Rice University

**Enhancing Stability and Expression of Recombinant Human
Hemoglobin in *E. coli*: Progress in the Development of a Recombinant
HBOC Source**

by

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ABSTRACT

Enhancing Stability and Expression of Recombinant Human Hemoglobin in *E. coli*: Progress in the Development of a Recombinant HBOC Source

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The commercial feasibility of recombinant human Hb (rHb) as an O₂ delivery pharmaceutical is limited by the production yield of holoprotein in *E. coli*. Currently the production of rHb is not cost effective for use as a source in the development of third and fourth generation Hb-based oxygen carriers (HBOCs). The major problems appear to be aggregation and degradation of apoglobin at the nominal expression temperatures, 28-37° C, and the limited amount of free heme that is available for holohemoglobin assembly. One approach to solve the first problem is to inhibit apoglobin precipitation by a comparative mutagenesis strategy to improve apoglobin stability. α Gly15 to Ala and β Gly16 to Ala mutations have been constructed to increase the stability of the A helices of both subunits of adult human hemoglobin (HbA), based on comparison with the sequences of the more stable sperm whale hemoglobin subunits. Human fetal hemoglobin is also known to be more stable than HbA, and comparisons between human β and γ (fetal Hb) chains indicate several substitutions that stabilize the $\alpha_1\beta_1$ interface, one of which, β His116 to Ile, increases resistance to denaturation and enhances expression in *E. coli*. These favorable effects of enhanced globin stability can be

augmented by co-expression of bacterial membrane heme transport systems to increase the rate and extent of heme uptake through the bacterial cell membranes. The combination of increased apoglobin stability and active heme transport may enhance holohemoglobin production to levels that may make rHb a plausible starting material for all extracellular Hb-based oxygen carriers.

ACKNOWLEDGEMENTS

“If you're going through hell, keep going.”

-Winston Churchill

“How few there are who have courage enough to own their faults, or resolution enough to mend them.”

-Benjamin Franklin

“The price of excellence is discipline. The cost of mediocrity is disappointment.”

-William Arthur Ward

“A lifetime of happiness? No man could bear it: it would be hell on earth.”

-George Bernard Shaw

“Beware of false knowledge; it is more dangerous than ignorance.”

-George Bernard Shaw

“My great concern is not whether you have failed, but whether you are content with your failure.”

-Abraham Lincoln

“Men can acquire knowledge, but not wisdom. Some of the greatest fools ever known were learned men.”

-Spanish Proverb

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CHAPTER 1

Introduction and Background

Introduction

A large array of hemoglobin-based oxygen carriers (HBOC) candidates have been examined, many developmental milestones have been met, and efficacy and side effects have been addressed through a variety of protein engineering and packaging strategies [1-7]. The first generation extra-cellular HBOCs encountered major obstacles due to short circulation half-lives, NO scavenging and blood pressure elevation, increased O₂ affinity and poor transport, renal toxicity, and symptoms suggestive of oxidative stress [1-5, 8-10]. These side effects initiated further development and optimization of potential HBOC products. Both micromolecular and macromolecular protein engineering approaches were used to construct second-generation HBOC prototypes to mitigate some of these clinical problems.

The micromolecular approach focused on side effects due to high rates of auto-oxidation, generation of radical oxygen species (ROS), too high or too low O₂ affinity (P_{50} adjustments), and unchecked scavenging of NO. These problems were addressed by site-directed mutagenesis using recombinant hemoglobin (rHb) technology. Detailed analyses of the oxidative stress caused by stroma free hemoglobin (SFH) have been published, and a template for focusing on the issues of ROS production has been described in detail in several comprehensive review articles by Alayash et al. [1, 6, 7].

Protein engineering and mutagenesis analyses of the distal pocket of recombinant Mb model systems and of rHb have led to detailed mechanisms for O₂ binding, auto-oxidation, heme loss, and scavenging of NO by dioxygenation to nitrate [10]. These mechanisms have been used to reduce ROS production, adjust P_{50} , and reduce the rate of NO scavenging over 30-fold [5, 8, 10-12].

The macromolecular approach focused on the reduction of renal toxicity, enhanced circulation life times, and inhibition of extravasation into the endothelium. These problems were addressed by the development of new protein crosslinking, polymerization, and decoration strategies. It has been recognized for over 40 years that clearance of SFH is caused by dissociation of extracellular hemoglobin into dimers when diluted in plasma [2, 6, 7]. Hb dimers are small enough to pass through the glomerulus of the nephrons in the kidneys and may play a role in acute renal toxicity due to rapid oxidation, heme loss, and precipitation [13-16]. Crosslinking, polymerization and increasing the overall molecular weight of SFH has been carried out to reduce tetramer dissociation, increase circulation half-life, and reduce rates of autooxidation, heme loss, and precipitation [1, 14, 17]. Stabilization of tetramers has been achieved by intramolecular genetic crosslinking, decoration with polyethylene glycol (PEG) polymers, intermolecular chemical crosslinking, and nanotechnology to create 'nanocapsules' or artificial cells with Hb encapsulated at high concentrations [14, 17-21].

Using either micro- or macromolecular approaches, various biotechnology companies and several of groups of basic research scientists have continued to develop HBOC technology for the production of a safe, effective, and commercially feasible

extracellular Hb-based blood substitute. However, to date no HBOC preparation has been approved for routine clinical use in the United States or Europe.

Background

1.1 Protein Engineering of Recombinant Hb-based Oxygen Carriers

With the discovery of the protein structure of hemoglobin by Perutz [22, 23], detailed analyses and studies of the effects of mutagenesis have been performed to elucidate the function of Hb and to develop protein engineering strategies to incorporate any desired effect. The study and examination of hemoglobin stability and folding represents a large portion of research on this family of proteins. Antonini, Brunori, Beychok, and others have attempted to characterize the stability and folding patterns of hemoglobin and its individual subunits for many years but with little success due to the inherent instability of these proteins at ambient temperatures [24, 25]. In contrast, sperm whale myoglobin (SWMb) is very stable, serves as a model system for stability studies of Hb monomers, and has allowed progress to be made toward understanding globin folding [26]. The extensive work on the mechanism of apo-myoglobin unfolding can be used as a model to interpret guanidine chloride (GdmCl) induced unfolding of apoHb because of the similarities in sequence and structure of SWMb and HbA and HbF subunits. GdmCl induced denaturation of apo and holo-Hb is reversible, whereas acidification, heating, and urea denaturation of HbA is irreversible [27, 28]

1.2 Cost Effective HBOC's: An Unlimited, Cheap Source is Possible

A major issue in the development of HBOCs is the cost, availability, and safety of the supply of hemoglobin. Currently, purified extracellular hemoglobin is obtained from human donors, cows, or heterologous expression in *E. coli*. Each Hb source comes with its own set of problems. Most of the first generation products were developed using human HbA obtained from outdated whole blood that can no longer be used by hospitals. The exception is the HBOC product from Biopure, Inc., which is based on bovine Hb purified from cow blood. The human sources of Hb still rely on donations and have the potential for retaining blood borne pathogens, particularly viruses (i.e. HIV, Hepatitis C and B, etc.). However, bovine Hb has the potential for contamination with prions, which cause Creutzfeldt-Jakob ("mad cow") disease [29, 30]. In contrast, a potentially limitless supply of Hb can be produced through recombinant technology in bacteria without the possibility of contamination by mammalian pathogens and viruses. In this case, the problem is production cost, which is formidable.

Recombinant Hb expression technology was first developed by Nagai and Thorgersen in 1984, who expressed α and β subunits separately as insoluble fusion proteins with an 31 amino acid λ phage N-terminal sequence, which allowed post-translational removal by Factor Xa. The denatured subunits were re-solubilized from inclusion bodies and then incubated with reduced holoprotein subunit partners and heme to form to the functional tetrameric holo rHb [31, 32]. Between 1988-1991, Nagai and coworkers at both Cambridge University and Somatogen, Inc. developed a simpler recombinant Hb system in which both genes were placed on single plasmid, co-expressed with V1M mutations for initiation, and heme was added externally to facilitate

holoprotein production directly in the bacterial cytoplasm without the need for modification or heme insertion [14, 33, 34]. In parallel, the workers at Somatogen, Inc. adopted the same strategy for expression of hemoglobin in the yeast *S. cerevisiae* [35].

A similar yeast expression system was developed a year later by Delta Biotechnology in England [36, 37]. Sligar and coworkers also developed a single plasmid recombinant hemoglobin expression system for *E. coli* at about the same time [29, 38], and Ho and Shen constructed a system based on the Somatogen work, in which an "extra" N-terminal Met was used for initiation and then cleaved off in the *E. coli* by overexpression of its methionine amino peptidase (MAP) [39, 40]. Reported values for holoHb formation were 3 to 5% of the total soluble proteins in each microorganism in the initial papers. A wide variety of studies were carried out using the yeast expression system both in the U.S. and in England to test the characteristics and to study certain hemoglobinopathies [41-45]. However, most workers abandoned the yeast system because of greater production of holo-rHb in *E. coli*, up to 10-30% of soluble protein [21, 46, 47] and the development of methods to remove free porphyrins and lipopolysaccharide contaminants [48] and to equilibrate the heme orientational conformers [40]. These downstream processing advances led all workers in the field to adopt *E. coli* as the organism of choice to produce recombinant hemoglobin as source material for HBOCs.

The proposed amount of extracellular rHb needed for a HBOC unit, which is equivalent to the O₂ transport capacity of whole blood, ranges from 30 to 75 grams [4]. The current cost of a unit of donated blood to hospitals ranges from \$150 to \$300 per unit or roughly \$5.00 per g of Hb [49]. Thus, recombinant hemoglobin needs to be produced

in pyrogen-free form for \$5 to \$10 per gm, which is a challenging goal for any recombinant protein produced in *E. coli*. To reduce production costs, both the amount of rHb that can be expressed and its stability must be maximized to maintain high levels of production without substantial losses during the downstream processing and purification to remove free porphyrins and adsorbed lipopolysaccharide antigens (pyrogens) [21, 40, 46-48]. This goal requires expression of high levels of a stable holoprotein that can be purified rapidly and efficiently with extremely high yields.

1.3 Factors Governing Heterologous holoHb Expression in *E. coli*

A molecular scheme for hemoglobin assembly is shown in Figure 1.1. The newly formed α and β polypeptides must fold and then associate to form a relatively stable $\alpha_1\beta_1$ dimer. These dimers (or the folded monomers) then bind heme to form hoglobin subunits, which in turn rapidly self-associate to form the final holo-tetramer that is stable for days at 37°C. In contrast, all the apoglobin forms are very unstable, denature rapidly at temperatures above 15°C, and in the absence heme during expression, are found as precipitates in inclusion bodies or not at all due to proteolysis [20, 21, 47, 50]. In our scheme, we have heme reacting first with apoHb dimers; but it is also possible that heme binds to the individual apoglobin subunits, presumably in folded states. However, we can only measure the stability apoHb dimers *in vitro*, and the apo-forms of the individual chains unfold and aggregate irreversibly in simple aqueous solutions, even at low temperatures.

Within the framework of Figure 1.1, a simple mathematical formula for the rate of holoHb expression is:

$$R_{net,holoHb} = k_{heme} \times \frac{K_{UN}}{1 + K_{UN}} - k_{degradation} \times \frac{1}{1 + K_{UN}} \quad (1)$$

where $R_{net,holoHb}$ represents the overall rate of holoHb expression in *E. coli*; k_{heme} is the rate of heme uptake or synthesis, $k_{degradation}$ is the rate of irreversible aggregation or proteolysis of unfolded rHb, and K_{UN} is the equilibrium constant for folding of the denatured or unfolded (U) state to the folded or native (N) apoprotein. In this model, the formation of holoHb is governed by the fraction of newly synthesized globin that is the native state ($K_{UN}/(1+K_{UN})$) multiplied by the rate of heme uptake or synthesis by the bacterial cells. The actual bimolecular rate constant for heme binding to globin is very large (~ 1 to $10 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$) and thus, the heme association reaction is not rate limiting [51]. Instead, k_{heme} is determined by the rate of heme synthesis or the rate of transport of exogenously added cofactor. Formation of holoprotein is opposed by aggregation and proteolysis of the unfolded apoglobin state. The rates of these latter processes must be large in the case of separately expressed α and β subunits because the yields of individually expressed holo subunits are either zero or very low, respectively [33]. Almost all workers lower the temperature of their bacterial growths from 37° to 28° immediately before induction and then maintain the lower temperature to maximize hemoglobin expression and minimize precipitation of apoglobin [47].

Equation 1 forms the basis for addressing problems associated with holoHb production. The first strategy is to inhibit denaturation by enhancing the stability of the native folded state (*i.e.* increasing K_{UN}) by mutagenesis. The second strategy is to increase the amount of heme available to the newly synthesized globin in the cell

cytoplasm (*i.e.* increasing k_{heme}). The group at Somatogen adopted the latter strategy by adding excess external heme to JM109 *E. coli* strains that are more permeable to heme than most other expression strains [33, 48]. More recently, we have adopted a more direct approach by co-expressing hemoglobin genes with heterologous heme transport genes from Gram negative bacterial genes. Douglas Goodwin's group at the University of Auburn developed the first heme transport co-expression system for large-scale production of catalases in *E. coli*. In their system, the ChuA heme transporter from *E. coli* was co-expressed with *kat* genes to facilitate heme uptake and holoprotein formation [52, 53]. As described below we have observed a similar increase in heme availability when co-expressing the heme utilization genes of *Plesiomonas shigelloides* with myoglobin and hemoglobin and then adding exogenous heme. The proteins and genes involved in *P. shigelloides* heme transport are shown in Figure 1.1, 4.1 and 4.2 respectively, and are discussed in Chapter 4.

Figure 1.1 Strategies to Increase Hemoglobin Synthesis in *E. coli*. This figure shows the heme transport system of *P. shigelloides*, uptake and diffusion of exogenous free heme, and heme synthesis during rHb expression and assembly in *E. coli*. The heme transport system consists of HugA (yellow), HugB (blue), HugC/D (green/pink), TonB (silver), and Exb B/D (lt. pink/lt. green). A detailed description of heme transport is given in Section 6

1.4 Increasing Heme Uptake in *E. coli*

Our current expression cell lines for expression of rHb (SGE1661 and BL21(DE3)) require the addition of a large amount of exogenous heme to achieve reasonable amounts of holo-hemoglobin for analysis. Increasing the amount of exogenous heme does not, above a certain level, significantly increase rHb production because of the high affinity of heme for lipid bilayers and its slow dissociation across bacterial membranes [23,24]. We chose to compare inefficient uptake of exogenous heme by *E. coli* with the use of the heme utilization genes (*hug*) genes from *Plesiomonas shigelloides*. In collaboration with Prof. Douglas Henderson at The University of Texas – Permian Basin, we examined the effects of incorporating the all of the *hug* genes from *Plesiomonas shigelloides* into our recombinant hemoglobin expression system.

The heme transport system is an intricate combination of transmembrane, periplasmic, and cytoplasmic proteins, which work in conjunction with each other to transport heme into the cell. The acquisition of heme begins with the outer membrane heme receptor protein hugA, which binds to exogenous heme (often from hemoglobin released by red cell lysis and intestinal bleeding caused by this pathogen) [54-57]. Once external heme has bound to the hugA receptor, the periplasmic tonB that is complexed to exbD/exbB in the inner membrane is coupled to the pore-occluding luminal domain of the heme receptor. The heme is then actively transported through hugA into the periplasm by use of a proton gradient generated by bacterial respiration [58, 59]. Recent molecular dynamic studies by Gumbart et al. have suggested that TonB pulls on the pore-occluding luminal domain causing it to unfold [60]. This unfolding of the luminal domain allows the heme to enter the periplasm. The heme shuttle protein hugB binds to heme and

transports it through the periplasm to another complex of transmembrane proteins, hugC/hugD, located in the inner membrane (an ABC transporter). This protein complex allows heme to enter the cytoplasm of the cell where the remaining *hug* proteins, hugW, hugX, and hugZ break down heme to acquire the Fe(II) and/or prevent heme toxicity in the cell (Figure 4.1) [54, 57].

1.5 Protein Engineering: Comparative Design of More Stable rHb

In the forty years since the determination of the high resolution structure of hemoglobin by X-ray crystallography, detailed structural and functional analyses have been performed to determine the mechanisms of ligand binding, discrimination, and cooperativity [22, 23]. Libraries of site-directed mutants have been constructed to test these ideas and to incorporate any desired effect with respect to O₂ affinity, NO dioxygenation, autooxidation, and heme loss [1, 5-7, 61-64]. In contrast, our understanding of apohemoglobin stability and folding has remained empirical and few detailed mechanistic studies have been reported. Antonini, Brunori, Beychok, and others have attempted to characterize the unfolding reactions of both holo- and apohemoglobin and its individual subunits [24, 25], but no detailed structural interpretations or mechanisms have been presented. Fortunately, the more stable holo- and apo-forms of myoglobin (Mb) can serve as model systems for globin folding *in vitro* and expression *in vivo*.

We have used the large amount of prior work on apomyoglobin to develop a comparative mutagenesis strategy to enhance the stability of apoHb. Prior work by Scott et al. [65] showed that, of the 13 mammalian myoglobins examined, sperm whale apoMb

is the most resistant to guanidine hydrochloride (GdmCl) denaturation, whereas pig and human myoglobin show the least resistance. By comparing sequences, they were able to construct five site-directed mutations in pig apoMb, which conferred an enhanced stability that matched that of wild type sperm whale apoMb. The 600 fold increase in stability of sperm whale apoMb, K_{UN} , compared to that of apoMbs from terrestrial animals appears to be a result of strong selective pressure to prevent denaturation of the whale globin by the severe muscle acidosis that occurs during deep dives when the animal remains under water for up to an hour at a time [65].

Our first mutagenesis strategy to improve the stability of apoHb is based on the assumption that hemoglobin from sperm whales must also be more stable to endure the denaturing conditions that occur during these deep dives. This idea was confirmed in preliminary denaturation experiments with recombinant sperm whale Hb expressed in *E. coli* in collaborative work with Roman Aranda and George Phillips at the University of Wisconsin. Consequently, we compared the sequences of the sperm whale α and β subunits with those for the subunits of adult human Hb and focused on those differences that might result in an increase of stability.

A second comparative approach to enhance stability is to compare adult β subunits with the γ subunit from fetal hemoglobin (HbF). Human HbF is known to be significantly more resistant to alkaline, acid, and alcohol denaturation than HbA [66, 67]. In addition, the $\alpha_1\gamma_1$ dimer has a threefold smaller dissociation rate constant than the $\alpha_1\beta_1$ dimer and an increased rate of holo-assembly [68-71]. HbF is composed of 2 α and 2 γ subunits and forms dimers and tetramer in roughly the same conformation as HbA [66]. Initially, several workers suggested that the replacements of Cys112 with Val and Tyr130

with Trp in the γ subunit were the major causes of the increased stability of HbF and the extra resistance of the $\alpha_1\gamma_1$ dimer to dissociation; however, these mutations had little effect on the stability of HbA [66, 67]. Adachi and coworkers did show convincingly that the β His116 to Ile replacement in γ subunit enhances the hydrophobic surface of the $\alpha_1\gamma_1$ interface, and when introduced into recombinant hemoglobin, increases the stability of the $\alpha_1\beta_1$ interface [66]. Thus, we added this replacement to human HbA to see if it stabilized the apoglobin by inhibiting dissociation of dimers into monomers.

CHAPTER 2

Materials and Methods

2.1 DNA Constructs and Expression Vectors

2.1.a Recombinant Hemoglobin Expression Vector

All recombinant hemoglobin, wild-type and mutants, used in this thesis were expressed using the rHb0.0 plasmid developed by Baxter Hemoglobin Therapeutics (formerly Somatogen, Inc.). The rHb0.0 construct is derived from pDLIII13e, which is based on the parent vector pKK223-3 [75], which uses the *tac* promoter and IPTG for induction of expression. The α and β subunits are located in the multi-cloning site between *Hind* III and *Bam* HI cut sites and expressed in succession. The tetracycline resistance gene is used for plasmid selectivity and the regulatory gene *lacI* to control expression of desired genes. The subunits were cloned into rHb0.0 with a valine to methionine replacement at their first positions of the α and β genes for expression in *E. coli*, and the rest of the sequences were optimized for *E. coli* codon bias. The resulting rHb0.0 expression vector is a high-copy plasmid (~400 copies per cell) that allows for high levels of recombinant protein expression [41]. A plasmid map of rHb0.0 is located in Appendix A.

2.1.b *Plesiomonas shigelloides* Expression Vector

The heme transport system proteins from *Plesiomonas shigelloides* are expressed from the pHUG21.1 plasmid (gift from D. Henderson UTPB). pHUG21.1 is derived from the low copy parent vector pACYC184 and contains the following heme transport genes: *hugA*, *hugB*, *hugC/D*, *tonB*, and *exbB/D*, which are located in the multi-cloning site between two *Hind* III cut sites [54]. The expression of these genes is controlled by the highly conserved ferric uptake repressor (FUR) promoter and induced by the addition of the iron chelator 2,2-dipyridine to deplete the media of free iron. The pHUG21.1 vector also contains the *cat* (chloramphenicol acetyl transferase) gene that for chloramphenicol resistance and plasmid selectivity [54]. pACYC184 is compatible with pMB1 (myoglobin expression), pHb0.0, and other ColE1 related plasmids and can therefore be co-transformed and maintained with the globin expression vectors in *E. coli* with rHb0.0. A plasmid map of pHUG21.1 is located in Appendix B.

2.1.c Site-Directed Mutagenesis using PCR

All of the single, double, and triple recombinant human hemoglobin mutations were created using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene Inc. La Jolla, CA). Using the rHb0.0 wild-type genes as the parental templates, complimentary synthetic oligonucleotides (~33 bases long) with the desired point mutations were ordered, being careful to maintain proper GC percentage and T_m [72]. Mutations were incorporated by using the polymerase chain reaction (PCR), which begins as the oligonucleotide primers anneal to the parent rHb0.0 DNA after it has been denatured. After the annealing step, the high-fidelity DNA polymerase included in the QuickChange™ kit extends and incorporates the mutation into each of the nascent

strands. After the all of the required temperature cycles are complete, digestion of the methylated, nonmutated parental DNA template is performed by the addition of the restriction enzyme *Dpn* I [72]. After digestion, these PCR products are then transformed into JM109 or DH5 α competent cells and grown at 37°C overnight on tetracycline treated LB agar plates to confirm transformation and selectivity. DNA was isolated from the resulting colonies and the desired mutations were confirmed by primer extension sequence analysis using sequencing primers designed in the Olson Lab for the entire cloned rHb gene. Multiple mutants for this study were constructed sequentially by starting with the wild-type vector confirming the new sequences after each round of mutagenesis.

2.2 Preparation of Recombinant Human Hemoglobin

2.2.a Expression of Recombinant Human Hemoglobin

Plasmids (pDLIII-13e) containing genes for prokaryotic expression of human hemoglobin were obtained through a collaboration with Baxter Hemoglobin Therapeutics (Boulder, Colorado) [34]. The plasmid contains the genes for tetracycline resistance and α and β subunits of human hemoglobin. Co-transcription of the hemoglobin genes is controlled by an inducible *tac* promoter [34].

The wild-type hemoglobin background contains a Val(1)→Met substitution at position 1 of each subunit to allow prokaryotic expression of the proteins. In hemoglobin, the Val(1)→Met substitution causes less conformation change from the native structure than an additional N-terminal Met, and results in only very small functional differences between the native protein and the recombinant wild-type control [73]. Protocols for transformation and media preparation are described in the lab manual Molecular Cloning

[74]. All wild type and mutated versions of rHb0.0 were transformed into the expression cell line, SGE1661 (Baxter/Somatogen), which is a variant of JM109 *E. coli* that allows more rapid uptake of extracellular heme. The cells were spread onto agar LB agar plates with 15 µg/mL tetracycline, incubated at 37°C for approximately 12 hours, and stored at 4°C.

The protocols for the growth of the cells and expression of hemoglobin was adapted from the method described by Looker and coworkers [75,76]. A single bacterial colony was removed from the agar plate and used to inoculate into 4 mL of LB, which was then incubated at 37°C overnight in a shaker/water bath. This growth was used to inoculate 1 L of defined media (DM1) and grown to log phase and then transferred to a Biostat C bioreactor (B. Braun). The instructions for making the defined media and supplements for cell growth and protein expression in the bioreactor can be found in David Maillett's thesis.

The bioreactor program contains a cell growth and protein expression phase executed in order, and can be adjusted for each growth. The growth phase temperature is monitored and maintained at 37°C by the B. Braun bioreactor software and is maintained by a water-jacket to create an adiabatic growth chamber. The O₂ level inside the chamber is monitored and maintained at 80% by a cascading increase correlated with the stirring rate and air flow levels. The pH level is monitored by an electrode and kept at pH 7.0 by addition of concentrated NH₄OH, which also serves as a nitrogen source for the bacteria. A 40% glucose solution is used for the carbon source during growth and is incrementally increased as bacterial optical density increases using the bioreactor software.

Once the bacterial cell optical density (O.D.) reached a level of 3.0, the O₂ level and spin-rate are usually at their highest levels (80% and 500rpm respectively) At this O.D. the inducing agent, isopropyl β -D-thiogalactopyranoside (IPTG), is added to a final concentration of ~100 mg/L. Once the expression phase has started, the batch temperature is lowered to 28°C by the bioreactor software. Lower temperatures are more optimal for expression of recombinant hemoglobin because of the instability of apoHb [47]. A basic hemin solution (1.5 g/L, with a few drops of 10M NaOH per liter to enhance solubility) is continuously added to the growth during the expression phase. The duration of the expression phase lasts between 8 and 12 hours after induction. The resulting bacterial growth is harvested (14 to 16 liters of growth) into 1 L bottles and chilled on ice while being bubbled with CO to prevent oxidation and heme degradation. The cell suspensions are then centrifuged in a swinging bucket Sorvall RC-3B Refrigerated Centrifuge (Sorvall, Newtown, CT) to harvest the cells. A typical growth produces roughly 40-50 g cell paste/L which is then frozen and stored at -20°C. This procedure is adapted from David Maillett's doctoral thesis with a description of the MFCS recipe in its Appendix [75].

2.2.b Purification of Recombinant Human Hemoglobin

The protocol for purifying recombinant hemoglobin was adapted from a procedure described by Baxter Hemoglobin Therapeutics [47]. First, the frozen cell paste was resuspended in chilled 40 mM Tris base, CO saturated buffer with 1 mM benzamidine HCl (protease inhibitor) by agitation at medium speed with an Osterizer blender. The ratio of break buffer to cell paste is 3 mL/g, with more buffer being added if

the resulting slurry is too viscous. The suspension was then strained through cheesecloth and fed through a cell press where the first pass results in ~90% lysed cells collected in a container chilled on ice. A second pass through the cell press is performed to ensure 100% destruction of the bacterial cell walls. Typically 5 mL of Cysep 390 (Cytex, NJ) flocculating agent is added to the 1.5 to 2 L of cell lysate to precipitate cell debris; zinc acetate was added to a final concentration of 2mM to bind high zinc affinity *E. coli* proteins; and NaOH was added drop-wise to adjust the pH to 8.0. The cell lysate is stirred to insure the flocculating agent aggregates all cellular debris. The resultant mixture is aliquoted into 250 mL bottles and centrifuged at 10,000 rpm for one hour using a Beckman J-21C centrifuge, and the resultant supernatant is strained through cheesecloth before loading onto the first column.

Purification of rHb begins with a Pharmacia XK50 column filled with ~400mL of Chelating Sepharose Fast Flow resin (GE HealthCare). The column is charged with Zn^{2+} by equilibration using 2 column volumes (CV) with 20 mM zinc acetate (ZnOAc). Access Zn^{2+} is removed by rinsing with 1 CV of 200 mM NaCl. The clarified lysate is loaded onto the column at a slow speed to ensure the formation of a tight protein band at the top of the column as rHb binds to the resin [76]. Once all of the lysate has been loaded on to the column, the large red band of rHb is subjected to a variety of rinses. The order of rinses is as follows: 1 CV of 20 mM Tris, 500 mM NaCl, pH 8.5 (removes genomic and recombinant DNA and *E. coli* proteins not interacting with the resin), 2 CV of 200 mM Tris, pH 8.5 (eludes weakly interacting *E. coli* proteins from the resin), 2 CV of 20 mM Tris, pH 8.5 (equilibrate the bound protein band in its storage buffer), and finally the protein is eluted with 15 mM EDTA, 20 mM Tris, pH 8.5 slowly to keep the

protein band concentrated as it moves down the column [76]. All rinsing and elution buffers are made with pre-chilled milliQ water, adjusted to the desired pH at 4°C after dilution of Tris using 2M Tris Base stock solution. Buffers are allowed to equilibrate overnight at 4°C, and pH is again checked before use in purification of rHb and is adjusted accordingly. Each buffer is saturated with 1 atm of CO immediately before use on the affinity column. An absorbance spectrum of the elutant was measured (600nm to 250nm) and the ration of A_{420} to A_{280} was used as a rough measure of purity. This first column usually results in ~80% pure rHb. The EDTA and Zn^{++} is removed from rHb by buffer exchange using a Sephadex®-G25 column equilibrated with 20 mM Tris, pH 8.5 (storage buffer) [76].

The next step to purify rHb is a heat step to quickly eliminate rHb with heme groups that lack the iron atom (protoporphyrin IX). The buffer exchanged rHb is concentrated to ~2 mM (per heme basis) and placed into a round bottom flask containing a stop-cock elbow joint which is then subjected to 5 vacuum argon purge cycles so that the vessel becomes anaerobic, and then in the last step 1 atm of CO is introduced above the solution. The anaerobic vessel containing rHbCO is then heated to 65°C for no more than 3 minutes, although aggregates may start to form earlier. Once the vessel is cooled and the rHb is equilibrated on ice, the precipitated protein is pelleted out and filtered through a 0.22 μ m syringe filter. At this step, the resulting filtered rHb is ~90% pure.

The rHb is then loaded onto a 20 mL Source Q anion exchange column (GE HealthCare) equilibrated with 20 mM Tris, pH 8.5 and attached to a GE HealthCare AKTA FPLC. The column is run at less than 400 psi at a flow rate of 10 mL/min using CO saturated H₂O for mixing with buffer stocks of 2.0 M Tris, pH 8.9 and 0.5 M NaCl.

The program runs a salt concentration profile that includes a single step to wash off non-specifically bound material, a gradual gradient to elute the band of interest, and a sharper gradient to remove a second major band. These bands were previously subjected to in-depth characterization by David Maillett who determined that peak 1 from the anion exchange column is unmodified $\alpha_2\beta_2$ tetramers and Peak 2 is a β_4 chain population using reverse phase chromatography [76]. Thus, there appears to be a net loss of α -globin during expression and purification.

Peak 1 from the anion exchange column is collected, concentrated to ~ 2 mM heme, flash frozen and stored in liquid nitrogen until use. A final UV-visible spectrum of CO bound wild-type recombinant human hemoglobin is performed to confirm CO binding and SDS-PAGE analysis is performed to determine final purity. The combined two column method can produce $\geq 95\%$ pure rHb.

2.3 Apo-preparation of Recombinant Human Hemoglobin

A major goal of this thesis was to investigate the effect of the single, double, and triple mutations on the stability of apohemoglobin. All of the globins used in this thesis were purified as intact recombinant human holohemoglobin expressed in *E. coli*, as explained in Section 2.2. Apoglobin was prepared using a modified form of the acid-acetone method described by Ascoli et al. [24]. The acid/acetone reagent was prepared by adding 2.5 mL of 2M HCl to 1L of analytical grade acetone and stored at -20°C [24].

The rHb used or apoHb preparation is first treated with a few pellets of potassium ferric cyanide ($\text{K}_3\text{Fe}(\text{CN})_6$) to oxidize the heme completely, which allows for easier removal of the Fe-porphyrin. Oxidation is confirmed by the change in color of the

protein sample from bright red (reduced CO form) to dark brown (oxidized aquomet form). The oxidized Hb sample is then desalted and excess $\text{K}_3\text{Fe}(\text{CN})_6$ removed by buffer exchange into water using a long, hand-poured Sephadex[®]-G25 column (Sigma-Aldrich, St. Louis, MO). The eluant was then re-concentrated to ~1mM on a per heme basis.

A ~1 mL aliquot of the prepared rHb sample is added drop-wise into a vigorously stirring threaded 30 mL glass centrifuge tube containing 25 mL of acid-acetone cooled and maintained at -20°C by a dry ice/acetone bath. After the rHb sample is added, the acid-acetone reagent is slightly reddish as a result of heme removal and the rHb protein was flash frozen and precipitated from the acetone solution. The stir-bar is removed and the centrifuge tube was placed in a JA-20 rotor pre-chilled to -20°C in a Beckman J2-HS floor centrifuge, and centrifuged at 5,000 rpm for 3 minutes to isolate the protein pellet. The low temperature must be maintained to prevent the sample from heating up the acetone and creating pressure and bubbling inside of the tube, which disturbs the fragile protein pellet. The supernatant is removed carefully, and the pellet is examined for any reddish color pigmentation to determine if treatment with acid-acetone has to be repeated to remove all traces of hemin.

The white-colored pellet is resuspended in 1 to 2 mL of double distilled deionized water and placed in a 3 mL Pierce Slyde-A-Lyzer (Pierce, Inc.) with a molecular weight cut-off (MWCO) of 3000. After placement into the dialysis cartridge, the nascent apoglobin is subjected to a series of flash dialysis steps, each lasting an hour beginning with 2 L of 0.1% sodium bicarbonate and then 5mM, 10mM, and ending with 50mM KPi, pH 7.4. Unfortunately, after the flash dialysis is complete only 20% to 30% of the sample

remains in solution with the rest irreversibly aggregating. The apoglobin that remained in solution is removed from the dialysis cartridge and filtered through a 0.22mm syringe filter and stored on packed ice. The apo-protein is concentrated only if necessary and is usually ready for experimentation.

2.4 Unfolding Analysis of rHb: *Circular Dichroism*

2.4.a Description of Automatic Titration Method

The AVIV Model 202-01 has an attachment that allows for automatic titration of unfolded apo-protein with denaturants, which in our work is GdmCl, directly in a 1.0 cm path length quartz cuvette. The protein solution in these experiments is normally $\sim 5 \mu\text{M}$ apoglobin in 1.8 mL of 50mM KPi, pH 7.4 with a round stir bar to mix the additions of the 5 μL of denatured protein (also 5 μM) in a supersaturated solution of GdmCl in 50 mM KPi, pH 7.4. The additions are made with a Hamilton Microlab 500 automatic titrator controlled by the AVIV software. CD readings at 222 nm are taken at 0.1 M intervals from 0 M to 5 M at 10°C with constant stirring and a equilibration time of 5 minutes between each titration step. To screen for the effects of the stability mutations, the titration data signals for all native, wild type, and apo-rHb variants were normalized and plotted. A significant relative increase in resistance to GdmCl induced denaturation was seen, even though the data did not allow a simple two-state analysis [77]. Because of worries about equilibration times and systematic errors, we also used the equilibration reaction method to obtain CD titration data, in which each point represents an independent solution mixture that had been equilibrated for ≥ 60 minutes.

2.4.b Description of Equilibration Reactions Method

The most favorable recombinant human apohemoglobin mutants were analyzed multiple times by the individual equilibrium reaction method, detecting loss of helicity by circular dichroism. All experiments were performed on the AVIV Model 202-01 spectropolarimeter with entire spectra being recorded in the far-UV region between 190 nm and 250 nm with data intervals between 0.5 or 1.0 nm respectively, and an averaging time of 5 seconds per data point. At these wavelengths, the chromophore is the peptide bond, and the signal arises when it is located in a regular, folded α -helical environment.

All rHb apo-protein samples were dialyzed into 50 mM KPi, pH 7.4 and subjected to a 2 to 3 hour incubation time in the denaturant guanidine hydrochloride (GdmCl) to allow for proper equilibration. Apo-protein concentration ranged from 2 to 30 μ M respectively with each reaction spanning a denaturant range from 0 M to 5 M GdmCl with 0.25 M intervals culminating in 21 separate reactions for each experiment. All equilibrium unfolding experiments using this method were performed at 10°C and in a 0.1 cm reduced volume quartz cuvette to allow higher concentrations. Each experiment began with a baseline spectra recorded to determine any effect of the buffer on the CD spectra and subtracted from all other samples in the experiment. A CD spectrum for each reaction mixture in the experiment was recorded to confirm changes in the secondary structure. The changes at 222 nm were then plotted versus concentration of GdmCl. All data output is recorded as molar ellipticity, with the units of [17] being $\text{deg cm}^2 \text{ dmol}^{-1}$.

2.5 Unfolding Analysis of rHb: *Fluorescence*

In addition to CD spectra, fluorescence data were also collected for most of the GdmCl titrations. Fluorescence readings were made with a Varian Cary Eclipse Fluorescence spectrophotometer using a 1.0 cm quartz cuvette with the temperature regulated at 10°C using the attached circulating water bath connected to the sample chamber. Each GdmCl/apo-rHb equilibrium mixture was excited at 285 nm, and emission spectra were recorded from 300 nm to 500 nm. A blank containing 50 mM KPi, pH 7.4 was scanned, and the resulting spectrum was subtracted to correct inherent scattering of the excitation light. A Savitzky–Golay digital filter was used to smooth output data using the Cary Eclipse software .

2.6 Preparation and Analysis of Co-Transformed *E. coli* Strains

2.6.a Co-Transformation of *E. coli* for Expression Study

To test the effects of the heme transport genes on holo-rHb expression *in vivo*, we constructed a strain of *E. coli* with and without a low copy plasmid containing the heme utilization genes (*hug*) of *P. shigelloides*. Analysis of the parent vectors used in this study confirmed no origin or replication site conflicts between the rHb0.0 and pHUG21.1 plasmids. Co-transformation was begun with the insertion of the pHUG21.1 plasmid into super-competent BL21(DE3) cells, which are grown at 37°C on a LB agar plate containing chloramphenicol (32mg/mL). Colonies from this plate are grown in 5 mL LB broth treated with chloramphenicol. Four mL of the growth was used for DNA isolation, and 1 mL was used to create a glycerol stock. The DNA was isolated by using the Eppendorf DNA Mini-Prep Kit (Eppendorf, Inc.) and subjected to a restriction enzyme

digestion using Hind III (New England BioLabs, Inc.) and then analyzed by gel electrophoresis to confirm that pHUG21.1 was present and successfully transformed into the bacteria. A stab of the glycerol stock was used to inoculate 50 mL of LB Broth treated with chloramphenicol and grown to an optical density of 0.4 to 0.6 at A_{600} . These cells containing pHUG21.1 were pelleted by centrifugation, turned into competent cells by the Rubidium chloride method [78], separated into 200 μ L aliquots, flash frozen in a dry ice/isopropanol bath, and stored at -80°C . These competent cells were subjected to a second transformation step [78] to insert the rHb0.0 expression vector. The resulting co-transformed strain, pHb0.0/pHUG21.1/BL21(DE3), was used to examine the effects of heme transport on the *in vivo* expression of wild-type holoHb and the stability mutants. All rHb mutation variants were transformed into the pHUG21.1/BL21(DE3) competent cell strain for these expression assays, except in a few cases where only protein mutation effects were being examined without the heme transport vector.

2.6.b *In vivo* Spectroscopic Assay for Expression of holo-rHb in *E. coli*

Competent BL21(DE3) cells were co-transformed with pHb0.0 (rHb α and β genes) and pHUG21.1 (*hug* genes) under chloramphenicol and tetracycline dual selectivity, with IPTG inducing rHb production and 2,2-dipyridine (DIP) inducing the *hug* genes by iron depletion. With independent induction for each set of genes, greater control is built in for optimization of proper growth condition. Expression of holoprotein was analyzed in cell suspensions by a low resolution spectral assay, developed by A.J. Mathews at Somatogen, Inc and used to determine the relative level of functional holo-rHb by converted the intracellular hemoglobin to the CO-form and measuring the height

of its unique Soret peak at 420nm. After induction, each expression growth was spun down and the cells resuspended to an OD₆₀₀ of 0.5 ± 0.02 in a quartz cuvette with 0.1M Tris pH 7.5 buffer saturated with CO. The visible spectra were recorded from 600 to 350 nm, and the first derivative was calculated using the Cary Eclipse software to produce the absorbance peaks and troughs used to calculate the relative holo-rHb yield.

2.6.c Quantitative *in vitro* Assay for Expression of holo-rHb in cell lysates.

Small-scale purifications were performed for each mutant expression system as detailed above in Section 2.6.c, and the amount of purified holo-rHb was correlated with the *in vivo* rHbCO spectral assays for the set of same cells. A culture flask containing 50 mL LB Broth was inoculated with *E. coli* transformed with the desired rHb mutant. The growth was pelleted, the weight of the “dry” cell paste was recorded, and the pellet was stored overnight at -20°C. The cells were then resuspended in 20mL of 40 mM Tris Base buffer and sonicated on ice to lyse the cells. Mini-prep purification and elution is performed exactly as described in Section 2.2.b (excluding ion-exchange) using a 5mL mini Zinc-Chelating column (GE HealthCare). The resulting isolated rHb protein was used to determine the amount of functional rHb isolated by UV-Vis spectroscopy. The concentration of rHbCO was determination by the absorbance of the Soret peak at 420 nm, and most samples were $\geq \sim 80\%$ pure.. The concentration of rHb was converted from mM heme to grams of rHb and divided by the “dry” weight of the cell paste to determine the relative amount of rHb produced by this small-scale growth (*i.e.* g of rHb/g of cells). As described in Chapter 4, there is a linear correlation between the *in vivo* derivative

signal and the amount of purified holoHb, and both increase for the stability mutants and when the heme transporter system is co-expressed with the hemoglobin genes.

CHAPTER 3

Designing and Screening of rHb Stability Mutants

3.1 Strategies for rHb Stability Design

In many cases, mimicking Nature's design is the goal in protein engineering; however, in the new age of recombinant DNA technology, it is now possible to generate new protein functions very quickly, instead of the millions of years required for evolution. The native structure of hemoglobin appears to have evolved to readily fall apart from stable tetramer to unstable dimers and monomers after red blood cell lysis. [1-5]. This evolved property must be changed to produce stable extracellular Hb-based blood substitutes. Simple human hemoglobin tetramers are not adequate because they fall apart, causing a variety of unwanted and dangerous side effects that lead to systemic hypertension and renal failure [1-5].

The most beneficial result of recombinant technology is the ability to create mutations in proteins at any desired position and this methodology has lead to complete characterization of the function of the native structure. The study and examination of hemoglobin stability and folding has represented a large portion of research in the globin family of proteins. Sperm whale myoglobin (swMb) stability and folding studies have served as a model system to help understand some of the issues involving hemoglobin stability and folding [26, 89].

3.1.a Sequence Alignment with Sperm Whale Hemoglobin

The same strategies developed by Scott et al. and Lucian Smith [65] for sperm whale Mb have been used to determine if stability can be added to recombinant human hemoglobin (rHbA). Like sperm whale apoMb, sperm whale apoHb is significantly more stable than human apohemoglobin (see Fig. 3.8, Aranda, Soman, Graves, Olson, and Phillips unpublished). Using sequence alignments we focused on the most logical differences that might result in an increase of stability.

Initial examination of the sequence alignment directed attention to the α Gly15 and β Gly16 positions on the A helices of both subunits of human Hb (Figure 3.1 and 3.2). In sperm whale Hb, these residues are alanines. We focused on these differences because glycine is a poor helix former and its presence may decrease the length of the A helix. Our studies of sperm whale mutations in rHb began with these glycine to alanine replacements. The hypothesis was that replacing the human Hb residues with the whale residues should enhance in the overall stability of apo-rHbA [10, 79].

Figure 3.1. Sequence alignment of human HbA and sperm whale Hb α subunits.

Conserved residues are shown in colored bars and residue differences are shown in white bars. Recombinant hemoglobin has a VIM replacement; however, for alignment purposes M was added in front of the valine at the N-terminus.

Figure 3.2. Sequence alignment of human HbA and sperm whale Hb β subunits.

Conserved residues are shown in colored bars and residue differences are shown in white bars. Recombinant hemoglobin has a VIM replacement; however, for alignment purposes M was added in front of the valine at the N-terminus.

3.1.b Sequence Alignment with Human Fetal Hemoglobin

A second comparative approach to increase stability is to compare adult β subunits with the γ subunit from fetal hemoglobin (HbF). HbF is composed of 2 α and 2 γ subunits (dimer interface at $\alpha_1\gamma_1$) and forms a tetramer in the same configuration as HbA [80]. HbF has been shown to be more resistant to alkaline, acid, and alcohol denaturation than HbA [67, 80], and the $\alpha_1\gamma_1$ dimer has a threefold smaller dissociation rate than the $\alpha_1\beta_1$ dimer of adult Hb and an increased rate of holo-assembly [68-71]. As described in Chapter 1, the residue differences at positions β Cys112 and Tyr130 (Thr112 and Trp 130 in γ subunits) were believed to be part of the cause for the increased stability of the intact tetramer and the resistance to dissociation of the $\alpha_1\gamma_1$ dimer into its individual subunits, but mutations at these positions in β subunits had little effect on $\alpha_1\beta_1$ dimer dissociation or overall stability [67, 80]. Figure 3.3 shows the sequence alignment between human rHbA β subunit versus human HbF γ subunit. Rational analysis also identified that the β His116 position could be altered to a non-polar, aliphatic residue to increase the hydrophobic surface of the $\alpha_1\beta_1$ interface and cause a marked decreases in the rate of $\alpha_1\beta_1$ dimer dissociation [80]. By using this comparative and rational design, the basic dimer unit of rHbA is strengthened.

Figure 3.3. Human HbA dimer interface and β to γ replacements from HbF.

Residue difference in HbA β and HbF γ subunits were taken for Bunn and Forget [80] with focus on residue differences in the dimer interface. Work concentrated on the residue G18 was changed from a histidine to an isoleucine. This residue change is supported by work done by Adachi et al. [67, 69].

3.2 Selection and Screening of Specific rHb Stability Candidates

Denaturant titrations of apoHb for mechanistic unfolding studies have not been attempted before. The major dilemmas were reversibility, the complexity of the reactions involving monomers, dimers and tetramers, the temperature instability of apoHb, which precludes experiments above 5-10° C, and the use of GdmCl with its effect on the dissociation of heterodimers. The two types of CD titrations are described in Chapter 2 and in our initial screens the automatic titrator was used to pick the key mutants with enhanced stability.

Proof of principle studies were conducted with the α G15A, β G16A, and β H116I mutations *in vitro* in initial GdmCl-induced unfolding experiments (10°C, 50mM KP_i, pH 7.4), with proteins containing single and multiple replacements as seen in Figure 3.5. The single α G15A and β G16A substitutions confer small shifts of the GdmCl induced transitions to higher denaturant concentrations as compared to the curve for the native apoprotein. The most encouraging result is that the relatively small effects of the individual Gly to Ala mutations appear to be additive, causing the [GdmCl]_{midpoint} to increase from ~1.2 M for rHb(0.0) to ~ 1.6 M for the α (G15A) β (G16A) double mutant. The single α (WT) β (H116I) apoglobin mutants shows an increase of [GdmCl]_{midpoint} to ~1.65 M. The triple α (G1A) β (G16A/H116I) mutant has the largest shift to ~1.85 M and, as will be discussed in the next section, shows the highest level of *in vivo* expression of all the mutants examined (Figure 4.5). Again, the effects of the β H116I mutation appear additive with the individual A helix glycine to alanine replacements, and as result the triple mutant is the most stable apoHbA that has been examined.

Figure 3.4. Comparison Curves of the Unfolding of rHb Wild Type and mutants.

This graph shows the apparent additive effect of resistance to the denaturant GdmCl. Single residue mutations on the A helix (αG15A and βG16A) and the combination of the two (αG15A/βG16A) show small but significant increases in [GdmCl]_{midpoint}. A larger increase was observed for the single mutant βH116I, and the triple mutant αG15A/βG16A/βH116I, increased the [GdmCl]_{midpoint} further from any other mutant.

3.3 Initial Mechanism for Recombinant Hemoglobin Folding

Two state mechanisms for fitting are reserved for small, monomeric or single domain proteins that have a high intrinsic level of stability. This situation is clearly not applicable to apoHb, which is a dimer in the native (N) state and unfolds to form two monomeric unfolded (U) states, perhaps even through a molten globule intermediate as is seen for apoMb. This postulate is supported by the broadness of the CD unfolding curves and their dependence on protein concentration. Thus, there is clearly one or more intermediate states during apoHb unfolding that need to be identified (Anson 1945, Jackson 1988). I attempted to address this issue by measuring equilibrium unfolding as function of protein concentration and changing the $\alpha_1\beta_1$ interfaces in an attempt to reveal the presence of these states.

3.3.a Examination of Concentration Dependence on rHb Folding

In order to examine the uncertainties in the unfolding curves, both denaturation and refolding experiments with recombinant and native apoHbA were performed using hand equilibration experiments by diluting denatured apoHb in 50mM potassium phosphate buffer, and allowing each sample long times to equilibrate at 5°C or diluting native apoHbA into various GdmCl solutions. Figure 3.5a shows both folding and refolding experiments. These experiments show that, if care is taken to keep the samples cold, apoHb can refold from a denatured state. This reversibility is important in the determination of the mechanism of apoHb folding *in vitro*, and assigning credibility to the interpretations. The refolding experiment was performed twice and showed

reasonable reproducibility. In general, these experiments show greater scatter than those observed for sperm whale apoMb. One major source of variability is inadvertent heating, which leads to cloudiness and precipitation and inaccurate CD readings, particularly at low [GdmCl]. In some cases, the precipitation appears irreversible even in high [GdmCl] and prevents even qualitative mechanistic analysis [22]. Because data for the refolding experiment is similar that for the unfolding experiments, we felt confident enough to try to analyze the unfolding process in terms multiple step schemes taking into account dimer dissociation and protein concentration dependence.

The unfolding data as function of total apoHb concentration suggest that refolded monomers of the α and β subunits can combine to reform the apodimers without irreversibly aggregating in the monomeric unfolded states. As apoHb is increased from 3 to 30 μM , there is progressive appearance of second phase that occurs at larger [GdmCl]. Even though there is still a large variability in the curves, this trend always occurs as the concentration of protein increases (Compare Figs. 3.5 and 3.6).

Figure 3.5 A. Reversible Folding Experiment of rHb Wildtype. Refolding experiments were performed (red, green) and show that apoHb does refold and regain CD signal to the original B. CD value. **Concentration Dependence of apoHb Unfolding.** With increasing apoHb concentration, broadening of the second transition occurs, and $[\text{GdmCl}]_{\text{midpoint}}$ shifts towards higher concentration of denaturant.

As shown in Fig. 3.5B, the $[\text{GdmCl}]_{\text{midpoint}}$ can be seen to shift from left to right as concentration of the protein increases suggesting that resistance to GdmCl denaturation is concentration dependent. It is equally clear that two unfolding transitions of unfolding occur and are pronounced at high protein concentration. The initial phase that is roughly the same for all the samples, whereas the second transition moves to higher $[\text{GdmCl}]_{\text{midpoint}}$ as the protein concentration is increased. A simple two step mechanism and analyses are given in Equations 2-4.

3.3.b Proposed Mechanism of apo Hemoglobin Dimer Disassembly

Until this work, there has been a lack of a sound theoretical framework for analyzing and interpreting the observed unfolding data for apoHb quantitatively. As mentioned, the simple two-state models used by most workers in the protein folding field are inadequate and misleading in part because they do not take into account dissociation of apoHb dimers into either folded or unfolded monomers. As shown in Figure 3.5B and Figure 3.6C, it is clear that increasing the concentration of apoHb from 3 to 30 μM leads to increased resistance to loss of the last 50 to 60% of the $\text{CD}_{222\text{nm}}$ change of the apoprotein. To describe these curves, we propose the following mechanism.

ApoHb is known to be dimer with an intact $\alpha_1\beta_1$ interface [67-79, 80]. The first phase of unfolding (K_{DI}) seen between [GdmCl] of 0 M to 1 M is noticeably independent of protein concentration. This result suggests that the initial step involves a first order "melting" of the heme pocket with retention of the $\alpha_1\beta_1$ interface and A, G, and H helices, suggested by what is seen in apoMb denaturation [26-27, 51, 89]. This dimeric "molten globule" intermediate (I), which is also analogous to the I state in apoMb, then unfolds by dissociation into monomers ($K_{2,1}$) and dependency on protein concentration has a transition of denaturation between 1 M and 3 M [GdmCl]. The latter monomers immediately lose all secondary structure to form the U state with the data showing an

approach to a post-transition baseline beginning at 3 M [GdmCl] to excess. This second step ($K_{2,I}$) is second order and inhibited as the protein concentration is increased to promote dimer formation. This mechanism is depicted in Equation 2, and the data for each phase of this mechanism can be seen in Figure 3.6C.

The fraction of CD change is given by:

$$Y_{CD} = \text{"Fraction of CD change, going from 0 to 1"} = \frac{F_{DI}2[I] + F_{DU}[U]}{([U] + 2[I] + 2[D])} \quad \text{Equation 3}$$

Y_{CD} is the fractional change in CD absorbance, F_{DI} is the fractional CD change that occurs for the D to I transition, and F_{DU} is the fractional change for complete unfolding, which is defined as one if the CD signal change is normalized. The final expression is a very complex function because the last step is second order. The exact expressions for the amounts of $[U]$, $[I]$, and $[D]$ as function of K_{DI} , $K_{2,I}$, and total protein, C_0 , are given in Equation 4. The values of for these protein species are then inserted into Equation 3 to give Y_{CD} or $(1-Y_{CD})$ as function of [GdmCl] (*i.e.* solid lines in Figures 3.8B and 3.8C).

The dependence of the unfolding or dimerization constants on [GdmCl] is given in Equation 4, based on the linear free energy relationship for each unfolding step:

$$\Delta G_{i,\text{unfolding}} = \Delta G_{i,\text{unfolding}}^0 - m_i [\text{GdmCl}].$$

$$[U] = \frac{-\frac{K_{2,I}K_{DI}}{1+K_{DI}} + \sqrt{\left(\frac{K_{2,I}K_{DI}}{1+K_{DI}}\right)^2 + 8\frac{K_{2,I}K_{DI}}{1+K_{DI}}C_o}}{4}; [I] = \frac{K_{DI}}{1+K_{DI}} \frac{1}{2}(C_o - [U]); [D] = \frac{1}{1+K_{DI}} \frac{1}{2}(C_o - [U])$$

Equation 4

$$K_{DI} = K_{DI}^0 \exp(m_{DI}[\text{GdmCl}]); K_{2,I} = K_{2,I}^0 \exp(m_{2,I}[\text{GdmCl}])$$

This mechanism is compelling for three reasons. (1) Equation 3 provides a reasonable fit to the observed data for wild-type apoHb (Figure 3.6C) and a plausible explanation for protein concentration dependence of the second unfolding phase. The fitted curves are preliminary and assignment of all parameters uniquely is difficult. However, the Equation 4 can simulate the appearance of a second phase at high protein concentration dependence.

(2) The increased stability of sperm whale hemoglobin can be explained by an ~ 3-fold decrease in the dimer intermediate dissociation constant (*i.e.* $K_{2,I} \approx 0.05 \mu\text{M}$ for human apoHb *versus* $\sim 0.02 \mu\text{M}$ for sperm whale Hb were used to compute the simulated curves in Fig. 3.6B with all other parameters remaining the same). (3) The mutants shown in Figure 3.6A stabilize the molten dimer intermediate by strengthening the A helices and the $\alpha_1\beta_1$ interface (*i.e.* decreasing K_{DI}) Together the new mutants and mechanism appear to provide a structural framework for further rational engineering of even more stable recombinant hemoglobins.

Figure 3.6. ApoHb unfolding curves at 5°C, pH 7. Panel A, GdmCl induced unfolding curves for mutant apo-rHbs (5 μ M). The fraction of folded protein ($1 - Y_{CD}$) is plotted vs. $[GdmCl]$. Y_{CD} is the fractional CD change at 222 nm. The data in panels A and B were collected with an automatic titrator. **Panel B,** comparison of native human apoHbA and recombinant sperm whale apoHb (both ~5 μ M). **Panel C,** Unfolding of wt apoHbA as function of total protein concentration using individual mixtures. The lines in panels B and C represent simulations (not fits) to the two-sep unfolding scheme. (Eqs. 2 and 4)

CHAPTER 4

Effect of *hug* Co-Expression on rHb Yield

4.1 The Heme Transport System (HTS): How It Works

A schematic mechanism for heme utilization by the heme transport system of *P. shigelloides* as well as many other pathogens is shown in Fig. 4.1 [54, 82, 83]. The expression of the HTS proteins is regulated by the presence of iron in conjunction with the transcription repressor protein FUR (ferric uptake repression). In high free Fe(II) conditions, the co-repressor Fe(II) is bound to the FUR protein, which enables transcription by binding to a highly conserved DNA sequence called the FUR binding site located inside the -35/-10 promoter region [84, 85]. Once FUR has bound to DNA, RNA polymerase cannot bind to the DNA promoter effectively and thus transcription, translation, and the resulting iron acquisition products are not synthesized. Alternatively, in low Fe(II) conditions, the co-repressor Fe(II) is scavenged from the FUR protein. This removal of the cofactor from FUR results in an allosteric change in the repressor protein. Once the allosteric change takes place, the FUR protein releases the DNA, and RNA polymerase is allowed to bind to the promoter of the heme utilization genes and begin transcription [84, 85].

A variety of pathogenic bacteria use a heme transport system to acquire free heme from the environment and use it as an iron source in low Fe(II) conditions [55]. The heme transport system is an intricate combination of transmembrane, periplasmic, and

cytoplasmic proteins, which work in conjunction to transport heme into the cell. The acquisition of heme begins with the outer membrane heme receptor protein hugA, which binds to heme from blood proteins after intestinal bleeding, e.g. from hemoglobins, albumin, and hemopexin [54-57]. The heme receptor hugA, is a transmembrane protein located in the outer membrane, which is characteristic of Gram negative bacteria. Once heme has bound to the hugA receptor, the tonB and exbD/exbB protein complex in the inner membrane use a proton gradient for active transport of heme through hugA into the periplasm [58, 59]. It is at this point the putative heme shuttle hugB binds to heme and transports it from the periplasm to another complex of transmembrane proteins, hugC/hugD, located in the inner membrane. This protein complex allows heme to finally enter the cytoplasm of the cell where it is believed that the remaining HUG proteins, hugW, hugX, and hugZ break down heme to acquire the Fe(II) [54, 57]. Figure 4.1 shows a detailed diagram of the active transport of heme in the pathogen *P. shigelloides*.

Figure 4.1. Heme Transport System and heme uptake of *Plesiomonas shigelloides*

This figure depicts the entry of free heme through the outer (*hugA*) and inner cell membranes (*tonB/exbD/exbB* and *hugC/hugD*) by transmembrane *hug* proteins and the periplasmic heme shuttle (*hugB*). Heme degradation enzymes (*hugW/hugX/hugZ*) breakdown free heme to release the Fe(II) atom. The plasmid used in this study (pHUG21.1) does not include these heme degradation enzymes.

4.2 Description of Heme Transport System Plasmid pHUG21.1

The plasmid used in this study for heme transport, pHUG21.1, was a gift from Professor Doug Henderson at the University of Texas – Permian Basin. The plasmid was designed using the parent vector pACYC184, which is a low copy plasmid that results in 5-8 copies per cell [54, 86-88]. Antibiotic selectivity for this plasmid is chloramphenicol with the origin site being compatible with the recombinant hemoglobin plasmid rHb0.0 (described in detail in Chapter 2). Specific *hug* genes were cloned into the multi-cloning site between the HindIII and EcoRI restriction sites. Figure 4.2 shows the direction of transcription of the specific *hug* genes and the FUR promoter binding site. Induction of the *hug* genes is controlled by a cloned FUR binding site. The iron chelators EDDA and 2,2-bipyridine scavenges free Fe(II) from the FUR protein, causing it to dissociate from DNA and allow for transcription of the *hug* genes by *E. coli* RNA polymerase [54, 86, 87]. Since IPTG is not used for induction of this plasmid, a co-transformed cell (depicted in Figure 4.3) can, in principle, have induction of its two plasmids at separate stages of cell growth. The expression of the *hug* genes can be initiated just prior to rHb0.0 induction and free hemin addition. As a result, the hemin can immediately be transported into the *E. coli* cell as rHb expression begins. The goal is to increase the rate of heme uptake, k_{heme} , in Equation 1.

Figure 4.2. Orientation of *hug* genes and FUR binding site in pHUG21.1 plasmid.

Specific genes used in pHUG21.1 (grey bar lines up with relevant genes) are in color and cloned between HindIII and EcoRI cut sites. When the FUR repressor has its iron removed, it no longer interacts with the FUR promoter and transcription begins.

Figure 4.3. Mechanism of Co-Transformation of *E. coli* using pHUG21.1 and rHb0.0

A. BL21(DE3) competent cells are transformed with pHUG21.1 plasmid. Once transformation is confirmed, the pHUG21.1/BL21(DE3) cell line is converted into competent cells using a rubidium chloride method [78]. **B.** pHUG21.1/BL21(DE3) competent cells are then transformed with rHb0.0. Transformation of both plasmids is confirmed by 1% gel electrophoresis.

4.3 Protein Expression Screening Using Derivative Assays

An examination of rHb production in *E. coli* was performed, with and without co-expression of the *hug* genes of *P. shigelloides*. In this expression assay BL21(DE3) cells were co-transformed with rHb and the pHUG21.1 plasmids. Parallel experiment were performed with BL21(DE3) co-transformed with wild-type rHb0.0 and pHUG21.1 as a positive control and with hemoglobin plasmids containing the β Gly16→Ala, α Gly15→Ala, and β His116→Ile mutations and pHUG21.1. To determine the effect of *hug* genes on the production of rHb, 2,2-bipyridine (DIP) is added to starve the cells of free iron. Induction of *hug* genes takes place immediately after inoculation of the media with this chelating agent. After the cells are allowed to grow for 8 hours, rHb and *hug* gene induction is initiated with IPTG and DIP and high concentrations of heme are added. After 8 to 10 hours of induction, the cells are examined by UV/Vis spectroscopy. The first derivative of the resulting scan (700nm-350nm) was calculated and gave rise to minima and maxima peaks. The “peak to trough” distance provides a relative measure of rHb production and has been well established by studies performed on myoglobin by former graduate student lab member Lucian Smith [89]. This assay is performed on each growth condition and an example of the raw data is shown in Figure 4.4. The resulting processed data show that when the system is starved of iron, the production of rHb increases significantly over the conditions that are not iron starved (Figure 4.5A/B versus Figure 4.5C). The data further suggest that the stability mutation has a higher degree of overall rHb production than that of wild-type Hb. This preliminary data clearly show that stability mutations positively affect the expression yield of holohemoglobin *in vivo*.

The key conclusions from the data in Figure 4.5 are: (1) rHb expression yields can be increased over 3-fold by stability mutations and the presence of heme transporters (i.e. rHb0.0 (IPTG+1x heme, panel B) *versus* triple mutant (DIP + IPTG + 1x heme, panel C); (2) the favorable effects of mutagenesis and heme transport appear roughly additive (panel B versus C); and (3) the expression differences between the more stable Hb mutants decreases significantly when the rate of heme transport is enhanced. Equation 1 predicts the latter effect. If k_{heme} is made large enough by efficient heme transport and the presence of large amounts of exogenous heme, the rapid rate of holoHb formation can overcome the rate of degradation even if the fraction of unfolded apoHb is significant.

Figure 4.4. UV-Visible Determination of rHb Expression *in vivo* *E. coli* BL21(D3) cells were co-transformed with rHb0.0/pHUG 21.1 plasmids and maintained on agar plates with tetracycline and chloramphenicol. Tubes containing 5 ml of LB broth were inoculated and then grown for 8 hours at 37° C. IPTG and DIP additions were made to the cultures to induce the rHb0.0 and *hug* genes. **A.** UV-visible spectral scans were taken with cells suspended at 0.5 OD₆₀₀, in Tris buffer, pH 7.5, and 1 atm of CO. An increase in the Soret peak at 420nm can be seen as a result of the co-expression of the *hug* genes. **B.** The 1st derivative of each spectrum in panel A was calculated to remove light scattering background and to amplify the HbCO signal. As shown, there is a pronounced increase in rHb Soret derivative peak for the co-expression conditions versus simple induction of rHb by itself.

Figure 4.5. Detailed Histogram of rHb Expression Levels. **A.** The first histogram shows the relative expression levels of wild type rHb (0.0 - black bars), single mutant ($\alpha G15A$ - white bars), and the more apoHb stable triple mutant ($\alpha G15A/\beta G16A/\beta H116I$ - gray bars) in *E. coli* in the absence of co-expression of the *P. shigelloides* *hug* genes. **B.** The second histogram shows the relative expression of wild type rHb (0.0 - black bars) in *E. coli* with co-expression of the *hug* genes induced by 2,2'-dipyridine addition (+DIP). **C.** The third histogram shows the effect of the stability mutants with the co-expression of the *hug* genes. (Note: Control=Luria broth only with no induction additives.)

4.4 Correlation of Expression/Mini-Prep Data

The HbCO derivative spectrum assay defines the relative amount of rHb expression *in vivo* and can be used for high through put screening of conditions, transport systems, and stability mutants. However, to obtain a better absolute estimate of the amount of holoHb production, a second, more quantitative *in vitro* assay was developed and is based on small-scale "mini-prep" purification protocols developed by A. J. Mathews and T. Fattor at Somatogen, Inc. (A. J. Mathews, personal communication and [33]). *E. coli* growth cultures (500mL) are harvested after induction of rHb with and without co-expression of the *hug* genes as described in the previous section. The cells are pelleted, resuspended in breaking buffer, lysed, and flushed with 1 atm of CO for conversion to rHbCO. The resulting supernatant is centrifuged to remove cellular debris and then the clarified lysate is run through a small 5 ml fast flow Zn column (GE/Amersham) in the exact manner described for purification of rHb in Chapter 2. The total amount of holoHb expressed is calculated from the total volume of the eluted protein times the concentration of HBCO determined by the absorbance of the Soret peak at ~420 nm. The grams of hemoglobin produced are then divided by the grams of packed cells that were in the original suspension before lysis.

The correlation of the amount of partially purified rHb (g Hb/g cells) with the amplitude of the CO derivative spectrum is shown in Figure 4.6. Small-scale purification was performed for each mutant immediately after the amount of *in vivo* expression was estimated in the HBCO derivative assay. The data in the figure shows that there is a reasonable linear correlation between the *in vivo* spectral assay and the amount of purified Hb obtained per g of cells. These results also indicate that the combination of

stability mutations and heme transport can increase the production of partially purified rHb 2 to 3-fold (open vs. closed circles Fig. 4. 6).

Figure 4.6. Correlations between the UV-Visible Derivative HbCO Signal and Small-Scale Purification of Holohemoglobin. Expression yields from mini-prep purification of rHb0.0 (open circles, IPTG conditions) and three stability mutants plus or minus co-expression of the *hug* genes (closed circles, IPTG, 1x heme, DIP). A linear correlation can be seen, indicating expression levels are increasing with the co-expression of rHb and *hug* genes.

CHAPTER 5

Conclusions

Conclusion

As described in the introduction, second-generation of HBOC's were re-engineered to solve problems associated with low circulation half-lives, inefficient O₂ transport, and rapid rates of NO dioxygenation leading to both systemic and pulmonary vasoconstriction. Recombinant DNA technology offers the greatest potential for solving all of these problems and eliminating the need for using donated human or animal blood as the source material for the HBOC. Recombinant Hb produced in bacteria offers a potentially unlimited and virus-free source for all HBOC products, but its production in bacteria is costly and currently inefficient. Our goal has been to enhance production yields by (1) increasing apoglobin stability by a comparative mutagenesis strategy and (2) enhancing the rate of heme uptake in *E. coli* by heterologous expression of heme transport genes from other Gram-negative, pathogenic bacteria. The experiments and analyses in this study indicate that this combined approach can increase the level holo-rHb production over three-fold compared to current wild-type rHb expression systems.

5.1 Effect of Protein Engineering on rHb Production

Although highly significant, these initial experiments represent only a small step forward, and, in our view, even greater enhancements can be achieved. Our initial

attempt to enhance apoHb stability involved sequence comparisons between adult human α and β chains and the more stable sperm whale and human fetal Hbs. Both rational and random mutagenesis can be applied to enhance apoglobin stability and solubility post-expression, using known principles of protein folding and absorbance screening for rHb expression. Optimization by these protein engineering and molecular biology methods is at the forefront of current technology. ApoHb stability also needs to be considered when selecting functional mutations. In a screen for mutants with high P_{50} values, Weickert et al. [21] discovered that the Hb Providence mutation(β K82D) enhanced the expression yield of wild-type holo-rHb and rescued the poor yields of Hb Presbyterian (β N108K). Thus, we are confident that even greater enhancements of apoHb stability than those shown in Figure 3.5 can be achieved.

5.2 Factors Governing rHb Production Yield - Heme Transport

In addition to optimization of rHb production through protein engineering, it is also very likely that more efficient heme transport systems and expression vectors can be developed. In addition to the *hug* genes, The Olson Lab and others are examining the efficiencies of globin co-expression with the *shuA* genes from *Shigella dysenteriae* and *chuA* from *E. coli* O157:H7 [52]. Unfortunately, the current co-expression systems are cumbersome, involving maintenance of two plasmids in each bacterial cell, and these systems almost certainly diminish rates of cell growth and hemoglobin synthesis. Ultimately, it will necessary to incorporate an efficient heme transport system in the *E. coli* chromosome under control of a transcription factor that can regulated independently from the *lac I* IPTG system used to induce globin expression into commercial expression

strains of *E. coli*. Most of the technology to solve these problems already exists [52]. Thus, we suggest that rHb can be produced in *E. coli* at levels high enough to allow commercialization and that, in the future, genetically engineered Hb will be the starting material of choice for all HBOC products.

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APPENDIX A

Plasmid Map of rHb0.0

APPENDIX B

Sequence of rHb0.0 Plasmid in FASTA Format

RED = Hba alpha subunit
 BLUE = Hba beta subunit
 GREEN = tetr
 BLACK = rHb0.0 vector
 PURPLE = tac promoter
 MAROON = Stop Codon
 GOLD = Shine-Delgarno Sequence
 PINK = Lac Repressor

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