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Development of Recombinant Hemoglobin-Based Oxygen Carriers

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Abstract

Significance: The worldwide blood shortage has generated a significant demand for alternatives to whole blood and packed red blood cells for use in transfusion therapy. One such alternative involves the use of acellular recombinant hemoglobin (Hb) as an oxygen carrier. **Recent Advances:** Large amounts of recombinant human Hb can be expressed and purified from transgenic *Escherichia coli*. The physiological suitability of this material can be enhanced using protein-engineering strategies to address specific efficacy and toxicity issues. Mutagenesis of Hb can (i) adjust dioxygen affinity over a 100-fold range, (ii) reduce nitric oxide (NO) scavenging over 30-fold without compromising dioxygen binding, (iii) slow the rate of autooxidation, (iv) slow the rate of heme loss, (v) impede subunit dissociation, and (vi) diminish irreversible subunit denaturation. Recombinant Hb production is potentially unlimited and readily subjected to current good manufacturing practices, but may be restricted by cost. Acellular Hb-based O₂ carriers have superior shelf-life compared to red blood cells, are universally compatible, and provide an alternative for patients for whom no other alternative blood products are available or acceptable. **Critical Issues:** Remaining objectives include increasing Hb stability, mitigating iron-catalyzed and iron-centered oxidative reactivity, lowering the rate of heme loss, and lowering the costs of expression and purification. Although many mutations and chemical modifications have been proposed to address these issues, the precise ensemble of mutations has not yet been identified. **Future Directions:** Future studies are aimed at selecting various combinations of mutations that can reduce NO scavenging, autooxidation, oxidative degradation, and denaturation without compromising O₂ delivery, and then investigating their suitability and safety *in vivo*. *Antioxid. Redox Signal.* 18, 2314–2328.

Introduction

GLOBALLY, AN ESTIMATED 103 million units of whole blood and packed red blood cells are donated every year (161a). In the United States alone, over 15 million units are transfused annually (159). These transfusions are often life saving (27, 29, 106, 154) and rarely cause serious adverse events, although side effects do occur (23, 37, 70, 71, 82, 105). However, most U.S. hospitals consistently report blood shortages (159), and studies predict that these shortages will worsen as the population ages (53, 130, 144, 164). This situation is more troubling in less-developed countries where blood donation rates are as low as 0.4 units per thousand people compared to 85 units per thousand in the United States and blood-borne diseases are much more prevalent (159, 161a). Costly diagnostics and handling regulations, increasingly stringent

donor deferral criteria, and a lack of blood donors partly explain this problem (17, 51, 70, 161). However, other issues, including emergent pathogens and natural disasters are of concern (1, 24, 25, 32, 34, 50, 59, 60, 89, 128, 141, 161). Disease transmission and adverse reactions from mistransfusions remain problematic even with best practices in place (30, 103, 129, 133, 161). These and other factors have led to a cost explosion in the industry (105), with the price of blood units increasing significantly in recent years (7, 11, 134).

Recombinant hemoglobin-based oxygen carriers (rHBOCs), along with many other naturally occurring Hb-based products, provide an alternative transfusion strategy (46, 100, 124). rHBOCs consist of concentrated solutions of purified acellular human Hb, which has been heterologously expressed in transgenic bacteria, mice, swine, yeast, and other organisms (2, 12, 28, 31, 52, 62, 78, 79, 85, 95–97, 123, 135, 136, 145,

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Figure 1 outlines the events that extracellular Hb undergoes *in vivo*. These include nitric oxide (NO) scavenging, tissue-damaging oxidative reactions, heme loss, denaturation, aggregation, precipitation, and uptake by macrophages *via* the CD163 haptoglobin-Hb receptor, internalization, and proteolysis of the globin. The erythrocyte membrane and endogenous reduction systems mitigate or prevent these reactions in intact erythrocytes. Outside the red cells, however, all levels of acellular Hb are associated with some degree of enhanced morbidity and mortality rates (122, 138). Small-to-moderate levels of hemolysis (10–100 μM free Hb) can occur after red blood cell transfusions, chronic inflammatory disorders, myocardial infarction, septic shock, sickle cell crises, and physical injury (19, 67). Ten-fold higher levels of free Hb (200–1000 μM) can occur due to acute hemolytic disorders, hemorrhaging, or trauma (122), and even higher levels of acellular Hb (2000–6000 μM) are generated after HBOC infusion (19, 67). Regardless of dosage, there is a general consensus that toxic reactions associated with acellular Hb are either the direct cause of the pathology or an enhancer of pre-existing clinical conditions.

Experiments with recombinant Hb first began in the 1980s when investigators created transgenic organisms capable of producing high yields of human Hb (75). Transgenic pigs were produced that expressed up to 32 g of human Hb per liter of hemolysate, amounting to 24% of the total Hb content

in the erythrocytes (135). Expression of rHb in *E. coli* and *S. cerevisiae* was also accomplished with impressive yields (*i.e.*, 2%–10% of the total cellular protein content consists of Hb) (2, 28, 52, 62, 74, 79, 96, 97, 136, 156). Although each system has its own set of advantages and drawbacks, most recent efforts have focused on *E. coli* production systems.

In the early 1980s, Nagai *et al.* showed that the unfolded β -subunits from human Hb could be expressed and isolated from inclusion bodies in *E. coli*. The denatured globin could be refolded and reconstituted with hemin in the presence of native, reduced α -chains to produce functional, tetrameric Hb (47, 90–92). Subsequently, Nagai developed a similar system for obtaining unfolded α -globin and reconstituting it with native β -chains (92, 125, 146). Two years later, Somatogen's and Nagai's groups developed an Hb operon for coexpression of α - and β -chains with the addition of exogenous heme to produce large amounts of intact, soluble tetrameric Hb in *E. coli* (62, 79, 80). Sligar's group developed a similar Hb operon system to express human adult human Hb A (HbA) (57, 58). The initial expression systems used an extra N-terminal methionine to facilitate bacterial translation of the subunit genes. However, this approach led to heterogeneity due to incomplete processing of the N-terminal Met, and as a result, both groups adopted V1M mutations, for which no cleavage of the N-terminal Met occurs. A few years later, T-J. Shen and C. Ho developed a system in which α - and β -chains containing an extra N-terminal Met were coexpressed with *E. coli* methionine aminopeptidase to allow complete removal of the initiator amino acid and generation of recombinant Hb identical in a primary structure to native HbA (136, 137). The Somatogen and Nagai groups also developed a fused di- α gene, which was inserted in an operon with a copy of the β -gene to express a tetramer that does not dissociate into $\alpha_1\beta_1$ dimers under normal physiological conditions, even when very dilute (79). The latter genetically crosslinked tetramer was called rHb0.1, with the 0.1 referring to one glycine linker between the two α -polypeptides, and this recombinant protein served as the genetic background for their initial rHBOC products. The first prototype for initial animal and phase I human trials was rHb1.1, which had the V1M mutations for expression in *E. coli*, the fused di- α -subunits with one glycine linker, and the Hb Presbyterian mutation (N108K) to decrease oxygen affinity to a value similar to that observed for intact human red blood cells (79).

Engineering Principles for rHBOC Products

Early studies showed that transfusing stroma-free, unmodified, extracellular Hb into humans results in renal dysfunction, hypertension, and abdominal pain (87, 94, 126). Subsequent work indicated that free HbA tetramers dissociate into dimers when diluted into plasma. These dimers pass through the kidney glomerular filters, resulting in an initial rapid clearance. Because the dimers are relatively unstable, autooxidation, hemin loss, and precipitation also occur, leading to obstruction of the kidney pores and oxidative stress with attendant renal failure (3, 21, 46, 74, 124). Another problem is that extracellular Hb rapidly scavenges endothelial NO and interferes with vasoregulation. In addition, the oxygen transport behavior of cell-free Hb is substantially different from that of erythrocyte-encapsulated Hb.

To address clearance issues associated with tetramer dissociation into dimers, investigators initially experimented with ways to inhibit Hb dissociation by chemical, physical, or

genetic methods, including polymerization, conjugation, encapsulation into vesicles, and fusion of the subunit genes (94). Although polymerization and conjugation with polyethylene glycol polymers (PEGylation) strategies significantly mitigated problems associated with short circulation life spans and renal toxicity, there does not appear to be a consensus in the field as to the ideal HBOC formulation. The most successful strategy for rHBOCs has historically involved the use of a di- α gene with a single glycine linker between the C-terminal end of one α -subunit and the N-terminal end of the other and coexpression with V1M β subunits (79). Initial studies with these genetically crosslinked tetramers suggested that a number of biochemical properties needed to be optimized. These include (i) adjusting oxygen affinity and binding rate constants for efficient O₂ transport, (ii) reducing rates of NO dioxygenation (NOD), (iii) increasing resistance to auto-oxidation and hemin loss, and (iv) enhancing globin stability for both high levels of expression during production in *E. coli* and inhibited denaturation during storage or in blood.

Rational mutagenesis can be used to optimize these properties, although alternative engineering approaches could be used, including directed evolution or random mutagenesis to select or screen for new rHb molecules with more desirable properties. Random mutagenesis is a very powerful approach if little is known about a protein's structure and function. Studies of random substitutions at the 64 (E7), 67 (E10), and 68 (E11) positions have been evaluated in sperm whale myoglobin. In this work, colonies were screened for red color as an indication of globin stability and efficient expression. Interestingly, these studies provided little new information about apomyoglobin stability and hemin affinity and confirmed what had already been established from rational mutagenesis experiments (98, 140). Although significant technical advancements have been made in the area of directed evolution, screening a large library of Hb variants simultaneously for four to five distinct properties in bacteria is not yet technically feasible with current methods. Highly complex expression systems with appropriate selective pressures have not yet been devised, and as a result, essential experiments cannot be performed without labor-intensive protein purification steps. Thus, over the past 20 years, only rational and comparative mutagenesis approaches have generated useful and practical results. However, directed evolution- or library-screening approaches could potentially advance the field more rapidly if an appropriate screening methodology were developed.

Figure 2 depicts a hypothetical rHBOC prototype that contains 10 mutations designed to optimize several properties. The purpose of each mutation is described briefly in the legend and in the figure. The initial second-generation rHBOC, which was developed by Baxter Hemoglobin Therapeutics (formerly Somatogen) and Olson's group at Rice University, contained the di- α -Gly linker, V1M mutations for expression in *E. coli*, β V67W and α L29W mutations to reduce NO scavenging $\sim$ 30-fold, and the α H58Q mutation to enhance O₂ dissociation from α -subunits (33, 35, 99). The genetically crosslinked tetrameric prototype was named rHb3011 and then polymerized and PEGylated to generate rHb2.0 by Baxter Hemoglobin Therapeutics (56, 99, 110, 116). Unfortunately, there were no systematic attempts to determine quantitatively the benefit of the additional polymerization and PEGylation steps compared to the simple genetically crosslinked tetramer with low rates of NOD and moderate O₂

FIG. 2. Structure of human rHb containing mutations designed to optimize specific properties. The structure is a composite of the following rHb protein data bank (PDB) files: 101L [di- α Gly(L29F)/ β (WT)], 101M [di- α -Gly(WT) β (V67W)], and 1VWT [α (V96W)/ β (WT)]. The remaining mutations were modeled into the composite structure. The mutations shown were selected to: (i) crosslink the tetramer by creating a di- α gene with a single glycine linker (di- α Gly); (ii) inhibit the rate of nitric oxide dioxygenation (NOD) ≥ 20 -fold by reducing the capture volume in the distal portion of the heme pocket (α L29F or L29W and β V67W or β V106W); (iii) raise P_{50} (lower O_2 affinity) directly by weakening stabilization of the bound ligand (α H58Q) or allosterically stabilizing the T or low affinity quaternary state of the tetramer (α V96W or β K82D [Hb Providence]); (iv) reduce autooxidation rates (α L29F) and inhibit hemin loss (β S44H); (v) reduce production of reactive ferryl species and protein radicals, enhance pseudoperoxidase activity by the α H58Q mutations, and inhibit protein radical formation by Y to F, C to A, and M to F at key locations; and (vi) enhance apoHb stability by strengthening the $\alpha_1\beta_1$ interface (α G15A, β G16A, and β H116I) as described in the text. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

affinity. Both PEGylation and polymerization lower the extent of the hypertensive effect of acellular HBOC products by themselves and so do the mutations, but it is not clear if the effects are additive.

Unfortunately, the L29W(B10) mutation, which lowers the rate of the NOD reaction in α -subunits, also decreases the stability of apoHb, reduces expression yields, and increases autooxidation and hemin loss, even though NO scavenging is reduced, and O_2 binding is optimized (see Figs. 5 and 6). The similar L28W(B10) mutation in β -subunits creates an even more unstable Hb, which is why this replacement was not used in the second-generation product.

The other mutations in Figure 2 have not been used in a product. However, we and others have investigated them as potentially useful substitutions that can (i) increase P_{50} by selectively stabilizing the low-affinity (T) quaternary state by substitutions at allosteric sites [α V96W or β K82D (83, 158)]; (ii) decrease the rate of hemin dissociation from β -subunits [β S44H (160)]; (iii) increase the resistance of apoHb to unfolding [β G16A, β H116I, and α G15A (52)], and (iv) decrease the lifetimes of destructive protein radicals and ferryl species

after treatment with H_2O_2 by changing tyrosines to phenylalanines and *vice versa*, removing cysteines to prevent thiol radicals and oxidation to cysteic acid, and methionines to phenylalanines to prevent degradation to sulfones and other breakdown products (113, 114). It is not clear how many of these mutations are needed for a safe, effective, and commercially feasible rHBOC.

Controlling O_2 Affinity and NOD

The affinity and rate constants for O_2 binding are crucial properties that need to be engineered for rapid uptake and release by Hb in response to changing partial pressures of oxygen in the microcirculation. The optimum parameters for oxygen transport by Hb in human red blood cells appear to be a $P_{50} = 30\text{--}50\text{ }\mu\text{M}$ (20–40 mm Hg) at 37°C and association (k'_{O_2}) and dissociation (k_{O_2}) rate constants $\geq 1\text{ }\mu\text{M}^{-1}\text{s}^{-1}$ and $\geq 2\text{ s}^{-1}$, respectively (22, 35, 63, 76, 93, 104, 150). The mechanisms that govern O_2 binding have been established in detail and used to design recombinant Hb molecules with P_{50} values that range from ≤ 0.2 to $\geq 200\text{ }\mu\text{M}$, using both allosteric and active site mutations (14, 35, 83). The more difficult challenge has been to inhibit the rate of NO scavenging without markedly altering the rates of O_2 uptake and release. The key reactions of NO with reduced Hb are shown in Figure 3A (69, 99). Even if some free NO binds to deoxyHb, its ultimate fate will be irreversible dioxygenation to nitrate if any O_2 is present (45). To reduce the rate of NO scavenging, strategies had to be devised to selectively inhibit NOD.

Both the NOD reaction to produce nitrate and bimolecular NO binding to Fe(II) are limited by the rate of entry and capture of the ligand (38, 101). The distal histidine (E7) acts like the thumb of a baseball glove, rotating outward to allow ligand entry, which is then captured in the internal pocket surrounded by Leu(B10), Phe(CD1), Val(E11), and Leu (G8) (Fig. 3B) (13–15, 131). Both NOD and bimolecular ligand binding can be inhibited by filling the back of the pocket with large Phe or Trp replacements at these positions (Fig. 3B) (15, 35, 38, 101), which cause the incoming ligand to reflect back out of the pocket before the His(E7) gate closes. Insertion of large aromatic side chains at the B10 position are much more effective at reducing k'_{NOD} in α -subunits than mutations at the E11 and G8 positions, whereas the opposite is true in β -subunits (13, 38). The largest reductions in the rate of NO scavenging in β -subunits have been achieved with Trp replacements at the E11 position (13, 38, 99).

In α -subunits, the bimolecular rates of NO and O_2 binding are reduced to almost the same extent as the rates of the NOD reaction by single Trp or Phe mutations at the B10, E11, and G8 positions. However, replacement of His(E7) with Gln in these space-filling mutants allows discrimination between ligand binding and the NOD reaction. The lack of ordered water in the distal pocket of Gln(E7) deoxy- α -subunits (39) allows more rapid bimolecular ligand binding, but hydrogen bonding between Gln(E7) and bound O_2 still occurs and acts as a barrier to NO entry and dioxygenation. The hydrogen bond donated by Gln(E7) to bound O_2 is weaker than that donated by His(E7), which reduces O_2 affinity, increases the rate of O_2 release, but, combined with Trp(B10), maintains a low value of k'_{NOD} (Fig. 4B) (35).

This multiple-mutation strategy was used to construct prototype rHb molecules with both high and low O_2 affinity

FIG. 3. O_2 binding, NO dioxygenation, and active site of Hb subunits. (A) Rates of reaction of NO and O_2 with adult human Hb A (HbA) (35, 45, 99). (B) Structure of the active site of oxygenated human HbA β subunit (PDB 2DN1). The helical positions LeuB10, HisE7, ValE11, HisF8, and LeuG8 correspond to the 29, 58, 87, 62, and 101 sequence positions, respectively, in α subunits and the 28, 63, 67, 92, and 106 positions in β subunits of human HbA. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

and systematically reduced NOD rates and blood pressure effects when transfused into rats (Fig. 4B) (33, 99). The correlation between k'_{NOD} and changes in mean arterial blood pressure (MAP) or total peripheral resistance (TPR), ΔMAP or ΔTPR in Figure 4B, provides strong, if not unambiguous, evidence that the blood pressure effect caused by acellular Hb is due to NO scavenging. The most successful rHBOC prototype was called rHb3011 [di- α (L29W/H58Q) β (V67W) in Fig. 4B] and has a markedly reduced k'_{NOD} , a high P_{50} , and moderate rates of O_2 binding and release (33, 99). Acellular rHb3011 does not cause a rapid increase in TPR (MAP/cardiac output) when infused into rats at a level of ~ 0.35 g of rHb per kg of animal, which increased the blood volume by 10% (99), whereas the control, rHb1.1, with a high rate of NOD, causes a $\geq 30\%$ increase (Fig. 4C). The simplest interpretation of Figure 4C is that the low rate of NOD reduces the toxicity of rHb3011 with respect to hypertension. The rHb3011 molecule has also been shown to reduce or prevent

gastrointestinal dysmotility (54), and its genetic background was built into the last Baxter HBOC prototype, rHb2.0, which also showed no hypertensive side effect (56, 84, 110, 116, 142, 155). However, rHb2.0 was later shown to cause complement activation in initial safety trials in humans (40), which, as shown in Figure 5, appears to be due to high rates of auto-oxidation, heme loss, and/or globin denaturation, properties that were not optimized in the second-generation product, but have been shown to lead to endothelial damage and inflammatory responses (8–10).

Autooxidation, Heme Loss, and Reactivity with H_2O_2

Recent work has demonstrated that, in addition to NOD activity, spontaneous and H_2O_2 -induced oxidation of Hb iron atoms to the ferric and ferryl states can lead to diverse pathophysiologies that are thought to be related to heme loss and destruction, iron-catalyzed redox reactions, and globin

FIG. 4. Successful variants for independently varying P_{50} (83) and NOD rates (99) and for reducing the blood pressure effect in rat model systems by lowering k'_{NOD} (36, 99). rHb1.1 contains the Hb Presbyterian mutation β N108K to maintain a high P_{50} . HSA refers to infusion of human serum albumin instead of a recombinant hemoglobin-based oxygen carrier (rHBOC) (36, 99). (A) Increasing P_{50} with allosteric mutations (83); (B) Decreasing mean arterial blood pressure (MAP) and/or total peripheral resistance (TPR) by reducing k'_{NOD} , the bimolecular rate constant for NO dioxygenation (99); (C) Eliminating the blood pressure effect by distal pocket mutations (99).

FIG. 5. Autooxidation, heme loss, aggregation, and precipitation of rHb3011 at 37°C, pH 7. (A) Spectra of oxy-rHb0.1, bold lines every 1 h; gray lines every 5 h; *insets*, time courses for $A_{576\text{nm}}$. **(B)** Same data for oxy-rHb3011. In both **(A)** and **(B)** the initial $A_{576\text{nm}}$ was normalized to 1.0, and catalase and superoxide dismutase were present at 12 and 6 mmol/mol of heme respectively. **(C)** Time courses for autooxidation, showing the normalized change of the ratio $A_{576\text{nm}}/A_{630\text{nm}}$; **(D)** time courses for heme loss measuring the decrease in $A_{410\text{nm}}$ as heme transferred to the H64Y/V68F apoMb reagent. The increase at long times for rHb3011 is due to aggregation of the apoglobin and an increase in turbidity.

FIG. 6. Autooxidation and denaturation of α B10 and β G8 mutants in wt/mutant hybrid rHbO₂ tetramers at pH 7, 37°C. Spectral changes following autooxidation of (A) α (L29F) β (wt); (B) α (L29W) β (wt); (C) α (wt) β (L106F); and (D) α (wt) β (L106W) recombinant human Hb. The *black lines*, spectra collected every 1 h for the first 10 h; gray lines, every 5 h afterward; *insets*, time courses for $A_{576\text{nm}}$. Only half the expected absorbance change at $A_{576\text{nm}}$ is observed for α (L29F) β (wt) after 50 h, suggesting very slow autooxidation of the mutated subunit. In all cases, the initial $A_{576\text{nm}}$ was normalized to 1.0. Catalase and superoxide dismutase were present at 12 and 6 mmol/mol of heme, respectively.

denaturation (112, 115). These events may be linked to the adverse events observed after HBOC infusion and must also be addressed in rHBOC prototypes. Baldwin, Alayash, and others have shown that these oxidative processes can damage the endothelial cells of blood vessel walls and the epithelial cells lining intestinal villi after transfusion with simple tetrameric HBOCs (4, 5, 8–10, 20). Thus, in addition to reducing NO scavenging, protein-engineering strategies also need to generate rHbs that are more resistant to oxidation and denaturation than the native globin.

One simple method for screening resistance to auto-oxidation and denaturation is to incubate rHb variants in an air-equilibrated buffer and follow autooxidation and precipitation over a period of days at pH 7 at 37°C. Sterile conditions are required to prevent bacterial growth, and catalase and superoxide dismutase are necessary to prevent acceleration due to H₂O₂ accumulation. Evaluations of several candidate rHbs are shown in Figure 5. The results show that oxygenated rHb3011 is unstable for long periods of time at 37°C. The absorbance spectra for rHb3011 are suggestive of the formation of soluble aggregates, which then precipitate within 30 h (Fig. 5B and inset). By contrast, rHb0.1 exhibits a slow autooxidation process, with isosbestic points and a two exponential decay curve (Fig. 5A) representing differences between the α - and β -subunits (148, 162). This behavior is similar to that observed for wild-type HbA obtained from donated red blood cells. The samples in Figures 5 and 6 had not been subjected to a final removal of LPS. When LPS levels are lowered to <1 E.U. per ml of concentrated protein (~1 mM heme), the rates of precipitation after oxidation and heme loss are slowed, but the relative behavior remains the same, with rHb3011 being very unstable at 37°C (data not shown). Thus, LPS appears to facilitate unfolding of apoglobin, perhaps by either binding to the empty heme pocket or acting as a surfactant (66, 120, 121).

An estimate of the rate of autooxidation for rHb3011 can be obtained by plotting the change in the ratio $A_{576\text{nm}}/A_{630\text{nm}}$ (~[HbO₂]/[metHb]) as a function of time to compensate for changes in turbidity and precipitation of the sample. As shown in Figure 5C, rHb3011 autooxidizes 5-fold to 20-fold more rapidly than rHb0.1. The instability of the resultant met-rHb3011 is most likely due to rapid heme loss and unfolding. When we looked at heme dissociation directly (Fig. 5D), rHb3011 loses its prosthetic group about two-to-four times more rapidly than rHb0.1. The resultant apo-rHb3011 also rapidly denatures, increasing the turbidity of the solution within 1–2 h, as shown by the almost linear slow increases in absorbance at 410 nm (Fig. 5D). These detrimental properties of the TrpE7 and TrpE11 mutations probably account for the unsuccessful initial human safety trials of rHb2.0 that was based on the rHb3011 design. The oxidative stress and inflammation resulting from plasma Hb denaturation seem a likely cause of complement activation, although this conclusion needs further testing (40). Regardless, it is clear that alternative strategies are needed for lowering NO scavenging while retaining low rates of autooxidation, heme loss, and globin unfolding.

The instability of rHb3011 is due to the presence of Trp29(B10) in α -subunits and Trp67(E11) in β -subunits. Similar rapid rates for the appearance of turbidity and precipitation occur for α (L29W) β (wt), but not α (L29F) β (wt) (Fig. 6A, B), and for α (wt) β (V67W), but not α (wt) β (V67F), rHb

(data not shown). In contrast, the α (wt) β (L106W) mutant was resistant to unfolding and precipitation (Fig. 6C, D), and thus Trp106(G8) can be used in β -subunits to slow NO scavenging without compromising stability. One lesson learned from these simple experiments is that NO scavenging can be reduced without compromising stability by using α Phe29, β Leu67, and β Trp106 insertions. Individually, the β V67L and β L106W mutations reduce k'_{NOD} roughly ~4-fold (data not shown), and in combination, they are expected to achieve a ≥ 16 -fold decrease. The cause for the reduction of capture volume in deoxy- β TrpG8 subunits is the presence of a water molecule in the distal pocket, which is held in place by three hydrogen bonds, including one from the indole N–H atoms (13).

Alayash's group and many others (5, 18, 20, 149) have suggested that the ferryl species and protein radicals produced by the reaction of H₂O₂ with acellular Hb cause many of the side effects associated with acellular Hb. This same group has shown that administration of haptoglobin can mitigate the oxidative stress caused by acellular Hbs (6, 20, 127). Alternatively, Reeder, Cooper, Wilson, and colleagues have suggested that Hb can be engineered to be less toxic with respect to the generation of long-lived protein radicals by engineering electron transfer pathways that enhance pseudoperoxidase activity by speeding up reduction of ferryl species to more-inert ferric states (112–114). For example, they have shown that α -Tyr42 facilitates reduction of ferryl α -subunits back to the ferric state in the presence of reducing agents found in plasma, including ascorbate, and suggested that engineering more efficient reduction pathways would stabilize acellular Hb (113). Similarly Alayash has also suggested that replacing more reactive amino acids with more inert ones might also prevent Hb damage and radical generation (*i.e.*, C to A, and M to F in Fig. 2)

Increasing Expression Yields with Heme Transporters

A major limitation in the production of recombinant Hb stems from its instability during expression in *E. coli*. Although recombinant technology allows for expression of large amounts of protein, often the majority of the globin lacks the heme prosthetic group that is required for function and stability (57, 157, 158). Heme synthesis by *E. coli* is simply too slow to keep up with induced synthesis of recombinant heme proteins from high-copy-number plasmids, and thus a high apo-/holoprotein ratio is observed in the absence of added heme. The availability of heme is the most dominant factor limiting the high-level expression of recombinant Hb in *E. coli*. Hb expressed without adequate amounts of heme results in both proteolysis and precipitation into inclusion bodies, from which reconstitution of holoHb is inefficient, time consuming, and costly (57, 157).

In current rHBOC production protocols, holoHb expression is maximized by adding ~160–320 μ M free heme to the growing culture after induction of the Hb genes (Fig. 7). Normal *E. coli* strains do not have an outer membrane heme transporter and cannot use heme as an iron source. Thus, very high levels of exogenous heme are needed for passive diffusion, and only certain strains, presumably with smaller, more porous outer membranes, can be used (primarily based on *E. coli* JM109 strains). This protocol was developed by Somatogen, Inc., in the early 1990s (80) and is also used by

FIG. 7. Strategies for co-expressing recombinant hemoglobin and bacterial heme transporter genes. Relative expression of wild type rHb0.1 (di- α Gly/ β wt) in *Escherichia coli* as a function of media heme concentration with and without co-expression of the *Plesiomonas shigelloides* heme transport system [pHUG(IR)] or with *chuA* from *E.coli*O157:H7 [pHPEX(IR)] where IR is the iron regulated promoter. Expression was measured using the CO derivative assay described by Graves *et al.* (52). The expression levels are relative to that measured for rHb0.1 expression in 10 μM heme with no heme transport co-expression. **(A)** Expression in the JM109-based *E. coli* cells developed by Somatogen, SGE 1661 cells using the pRHBOC plasmid with the α and β HbA genes under *P_{tac}* control and the pHUG21 plasmid with the *hug* genes under *P_{IR}* control. **(B)** Expression in BL21 DE3 *E. coli* using the pGlobin plasmid with the α and β genes under *P_{T7lac}* control and the same pHUG21(IR) plasmid for *hug* gene expression. The expression levels are normalized to the control in **(A)**, which is our standard system. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Chien Ho's group for producing recombinant Hb in *E. coli* (137). Stimulation of endogenous heme synthesis by *E. coli* can be achieved by the addition of δ -aminolevulinic acid, but this reagent is too expensive to use routinely for large-scale production, and the enhancement of rHb production is not as great as adding high levels of exogenous hemin. However, the addition of large amounts of hemin leads to the generation of degradation products that contaminate the rHb preparations, including metal-free porphyrins that must be removed to prevent photoinduced oxidation of the samples (109).

Attempts have been made to address the heme uptake problem by coexpressing heterologous heme transport systems with the Hb genes, so that less hemin has to be added and uptake is more efficient. We have examined two systems: (i) the pHUG system developed originally by D. Henderson at the University of Texas, Permian Basin, which expresses the complete *Plesiomonas shigelloides* heme utilization gene (*hug*) operon (52, 153); and (ii) the pHPEX system, which was developed by C. Varnado and D. Goodwin at Auburn University (151). The latter system coexpresses the apoglobin genes with the *chuA* outer membrane receptor from pathogenic *E. coli* O157:H7. Two sample coexpression experiments are shown in Figure 7. In panel A, the *E. coli* strain SGE1661 (JM109 based) was grown with the rHb vector pRHBOC

alone or cotransformed with either the *hug* vector pHUG21 or the *chuA* vector pHPEX. In panel B, the *E. coli* strain BL-21 DE3 was grown with the pET-based rHb vector pGlobin or cotransformed with the pHUG21.

In each case, rHb expression was induced by IPTG addition. The heme transport genes are regulated by the iron-regulated (IR) promoter and induced by iron depletion using the iron chelator ethylenediamine-N,N'-diacetic acid (52, 153). As shown, much more Hb is produced at low (20–40 μM) external heme concentrations when the heme transporters are coexpressed, and the pHUG system is more effective than the pHPEX system. High levels of expression are also seen at larger concentrations of external heme, and, under these conditions, coexpression of the *hug* genes does not significantly increase the apparent expression levels. However, samples from the high heme incubations contain significant levels of sulfheme and partially denatured holoprotein, which lead to large decreases in final product yields.

Sulfhemoglobin, having a characteristic absorbance peak in the visible spectrum around 620 nm, is a result of the reaction of inorganic sulfides with iron-protoporphyrin IX. The modified heme is likely created as a result of redox chemistry from reactive oxygen species (ROS, *i.e.*, $\text{O}_2^{\bullet-}$, H_2O_2 , and Fe(IV)=O) generated in the cells. ROS levels are increased by

the presence of high amounts of the highly reactive free heme. Reports of sulf- and other modified hemes have also been described for the expression of recombinant myoglobin variants with green to blue colors (49, 77). Although heating steps or redox recycling can be used to remove protein with modified heme, these procedures often lead to lower protein yields. In our hands, optimal holoHb production requires coexpression with the *hug* genes at low exogenous heme levels ($\sim 20\text{--}40\ \mu\text{M}$).

Expression of Hb in *E. coli* cell lines that are less porous (*i.e.*, BL-21) than JM109 cells requires the presence of an outer membrane heme receptor to obtain measureable amounts of soluble holoHb, regardless of the concentration of heme added (Fig. 7B). This need for heme transport has also been shown for the expression of high levels of other heme proteins in BL-21 cells (117, 151). Expression of Hb with a heme transporter and low concentrations of heme result in an increase in holoprotein production, and at the same time limits the fraction of modified heme complexes, which decrease the yield of functional and stable human Hb.

Increasing Expression Yields with α -Hb Stabilizing Protein

α -Hemoglobin stabilizing protein (AHSP) is a conserved molecular chaperone that is thought to facilitate HbA expression in mammalian erythroid precursors (48, 68). Work characterizing this protein suggests that it facilitates HbA α -subunit structure acquisition and diminishes α -subunit reactivity with ROS by stabilizing a hemichrome-folding intermediate (42, 43, 163). These findings indicate that coexpressing AHSP with rHb in *E. coli* might result in greater production yields of rHb. Vasseur-Godbillon *et al.* (152) investigated this hypothesis by coexpressing AHSP with α -subunits. Although β -subunits were not included in this study, it was found that AHSP enhanced α -subunit expression yields dramatically. More recently, Faggiano *et al.* (41) coexpressed AHSP with both α - and β -subunits to produce a self-polymerizing bovine rHb in *E. coli*. In contrast to the work of Vasseur-Godbillon *et al.* (152) on the expression of α -subunits alone, Faggiano *et al.* (41) found that AHSP impedes rHb production when expressed with the α - and β -subunits in

equimolar amounts. We have independently conducted co-expression assays in our laboratory using rHb in pET and AHSP in pBAD expression vectors to vary the levels of AHSP with respect to the α - and β -subunits. Our preliminary results also suggest that high [AHSP] may inhibit rHb formation, but low catalytic levels of AHSP may facilitate production of holo-rHb (86). However, much more work is needed to verify these observations.

Enhancing apoHb Stability

In 2000, we discovered that apoMb and apoHb stability varies widely among different mammalian species and that it is a major limiting factor in the production of intact holoproteins in *E. coli* (52, 132, 140). Sperm whale myoglobin, sperm whale Hb, and human fetal Hb are all more stable than human Hb A (52). By comparing the primary amino acid sequences of these proteins, Graves *et al.* (52) identified three mutations that markedly increase the resistance of apoHbA to GdmCl-induced unfolding (Fig. 8A). These mutations include α G15A, β G16A, and β H116I. Although β H116I is located directly in the $\alpha_1\beta_1$ interface within HbA, the other mutations are solvent-exposed residues of the A-helices very near the AB loop at the edges of the interface. Therefore, they most likely exert their effects by increasing the stability of the A helix secondary structure.

These mutations were incorporated into rHb expression vectors, and the proteins produced using these constructs were purified and characterized by Graves *et al.* (52). As shown Figure 8A, the stabilizing effects of these mutants are additive, and each mutation progressively increases rHb resistance to chemical denaturation by GdmCl. The expression yields of these rHbs were also found to be variable, with the incorporation of each mutation resulting in predictable increases in rHb expression levels. These results are shown in Figure 8B, which is a plot of expression level *in vivo* against GdmCl-induced denaturation midpoint measured *in vitro* [data taken from Graves *et al.* (52)]. These gains appear to be additive to the enhancement obtained with the use of the *P. shigelloides* heme transport (*hug*) genes discussed in the previous section; however, the standard errors in the small growth assay are

FIG. 8. Correlation between apoglobin stability and expression yield of holoHb. (A) GdmCl-induced unfolding curves of wild-type human rHb and three mutants containing replacements based on comparisons between sperm whale, adult human, and fetal human Hb sequences (52). There is a clear progressive increase in stability, with the [GdmCl]_{midpoint} increasing from ~ 1.2 to $1.9\ \text{M}$. **(B)** Correlations between the grams of holohemoglobin isolated per gram of cell paste with GdmCl midpoint concentrations of apoHb unfolding curves (52). Closed circles, co-expression of rHbs with *P. shigelloides* *hug* genes and in the presence of $60\ \mu\text{M}$ external heme. Open circles, yields without expression of *hug* genes $\pm$ added external heme. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

very large and much more systematic work in bioreactors is needed (Fig. 8B).

Bioreactor Protocol Optimization

Previous work using small scale assays have shown that Hb production can be increased by ten-fold in *E. coli* BL21(DE3) transformed with a single plasmid containing human Hb genes and *P. shigelloides* heme transport genes, versus the strain containing only the Hb genes (153). As described in Figure 8, Graves *et al.* (52) found that modifying the Hb genes to generate a more stable form of Hb also increased Hb produced in *E. coli*. Recently, Smith *et al.* (139) described a protocol to produce Hb in a bioreactor, which combined both approaches. Their procedure included the following important features: (i) expression of the human Hb variant with the stability mutations on the same plasmid as the *P. shigelloides* heme transport genes (52); (ii) the use of a mass flow equation for glucose addition (73) and close monitoring of glucose levels to optimize cell growth and plasmid retention; (iii) addition of phosphate later in the run to better enable the transport and metabolism of glucose (118); and (iv) induction of the culture during the mid log phase of growth and the addition of a heme/base feed at the time of induction that is controlled by pH.

Using this procedure, the cultures reached an average OD₆₀₀ of 281 and a dry cell weight of 84 g/L, with the OD₆₀₀ and dry cell weight increasing threefold and twofold, respectively, over what was seen at induction. In addition, the cultures produced 6.6 g/L of Hb in clarified lysates or ~0.08 g of Hb/g of dry weight cells. This production level is similar to that shown in Figure 8 for the triple stability mutant in small scale 50 ml cultures without purification [~0.06 g/g dry wt, assuming that the dry weight of cells is 0.3 that of the wet weight of paste (16)]. Both results are highly encouraging and suggest that the production of recombinant Hb in *E. coli* may be an economically viable method of obtaining therapeutic Hb, as was argued previously by Baxter Hemoglobin Therapeutics (109, 158).

Cost of rHb Production

One of the major issues with rHBOCs is production cost. In the United States, one unit of whole blood can vary from 405 to 550 ml and is collected from adult donors whose Hb concentration can range from 12.5 to 18.0 g/dL (119). A single unit of blood can therefore contain 50.6–99.0 g of Hb. In 2011, the United States Centers for Medicare and Medicaid Services fixed reimbursement prices at \$202.83/unit for whole blood and \$154.32/unit for packed red blood cells (www.aabb.org/programs/reimbursementinitiatives/Pages/12hoppsrule.aspx). These prices do not include costs that arise subsequent to purchase from blood centers (*e.g.*, those associated with blood administration, storage, transport, disposal, and adverse event treatment), and blood centers may of course charge more or less than the fixed reimbursable costs. However, these reimbursable prices suggest that the cost of Hb on a per unit mass basis is roughly \$2/g to \$4/g. As a comparison, current rHb expression methods using *E. coli* can generate 0.38–6.6 g of rHb per liter of clarified bacterial lysate (80, 109, 139, 158). Based on nonbulk costs of growth media, hemin, antibiotic, glucose, and expression inducer and as-

suming a 60% purification recovery we estimate that it is possible to produce rHb at a cost of approximately \$11/g. However, if we include costs of equipment investment and maintenance, utilities, labor, waste disposal, purification, downstream processing, and other operating expenses, the cost increases to ≥\$200/g. However, with further optimization and economies of large-scale production, rHb could become comparably priced or only moderately more expensive than whole blood and packed red blood cells as a source of Hb.

Endotoxin Removal

Although expression of rHb in bacterial systems has the potential for large-scale applications, endotoxin levels are increased due to antigenic LPS present in the bacterial cell wall that bind to Hb (66, 120). In addition, Levin and coworkers have provided evidence that Hb binding enhances the inflammatory responses caused by LPS (66, 121). The standard purification procedure for rHb decreases endotoxin levels by several orders of magnitude. However, additional steps are needed to lower endotoxin levels for safe use in humans.

In the early 1990s, Somatogen, Inc. conducted a variety of initial safety trials with healthy human volunteers with their initial rHb1.1 (Optro) product. Many of the subjects developed pyrogenic responses and gastrointestinal problems, most of which were the result of bacterial endotoxin and protein contamination (109). These results led to the development of a “flash” heating step for the CO complex followed by a Q Sepharose FF column, both of which remove large amounts of weakly bound LPS, free porphyrin, and other contaminants and decrease the endotoxin levels to ≤2 EU/g (109). Polymyxin B agarose, diethylaminoethyl (DEAE) cellulose, or ε-poly-L-lysine cellulose can be used to safely and efficiently remove any remaining LPS as long as breakdown of the column matrix does not occur (61). These techniques led to the use of the first (rHb1.1) and second (rHb2.0) products in humans where, as described in previous sections, the observed side effects were much less severe and appeared to be due to NO scavenging (Fig. 4) and protein instability (Fig. 5). The latter are intrinsic properties of the Hb itself and not bacterial contaminants, although it is always difficult to exclude the possibility of contamination.

Although current LPS reduction techniques may be satisfactory, most pharmacopeias specify an upper limit of 5 EU per kg of body mass per hour for intravenous therapeutics (107). Infusion of therapeutic levels of rHBOCs in a trauma care context will require hundreds of grams of protein. Thus, LPS-mitigation requirements for this amount of a rapidly infused rHBOC are more stringent than most other therapeutic proteins, which are used in milligram-level quantities. Thus, although expression in *E. coli* is required for pilot-scale experiments and screening of small to medium sized libraries of prototype rHBOCs, it may eventually be more practical to use previously developed heterologous expression in mammalian systems for large scale manufacturing of a rHBOC product (135).

Future Prospects

The use of extracellular Hb as a blood substitute is probably the most economically and scientifically feasible way to produce an oxygen carrier when donated blood is not available.

Recombinant technology allows the use of rational protein engineering strategies to mitigate or eliminate toxic effects caused by the presence of acellular Hb in the circulation (*i.e.*, the problems shown in Fig. 1). Genetic crosslinking of α subunits prevents dissociation into dimers. Detailed mechanisms of O₂ binding to and NOD by Hb provide a blueprint for addressing efficacy and interference with vasoregulation. Similar protein engineering strategies can be used to inhibit autooxidation, heme loss, and globin unfolding, all of which can cause oxidative stress and inflammation. Future work should be aimed at reducing production costs and identifying the specific ensemble of suitable rHb mutations.

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Abbreviations Used

AHSP = α -hemoglobin stabilizing protein
 Apo = heme-lacking
 Hb = hemoglobin
 HbA = adult human hemoglobin A
 HBOC = hemoglobin-based oxygen carrier
 heme = ferroporphyrin IX
 hemin = ferriprotoporphyrin IX
 IR = iron regulated
 LPS = lipopolysaccharides
 MAP = mean arterial blood pressure
 met = ferric oxidation state
 NO = nitric oxide
 NOD = nitric oxide dioxygenation
 rHb = recombinant hemoglobin
 rHBOC = recombinant hemoglobin-based oxygen carrier
 ROS = reactive oxygen species
 TPR = total peripheral resistance