beta his^{63}) can be replaced with phe which results in very low O_2 affinity. The other likely residue for mutation is beta phe^{42}; replacement with trp is likely to result in low O_2 affinity. The corresponding residues of the alpha chain may be altered instead of, or in addition to, these preferred mutations of the beta chain.

<u>Replacing residues at the alpha$_1$ beta $_2$ contact and the central cavity</u>.

The oxygen affinity and cooperativity of hemoglobin depend on the relative stabilities of the T (low affinity) and R (high affinity) quaternary states. These two states are in equilibrium with each other but one state may be favored by mutations at the alpha$_1$ beta$_2$ contact or in the central cavity. There are many naturally occurring mutations at these sites, and

careful study of these should be of value in designing a hemoglobin molecule with the desired properties.

Thus alpha 1 residues 37(C2)Pro, 38(C3)Thr, 40(C5)Lys, 41(C6)Thr, 42(C7)Tyr, 44(CD2)Pro, 88(F9)Ala, 91(FG3)Leu, 92(FG4)Arg, 94(G1)Asp, 95(G2)Pro, 96(G3)Val, 97(G4)Asn, 140(HC2)Tyr, and 141(HC3)Arg are all known to lie within 4 angstroms of at least one residue of the beta2 chain of human deoxyhemoglobin. Similarly, beta2 residues 146(HC3)His, 145(HC2)Tyr, 105(G7)Leu, 102(G4)Asn, 101(G3)Glu, 100(G2)Pro, 99(G1)Asp, 98(FG5)Val, 97(FG4)His, 43(CD2)Glu, 41(C7)Phe, 40(C6)Arg, 37(C3)Trp, 36(C2)Pro, 35(C1)Tyr, and 34(B16)Val lie on the other side of the alpha1beta2 interface of human deoxyhemoglobin.

Mutation of beta102(G4)Asn is particularly preferred. Hg Kansas is a known low affinity mutant in which this residue is altered to Thr. As previously mentioned, the oxy state is stabilized by a hydrogen bond between beta Asn102 and alpha Asp94. The preferred mutation would disrupt this hydrogen bond.

Another favored mutation at this site is to beta Asp102. The negative charge-negative charge repulsion between this group and the alpha Asp94 would further destabilize the oxy state.

Still other preferred mutations at the beta102 locus would be to Ser (Hb Beth Israel), Lys (Hb Richmond) and Tyr (Hb St. Mande).

Increasing the stability of hemoglobin

Inside the erythrocyte the cytochrome b_5 and glutathione reduction systems serve to maintain hemoglobin in the active ferrous form. Free hemoglobin in the bloodstream would rapidly be at least partially oxidized to the ferric state since there is no such means of keeping it in the reduced form outside the red cell. It is possible to stabilize the ferrous form by replacing the Val-E11 residue with a large aliphatic amino acid such as Ile or Leu. A large side chain at this position prevents electron donors from reaching the iron atom and so slows the rate of autoxidation. The His63->Phe mutant is also of interest.

If hemoglobin is cross-linked both intermolecularly to form higher molecular weight aggregates and intramolecularly to prevent dissociation into dimmers then it will neither react with haptoglobin nor pass through the glomerular membrane of the kidney. If the oxygen binding properties of haemoglobin are to be unaffected it is important that the cross-linking does not prevent conformational change of the protein. This is because the heme-heme interaction arises from a reversible transition between tow quaternary structures: the T structure with low oxygen affinity. These two structures with high oxygen affinity. These two structures differ in the contact between the $alpha_1$ and $beta_2$ subunits. Therefore this contact should be allowed to undergo the conformational changes accompanying oxygen association and dissociation.

Site-directed mutagenesis can be used to replace certain surface residues of haemoglobin with cysteinyl residues. Protein engineering techniques similar to those used to introduce new disulfide bridges to

lysozyme, subtilisin and dihydrofolate reductase can be used. Hemoglobin molecules carrying such -SH groups can be cross-linked by disulfide bridges or via a bifunctional thiol reagent. It should also be noted that there is a natural mutant (Hb Ranier) in which His 143 beta is replaced by Cys and the newly introduced cysteine forms a disulfide bridge with Cys 93 beta in vivo. This mutant is more stable than native Hb.

Table 2 is a list of candidate, non-naturally occurring hemoglobins which are expected to exhibit a lower affinity for oxygen than does conventional hemoglobin.

After determining the amino acid sequence changes which distinguish the desired hemoglobin from conventional hemoglobin, it is necessary to design the expression vector. The most convenient starting point is a nucleotide sequence which codes upon expression for conventional hemoglobin. This sequence then may be modified by site-specific mutagenesis.

Techniques of site-specific mutagenesis are well known, and this invention is not limited to any particular technique. The two principal techniques are the gapped duplex A.A., Kruse, K. B., Brown, J.L. BioTechniques 6, 338-339, 1988) and M-13 (Zoller, M.J. and Smith, M. Meth. Enz. 100, 468-500, 1987) methods.

Alternatively, a subsequence with the desired mutation may be synthesized and then ligated to other subsequences to form the desired molecule.

The gene must be placed under the control of a promoter. Either a constitutive or an inducible

promoter may be used; the advantages and disadvantages of each are well known in the molecular biology art. A promoter must be chosen which is functional in the host. Thus, a bacterial promoter would be chosen if the expression vector is to be introduced into a bacterial host, a yeast promoter, if a yeast host, and a mammalian promoter, if a mammalian cell host. It should be understood that the promoter of a viral gene of a virus which infects the chosen host cell may also be used. The present invention does not depend upon the choice of either the promoter or the host. However, it is desirable to choose a host so that the subsequent purification of the mutant hemoglobin is not unduly complicated.

For the same reason, it is preferable, but not required, that the mutant hemoglobin be expressed as a moiety of a fusion protein. Attempts to express the alpha chain other than as a fusion protein were largely unsuccessful. However, the entire alpha chain sequence could be expressed as part of a fusion protein including a portion of the beta chain, and separated from the latter by a spacer providing a selective cleavage site. The hemoglobin is obtained merely by placing the secreted fusion protein in an appropriate cleavage environment. Numerous fusion protein/cleavage system combinations are now known in the art.

After expressing, recovering and purifying the mutant hemoglobin, its P_{50} is measured in accordance with the protocol set forth in the Reference Example. Preferably, its P_{50} is at least 10% higher than that of conventional hemoglobin A when measured in the same environment.

While it is not practical to combine stroma-free conventional hemoglobin with sufficient 2,3-DPG to restore its P_{50} to intra-erythrocyte levels, it may be possible to combine a modestly right-shifted mutant hemoglobin with a small amount of 2,3-DPG or functionally similar organic phosphate (e.g., pyridoxal phosphate or ATP-dialdehyde) so as to mimic the oxygen-carrying capacity of whole blood. The half life of the organic phosphate may be improved by encapsulating the organic phosphate and the mutant hemoglobin in a liposome to obtain a "simulated erythrocyte," or by covalently attaching the organic phosphate group to the hemoglobin.

Reference Example

Our preferred method of measuring the P_{50} of purified hemogoblin solutions for the purpose of the appended claims is as follows.

Hemoglobin-oxygen equilibrium data are measured using a thin film technique (Imai, K. Meth. Enz. 76, 438-449, 1981). A sample of hemoglobin (0.6 mM) in buffer (50 mM Bis-Tris or 100mM HEPES), pH 7.4, 0.1M NaCl, is applied to the cell and then equilibrated at 25°C. The hemoglobin is saturated with O_2 by a stream of air, or air/O_2 if the hemoglobin has low O_2 affinity or if the local barometric pressure prevents abient oxygen from achieving a high enough partial pressure to saturate the molecule. Deoxygenation is achieved by closing the cell to O_2 flow and flushing the cell with N_2 (>99.98% pure). The oxygen equilibrium curve is obtained by plotting the change in absorbance at 560 nm against the pressure of O_2 in the cell. Percent saturation is determined by measuring the A^{560} at a

given partial pressure (i) divided by the A^{560} of the beginning, totally saturated Hgb solution Hgb solution [A^{560} (i)/A^{560} (100%) x 100 = % saturation]. The P_{50} is defined as the partial pressure (i) of O_2 required to cause 50% saturation of O_2 binding sites.

P_{50} may also be measured under other conditions, but it should be noted that many environmental factors affect hemoglobin's oxygen affinity. The effect of Ph, CO_2, inorganic anions, organic phosphates and temperature on P_{50} are discussed in Bunn and Forget, HEMOGLOBIN:MOLECULAR, GENETIC AND CLINICAL ASPECTS 37-47, 95-98 (W.B. Saunders Co.: 1986).

Since many determinations of whole blood oxygen binding curves are made under standard physiologic conditions (37°C, pH=7.4, PCO_2=40 mm Hg), it may be necessary to adjust literature figures. In this context, it should be noted that a 10°C increase results in nearly a two-fold increase in P_{50}, while the dependence of P_{50}, while the dependence of P_{50} on pH is approximately given as delta log P_{50}/delta pH = -0.5.

Comparing P_{50} values of purified Hb preparation to P_{50} values of whole blood can be problematic. Whole blood, or isolated RBC's, contain many components that naturally modulate the shape of the hemoglobin-oxygen binding curve. The RBC encapsulates Hgb in the presence of a high concentration of the effector molecule 2,3-DPG; a molecule that causes Hgb to have a markedly lower affinity for O_2. Other intra-erythrocyte components also affect the shape of the binding curve: ATP, Cl^-, CO_2, H^+, orthophosphate, methemoglobin and carboxyhemoglobin. These substances are not normally present in purified HgB solutions and

thus, the P_{50} value of purified Hgb is lower than that found in whole blood. One very important modulator of Hgb-oxygen affinity is Cl^- ion. Cl^- ion is found outside the erythrocyte in the blood serum at a physiologic concentration of approximately 0.15M. Since Cl^- causes a lower O_2 affinity, a Hgb solution with a P_{50} measured in vitro may well have much lower O_2 affinity if infused into the blood stream. Another problem with measuring O_2 binding of whole blood is that RBCs are quite fragile and in the process of manipulating the erythrocyte into the instrument used to measure the O_2 binding it is inevitable that at least a small percentage of the RBCs will lyse. Lysed RBCs leak Hgb into the surrounding media away from 2,3-DPG; hence, since free Hgb has a higher affinity than intraerythrocyte Hgb, lysed RBCs will have a higher O_2 affinity and can cause a falsely low P_{50} value for whole blood P_{50} determinations. It is widely accepted that under physiologic conditions whole blood has a P_{50} value of 26-28 mmHg. When Hgb is isolated from whole blood, however, the measured P_{50} is on the order of 1-10 mmHg depending on the investigators experimental conditions. For these reasons it is most accurate to measure Hgb-oxygen equilibria with purified Hgb molecules under strict conditions of buffer, pH and salt concentration. Unfortunately, there are no accepted "standards" for all investigators to measure Hgb oxygen binding in in vitro systems.

Still, as many mutant hemoglobins are first identified in patient's whole blood, one would like to be able to compare the relative affinities of native and mutant Hgb for O_2, between whole blood and purified Hgb preparations. An example of this is Hgb Chico (beta lys^{66}-->thr) (Table 1). If one examined only the

P_{50} value of the purified mutant Hgb (10.1 mmHg) one would note that Hgb has a P_{50} value less than that for normal whole blood (27.2 mmHg). Still, when that hemoglobin is measured in RBCs under physiologic conditions it is apparent that it does have a higher P_{50} than normal whole blood (38 mmHg). One cannot predict the degree that the P_{50} value will change going from whole blood Chico to a purified Hgb Chico if it were infused into the bloodstream as a blood substitute. One can conclude, however, that the P_{50} will be higher than it is in pure form, and that by reacting the mutant Hgb with organic phosphates that P_{50} will be even higher.

Note also that whole blood oxygen binding curves are customarily determined under standard physiologic conditions (37°C, pH 7.4, PCO_2=40 mmHg) and red cell 2,3-PDG varies with age, sex and condition.

(Example 1: Production of Artificial Conventional Hemoglobins)

Construction of M13 mp11 FX

M13 mp11 FX encodes a sequence (Ile-Glu-Gly-Arg) including the recognition site for factor X_a. This M13 derivative can be used to join any coding sequence to the factor X_a recognition sequence. See Nagai, EP Appl 161,937 (CELLTECH LTD.) However, this invention is not limited to the use of either M13 or of the factor Xa cleavage system.

All DNA manipulations were carried out essentially as described by Maniatis et al ('Molecular Cloning' Cold Spring Harbour, New York, 1982). A temperature-sensitive lysogenic strain MZ-1 ($galK_{am}$ 8attL

BamN$_7$N$_{53}$cI857 H1, his-, ilv-, bio-, N$^+$, a gift from Dr. K. McKenney and available on request from Medical Research Council) was used as a host strain for plasmids containing lambda P_L promoter and transformation was carried out by the method of Remaut et al (Gene 15, 81-93 (1981)). Other promoters and host strains could have been employed.

T4 DNA ligase was prepared from strain NM989 (Murray et al, J Molec Biol 132, 493-505 (1979) and Tait et al, J Biol Chem 255, 813-815 (1980)). Restriction enzymes were purchased from New England BioLabs.

Two oligonucleotides dTACCCTCGATGGATC and dCATCGAGGGTAGGCC were synthesized by a phosphotriester method on a controlled pore glass support (Sproat et al, Tetrahedron Lett, 24, 5771-5774 (1983)) and purified by HPLC (Gait et al, Nucleic Acids Research 10, 6243-6254 (1982)). These oligonucleotides encode the peptide sequence (gly)-ser-ile-glu-gly-arg in a BamH1-Stu1 linker. The two oligonucleotides were allowed to anneal after phosphorylation with T4 polynucleotide kinase (P-L, Biochemicals) and r[gamma-^{32}P]ATP (3000 Ci/m mol, Amersham) and ligated to form concatamers. The DNA was then digested with Bam HI and cloned into the dephosphorylated Bam HI site of M13 mp11 (Vieira et al, Gene 19, 259-268 (1982)) to yield M13 mp11 FX, as shown in Figure 1a, which forms blue plaques in the presence of isopropyl-beta-D-thiogalacto-pyranoside and 5-bromo-4-chloro-3-indolyl-beta-d-galactoside (Sigma).

Construction of mp11 FX alpha-globin

40 micro g of cloned human alpha-globin cDNA were digested with the restriction enzymes Nco I and Apa I. The single-stranded ends of the excised alpha-globin fragment were trimmed by incubation at 0°C for 10 minutes with 200 units of mung bean nuclease (P-L Biochemicals) in 30 mM sodium acetate pH 4.6, 50 mM sodium chloride, 1 mM zinc chloride, 5% glycerol. The alpha-globin sequence was then cloned into the Stu I site of M13 mp11 FX described above (Nagai & Thogersen, Nature 309, 810-812). The DNA sequences of several clones were determined (Sanger et al Proc. Natl. Acad. Sci. USA, 74, 5463-5467 (1977)) and a clone in which the first valine codon of the alpha-globin gene was joined to the DNA sequence encoding the factor X_a recognition site (Ile-Glu-Gly-Arg) was named mp11 FX alpha-globin.

Construction of pLcII beta and pLcIIFX beta

Plasmids pLcIIFX beta and pLcII beta direct efficient production of a hybrid protein consisting of the 31 amino-terminal residues of the lambda CII protein and the complete human beta-globin, with and without the factor X_a cleavage site, respectively.

The Eco-Hind III fragment containing the multi-restriction sites was cut out from M13 mp10 (Vieira eta al, supra) and ligated to Eco RI-Hind III cut pLc245 (Remaut et al, supra) to form pLmp10. The 319 bp Alu I fragment containing the nutR, t_{R1} sites and a part of the cII gene was cut out from pKG1805 (McKenney, K PhD Dissertation, The Johns Hopkins University (1982)) and cloned into the Sma I site of M13 mp10 in the same orientation with respect to the beta-galactosidase alpha-peptide gene. The Eco RI-Hind III fragment

containing the lambda DNA sequence was then cut out and cloned into the Eco RI-Hind III site of pLmp10 to yield pLcII.

A complete human beta-globin cDNA sequence was reconstructed by joining restriction fragments prepared from an incomplete cDNA clone (pJW102) (Wilson et al, Nucleic Acids Research 5, 563-581 (1978)) and a genomic DNA clone (Lawson et al, Cell 21, 647-651 (1980)) and cloned into the Sma I-Hind III site in M13 mp9. M13 mp9 beta cDNA thus obtained was opened at the Nco I site which is located at the initiation codon and treated with Klenow DNA polymerase (Boehringer Mannheim) in the presence of 100 micro M 4dNTP to obtain flush ends. The beta-globin cDNA sequence was then cut out with Hind III and inserted into the Bam HI (filled-in)-Hind III site of pLcII so that the beta-globin gene was fused to the lambda cII gene in phase via a small linker DNA derived from M13 mp10.

In order to construct pLcIIFX beta, M13 mp9 beta cDNA was opened with Nco I and 40 micro g of DNA was treated with 200 units of mung bean nuclease (P-L Biochemicals) in 30 mM Na-acetate pH 4.6, 50 mM NaCl, 1 mM $ZnCl_2$, 5% glycerol at 0^oC for 10 min to remove the 5' protruding end. The beta-globin cDNA sequence was cut out with Hind III and cloned into the Stu I-Hind III cut M13 mp11 FX. The DNA sequence was determined by the dideoxy chain termination method (Sanger et al, PNAS 74, 5463-5467 (1977)) to ensure that the first valine codon of the beta-globin gene was preceded by the DNA sequence coding for Ile-Glu-Gly-Arg. Then, the Bam HI fragment containing a part of the beta-globin sequence was cut and cloned into Bam HI digested pLcII beta to form pLcIIFX beta, as shown in Figure 1b.

Construction of pLcII FX beta FX alpha

M13 mp11 FX beta-globin DNA was prepared in single-stranded form and a BglII site was introduced into the beta-globin sequence using a mutagenic oligodeoxynucleotide, dACCAACTTCAGATCTGTTACCTTG, called KN83, to obtain mp11 cII FX beta FX. The replicative form of this mutant clone was digested with SacI and Hind III, and the resulting cII FX beta FX fragment was cloned into Sac I/Hind III cut pLmpII to form pLcII FX beta FX. This recombinant plasmid was digested with BglII and the 5′ terminal phosphate groups of the linear DNA were removed with calf intestinal alkaline phosphatase. The replicative form of mp11 FX alpha-globin was digested with BamH1 and the FX alpha-globin containing fragment was ligated with the linearised pLcII FX beta FX to form pLcII FX beta FX alpha. This plasmid encodes a fusion protein consisting of the 31 amino-terminal residues of the lambda phage cII protein, the tetrapeptide Ile-Glu-Gly-Arg, the 20 amino-terminal residues of human beta-globin, the tetrapeptide Ile-Glu-Gly-Arg and human alpha-globin at the carboxyl end. Transcription of the fusion protein gene is initiated at the lambda P_L promoter and is regulated by lambda repressor.

Expression of Recombinant Conventional Alpha and Beta Globin

A defective lambda phage lysogenic strain of E.coli QY13 (a gift from S. Brenner, and available on request from Medical Research Council) harboring pLcIIFX beta FX alpha-globin or pLcIIFX beta-globin was grown at 30°C in 2xTY medium (16g tryptone, 10g yeast extract and 5g sodium chloride-litre) in the presence of ampicillin (25 micro g/ml). When the optical

density (600 nm) reached 1.5-1.6, the temperature was quickly raised and maintained at 42°C for 15 min, followed by further incubation at 37°C for 3-4 hr. The cells were harvested and frozen in liquid nitrogen.

The cells (100g) were thawed and suspended in 80 ml of 50 mM Tris-HCl (pH 8.0)/25% sucrose (wt/vol)/1 mM EDTA and lysed by addition of lysozyme (200 mg). Then, $MgCl_2$, $MnCl_2$ and DNase I were added to final concentration of 10 mM, 1mM and 10 micro g/ml, respectively. After 30 min incubation 200 ml of 0.2 M NaCl/1% deoxycholic acid/1.6 % Nonidet (RTM) P-40 (vol/vol)/20 mM Tris-HCl(pH7.5)/2 mM EDTA were added to the lysate, which was then centrifuged at 5000 x g for 10 min. Then the pellet was suspended in 0.5 % Triton (RTM) X-100/1 mM EDTA and centrifuged. This procedure was repeated until a tight pellet was obtained. The protein pellet was finally dissolved in 8 M urea/ 25 mM Tris-HOAc (pH5.0)/1 mM EDTA/1 mM dithiothreitol in the case of cIIFX beta-globin fusion protein. In the case of cIIFX beta-FX-alpha-globin fusion protein, the pellet was first dissolved in 6M guanidine hydrochloride/25 mM Tris-HOAc (pH 5.0)/1 mM EDTA/1 mM dithiothreitol.

The fusion protein solution was then applied to a 4 x 10 cm CM-Sepharose (RTM) (Pharmacia) column equilibrated with the same buffer. The fusion protein was eluted with a linear gradient formed with 500 ml of 8 M urea/25 mM Tris-HOAc pH 5.0/1 mM EDTA/1mm dithiothreitol and 500 ml of the same buffer with 0.2 M NaCl. The fusion protein was further purified on a 5 X 60 cm Sephacryl S-200 column equilibrated with 5 M guanidine-HCl/50 mM Tris-HCl/1 mM EDTA/1 mM dithiothreitol to remove any trace of impurities. The

combined fraction was extensively dialyzed against 50 mM Tris-HCl (pH 8.0)/0.5 M urea/1mM $CaCl_2$.

Protein Cleavage

The cIIFX beta FX alpha-globin or cIIFX beta-globin fusion protein was incubated at 0°C with blood coagulation factor X_a that had been activated with Russell's viper venom immobilized on cyanogen bromide-activated Sepharose-6B. 100 micro 1 aliquots were removed after intervals of 2, 5, 15, 30, 60 and 120 minutes. 100 ul of protein sample buffer (Laemmli, 1970) and 1 ul of 100 mM DTT were added to each aliquot, which was then boiled before being applied to an SDS polyacrylamide gel. Factor X_a cutting of the cIIFX beta FX alpha fusion protein gives rise to a number of polypeptide products. This is due to the presence of two recognition sites within the protein. Complete digestion releases three polypeptides, a cII protein fragment and a beta-globin fragment, both with the tetrapeptide Ile-Glu-Gly-Arg at the carboxyl terminus, and the desired alpha-globin. Partial digestion of the fusion protein gives two other products.

Factor Xa cleavage of cIIFX-beta yields two products, a cII fragment and the desired beta globin.

Formation of Semi-Artificial Hb with alpha-globin produced in E.coli

25 mg of hemin-Cl was dissolved in 2.5 ml of 0.1 N KOH and 30 diluted with 20 ml of water and 2.5 ml of 1 M KCN. The native beta chain was diluted in 20 mM K phosphate buffer pH 5.7, 1 mM EDTA, 1mM dithiothreitol

(DTT) and bubbled with CO. Alpha globin produced in E.coli was dissolved in 8 M urea/50 mM Tris-Cl pH 8/1 mM EDTA/1 mM DTT at the concentration of 5 mg/ml and incubated at room temperature for 3 hours. The alpha-globin solution was added dropwise to 20 volumes of 30 mM K phosphate buffer pH 5.7, 1 mM EDTA, 1 mM DTT with gentle stirring. The hemin-dicyanide solution (1.2 equivalent to the alpha-globin) was added dropwise to the alpha-globin solution and the beta chain was added in slight excess. The semi-artificial Hb was dialyzed overnight against 0.1 M K phosphate pH 7.6 1 mM EDTA, 1 mM KCN.

Formation of semi-artificial Hb with beta-globin produced in E.coli

Beta-globin (100 mg) was dissolved in 8 M urea, 50 mM Tris-Cl pH 8.0, 1 mM DTT, 1 mM EDTA at the concentration of 5 mg/ml and incubated at room temperature for 1 hr. The beta-globin solution was added dropwise to 16 volumes of alpha chain solution (either isolated from Hgb A, or produced by recombinant means) (3.2 mg/ml) in 10 mM Tris-Cl pH 8.0. The hemin-dicyanide solution (1.2 equivalents to beta-globin) was added dropwise with gentle stirring. The semi-artificial Hb was dialyzed against 2 changes of 0.1 M K phosphate pH 7.4 1 mM EDTA, 1 mM KCN.

Formation of wholly artificial hemoglobin

The lyophilized recombinant alpha and beta globins were dissolved in 8M urea/50 mM Tris-Cl, pH 8.0/1 mM EDTA/1 mM DTET, diluted to a concentration of 5 mg/ml and incubated at room temperature for 3-4 hours. The alpha globin was then diluted to 0.3 gm/ml with chilled

20 mM K_2HPO_4, pH 5.7/1 mM EDTA/1 mM DTT. Hemin (25 mg) was dissolved in 2.4 mg 0.1 M KOH, diluted with an equal volume of 1 M KCN; this solution was then made 0.1 mg.ml in hemin and 20 mM K_2HPO_4, pH 6.7 with stock phosphate buffer. Hemin from this solution was added to a 2.8 molar excess to chilled alpha-globin; and equal molar amount of beta-globin was added and the solution was dialyzed at 4°C overnight against 0.1 M K_2HPO_4, pH 7.6/1 mM EDTA/1 mM KCN.

Purification of the semi-artificial or wholly artificial Hb

The artificial Hb was concentrated by ultra-filtration using diaflo PM-10 membrane (Amicon) and transferred into a 200 ml screw-top test tube with a rubber septum. The hemoglobin solution was deoxygenated by evacuation and flushing with N_2, and then the solution was saturated with CO. 100 mM Sodium dithionite solution was prepared anaerobically in a 20 ml screw-top test tube with rubber septum. 4.5 equivalents of dithionite were added to the Hb solution with a syringe, and the mixture incubated on ice for 15 min.

The Hb solution was gel-filtered against 10 mM Na phosphate buffer pH 6.0 on a 4 X 40 cm Sephadex (RTM) G-25 (fine) column. The Hb was then applied to a 2 X 10 cm CM-52 (Whatman (RTM)) column equilibrated with the same buffer and the chromatography was developed with a linear gradient of 500 ml 10 mM Na phosphate buffer pH 6.0 and 500 ml of 70 mM sodium phosphate buffer pH 6.9. CO was removed from Hb by photolysis under a stream of oxygen. This Hb shows native oxygen binding properties.

A wholly artificial hemoglobin may be prepared by the combination of alpha-globin and beta-globin both produced in E.coli, or any other host of a non-erythroid nature, with a source of heme.

Example 2: Production of Low-Affinity Hemoglobin Mutants

Construction and Mutagenesis of pLcIIFXbeta-globin(Thr^{102})

A synthetic oligonucleotide of sequence dGGAGCCTGAAAGTCTCAGGA was designed from published mRNA sequence information [Bunn & Forget, eds., Hemoglobin: Molecular, Genetic and Clinical Aspects, W.B. Saunders Co., Philadelphia, PA, 169-222 (1986)] and synthesized on a controlled glass support. The oligonucleotide was gel purified [Lloyd et al., BioTechniques 4, 8-10 (1986)] and used to prime the site-specific mutagenesis of M13 mp10 cIIFXbeta -globin by the methods of Zoller and Smith [Methods in Enzymology 100, Academic Press, New York, 468-500 (1983)].

The mutagenic oligonucleotide was complementary to the beta-chain structural gene sequence flanking and centered upon the wild-type codon for Asn^{102}. At this triplet specific base substitutions were designed into the oligonucleotide to specify ACT-Thr^{102}, the amino acid sequence alteration which is characteristic of the Kansas beta-globin mutant [Bonaventura & Riggs, J. Biol. Chem. 243, 980-991 (1968)]. The particular Thr codon employed in this substitution, while differing from that found in the original Kansas isolate (Bonaventura and Riggs, supra), is preferentially

utilized in highly expressed E. coli genes [Grantham et al., Nucleic Acids Res. 9, r43-r74 (1981)].

Production of Mutant beta-Globin

The products of the in vitro mutagenesis reaction were transformed into competent E. coli ^{M}Z-1 [$galK_{am}$ 8attL $BAmN_7N_{53}cI857$ H1, his^-, ilv^-, bio^- N^+, a gift of Dr. K McKenney and available on request from the Medical Research Council), by standard procedures of $CaCl_2$-shock [Maniatis et al., Molecular Cloning, Cold Spring Harbor Laboratory, New York, 250-251 (1982); Nagai & Thogersen, Methods in Enzymology, supra].

Transformants harboring the desired mutant M13 bacteriophage constructs were then identified by differential plaque-hybridization screenings at high stringency using gamma[^{32}P]-end-labeled oligonucleotides as probes.

The reactants used to prepare each of the phosphorylated hybridization probes were 300 pM (2 ug) of oligonucleotide, 100 pM (0.7 mCi) gamma-[^{32}P]-ATP (specific activity approximately 6000 Ci/mM), and 15 units T4 polynucleotide kinase in a total reaction mixture of 50 ul. After 37° C incubation for 2 h, the end-labeled oligomers were purified away from orthophosphate and unincorporated precursor nucleotide using reverse phase C-18 sep-paks (R-TM) (Waters Associates Milford, MA). This latter procedure involved loading the phosphorylation reaction mixture onto the C-18 cartridge in an aqueous salt solution, eluting orthophosphate and unincorporated ATP with water followed by 10% methanol, and then eluting the purified oligomer with 60% methanol. The probes employed comparatively in the differential hybridization

analyses were both the mutagenic oligonucleotide and another 20-mer (dGGAGCCTGAAGTTCTCAGGA) which is perfectly complementary to the wild-type beta-chain DNA sequence in the same coding region.

After identification and plaque purification (Zoller & Smith, supra) of several of the desired M13 phage constructs, one of the resultant Thr^{102} mutants, termed M13 mp10 cIIFX beta-globin (Thr^{102}), was further verified by DNA sequence analysis [Sanger et al., Proc. Natl. Acad. Sci. USA 74, 5463-5467 (1977)] to contain the desired mutation at codon-102, and only that particular amino acid sequence alteration in the beta-chain structural gene coding sequence.

A large scale preparation of M13 mp10 cIIFX-globin(Thr^{102}) RF DNA was conducted as follows [Recinos, Ph.D. Dissertation, Vanderbilt University (1987)]. The host E. coli was grown at 37° C overnight in M9 minimal medium (Maniatis et al., supra) plus 2 ug/ml thiamine. 0.3 ml of this cell culture was then diluted (1:50) into 14.7 ml 2X YT medium and growth at 37° C was continued for an additional 2 h. The latter culture was again diluted (1:10) into a final volume of 150 ml 2X YT, and this cell solution was inoculated with the plaque purified mutant M13 phage construct at a multiplicity of infection of approximately one. This phage infection was then shaken vigorously at 37° C for 14 h, and cells for RF preparation were harvested by centrifugation (5000 X g, 10 min, 4° C). The mutant phage supernatant was stored at -20° C for use in scaled-up versions of the protocols (Zoller & Smith, supra) for phage purification and for single-stranded template preparation.

Double-stranded RF DNA was purified from the cell pellets as follows. Pellets were frozen in an alcohol dry-ice bath for 10 min, thawed at 20° C and completely resuspended on ice in 10 ml 25% sucrose, 50 mM Tris-HCl (pH 8.0). Lysozyme was added to a final concentration of 4 mg/ml, and incubation was continued on ice for 5 min. EDTA was then added to a final concentration of 80 mM, and again after 5 min on ice, an equal volume of 0.5% Triton X-100, 50 mM Tris-HCl (pH 8.0), 62.5 mM EDTA was added. This solution was kept on ice for 15 min more, and then 5 M NaCl was added to a final concentration of 1 M. The last solution was loaded into Beckman (RTM) Type 70 Ti rotor bottles, and after a further incubation on ice for 3 h, was centrifuged at 40,000 rpm for 75 min at 15° C. RF DNA was decanted with the supernatant and precipitated at -20° C for 20 min with the addition of an equal volume of isopropanol. DNA precipitates were pelleted and resuspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100mM NaCl, and treated with RNase (final conc. 100 ug/ml) for 2 h at 37° C. This solution was phenol and chloroform extracted (one time each), and the DNA was ethanol precipitated and resuspended in 30 ml 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. The DNAs were twice banded by CsCl-ethidium bromide density gradient equilibrium centrifugation. Form I DNA bands were extracted 4X with CsCl-saturated isopropanol to remove ethidium bromide, and DNA and CsCl were ethanol precipitated. CsCl was removed from DNA pellets by resuspension in and dialysis against 10 mM Tris-HCl (pH 8.0), 0.2 mM EDTA. A final ethanol precipitation and resuspension in 0.5 ml Tris-HCl (pH 8.0) yielded 150 ug of purified M13 mp10 cIIFXbeta-globin(Thr^{102}) RF DNA for use in subcloning the mutant beta-globin

structural gene into the beta-globin expression construct.

The mutated beta-chain sequence was moved into the beta-chain expression vector, pLcIIFXbeta-globin (nic$^-$), by the following procedures. Mutant clone RF (50 ug) was restriction enzyme digested with Sac I and Hind III, and the resultant cIIFX -globin (Thr-102) fragment was isolated from a 1% preparative agarose gel (Maniatis et al., supra) and gel-purified by a phenol freeze-thaw procedure [Benson, BioTechniques 2, 77-78 (1984)]. Approximately 200 ug of expression vector DNA was isolated and purified from E. coli QY13 transformant cell pellets by methods nearly identical to those described above for the Rf preparation. This plasmid DNA (20 ug) was similarly restricted with Sac I and Hind III and further treated with bacterial alkaline phosphatase (Bethesda Research Laboratories, Gaithersburg, MD, as directed) to remove the 5' phosphates from the vector DNA, thereby preventing recircularization without insert DNA in subsequent ligation reactions.

The purified mutant insert DNA fragment was then ligated back into the expression construct at moderate insert end-concentration, replacing the wild-type protein coding sequences previously therein. Ligation reaction conditions (modified from procedure of New England Biolabs, Inc., Beverly, MA) were: 11 ug vector DNA and 2.1 ug purified insert DNA in 500 mM Tris-HCl (pH 7.8), 100 mM $MgCl_2$, 6 mM ATP, 2 mM dithiothreitol; total reaction volume = 125 ul. Incubation for the ligation was at 16° C for 10 h. The final ligation reaction mixture was used to transform competent E. coli QY13 with selection for ampicillin resistance.

Transformants harboring the desired plasmid construct for the expression of mutant beta-chain were identified by differential colony hybridization screenings [Grunstein & Hogness, Proc. Natl. Acad. Sci. USA 72, 3961-3965 (1975), with modifications] using the end-labeled mutagenic and wild-type oligonucleotides described above as probes. The correct plasmid construct was further verified by restriction analysis and by its expression of a protein which is chromatographed differentially from the wild-type cIIFX beta-globin fusion product by HPLC. The mutant beta-globin was produced, purified and combined with alpha-globin as described for native beta-globin.

Construction and Mutagenesis of pLcIIFXbeta-globin(Ile^{67})

Mutation of the val^{67} codon was introduced into the beta-globin cDNA sequence in M13 mp10 cIIFXbeta-globin using the mutagenic primer (dGCACCGAGGATTTTCTTGCC) as above. The mutant beta-globin was produced, purified and combined with alpha globin as described for native beta-globin to obtain a mutant hemoglobin.

Construction and Mutagenesis of pLcIIFXbeta-globin(phe^{63})

Mutation of the his^{63} codon was introduced into the beta-globin cDNA sequence in M13 mp10 cIIFXbeta-globin using the mutagenic primer (dTTCTTGCCGAAAGCCTTCA) as above. The mutant beta-globin was produced, purified and combined with alpha globin as described for native beta-globin to obtain a mutant hemoglobin.

Characterization of Mutant Hemoglobin

Oxygen equilibrium studies for Hgb (beta phe^{63}) Hgb (beta ile^{67}) were performed in 0.05 M bis-Tris pH 7.4, 0.1 M NaCl, at 25°C using the automated recording apparatus of K. Imai (Meth. Enz. 76, 438-449, 1981) and for Hgb (beta thr^{102}) in 0.1 M HEPES pH 7.4, 0.1 M NaCl using a thin layer optical cell (Gill, S.J. Meth. Enz. 76, 427-438, 1981). Results are shown in Table 3.

Example 3: Blood Substitute Solution

The purified hemoglobin is incorporated into a physiologically acceptable blood substitute solution. A preferred solution includes the following components:

Hgb (gm/l)	60-120
Sodium (mEq/l)	135-145
Potassium (mEq/l)	3.5- 4.5
Chloride (mEq/l)	90-110

Preferably, the solution has a pH of 7.3-7.5, an osmolality of 280-310, and an oncotic pressure of 20-30 mm Hg. Osmolality is controlled by concentration of hemoglobin and of the electrolytes, as well as by the optional ingredient glucose (preferably 0-30 gm/l). The oncotic pressure is controlled by the concentration of the hemoglobin and by its degree of crosslinking. Agents, such as albumin (0-70 gm/l), dextran (0-100 gm/l) and polyethylene glycol (0-25 gm/l) may be added to increase oncotic pressure. Moreover, to reduce the degree of methemoglobin formation, anti-oxidant or free radical scavengers, such as mannitol (0-20 gm/l),

glutathione (0-4 gm/l), ascorbic acid (0-0.3 gm/l) and vitamin E (0-100 IU/l) may be provided.

If a low oxygen affinity mutant hemoglobin is employed, it may be desirable or necessary to adjust the P_{50} of the solution to the preferred level by suitable choice of electrolytes, pH and other characteristics of the composition. Preferably, the final solution has a P_{50} of 24-32 torr under standard physiological conditions.

TABLE 1: NATURAL LOW AFFINITY HEMOGLOBIN MUTANTS

Hemoglobin	Alpha Mutant	P_{50}* RBC-Free Hgb	P_{50}* Whole Blood (nl)	Area of Mutant	Reference
Hirosaki	43(CD1) phe-->leu	n/a		heme	1,2
Torino	43(CD1) phe-->val	n/a		heme	1,3
Moabit	86(F7) leu-->arg		30.6 (26.4-29.2)	heme	4
Titusville	94(G1) asp-->asn	15.8(4.7)		$\tilde{a}_1\beta_2$	5

Hemoglobin	Beta Mutant	P_{50}(mmHg) Hgb (nl)	P_{50}(mmHg) Whole Blood (nl)	Area of Mutant	Reference
Raleigh	1 val-->acetyl ala	4.0(2.2)		DPG site	6

Connecticut	21(B3) asp-->gly	5.0(2.2)		B-E helices	7
Moscva	24(B6) gly-->asp	14.8(12.6)		B-E helices	8
Rothschild	37(C3) trp-->arg	3.5(2.0)		$\tilde{a}_1\beta_2$	9
Hazebrouck	38(C4) thr-->pro		36 (27-29)	$\tilde{a}_1\beta_2$	10
Hammersmith	42(CD1) phe-->ser	n/a		heme/$\tilde{a}_1\beta_2$	1,11
Louisville	42(CD1) phe-->leu	24(21)		heme/$\tilde{a}_1\beta_2$	12,13
Sendagi	42(CD1) phe-->val	3.75(3.05)		heme/$\tilde{a}_1\beta_2$	14
Cheverley	45(CD4) phe-->ser		38.7 (28.7)	heme	15
Okaloosa	48(CD7) leu-->arg	0.95(0.7)	30(26)	C-D helices	16
Bologna	61(E5) lys-->met		37.6 (27.0)	B-E helices	17

Cairo	65(E9) lys-->gln		41(31)	heme	18
Chico	66(E10) lys-->thr	10.1(5.0)	38.0 (27.2)	heme	19
Bristol	67(E11) val-->asp		25.0 (19.0)	heme	20
Seattle	70(E14) ala-->asp		43.5 (28.1)	heme	21,22
Vancouver	73(E17) asp-->tyr	n/a			1,23
Korle-Bu	73(E17) asp-->asn	n/a			1,24
Mobile	73(E17) asp-->val	n/a			
Rahere	82(EF6) lys-->thr	15.5(11.0)		DPG site	26
Pyrgos	83(EF7) gly-->asp			Externa	27
Roseau-Pointe	90(F6) glu-->gly		38(28)	$\tilde{a}_1\beta_2$	28
Agenogi	90(F6) glu-->lys	9.0(6.8)		$\tilde{a}_1\beta_2$	29

Caribbean	91(F7)	leu-->arg	28.0(21.0)		heme	30
Kansas	102(G4)	asn-->thr	28.0(9.0)		$\tilde{a}_1\beta_2$	31
Beth Israel	102(G4)	asn-->ser		88.0 (26.0)	$\tilde{a}_1\beta_2$	32
Saint Mande	102(G4)	asn-->tyr		52 (28)	$\tilde{a}_1\beta_2$	33
Richmond	102(G4)	asn-->lys	n/a		$\tilde{a}_1\beta_2$	1,34
Burke	107(G9)	gly-->arg	9.3(7.7)		heme	35
Yoshizuka	108(G10)	asn-->asp	12.9(9.0)		$\tilde{a}_1\beta_1$	36
Presbyterian	108(G10)	asn-->lys	6.3(2.5)		$\tilde{a}_1\beta_1$	37
Peterborough	111(G13)	val-->phe	14.0(9.0)		$\tilde{a}_1\beta_1$	38
New York	113(G15)	val-->glu	n/a		G-helix	1,39

Hope	136(H14)	gly-->asp	n/a	heme	1,40
Himeji	140(H18)	ala-->asp	5.8(4.5)		

* Parenthetical values are that investigator's measured P_{50} for conventional Hgb A in RBC-free or RBC-bound state, as indicated

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Table 2: Candidate Non-Naturally Occurring Low Affinity Hemoglobin Mutants

alpha chain

46 phe-->thr
46 phe-->ser
46 phe-->ala
58 his-->phe
58 his-->trp
61 lys-->thr
61 lys-->ser
61 lys-->met
61 lys-->asn
62 val-->leu
62 val-->ile
62 val-->phe
62 val-->trp
65 ala-->asp
94 asp-->gln
94 asp-->thr
94 asp-->ser
94 asp-->lys
94 asp-->gly
94 asp-->arg

beta chain

21 asp-->ala
21 asp-->ser
45 phe-->ala
45 phe-->thr
45 phe-->val
63 his-->phe
63 his-->trp
66 lys-->ser

66 lys-->asn
67 val-->phe
67 val-->trp
67 val-->ile
70 ala-->glu
70 ala-->ser
70 ala-->thr
96 leu-->phe
96 leu-->his
96 leu-->lys
98 val-->trp
98 val-->phe
102 asn-->asp
102 asn-->glu
102 asn-->arg
102 asn-->his
102 asn-->gly
108 asn-->arg
108 asn-->glu

Table 3: Oxygen Affinity Values for Mutant Hemoglobins

Hemoglobin Mutant	P_{50} (mmHg)	P_{50} mutant/P_{50} wild type
Hgb (beta phe^{63})	36.0	7.5
Hgb (beta ile^{67})	9.4	2.0
Hgb (beta thr^{102})	11.1	4.6

* C L M S *

CLAIMS

1. The use of cell-free mutant (as defined herein) hemoglobin in the manufacture of a non-toxic composition for administration to a patient so as to supplement the oxygen-carrying capacity of the patient's blood.

2. The use claimed in claim 1 wherein the mutant hemoglobin is a naturally-occurring hemoglobin.

3. The use claimed in claim 1 wherein the mutant hemoglobin is a non-naturally-occurring hemoglobin.

4. The use claimed in any preceding claim wherein the cell-free mutant hemoglobin has a P_{50} of at least about 10% greater than that of cell-free conventional hemoglobin under the same conditions.

5. The use claimed in any preceding claim wherein said mutant hemoglobin is an alpha chain hemoglobin mutant.

6. The use claimed in any preceding claim wherein the mutant hemoglobin contains a mutation which affects the equilibrium between the R state and the T state of the hemoglobin molecule.

7. The use claimed in claim 1 wherein the mutant hemoglobin contains a mutation of the beta Asn102 (G4) residue.

8. The use claimed in claim 7 wherein the mutant hemoglobin contains a beta Asn102 (G4) -> Thr mutation.

9. The use claimed in claim 1 wherein the mutant hemoglobin contains a His 63 Phe mutation.

10. The use claimed in claim 1 wherein the mutant hemoglobin contains a mutation of an amino acid residue within 4 angstroms, as determined on a nearest atom-to-nearest atom basis, of the heme moiety.

* C L M S *

11. The use claimed in claim 1 wherein the mutant hemoglobin contains a mutation of a first amino acid residue on a first chain which is within 4 angstroms of a second residue on a second and distinct chain, as determined on a nearest atom-to-nearest atom basis, wherein said first and second residues are a part of the alphalbeta2 or alpha2beta1 interfaces.

12. The use claimed in claim 11 wherein the mutant hemoglobin contains a mutation of beta residue 45 (CD4).

13. The use claimed in claim 11 wherein the mutant hemoglobin contains a mutation of beta residue 70 (E14).

14. The use claimed in claim 1 wherein the mutant hemoglobin contains a mutation of beta 67 Val.

15. The use claimed in claim 14 wherein the mutant hemoglobin contains a mutation of beta 67 Val -> Ile.

16. The use claimed in any preceding claim wherein the composition further comprises an organic phosphate which stabilizes the T state of hemoglobin.

17. The use claimed in any preceding claim wherein the mutant hemoglobin contains a mutation wherein another amino acid residue is converted to cysteine, and wherein said cysteine is cross-linked to another cysteine residue.

18. The use claimed in claim 2 wherein the mutant hemoglobin has the structure of a natural low affinity hemoglobin mutant selected from the group consisting of the mutants identified in Table 1.

* C L M S *

19. The use claimed in claim 3 wherein the mutant hemoglobin is a low affinity hemoglobin mutant selected from the group consisting of mutants having the mutation identified in Table 2 and combinations thereof.

20. The use claimed in any preceding claim wherein said mutant hemoglobin is wholly artificial.

21. A nontoxic pharmaceutical composition comprising a non-naturally occurring mutant (as defined herein) hemoglobin in a pharmaceutically acceptable carrier.

22. A recombinant DNA molecule comprising a fused gene, said fused gene comprising a first nucleotide sequence which codes on expression for an alpha globin, and a second nucleotide sequence which codes on expression for at least a 10 amino acid segment of a beta globin.

23. The molecule of claim 22 wherein said first and second nucleotide sequences are separated by a first spacer DNA sequence which codes on expression for a sequence of amino acids which provide a selective cleavage site for a site-selective protease.

24. The molecule of claim 22 wherein the first spacer DNA codes for a cleavage site which is selectively cleaved by Factor Xa.

25. The molecule of any of claims 22 to 24 wherein the second nucleotide sequence is positioned so that its first codon is read prior to the reading of the first codon of the first nucleotide sequence in the course of the expression of the fused gene.

26. The molecule of any of claims 22 to 25 wherein the second nucleotide sequence codes on expression for the N-terminal of a beta globin.

27. A method of producing hemoglobin which comprises (a) producing alpha globin by (i) introducing a recombinant DNA molecule according to claim 22 into a suitable host other than an erythrocyte, (ii) expressing alpha globin either in free form or as part of a fusion protein, (iii) if need be, cleaving said fusion protein at a selective cleavage site to liberate the alpha globin, and (iv) recovering and purifying said alpha globin; (b) obtaining beta globin from a source other than blood; and (c) preparing hemoglobin by combining said alpha and beta globin and a source of heme.

28. A hemoglobin composition comprising cell-free wholly artificial, biologically functional hemoglobin characterised in that both the α- and the β-globin subunits are derived by expressing recombinant DNA coding sequences.

29. The hemoglobin composition of Claim 28 wherein said hemoglobin has an affinity for oxygen which is lower than that of the conventional hemoglobin depicted in Figure 1

30. The hemoglobin composition of claim 28 wherein said hemoglobin is an alpha chain hemoglobin mutant.

31. The hemoglobin composition of claim 28 wherein the hemoglobin has the structure of a natural low affinity hemoglobin mutant selected from the group consisiting of the mutants identified in Table 1.

32. The hemoglobin composition of any of claims 28 to 31 wherein the α-globin subunit is derived by cleavage of a chimeric protein including at least a 10 amino acid segment of a β-globin.

33. A non-naturally occurring low hemoglobin mutant selected from the group consisting of mutants having the mutation identified in Table 2 and combinations thereof.

34. A hemoglobin composition comprising a solution of cell-free, mutant (as defined herein) hemoglobin in a pharmaceutically acceptable carrier, said solution having a P_{50} under standard physiological conditions of 24-32 torr.

* C L M S *

35. The composition of claim 34 wherein said composition further comprises sodium ions, potassium ions and chloride ions.

36. The composition of claim 35 wherein said composition further comprises an oncotic agent.

37. The composition of claim 35 wherein said composition further comprises an antioxidant.

38. A polypeptide comprising a covalent conjugate consisting of an α-globin moiety and at least a 10 amino acid segment of a β-globin.