INCREASING THE STABILITY OF RECOMBINANT ADULT HUMAN APOMEGLOBIN

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Adachi et al. “Significance of β16 His (G18) at αβ1 Contact Sites for αβ Assembly and Autoxidation of Hemoglobin” Biochemistry, vol. 42 (pp. 10252-10259), Apr. 16, 2003.

(Continued)
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ABSTRACT
The disclosure relates to recombinant adult human apohemoglobin (apo-rHb) in which the stability has been increased by replacement of an amino acid with a counterpart from another organism, such as a deep sea diving mammal. This mutated apo-rHb may be more stable and/or give higher production yields than unmutated adult human apo-rHb. The mutated apo-rHb may be produced in microorganisms, such as E. coli or yeast cells, or animal erythroid cells. Some apo-rHb of the present disclosure may be used as part of a blood substitute.

11 Claims, 6 Drawing Sheets
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Snyder “Respiratory Adaptions in Diving Mammals” Respiration Physiology 54 (pp. 296-294), 1983.


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Figure 3
Figure 4A

Figure 4B

SEQ ID NO: 1

SEQ ID NO: 2

SEQ ID NO: 3

SEQ ID NO: 4
c. Comparison of sperm whale and human apoHb unfolding, pH 7, 10°C

Native human apoHbA

Sperm whale apo-rHb

d. Apo α(WT)β(mutant) unfolding, pH 7, 10°C

β P(D2)A

WT rHb0.0

β C(G14)V

β G(E13)A

β G(A13)A

Figures 4C and 4D
Figure 5

A. Expression of rhb alone
- rhb0.0 in BL21
- α(WT)/β(G16A) in BL21

Expression Conditions:
- None
- heme
- Dps
- IPTG
- IPTG+2x-heme

Derivative Signal at 430 nm (OD570nm - 0.6)

Figure 6

Potential stabilizing HbF replacements in the α-β interface:

SEQ ID NO: 5

Figures 6

SEQ ID NO: 6
INCREASING THE STABILITY OF RECOMBINANT ADULT HUMAN APOHEMOGLOBIN

RELATED APPLICATION

This application is a continuation of International Application No. PCT/US2005/032627, filed on Sep. 15, 2005, which claims priority to U.S. Provisional Patent Application No. 60/610,110, filed on Sep. 15, 2004 and U.S. Provisional Patent Application No. 60/610,110, filed on Sep. 15, 2004, the full disclosures of which are incorporated herein by reference.

STATEMENT OF GOVERNMENT INTEREST

The present invention was made with United States government support awarded by the following agencies: NIH AR040252, NIH R01 HL047020 and NIH GM35649. The U.S. Government has certain rights in this invention.

TECHNICAL FIELD

The present disclosure relates to compositions and/or methods of producing compositions that include a form of hemoglobin.

BACKGROUND

Hemoglobin (Hb) is responsible for carrying and delivering oxygen to tissues and organs in animals and has been used in development of an effective and safe oxygen carrier as an alternative to blood transfusion. Hb can be obtained easily in large quantities from bovine sources, or can be produced transgenically, so the raw material is not limiting. Such forms of Hb, however, may have numerous serious side effects when transfused into a human patient. For example, raw Hb may cause vasoconstriction, abdominal pain, and acute kidney failure. In addition, products may cause elevation of blood pressure and other problems associated with interference with smooth muscle regulation.

Some of these effects may stem from the toxicity of Hb when it is outside of a red blood cell (erythrocyte). In addition, Hb outside of a red blood cell is rapidly broken down from its tetrameric form into dimers and monomers. These products may be taken up by the kidney and impair nephrological functions.

SUMMARY

Therefore, a need exists for oxygen delivery compositions that are safer, more clinically effective, and/or more economically produced.

The present disclosure, according to some example embodiments, relates to hemoglobin (rHb) and/or apohemoglobin (apo-rHb) in which at least a portion of the amino acid sequence (e.g., one or more amino acids) has been modified to match a counterpart from another amino acid sequence (e.g., another metal-binding protein). If more than one counterpart amino acid is used, the amino acids may be contiguous or non-contiguous. According to some embodiments, a counterpart may include any metal-binding protein from any species. For example, counterparts may include human or non-human iron-binding proteins.

In specific example embodiments, at least one of the following amino acid mutations may be made (the amino acids are specified by their helical location, i.e., A13 represents the thirteenth position along the A helix as indicated in FIG. 3):

- Gln13 to Ala (Ser)
- GlyB3 to Ala (Asp, Glu, Asn)
- CE corner mutations
- CysG11 to Ser, Thr, Val
According to some embodiments of the disclosure, at least one amino acid is modified to match the hemoglobin of another species. In some embodiments, two or more amino acids are modified to match the hemoglobin of another species. In some embodiments, three or more amino acids are modified to match the hemoglobin of another species. In some embodiments, no more than five amino acids are modified to match the hemoglobin of another species. In some embodiments, no more than ten amino acids are modified to match the hemoglobin of another species. In some embodiments, no more than fifteen amino acids are modified to match the hemoglobin of another species. In some embodiments, no more than twenty amino acids are modified to match the hemoglobin of another species.

**BRIEF DESCRIPTION OF THE DRAWINGS**

The present disclosure may be better understood through reference to the following detailed description, taken in conjunction with the following figures in which:

FIG. 1 illustrates a scheme for hemoglobin assembly in both E. coli, other microorganisms, and erythroid cells.

FIG. 2 illustrates the differences in stability of sperm whale and pig apomyoglobin.

FIG. 3 illustrates in vivo expression of holomyoglobin (holoMb) and correlations with the rates of and apomyoglobin (apoMb) stability.

FIG. 3A illustrates the expression of wild-type myoglobin (Mb). The top graph shows the raw absorbance data for a typical assay of E. coli cells expressing wild-type sperm whale myoglobin. The bottom graph shows the free CO-heme has a broad Soret absorbance band at 412 nm, readily oxidizes to 4-coordinate hemin with a very broad peak at ~380 nm, and does not interfere with the HbCO derivative spectrum.

FIG. 3B illustrates the correlation between the $-\log(K_{d,CO})$, which is a direct, in vitro measure of the stability of the apoprotein, measured in 200 m$^2$ KPI and the $\log$ (relative expression level) for 35 single, double, and triple mutants of sperm whale myoglobin.

FIGS. 4A and 4B provide a sequence comparison between human and sperm whale $\alpha$ and $\beta$ hemoglobin genes. Arrows indicate possible mutations to stabilize the human subunits.

FIG. 4C provides a comparison of the GdmCl unfolding curves of native human and recombinant sperm whale apomyoglobin. The broad sperm whale apomyoglobin curve is completely reversible and independent of total protein concentration in the range 2.5 to 10 $\mu$M (data not shown).

FIG. 4D illustrates the unfolding curves for four $\alpha$ (wild-type)$\beta$ (mutant) hybrid human apomyoglobins. The $\beta$G16(A13)A mutation causes a marked increase in $K_{d, GdmCl}$, increasing from $-1.4$ to 2.1 M. 

FIG. 5 illustrates measurement of holo-$\alpha$Hb0.0 and holo-$\beta$Hb0.0 ($\alpha$G16(A13)A) production in E. coli BL21 cells with and without co-expression of the hgb genes on pFHUG21.1.

The present disclosure relates, in some embodiments, modified forms of hemoglobin and/or myoglobin (e.g., apohemoglobin) and/or methods for producing modified forms of hemoglobin and/or myoglobin (e.g., apomyoglobin). Modified forms of hemoglobin and/or myoglobin (e.g., apohemoglobin) may have improved stability and/or may be suitable for use in blood substitutes. In some embodiments, methods of producing modified forms of hemoglobin and/or apohemoglobin may be produced may result in better yields (e.g., more protein is produced, more of protein produced is functional, and/or protein is produced more cost effectively).

In some embodiments of the disclosure, modified forms of hemoglobin and/or myoglobin (e.g., apohemoglobin) may be administered to a subject. For example, an amount sufficient to improve oxygen delivery may be administered to a subject in conjunction with a blood substitute. Subjects may include humans and non-human mammals. In some embodiments, administration of modified forms of hemoglobin and/or myoglobin (e.g., apohemoglobin) may be associated with little or no hypertensive side effects relative to administration of corresponding unmodified forms. According to one example embodiment, the disclosure relates to recombinant adult human apohemoglobin (apo-Hb) in which the stability has been increased by replacement of at least one amino acid with a counterpart from sperm whale $\alpha$ hemoglobin or human fetal $\gamma$ hemoglobin. This mutated apo-$\alpha$Hb may be more stable and/or give higher production yields than unmutated adult human apo-$\alpha$Hb. Some apo-$\alpha$Hb of the present disclosure may be used as part of a blood substitute.

In specific embodiments, more stable human $\alpha$ and $\beta$ globins may be constructed by modification (e.g., mutation) of adult human $\alpha$Hb that are based on the naturally occurring amino acids found in adult hemoglobins of sperm whales (SW Hb) (and other deep diving mammals) and replacements found in fetal human hemoglobin (HbF). Both SW Hb and HbF are much more resistant to denaturation than native adult human hemoglobin (HbA). Other deep diving mammals may exhibit similar resistance. Additionally, the fetal form of
hemoglobin in these mammals may provide even greater resistance to degradation. Resistance to unfolding, denaturation, and precipitation may increase production yields in E. coli and other microorganisms, including other bacteria and yeasts, and in animal erythroid cells, such as mammalian erythroid cells.

Based on studies with Mb, enhancement of degradation resistance of heme-free (apo) globin increases the level of production of intact protein by 50 to 100% in E. coli. A similar enhancement of expression level by stabilizing the subunits and interfaces of recombinant hemoglobin may make its production in E. coli not only feasible but also profitable. In some example embodiments, the production of intact, usable rHb may be increased from the current level of 5-10% of E. coli total soluble protein to 30% or more.

Thus, example embodiments of the present disclosure relate to rHb production cells, tissues, or animals in which apo-rHb contains at least one amino acid mutation in the adult human α or β hemoglobin subunit introduced from a sperm whale or deep diving mammal hemoglobin or human fetal hemoglobin, such that the mutated apo-rHb may be more resistant to denaturation and thus may be more stable than unmutated adult human rHb.

Other example embodiments may relate to nucleic acids that encode mutated apo-rHb. These embodiments may also encode at least two different hemoglobin subunits for co-expression in the same cell to produce apo-rHb. Still other example embodiments relate to systems including cells, such as E. coli cells, other microorganisms, or animal erythroid cells, for production of a more degradation-resistant mutated apo-rHb. These systems may also exhibit increased rHb production and fewer degradation products when compared with similar systems for production of unmutated adult human apo-rHb. Other example embodiments relate to methods of making the above cells and nucleic acids as well as to methods of producing mutated apo-rHb.

The present disclosure, according to some example embodiments, may be used in conjunction with existing rHb technologies. For example, it may be used in connection with two co-filed applications U.S. provisional patent application Ser. Nos. 60/610,110 and 60/610,109, as well as U.S. Pat. Nos. 6,455,676; 6,204,009; 6,114,505; 6,022,849; and U.S. patent application No. 2003 0017537.

The following discussion relates to specific example embodiments of the disclosure.

The assembly of hemoglobin in either bacteria or in animal erythroid cells is a complex process involving ribosomal synthesis of two different protein chains or subunits (α, 141 amino acids and β, 146 amino acids). The newly synthesized α and β subunits do not appear to have any well-formed structure in the absence of a partner and first assemble to form an αβ dimer, which itself is also very unstable (apo αβ dimer in FIG. 1, where the suffix apo means no heme is bound and the protein has no “red” color). Only after heme (iron containing red pigment) is bound is the protein stabilized and resistant to degradation. Hemoglobin synthesis in bacteria may be limited by the availability of heme, and as a result, newly formed α and β proteins that are unable to find heme may tend to precipitate or be degraded by bacterial enzymes, particularly α subunits.

Apomyoglobins from deep diving whales are significantly more resistance to denaturation by chemical agents (i.e., guanidinium chloride, urea) than those from the terrestrial or surface swimming mammals, and these proteins may be easily expressed as intact myoglobins in E. coli (FIG. 2). There may be significant selective pressure for increased resistance of Mb to denaturation during the sustained hypoxic and acidoic conditions that occur in whale skeletal muscles during deep and prolonged dives (Zapol, W. M. et al., (1979) J Appl Physiol 47, 968-973; Snyder, G. K. (1983) Respir Physiol 54, 269-294; Kooyman, G. L. et al., (1998) Annu Rev Physiol 60, 19-32; Tang, Q. et al. (1998) Biochemistry 37, 7047-7056).

As shown in FIG. 2, CD titration curves are shown in the left panel for unfolding of wild-type pig apomoglobin (open circles), wild-type sperm whale apomoglobin (filled circles), and pig apomoglobin with five replacements based on the sequence of the sperm whale protein: G5A/S51T/D53A/G74A/T87K (open triangles) in the titration curve. The two step apoglobin unfolding mechanism is shown at the bottom of the figure (Barrick, D. et al., (1993) Biochemistry 32, 3790-3796; Eliezer, D. et al. (1997) FEBS Lett 417, 92-96). The thickness of the ribbons indicates the amount of helical structure. Native apomyoglobin (N) retains most of the secondary and tertiary structure present in holomyoglobin except for the F helix. Addition of denaturant unfolds the B, C, D, and E helices to give a molten globule intermediate (I) composed of folded A, G, and H helices. Further addition of denaturant results in the completely unfolded state (U). The spheres in the RIBBONS drawing show the location of the mutated residues. The solid and dashed lines represent global fits to the observed CD and fluorescence changes as described in Scott et al (Scott, E. et al. (2000) J Biol Chem 275, 27129-27136).

Apomyoglobin stability correlates quantitatively with expression, the yield of myoglobin production in vivo with the apomyoglobin stability of ≥35 different mutants that were designed to have widely different heme binding and protein folding properties was measured. (See FIG. 3.) Thus, apogoblin stability has been shown quantitatively to be a major limiting factor in the production of intact heme proteins in E. coli using recombinant myoglobin as a model system (Scott, E. E. et al. (2000) J Biol Chem 275, 27129-27136; Smith, L. P. (2003) PhD Dissertation Biochemistry & Cell Biology, Rice University Houston, Tex.; and Olson, J. S. et al. (1997) Artificial Cells, Blood Substitutes, and Immobilization Biotechnology 25, 227-241).

In FIG. 3A, the top graph shows the raw absorbance data for a typical assay of E. coli cells expressing wild-type sperm whale myoglobin. For these assays 5 ml cultures were grown overnight using the constitutive expression system of E. coli (Smith, L. P. (2003) PhD Dissertation Biochemistry & Cell Biology, Rice University Houston, Tex.). The cells were spun down and resuspended to an OD_600 of 0.5 to normalize the number of cells in each assay. The suspensions were flushed with 1 atm of CO and reduced with a small amount of dithionite. Visible spectra were recorded from 600 to ~350 nm and the derivative spectra were calculated numerically as shown. The ratio of the peak to trough absorbance derivative signal for mutant Mb was divided by that for wild-type Mb to obtain relative expression yield used in

Also in FIG. 3A, the bottom graph shows the free CO-heme has a broad Soret absorbance band at 412 nm, readily oxidizes to 4-coordinate heme with a very broad peak at ~380 nm, and does not interfere with the HbCO derivative spectrum (Looker, D. et al. (1994) Methods Enzymol 231, 364-374). The peak to trough difference at ~420 nm was used as a measure of holoMb expression level.

The unfolding constant, K_{unf}, represents the ratio of denatured unfolded state (U) to the native folded state (N) and can be obtained from titrations with a highly soluble denaturant like guanidinium chloride (GdmCl) or urea which facilitates the N to U reaction. The reciprocal of this value is the folding
constant, $K_{EN}=1/K_{NEN}$ which indicates how stable the apoglobin is. It is the equilibrium constant for the U to N reaction. For example, wild-type sperm whale myoglobin has $K_{EN}=12,000$ so that at equilibrium 12,000 molecules are folded and 1 is unfolded at room temperature. This number is often expressed on a logarithmic scale as $\log K_{EN}$ or from the experimentally determined unfolding constant, $-\log K_{NEN}$, for which the negative sign indicates inversion of the constant. The large and more positive the value of $-\log K_{NEN}$ the more stable the protein. Thus in FIG. 3B, the points at $-\log K_{NEN}$ values equal to +6, indicate a folding constant of 1,000,000 and very stable protein structures which correlate with high levels of expression in *E. coli*.

FIG. 3B shows that apoglobin stability is necessary, but not always sufficient, to achieve high production yields. For example, there are two major apoMb outliers below the lower 90% regression line, indicating that these proteins have reasonable folding constants but express poorly, perhaps due to higher rates of proteolytic degradation and aggregation. In contrast, there are no outliers above the upper dashed line, indicating that no unstable apobMs express well. Thus, stable apoglobin structures may be required for good production in *E. coli*.

In FIG. 3B, the correlation explains 52% of the total variance and has a $p$ value of 0.0000009. The linear regression between these two parameters is $\log\text{(expression)}=1.26+0.27\%(-\log K_{NEN})$. The dashed lines encompass 90% of the data points and are $+/-.0.42$ from the regression line (Smith, L. P. (2003) Biochemistry & Cell Biology, Rice University Houston, Tex.).

Accordingly, without being limited to any particular mechanism of action or theory, some example embodiments of the present disclosure relate to the creation of more stable $\alpha$ and $\beta$ subunits that may have strengthened tertiary structures and interactions in the $\alpha\beta$ interface. For example, dimers may be more resistant to degradation and precipitation while waiting for heme insertion. In some specific example embodiments, amino acid substitutions may include, without limitation, the example substitutions shown in Table 1.

### TABLE 1

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Stabilize the $\alpha$ Glys13→Als(Ser)</td>
<td>$\beta$ Glys13→Als(Ser) $\beta$ Glys2→Als $\beta$ Glys17→Lys</td>
</tr>
<tr>
<td>folded state of human apoglobin using mutations $\alpha$ CE corner $\beta$ Glys13→Als (Thr, Asp) based on $\alpha\beta$ mutations $\gamma$ GlyGln→Val, Thr, Ser, Cys</td>
<td>$\alpha$ CysGln→Ser, Thr $\beta$ ProH3→Glu, Ala (Gln)</td>
</tr>
<tr>
<td>comparisons with sperm whale Hb. $\alpha$ Thr, Val $\beta$ ProH3→Glu, Ala (Gln)</td>
<td></td>
</tr>
<tr>
<td>2. Strengthen the $\alpha\beta$ interface with mutations based on comparisons between the sequences of adult $\beta$ chains and fetal $\gamma$ chains. $\beta$ CysGlu→Thr $\beta$ HisGlu→Ile(Leu, Ala) $\beta$ ProH3→Glu $\beta$ TyrH8→Trp(Leu) $\beta$ ValH11→Met(Leu, Phe)</td>
<td></td>
</tr>
</tbody>
</table>

As shown in Table 1, the first set of mutations are designed to increase the stability of the individual subunits based on sequence comparisons between sperm whale and human hemoglobin. This first strategy is based on the assumption that sperm whale hemoglobin is under selective pressure to be more resistant to denaturation.

The second set is designed to strengthen the $\alpha\beta$ interface based on comparisons between adult $\beta$ hemoglobin and fetal $\gamma$ hemoglobin. This strategy is based on the observation that fetal hemoglobin is significantly more resistant to both acid and alkaline denaturation (Bunn, H. F. et al. (1986) *Hemoglobin: Molecular, Genetic, and Clinical Aspects*, W. B. Saunders, Philadelphia). The rate of dissociation of $\alpha\beta$ dimers may be at least 3-fold smaller than that of $\alpha\beta$ dimers and the rate of assembly of holo-$\alpha$ chains with holo-$\beta$ chains containing $G$ and $H$ helical substitutions based on $\gamma$ chains may have significantly higher bimolecular rates of dimer formation (Mrabet, N. T. et al. (1986) *J Biol Chem* 261, 1111-1115; McDonald, M. J. et al. (1987) *J Biol Chem* 262, 5951-5956; Adachi, K. et al. (2001) *Biochim Biophys Acta* 1529, 75-79; Adachi, K. et al. (2003) *Biochemistry* 42, 10252-10259; Joshi, A. A. (1994) *J Biol Chem* 269, 8549-8553). The replacements found in $\gamma$ chains significantly stabilize the $\alpha\beta$ dimer interface and increase resistance to apo-dimer unfolding, which, when used in the context of the present disclosure may enhance expression in a microorganism such as *E. coli*.

Over ten years ago, it was discovered that sperm whale apomyoglobin is 20 to 100 times more resistant to GdmCI-induced denaturation than most other mammalian apoMbs (FIGS. 2 and 3, and (Scott, E. E. (2000) *J Biol Chem* 275, 27129-27136; Hargrove, M. S. et al. (1994) *Biochemistry* 33, 11767-11775)). This observation has been discussed anecdotally in the literature and accounts for why sperm whale apoMb was chosen for detailed unfolding studies (Hughson, F. M. et al. (1990) *Science* 249, 1544-1548; Nishimura, C. et al. (2003) *J Mol Biol* 334, 293-307; Nishimura, C. et al. (2000) *Nat Struct Biol* 7, 679-686; Garcia, C. et al. (2000) *Biochemistry* 39, 11227-11237; Eliezer, D. et al. (2000) *Biochemistry* 39, 2894-2901). Sperm whale holomyloglobin can be expressed constitutively in large amounts in *E. coli* without adding heme and without producing large amounts of unfolded apoprotein in inclusion bodies. In contrast, pig and human myoglobin generally cannot be expressed readily as holoproteins without adding heme (Varadarajan, R. et al. (1985) *PNAS* 82, 5681-5684; Dodson, G. et al. (1988) *Protein Eng* 2, 233-237; Springer, B. et al. et al. (1987) *PNAS* 84, 8961-8965; Lloyd, E. et al. (1994) *FEBS Lett* 340, 281-286). The underlying physiological cause of these differences was discovered in a study of the unfolding properties of 13 different mammalian Ms (Scott, E. E. et al. (2000) *J Biol Chem* 275, 27129-27136). ApoMbs from deep diving whales are significantly more stable than those from the terrestrial or surface swimming mammals that were examined. These results indicate, among other things, that there is significant selective pressure for increased resistance of Mb to denaturation during the sustained hypoxic and acidotic conditions that occur in whale skeletal muscles during deep and prolonged dives (Zapol, W. M. et al. (1979) *J Appl Physiol* 47, 968-973; Snyder, G. K. (1983) *Respir Physiol* 45, 269-294; Kooyman, G. L. et al. (1998) *Ann Rev Physiol* 60, 19-32; Tang, Q. et al. (1998) *Biochemistry* 37, 7047-7056). GdmCI-induced unfolding curves for 28 different apoMbs were analyzed in terms of the two-step, three-state mechanism first described by Barrick, Baldwin, and Wright (FIG. 2, and Barrick, D. et al. (1993) *Biochemistry* 32, 3790-3796; and Hughson, F. M. et al. (1990) *Science* 249, 1544-1548), using algorithms devised by Elfink’s group to analyze combined CD and fluorescence data (Ramsay, G. et al. (1995) *Biophys J.* 69, 701-707). The fitted values of $K_{EN}$ and $K_{NEN}$ represent equilibrium constants for the native (N) to intermediate (I)
and intermediate (I) to unfolded (U) transitions in the absence of denaturant. NMR and mutagenesis studies have shown that the first transition involves “melting” of the heme pocket, with little change in secondary structure of the A, G, and H helical core (Hughson, F. M. et al. (1990) Science 249, 1544-1548; Nishimura, C. et al. (2003) J Mol Biol 334, 293-307; Garcia, C. et al. (2000) Biochemistry 39, 11227-11237; Eliezer, D. et al. (1998) Nat Struct Biol 5, 148-155). Higher concentrations of GdmCl are required to melt this more stable region in the second transition. The overall stability of native apoMb can be measured empirically as the concentration of GdmCl that causes 50% of the overall CD change, [GdmCI]midpoint, or as logKstability which is calculated as -log(Kstability).

Comparisons of the amino acid sequences of pig and sperm whale myoglobin suggest several substitutions that might account for the differences in stability. As shown in FIG. 2, five replacements are sufficient to increase the stability of pig apoMb to that of wild-type sperm whale apoMb. The three alanine mutations, G5A, D53A, and G74A, appear to stabilize the native state by elongating and strengthening the A, D, and E helices. Scott et al.’s results, however, do not show that mutation of human hemoglobin will produce useful, stabilizing changes, which sites should be mutated, or that any mutants will actually increase production yield in bacteria, such as E. coli.

Sperm whale and human hemoglobin subunits may be compared and used to selectively mutate human hemoglobin subunits based on the assumption that sperm whale hemoglobin will be more resistant to unfolding as a result of the same selective pressure that caused whaleMb to be more stable. Thus, a comparison between the primary sequences of SW and human β and α chains was made and mutations were selected based on increases in helix propensities. Synthetic sperm whale α and β genes based on the naturally occurring sequences were constructed and expressed and the recombinant whale hemoglobin was purified. The sequence comparisons, proposed mutations, and results with recombinant SW apoHb and four human β chain mutants are shown in FIG. 4 and Table 1. As shown in FIG. 4C, sperm whale apoHb is more resistant to unfolding induced by GdmCl than the human apoprotein at high denaturant concentrations and exhibits a very broad CD transition, suggesting that the whale apoHb has more stable folding intermediates than the human protein.

Some α hemoglobin mutations of some example embodiments of the present disclosure include, but are not limited to, Gly15 to Ala and Gly22 to Ala. These specific replacements were chosen because they suggest that the carbonyl-terminus of the A helix and the amino-terminus of the B helix are stabilized by alanyl side chains in whale α subunits. The same Gly to Ala mutation at the β hemoglobin A13 helical position causes a marked enhancement in human apoHb stability (β G(A13)A curve in FIG. 4D). β subunit mutations of other example embodiments of the present disclosure are shown in FIG. 4B, and four of these replacements have been made in α(wild-type)β(mutant) rhB tetramers. The glycine 16 to Ala mutation at the A13 helical position is very successful in enhancing the resistant of human apoHb to unfolding. The shift in midpoint GdmCl concentration for β G16A apoHb suggests an ~50-fold increase in overall stability, and remarkably, this human rhB single mutant appears to be more stable than sperm whale hemoglobin itself. As shown in FIG. 5, the measured expression level of the β Gly(A13)Ala mutant is ~2 times greater than that of the simple wild-type rhB0.0. This two-fold enhancement of expression occurs in the absence and presence of heme (FIG. 5A). Co-expression of the heme utilization (hug) genes (+DIP) enhances the production of both proteins significantly, but again the more stable β (Gly16 (A13)Ala) mutant still expresses to a much higher level (FIG. 5B). These results demonstrate, among other things, that enhanced resistance to denaturation does result in higher expression levels.

Briefly, in FIG. 5 E. coli BL21(D3) cells were co-transformed with PHUG 21.1/prlβ0.0 plasmids and maintained on agar plates containing tetracycline and chloramphenicol. Tubes containing 5 ml of LB broth were inoculated and then grown overnight at 37°C. Various additions were made to the cultures including IPTG, heme (increments of 10 μM total=1X), and 2,2-dipyridine, DIP (63 μM total), and the cultures were incubated at 37°C for another 16 hours. Then the cells were pelleted, resuspended to 0.5 absorbance units at 700 nm in Tris buffer, pH 7.5, and equilibrated with 1 atm of CO for 15 minutes to ensure HbCO formation and no further cell growth. Spectra of these samples were recorded and first derivatives of the observed spectra were calculated. No hRHBhCO is detected in the absence of IPTG induction, regardless of whether heme or DIP is added to the cultures.

Bunn, McDonald, Adachi, and co-workers have shown that the rate of dissociation of αβ, dimers is at least 3-fold smaller than that of αβ dimers, and that the rate of assembly of holo-α chains with holo-β chains containing G and H helical substitutions based on γ chains can have significantly higher bimolecular rates of dimer formation (Mrabet, N. T. et al. (1986) J Bioi Chem 261, 1111-1115; Joshi, A. A. et al. (1994) J Bioi Chem 269, 8549-8553; Adachi, K. et al. (2001) Biochem Biophys Res Commun 289, 75-79; and Adachi, K. et al. (2003) Biochemistry 42, 10252-10259).

As mentioned above, the sequences of human adult β chains may be compared with the human γ chains from fetal hemoglobin, which is known to be more stable (FIG. 6). A similar approach may be used to exam fetal whale hemoglobin, or fetal hemoglobins from other organisms. The highlighted replacements were selected to stabilize the αβ, interface, partially based on mutagenesis and kinetic studies of subunit assembly and dissociation by Bunn, McDonald, Adachi and co-workers (Mrabet, N. T. et al. (1986) J Bioi Chem 261, 1111-1115; McDonald, M. J. et al. (1987) J Bioi Chem 262, 5951-5956; Adachi, K. et al. (2001) Biochem Biophys Res Commun 289, 75-79; Adachi, K. et al. (2003) Biochemistry 42, 10252-10259; Joshi, A. A. et al. (1994) J Bioi Chem 269, 8549-8553). β Cys112 to Ser and Thr mutations increase the rate of formation of holo-dimers and have the advantage of removing a potentially reactive thiol group. The β His16 to Leu mutation facilitates subunit assembly by enhancing the apolar surface of the αβ, interface. The β Pro125 to Gli mutation is based on the presence of Gli, Gli, Gli, and Gli at this position in human β, γ, and ε chains, respectively, and removal of a Pro should strengthen the H helix. The remaining β Tyr130 to Trp (H8) and Val133 to Met (H11) mutations are partially based on suggestions by Bunn and Forget (1986) Hemoglobin: Molecular, Genetic, and Clinical Aspects, W. B. Saunders, Philadelphia) that these naturally occurring replacements enhance the hydrophobicity of the interior of the αβ, interface. The corresponding amino acids in human α, β, γ, and ε chains are Leu, Trp, and Trp, respectively, at the H8 helical position and Phe, Phe, and Leu, respectively, at the H11 position, implying that there may be selection for large apolar residues.

While not meant to be limited by theory, newly translated apoprotein generally should remain in solution and be resistant to proteolysis long enough for heme to be made available by either bacterial synthesis or transport of externally added heme. In this model, there is competition between precipita-
tion and proteolysis of the unfolded states and heme binding to the native state. If the fractions of the unstable I and U states are relatively high and the rate of heme transport and/or synthesis is low, little holoprotein will be expressed. Lucian Smith verified this model using an in vivo assay for holomoglobin production in E. coli and comparing the observed expression levels with the stabilities (logK


expression levels of the corresponding apoglobin mutants (Smith, L. P. (2003) The Effects of Amino Acid Substitution on Apomyoglobin Stability, Folding Intermediates, and Holoprotein Expression. PhD Dissertation, Biochemistry & Cell Biology, Rice University Houston, Tex.). A similar comparative mutagenesis strategy may be used to enhance the stability and expression of recombinant human hemoglobin.


One example embodiment of the present disclosure thus relates to Hb mutants whose apoglobin subunits are stable at room temperature. Studies of the folding characteristics of
apo-α and β chains allow more sophisticated analyses of the overall apoHb unfolding curves. Similarly, a more stable apoHb dimer allows direct comparisons between GdmCl, acid, and thermally induced folding, adding more physiological relevance to stability measurements. In addition to creating a much more stable and highly expressing rHb molecule, some embodiments of the disclosure also focus on doing so without creating antigenic sites and compromising reduced rates of NO scavenging and efficient O₂ transport.

Finally, a comparison of the holoprotein yields of wild-type and α((wt))β(G16A)rHb in small cultures in the absence and presence of heme and heme transport genes, for example, hug genes from Plesiomonas shigelloides, is shown in FIG. 8. FIG. 8 confirms that enhancing apohemoglobin stability increases holoprotein expression levels. In the absence of the hug genes, the mutant expression level was roughly twice that of the wild-type protein. This ratio became smaller as heme transport efficiency was increased by the hug transport system, but in all cases, more intact mutant protein was made. Thus, according to some example embodiments of the present disclosure, the mutant α and β hemoglobin may be usefully combined with other methods of increasing hemoglobin production, such as co-expression of heme transport genes to increase hemin uptake.

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  20  25  30
MET PHE LEU SER PHE PRO THR THR LYS THR TYR PHE PRO HIS PHE ASP
  35  40  45
LEU SER HIS GLY SER ALA GLN VAL LYS HIS GLY LYS LYS VAL ALA
  50  55  60
ASP ALA LEU THR ASN ALA VAL HIS VAL ASP ASP MET PRO ASN ALA
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  20  25  30
PHE MET SER PHE PRO SER THR LYS TYR THR PHE SER HIS PHE ASP LEU
  35  40  45
GLY HIS ASN SER THR GLN VAL LYS GLY HIS GLY LYS LYS VAL ALA ASP
  50  55  60
ALA LEU THR LYS ALA VAL GLY HIS LEU ASP THR LEU PRO ASP ALA LEU
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20 25 30
LEU VAL VAL TYR PRO TRP THR GLN ARG PHE PHE GLU SER PHE GLY ASP
35 40 45
LEU SER THR PRO ASP ALA VAL MET GLY ASN PRO LYS VAL LYS ALA HIS
50 55 60
GLY LYS LYS VAL LEU GLU ALA PHE SER ASP GLY LEU ALA HIS LEU ASP
65 70 75 80
ASN LEU LYS GLY THR PHE ALA THR LEU SER GLU LEU HIS CYS ASP LYS
85 90 95
LEU HIS VAL ASP PRO GLU ASN PHE ARG LEU LEU GLY ASN VAL LEU VAL
100 105 110
CYS VAL LEU ALA HIS HIS HIS PHE GLY LYS GLU PHE THR PRO PRO VAL GLN
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20 25 30
VAL VAL TYR PRO TRP THR GLN ARG PHE PHE GLU HIS PHE GLY ASP LEU
35 40 45
SER THR ALA ASP ALA VAL MET LYS ASN PRO LYS VAL LYS LYS HIS GLY
50 55 60
GLN LYS VAL LEU ALA SER PHE GLU GLY GLU LEU LYS HIS LEU ASP ASN
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LEU LYS GLY THR PHE ALA THR LEU SER GLU LEU HIS CYS ASP LYS LEU
85 90 95
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100 105 110
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20 25 30
VAL VAL TYR PRO TRP THR GLN ARG PHR PHE GLU SER PHE GLY ASP LEU  
35 40 45
SER THR PRO ASP ALA VAL MET GLY ASN PRO LYS VAL LYS ALA HIS GLY  
50 55 60
LYS LYS VAL LEU GLY ALA PHE SER ASP GLY LEU ALA HIS LEU ASP ASN  
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LEU LYS GLY THR PHE ALA THR LEU SER GLU LEU HIS CYS ASP LYS LEU  
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Tyr His
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<210> SEQ ID NO 6
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<400> SEQUENCE:

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20 25 30
VAL VAL TYR PRO TRP THR GLN ARG PHR PHE GLU SER PHE GLY ASN LEU  
35 40 45
SER SER ALA SER ALA ILE MET GLY ASN PRO LYS VAL LYS ALA HIS GLY  
50 55 60
LYS LYS VAL LEU THR SER LEU GLY ASP ALA THR LYS HIS LEU ASP ASP  
65 70 75 80
LEU LYS GLY THR PHE ALA GLN LEU SER GLU LEU HIS CYS ASP LYS LEU  
95 100 105 110
HIS VAL ASP PRO GLU ASN PHE LYS LEU GLY ASN VAL LEU VAL THR  
115 120 125
VAL LEU ALA ILE HIS PHE GLY LYS GLU PHE THR PRO GLU VAL GLN ALA  
130 135 140

Tyr His
145
What is claimed is:

1. A method of producing a stabilized apohemoglobin subunit comprising modifying at least two to up to 15 amino acids of the amino acid sequence of adult human apohemoglobin subunit to match a counterpart from an apohemoglobin subunit from a deep sea diving mammal, wherein a stabilized apohemoglobin subunit exhibiting improved resistance to degradation or precipitation prior to heme insertion as compared to unmodified adult human apohemoglobin is produced.

2. A method according to claim 1, wherein said deep sea diving mammal is a sperm whale.

3. A method according to claim 1, wherein said modifying comprises forming a nucleic acid encoding an adult human apohemoglobin subunit with at least two to up to 15 variant amino acids that match their counterpart amino acids of an apohemoglobin subunit from a deep sea diving mammal and expressing said nucleic acid in E. coli, another microorganism, or animal erythroid cells.

4. A recombinant adult human apohemoglobin production cell comprising:
   a nucleic acid encoding an adult human apohemoglobin subunit with at least two to up to 15 variant amino acids that match their counterpart amino acids of an apohemoglobin subunit from a deep sea diving mammal that is more resistant to degradation or precipitation prior to heme insertion than an unmodified adult human apohemoglobin subunit; and
   other cellular components sufficient to produce a recombinant adult human apohemoglobin.

5. A production cell according to claim 4, wherein the deep sea diving mammal is a sperm whale.

6. A production cell according to claim 4, wherein the production cell is an E. coli cell, a cell from another microorganism, or an animal erythroid cell.

7. A production cell according to claim 4, further comprising an expressible nucleic acid comprising the nucleic acid encoding an adult human apohemoglobin subunit.

8. A system for recombinant adult human apohemoglobin production comprising:
   a plurality of production cells; and
   a nucleic acid encoding a stabilized recombinant adult human apohemoglobin subunit more resistant to degradation or precipitation prior to heme insertion than unmodified adult human apohemoglobin;
   wherein the nucleic acid encodes an adult human apohemoglobin subunit with at least two to up to 15 variant amino acids that match their counterpart amino acids of an apohemoglobin subunit from a deep sea diving mammal.

9. A system according to claim 8, further comprising a second nucleic acid encoding a second recombinant adult human apohemoglobin subunit.

10. A system according to claim 8, further comprising a second nucleic acid encoding a wild-type adult human apohemoglobin.

11. A system according to claim 8, wherein the system produces recombinant adult human apohemoglobin that may be used as part of a blood substitute product.

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