Globin engineering studies: optimizing the designs of circularly permuted myoglobin and single-chain hemoglobin

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Globin engineering studies: Optimizing the designs of circularly permuted myoglobin and single-chain hemoglobin

By
Jamie M. Apperson

Accepted in Partial Completion
of the Requirements for the Degree
Master of Science

Kathleen L. Kitto, Dean of the Graduate School

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Jamie M. Apperson
November 15, 2013
Globin engineering studies: Optimizing the designs of circularly permuted myoglobin and single-chain hemoglobin

A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

by
Jamie M. Apperson
November 2013
Abstract

We are working to produce a stable and effective hemoglobin-based oxygen carrier (HBOC) for critical care. Mammalian myoglobins are good model systems for the protein engineering of human hemoglobin, and in the current work, our aim is to generate a circularly permuted myoglobin with increased thermodynamic stability compared to previous permuteins characterized by our lab. Our initial permuted myoglobin, HGL16, includes a 16-residue Gly-Ser linker (SGGG)_4 between the A and H helices in sperm whale myoglobin (swMb). Although HGL16 was shown to fold and function like wild-type swMb, its stability was reduced significantly. In the current work, computational design of the linker was employed, with the aim of increasing the stability of the permutein. The design modeled the linker as a helix, and includes novel interactions with the swMb framework. The resulting permutant, ML1, appears to be less stable than HGL16, but appears to refold properly from inclusion bodies based on the visible spectrum of the cyanomet isoform. In addition, we have generated a single-chain human hemoglobin (scHb) using shorter linkers between subunits. The scHb design includes a single glycine residue as the linker between the two α-globins and novel covalent connections between each α-globin and a permuted β-globin. To be utilized as the framework for a therapeutically useful HBOC, scHb must possess similar function and structure to authentic human hemoglobin (HbA). Functional studies indicate that scHb possesses highly similar ligand affinity to HbA in the R-state, but has an iron with increased reactivity in the T-state. Analysis by ^1H NMR indicates that the heme binding pocket and the α₁β₁ interface in scHb have structures similar to those in recombinant human hemoglobin (rHb).
Acknowledgements

I would like to express my sincere gratitude to the following individuals:

My advisor, Dr. Spencer Anthony-Cahill for his wisdom, guidance, and inspiration in fueling my desire to increase my knowledge and continually challenge myself.

My committee members, Dr. P. Clint Spiegel and Dr. John Antos for their availability and support.

Past and present group members for creating a positive and effective team.

Dr. John Olson at Rice University for providing the rHb0.0 clone, allowing us access to his laboratory for the ligand binding studies, and for the advice on the data analysis and interpretation.

WWU Sci. Tech Services - Erin Macri for her assistance with the ESI-MS and Clint Burgess for his great technical support.

Hla Win-Piazza and Peter Brzovic for their training and support with the NMR.

This work was funded by NIH grant 2R15HL081068
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Introduction

*Necessity for a red blood cell substitute.* The 2011 national blood collection and utilization survey reported a decreasing margin between available units of blood collected and number of units transfused. Between 2001 and 2008, the rate of units transfused was increasing at a slower rate than available supply (1). Between 2008 and 2011, there was a comparable decrease in the supply and units transfused (1). However, data from 2011 showed a greater decrease in available supply due to rejected units, leaving a margin of 5.2% of the available supply, Figure 1 (1).

![Figure 1](image1.png)

**Figure 1.** Allogeneic whole blood and red blood cell collections and transfusions from 1989-2011. The figure compares total donated blood (diamonds), total available supply (excludes supply rejected after testing – triangles), and the number of units transfused (circles) (1).

In response to this shrinking margin, significant research has focused on the development of a red blood cell (RBC) substitute. RBCs possess several distinct characteristics that make them ideal for their primary function of transporting oxygen throughout the body. They contain high concentrations of hemoglobin (Hb) encapsulated within a cell that protects the protein from degradation, as well as protecting tissues from oxidative damage (2). RBCs also encapsulate a number of other proteins that assist in function and prevention of toxicity, such as methemoglobin
reductase. Methemoglobin reductase prevents buildup of the oxidized form of Hb (Fe$^{3+}$ vs. Fe$^{2+}$), which is unable to bind oxygen. Another important enzyme found in RBCs is superoxide dismutase (SOD) which catalyzes the breakdown of superoxide ($O_2^-$). In addition, RBCs contain high concentrations of allosteric effectors such as 2,3-bisphospho-D-glycerate (2,3-BPG) that help modulate oxygen binding affinity. An ideal RBC substitute must be manufactured economically and in large quantities, be stable in vivo, possess oxygen carrying properties comparable to whole blood, and be non-toxic (2-7).

*Approaches to creating an RBC substitute.* Three main classes of RBC substitutes have actively been pursued. The first are perfluorocarbon emulsions, typically composed of a linear or cyclic carbon backbone highly substituted with fluorine and occasionally other halogens (Figure 2). Perfluorocarbons are chemically and biologically inert and have the ability to transport gases, including $O_2$ and $CO_2$, at significantly higher concentrations than aqueous buffers. Considerable interest has been generated by perfluorocarbon emulsions because they are relatively cheap, easy to produce in large quantities, and have minimal risk of infection and immunogenicity (2). An apparent drawback to the use of perfluorocarbons for $O_2$ transport is that they are insoluble in aqueous buffers and therefore not well-suited for intravenous administration. To overcome their insolubility, perfluorocarbons are prepared as emulsions by mixing with a surfactant, most commonly phospholipids (2). Although the perfluorocarbons are biologically inert themselves, toxicity issues arise from the surfactant used to prepare the emulsion. The emulsions’ capacity for $O_2$ is 3 mL/dL, a considerable increase compared to plasma (2). The amount of $O_2$ dissolved by perfluorocarbons is directly proportional to the p$O_2$ (2). This quality has the potential to be advantageous if the patient can be provided with high levels of fractional inspired oxygen (Fi$O_2$) in an oxygen tent, however this limits perfluorocarbon use to hospitals.
Figure 2. Examples of perfluorocarbon O\textsubscript{2} carriers studied in clinical trials: A) perfluorodecalin; B) Fluosol-DA; C) dichloroperfluoroctane; and D) perfluoroctyl bromide (perflubron).

Another class of RBC substitutes is liposome encapsulated Hb (LEHb), typically composed of a phosphatidylcholine bilayer containing a concentrated Hb solution (Figure 3). The appeal of LEHb is that it more closely mimics the size of RBCs. There is a protective membrane that prevents Hb from degradation. The liposome encapsulation also serves to protect tissue from oxidative damage and results in an increased circulatory half-life compared to other RBC substitutes (2). A large advantage is the ability to include the alloseric effector 2,3-BPG, methemoglobin reductase, and SOD within the liposome. A major drawback is the difficulty of creating liposomes of uniform size (2). Furthermore, LEHb is cleared by the reticuloendothelial system (RES), which can potentially pose an issue with the large clearance of phosphatidylcholine (2).

Figure 3. Cross-section of a liposome encapsulated hemoglobin RBC substitute (2).
The most-studied class of RBC substitutes is extracellular hemoglobin-based oxygen carrying (HBOC) solutions. The primary design criterion for an HBOC is that it possesses reversible O\textsubscript{2} binding behavior that mimics O\textsubscript{2} delivery by RBCs. As shown in Figure 4, the primary advantage of HBOCs compared to perfluorocarbons, is their efficient transport of O\textsubscript{2} under physiological conditions (2). The major shortcoming of donated blood is that it has a shelf life of only a few weeks, with mounting evidence to suggest that the incidence of adverse events following transfusion is correlated with increased time between collection and administration of a unit of blood (3,4). Because cell-free HBOCs are less complex in composition, and can be formulated with anti-oxidants, they have a longer shelf-life than RBCs (5). HBOCs also eliminate the need for blood-typing or cross matching, and could therefore be administered by emergency medical personnel at the site of an accident, rather than after transport to a medical facility (3). Thus, a trauma patient would receive oxygen-carrying fluids significantly earlier than is possible by current transfusion protocols.

![Figure 4](image-url)  
**Figure 4.** Comparison of O\textsubscript{2} dissociation curves of several types of RBC substitutes and human RBCs. Represented are PEG-Hb (an HBOC conjugated with polyethylene glycol), human RBCs, αα-crosslinked Hb (a covalently crosslinked HBOC generated by recombinant technology), O-raffinose polymerized Hb (chemically polymerized HBOC), and a perfluorocarbon emulsion (2).
Challenges associated with HBOCs. The first challenge associated with the development of HBOCs is the source of starting material. Three primary sources exist: human RBCs from outdated donated blood, bovine RBCs, and hemoglobin produced by recombinant technology (6). The product manufactured from human RBCs is limited by the availability of an outdated supply. Approximately 5-10% of human RBCs become outdated (11), leaving very little starting material to satisfy the need for an HBOC manufactured by this method. In contrast, bovine RBCs are plentiful. Rigorous testing is performed on donated blood to essentially eliminate the possibility of infection by human pathogens. However, both human and bovine RBCs are derived from sources which can be infected by mammalian pathogens (2,6). Such screening makes it unlikely that contaminated human RBCs will be administered to a patient. Nonetheless, even the remote possibility of infection by HIV or hepatitis viruses has resulted in a significant change in transfusion practices. Transfusion is viewed as a high-risk procedure by both patients and physicians (1,11). Concerns with using bovine Hb to make an HBOC include the possibility of contamination with bovine prions, which can cause variant Creutzfeldt-Jakob disease in humans (9,12).

An alternate starting material, recombinant Hb, was first described by Nagai and Thorgersen in 1984 (13,14). Their strategy was to express β-globin as a cleavable fusion protein, purify the fusion protein, and cleave the fusion junction using blood coagulation factor X (14). The isolated β-globin was reconstituted with heme and α-subunits to form fully folded α₂β₂ tetramers (14). In 1990, a simplified method for recombinant Hb expression was developed by Hoffman et al. (15). This method utilized an expression plasmid encoding both β- and α-globin genes, which were coexpressed in E. coli with addition of supplemental hemin. α- and β-chains folded correctly into fully functioning holo-rHb tetramers in vivo (15). This method was also successfully applied to Hb expression in S. cerevisiae (16), however most researchers abandoned the yeast system due to better holo-rHb yields from the E. coli expression system. Yields of 10-30% of total soluble protein
were reported for holo-rHb expression in *E. coli* (17,19). This method of production minimizes exposure of the HBOC preparation to human pathogens; however, the bovine hemin used to supplement the bacterial culture must be treated to remove any potential pathogens (2).

Although bacterial fermentation presents the possibility of a limitless supply of hemoglobin as starting material for an HBOC, the purification of recombinant Hb from bacterial lysate is significantly more complex than purifying Hb from erythrocytes. Because a high dosage of the recombinant protein is required, very little contaminant can be tolerated (2). Recombinant hemoglobin must be 99.999% pure to be safe for injection in humans.

Cell-free hemoglobin has a higher affinity for oxygen compared to hemoglobin in RBCs, because the negative allosteric effector 2,3-BPG exists in high concentrations in erythrocytes and functions to decrease O₂ affinity to the physiologically optimum range (2,9). Cell-free Hb will not be bound by 2,3-BPG due to its low concentration in blood plasma. Thus a cell-free Hb must be chemically modified, or contain specific mutations, to decrease its O₂ affinity. Both strategies have been employed in the development of HBOCs (2,7,9).

The formation of methemoglobin, the Fe³⁺ form of Hb, is increased in cell-free Hb solutions. Unlike Fe²⁺ Hb, methemoglobin cannot bind O₂ reversibly. Within RBCs, the presence of methemoglobin reductase and other antioxidants act to reduce the ferric heme to the functional ferrous form, and also protect against the formation of reactive oxygen species and heme release. When the oxidation of iron from Fe²⁺ to Fe³⁺ occurs, the bond between the heme and the ε-NH of the proximal histidine weakens, resulting in increased rates of heme dissociation (22). Free heme catalyzes reactions that produce reactive oxygen species (8) which are very damaging to cells (2,7,8).
There are two mechanisms of reaction that lead to the autooxidation of oxy-Hb. The first is direct dissociation of a superoxide anion (20):

\[
(1) \quad \text{Hb}(\text{Fe}^{2+})\text{O}_2 \rightarrow \text{Hb}(\text{Fe}^{3+}) + \text{O}_2^-. 
\]

The second mechanism for autoxidation is through nucleophilic displacement, where \( \text{O}_2 \) dissociates from ferrous heme and a nucleophile (Nu; the distal histidine is thought to act as the nucleophile) binds to the unliganded iron ion. An electron from the ferrous iron is then transferred to the dissociated \( \text{O}_2 \), forming the ferric state (21):

\[
(2) \quad \text{HbO}_2 \leftrightarrow \text{Hb}(\text{Fe}^{2+}) + \text{O}_2 \\
(3) \quad \text{Hb}(\text{Fe}^{2+}) + \text{Nu} \leftrightarrow \text{Hb}(\text{Fe}^{2+})(\text{Nu}) \\
(4) \quad \text{Hb}(\text{Fe}^{2+})(\text{Nu}) + \text{O}_2 \rightarrow \text{Hb}(\text{Fe}^{3+})\text{Nu} + \text{O}_2^-.
\]

If superoxide formation occurs within RBCs, superoxide dismutase (SOD) is present to break down this free radical. However, the mass of Hb administered in a single therapeutic dose (~ 100 g of Hb) could overwhelm the antioxidants in the plasma if a significant fraction of the cell-free Hb were oxidized. Thus, any modifications made to Hb to optimize functional properties (e.g., mutations or chemical crosslinks) must not increase the rate of methemoglobin formation.

Intravenous injection of 100 g of cell-free Hb will cause renal damage (2,3,5,7-9). It is normal to find free Hb at low concentration in blood plasma, due to the steady rate of hemolysis of RBCs. As RBCs age, they are more susceptible to hemolysis, and the Hb released from lysed RBCs acts to stimulate the production of new RBCs. Typically, Hb and its breakdown products in circulation are removed by other proteins in the plasma. Haptoglobin, hemopexin or albumin, and transferrin are able to bind, respectively, to circulating Hb dimers, heme, and iron (8,23). The resulting Hb-bound complexes are transported to specific tissues where they go through metabolic processing followed
by excretion. However, if these plasma proteins become saturated as a result of excess Hb in circulation, the persisting Hb accumulates in kidney tissue (8,24,25). Within erythrocytes, Hb is densely packed and stable as a 64 kDa heterotetramer ($\alpha_2\beta_2$). Cell-free Hb is more dilute in blood plasma, thus it has greater propensity to dissociate into 32 kDa $\alpha\beta$ heterodimers (24). The heterodimer is small enough to be filtered by the kidney and accumulate in the proximal tubule, causing renal toxicity (24,26).

In preclinical studies, and in human safety trials, the intravenous (IV) injection of extracellular Hb has been shown to cause hypertensive effects (also known as a “pressor response”) (2,7-9,27). Further investigation has revealed the pressor response to be the result of nitric oxide (NO) scavenging in extravascular tissues (7,28). NO participates in diverse biological functions, including the regulation of blood pressure, as a chemical messenger and as an initiator of biochemical cascades (29). The major mechanism of NO scavenging is the oxidation of NO to $\text{NO}_3^-$ by oxyhemoglobin (30). In addition, both ferrous and ferric Hb can bind NO tightly, resulting in further depletion of NO in the endothelial cells surrounding the vasculature (31). This presupposes that Hb extravasation is the first step in a series of events that leads to the pressor response. If so, decreasing the rate of HBOC extravasation should attenuate the pressor response (32,33).

Support for this hypothesis comes from the data shown in Figure 5, which show that larger molecular weight Hbs attenuate the pressor response in conscious rats (34). The HBOCs used in this study included the 64 kDa rHb1.1, a 130 kDa “di-Hb”, and a 260 kDa “tetra-Hb”. The two higher mass Hbs showed marked reduction of the pressor response compared to the 64 kDa Hb.

A similar reduction in the pressor response has been reported for ultrahigh molecular weight Hbs produced via chemical crosslinking with glutaraldehyde (35,37). Thus the hypothesis that a polymeric Hb reduces side effects associated with NO scavenging is well supported. Preparations
of chemically crosslinked Hbs yield a polydisperse product containing a wide range of polymeric species. The use of recombinant technology would allow the generation of poly-Hbs of defined molecular weight, as well as allowing site-directed mutations to improve HBOC function (6).

Figure 5. Changes in mean arterial pressure induced by intravenous introduction of HBOCs into conscious rats. Represented are a 64 kDa HBOC – rHb 1.1 (orange triangles); a 130 kDa “di-Hb” (blue circles); a 260 kDa “tetra-Hb” (green crosses); and human serum albumin to serve as a volume control (50 mg/mL, purple squares) (34).

The ideal HBOC. An ideal HBOC must address all challenges having to do with production, stability and function, toxicity, and side effects associated with NO scavenging (e.g., the pressor response). An HBOC must be manufactured in massive quantities at a unit cost comparable to that for a unit of donated blood (~ $600-1000) (6). Its functional characteristics should mimic those of whole blood, such that its binding and release of O$_2$ occur in a physiologically efficacious range (2,9). The formation of methemoglobin and free radicals must also be minimized, possibly by the incorporation of superoxide dismutase linked to the HBOC. The HBOC must not be filtered by the kidneys, and NO scavenging must be attenuated. Both of these issues may be resolved by the addition of novel covalent linkages between subunits.
Types of modified Hb. Chemical crosslinking of Hb is the most common method employed for generating novel intra- and intermolecular covalent linkages in HBOCs. The HBOCs produced in this fashion are shown schematically in Figure 6, and include surface-modified, cross-linked mono-Hbs, and polymerized Hbs (2,35). Surface-modified Hb is produced by conjugation of a macromolecule, such as polyethylene glycol (PEG) or polyoxyethylene (POE), to lysine residues on the Hb surface (2). Conjugation with the macromolecules serves two primary purposes: to stabilize the tetramer against subunit dissociation, and increase the hydrodynamic radius (to slow extravasation). Added benefits to this design include a decrease in immunogenicity (38), and an increase in viscosity and oncotic pressure, which may prove to be clinically useful traits (2).

Chemically cross-linked hemoglobins include intramolecular linkages that stabilize the Hb tetramer (Figure 6). One example of a chemical cross-linker is bis-(3,5-dibromosalicyl) fumarate, which reacts with lysine residues in the α-globins (39). This chemical linker is able to specifically bind \( \alpha_1 \text{Lys-99} \) and \( \alpha_2 \text{Lys-99} \) when Hb is in the deoxy form. The cross-linked Hb remains highly cooperative with a Hill coefficient of 2.6 (39). The oxygen affinity is decreased approximately 2-fold, whereas the \( P_{50} \) value increases to 13.9 mmHg compared to 6.6 mmHg for HbA (39).
Intrasubunit crosslinks can also be incorporated by gene fusion. As shown in Figure 7, the recombinant human hemoglobin produced by Somatogen Inc., rHb1.1, uses a single glycine residue to link the $\alpha_1$$\alpha_2$ termini (40). The covalent fusion of the $\alpha$-globins prevents dissociation of rHb1.1 into $\alpha\beta$ dimers, and thereby prevents renal toxicity (40). The $\beta$ globin subunits in rHb1.1 also carry a mutation that reduces $O_2$ affinity to a level that is close to that for whole blood.
The crosslinking of surface lysines by glutaraldehyde results in formation of Hb oligomers of various structures and molecular weights (2). The advantage of polymerized Hbs is their increase in size, which helps prevent extravasation and kidney filtration. A common problem is the difficulty to control precisely the molecular weight distribution of the final product (2,42).

**Engineering and design strategies – single-chain hemoglobin.** The studies reviewed above illustrate the challenges and advantages of fusing the α- or β-chains using recombinant or chemical crosslinking methods; however, no study has yet examined the effect of fusing all four Hb subunits to create a single-chain Hb (scHb). Additionally, none of these studies has described the consequence of directly fusing the α- and β-chains. There are several potential advantages to the generation of a cell-free HBOC derived from a scHb. First, like rHb1.1, a recombinant scHb would prevent the dissociation of the tetramer into αβ dimers. Second, a scHb simplifies the production of genes for ultrahigh molecular weight poly-Hbs and guarantees a monodisperse product. Third, scHb represents a therapeutically useful scaffold to be used for fusion to other proteins such as superoxide dismutase, which would increase the size of the protein as well as prevent buildup of harmful superoxide anions in cases of tissue reperfusion following prolonged ischemia (O₂ starvation).
The most straightforward method of connecting the four globins is to link the termini in series with three peptide linkers. The termini between the two α-globins are approximately 2 Å apart (Figure 8) and have been linked successfully by Looker et al. using a single glycine residue (40). Insertion of a linker between the α- and β- termini has not been reported, and may prove difficult because the distance between them is approximately 50 Å (Figure 8). A linker of that length is likely to complicate the folding of a polymeric Hb, due to many opportunities for “domain swapping” (41).

Figure 8. Illustration of distances between termini of Hb subunits, as observed in crystal structures. Left: Approximately 2 Å separates the α1-α2 termini, which can be linked by one or two residues. Right: Approximately 50 Å distance separates the α1-β1 termini.

Our strategy to achieve covalent connection of the α- and β-globins is to employ circular permutation of the β-globin. The effect of circular permutation can be understood by imagining the connection of the native N- and C-termini with a novel peptide linker, followed by cleavage of the resulting circularized protein to form novel termini (see Figure 9).
Figure 9. A cartoon representation of the circular permutation process of β-globin. The letters A-H signify different helices within the subunit. The bottom figure represents a hypothetical circularized protein.

By introducing a linker between native N- and C-termini, the β-globin termini can be relocated to the α₁-β₁ interface in hemoglobin, allowing for insertion of the entire permuted β-globin sequence into the G-H loop of the α-globin. Closer examination of the Hb crystal structure shows that the G-H loops of both α₁ and β₁ subunits are located on the globin surface and are separated by less than 15 Å (Figure 10). In the past, circular permutation has been successfully applied to both human α-globin (43) and sperm whale myoglobin (swMb) (44,48), which demonstrates that globins can tolerate permutation. Another reason to fuse the globins at the α₁β₁ interface is that this interface remains essentially static during R to T transitions, unlike the α₁β₂ interface, which is extensively remodeled (49). Additionally, the globin fold and function has been shown to tolerate circular permutation and extensive crosslinking (43,44,48,50-55).
Figure 10. The α₁β₁ subunits above (the image on the right is a 90° rotation of the image on the left) highlight the proximity of the G-H loops in the α- and β-subunits. The G-H loop in β-globin, which will serve as the new location of termini in the circular permutant, is colored orange. The G-H loop in α-globin is colored green. These loops are approximately 15 Å apart and can be fused by a peptide linker of 5 amino acids in length once the β-globin is circularly permuted.

Myoglobin as a model system. To assist in the design of an optimal β-hemoglobin permutein, myoglobin has been employed as a model system. Sperm whale myoglobin (swMb) is a well-characterized structural and functional homolog of human hemoglobin. As such, it has been shown to be a good model to understand the effects of point mutations on hemoglobin function (10). Thus, we have used Mb to test the effects of permutation on globin structure, stability, and function (44).

Properties of an optimal linker. When designing a circular permutein, a peptide linker of the proper length and flexibility must be inserted between the N- and C-termini of the parent protein. If the linker is too short, it induces conformational strain. If it is too long, increased conformational entropy can destabilize the permutant as well as reducing control over subunit assembly.

The first circularly permuted swMb produced in our lab, called “HGL16”, included a flexible 16 residue linker with the sequence, (GGGS)₄, inserted between the native termini (44). Figure 11 shows a schematic model of HGL16. HGL16 possesses a structure similar to that of wild-type swMb,
as determined by one- and two-dimensional NMR (44). Stopped-flow methods were utilized to assess ligand binding function, which proved to be essentially identical to that for wild-type swMb (see Table 1). However, the permutein showed a decreased stability by ~5 kcal/mol (Table 2). Several HGLx permutants were generated to assess the effects of varying linker length and amino acid composition on permutein stability. The stabilities of these linker variants were not significantly altered by the changes (Table 3).

Figure 11. Left: the X-ray crystal structure of wild-type swMb. Right: a model of circularly permuted swMb (“HGL16”) with a 16 residue linker inserted between the native N- and C- termini, and novel termini located between the G-H loop.
Table 1. Ligand binding rate constants and equilibrium constants for HGL16 and wild-type swMb. Ligand binding data were measured by stopped flow methods (44). Numbers in parenthesis are values reported by Rohlf et al. (57).

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<thead>
<tr>
<th>Parameter</th>
<th>HGL16</th>
<th>wt swMb</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO $k_{on} \cdot 10^6$ (M$^{-1}$s$^{-1}$)</td>
<td>0.63</td>
<td>0.50 (0.50)</td>
</tr>
<tr>
<td>CO $k_{off}$ (s$^{-1}$)</td>
<td>0.017</td>
<td>0.021 (0.018)</td>
</tr>
<tr>
<td>$K_{CO} \cdot 10^6$ (M$^{-1}$)</td>
<td>37</td>
<td>24 (28)</td>
</tr>
<tr>
<td>$O_2$ $k_{on} \cdot 10^6$ (M$^{-1}$s$^{-1}$)</td>
<td>18</td>
<td>16 (16)</td>
</tr>
<tr>
<td>$O_2$ $k_{off}$ (s$^{-1}$)</td>
<td>15</td>
<td>18 (17)</td>
</tr>
<tr>
<td>$K_{O2} \cdot 10^6$ (M$^{-1}$)</td>
<td>1.2</td>
<td>0.89 (0.9)</td>
</tr>
</tbody>
</table>

Table 2. Thermodynamic stability parameters for HGL16 and wild-type swMb calculated from urea denaturation experiments. Values listed are averaged over 3-4 separate experiments (44).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HGL16</th>
<th>wt swMb</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta G^\circ_{H2O}$ (kcal·mol$^{-1}$)</td>
<td>7.2 ± 0.5</td>
<td>12.4 ± 1.6</td>
</tr>
<tr>
<td>$m_g$ (kcal·mol$^{-1}$·M$^{-1}$)</td>
<td>-1.65 ± 0.11</td>
<td>-1.83 ± 0.23</td>
</tr>
<tr>
<td>$C_m$ (M urea)</td>
<td>4.19 ± 0.05</td>
<td>6.90 ± 0.06</td>
</tr>
</tbody>
</table>
In the current work, focus is shifted towards the design of a structured linker in the hope that it will increase permutein stability. Computational methods were employed to assist in the design of more complex linkers with stable structures (45, 46). Using RosettaDesign software, three circularly permuted swMb models were generated. All three of these designs include an α-helical linker between native termini, rather than a long flexible chain as in HGL16. Additionally, the designs include mutations made to the Mb framework to create novel interactions with the linker (Figure 12). If any of these models prove to increase the stability of the permutein, their design will inform a similar linker re-design for permuted Hbs.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Linker</th>
<th>ΔΔG°H2O (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>HGL 6</td>
<td>SGGSGG</td>
<td>-6.2 ± 0.8</td>
</tr>
<tr>
<td>HGL 7</td>
<td>SGGSGGG</td>
<td>-5.4 ± 0.9</td>
</tr>
<tr>
<td>HGL 8</td>
<td>SGGSGGG</td>
<td>-4.2 ± 0.7</td>
</tr>
<tr>
<td>HGL 9</td>
<td>SGGSGGGG</td>
<td>-4.8 ± 0.9</td>
</tr>
<tr>
<td>HGL 16</td>
<td>(SGGG)₄</td>
<td>-5.0 ± 0.7</td>
</tr>
<tr>
<td>HGL PP12</td>
<td>(P)₁₂</td>
<td>-7.0 ± 1.5</td>
</tr>
<tr>
<td>HGL EAAAS</td>
<td>EAAAS</td>
<td>-5.9 ± 0.6</td>
</tr>
</tbody>
</table>
Figure 12. Representations of linker structures as predicted by RosettaDesign are shown above. Panels A, B, and C (ML1, ML2, and ML3, respectively) illustrate circularly permuted swMb with the designed linker shown as spheres. The ruby spheres indicate hydrophilic residues and the yellow indicate hydrophobic residues within the linker. The dark blue spheres represent hydrophilic mutations and the light blue spheres are hydrophobic mutations made to the swMb framework. The mutations have been made in such a way to support the hydrophobic packing between the linker and the permutein. Panel D highlights the helical design of ML1 (47).

*Progress toward the generation of scHb.* To achieve the goal of generating a therapeutically useful scHb, our lab has progressed rationally through a series of circularly permuted globins (Figure 13).
Figure 13. Scheme for the generation of scHb: starting with αcpβ (top), then linking α- and cpβ-globin to create sc-αcpβ (middle), followed by the insertion of a linker between α1-α2 termini to create scHb (bottom). See text below for the definitions of these terms.

The initial design included circular permutation of the β-globin (cpβ) to allow co-expression with α-globin (acpβ) (58). The linker between native N- and C-termini is eight residues in length with the sequence Gly-Ser-Gly-Gln-Gly-Gly-Gly-Gly. The αcpβ construct is significantly less stable than recombinant wild-type hemoglobin (as shown by decreased soluble protein yields) (58). According to analytical HPSEC, co-expression appears to yield α1cpβ1 dimers, rather than a tetramer (58). αcpβ appear to bind ligand reversibly based on flash photolysis and stopped-flow (SF) data (Table 4). αcpβ exhibits biphasic ligand binding kinetics, with a significant shift to R-state vs. T-state compared to wild-type Hb (see Figure 14) (58).
Table 4. Rate constants of CO and O\textsubscript{2} binding to HbA and \(\alpha\text{cp}\beta\) (58).

<table>
<thead>
<tr>
<th>CO binding</th>
<th>(k'_T) ((\mu\text{M}^{-1}\text{s}^{-1}))</th>
<th>(k'_R) ((\mu\text{M}^{-1}\text{s}^{-1}))</th>
<th>O\textsubscript{2} binding</th>
<th>(k'_T) ((\mu\text{M}^{-1}\text{s}^{-1}))</th>
<th>(k'_R) ((\mu\text{M}^{-1}\text{s}^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA published values (64)</td>
<td>0.12</td>
<td>6</td>
<td>HbA published values (64)</td>
<td>5-10</td>
<td>66</td>
</tr>
<tr>
<td>HbA 100% photolysis</td>
<td>0.09</td>
<td>8.3</td>
<td>HbA 100% photolysis</td>
<td>7.09</td>
<td>59.71</td>
</tr>
<tr>
<td>HbA 4% photolysis</td>
<td>-</td>
<td>4.7</td>
<td>HbA 11% photolysis</td>
<td>-</td>
<td>42.85</td>
</tr>
<tr>
<td>(\alpha\text{cp}\beta) 100% photolysis</td>
<td>0.72</td>
<td>6.58</td>
<td>(\alpha\text{cp}\beta) 100% photolysis</td>
<td>19.19</td>
<td>61.7</td>
</tr>
<tr>
<td>(\alpha\text{cp}\beta) 12% photolysis</td>
<td>-</td>
<td>5.2</td>
<td>(\alpha\text{cp}\beta) 11% photolysis</td>
<td>-</td>
<td>45.67</td>
</tr>
</tbody>
</table>

Figure 14. Time courses for full photolysis of CO from HbA and \(\alpha\text{cp}\beta\) (panel A) and partial photolysis (B) of CO from HbA and \(\alpha\text{cp}\beta\). Experiments were conducted in 0.1 M potassium phosphate, 1 mM EDTA, pH 7.0 at 20 °C (58).

Not only does \(\alpha\text{cp}\beta\) retain similar function with reversible ligand binding to wild-type Hb, it also possesses similar fold and structure. A crystal structure, shown in Figure 15, was solved for \(\alpha\text{cp}\beta\) to 2.7 Å resolution. The crystal structure confirms association of the four subunits with an \(\alpha\text{i}/\text{cp}\beta\text{j}\) interface that is analogous to the wild-type \(\alpha\text{i}/\beta\text{j}\) interface. The structure also shows that the \(\beta\) heme-pocket is not perturbed by the circular
permutation. Additionally, the R-state Hb conformation is favored. The key conclusion from this study is that the circularly permutated Hb has the expected structure: The \( \text{cp}\beta \) termini were successfully localized to the \( \alpha_\beta \) interface, and the permutation did not significantly perturb the \( \beta \)-globin structure.

![Figure 15](image)

**Figure 15.** Crystal structure of \( \alpha\text{cp}\beta \). The blue subunits are \( \text{cp}\beta \)-globin and red subunits are \( \alpha \)-globin. The two light blue loops on the \( \text{cp}\beta \)-globins are the locations of the inserted linker between native N- and C-termini. Unpublished data by Michael Murphy and Clint Spiegel.

The next phase in the design of a single-chain Hb requires the introduction of novel covalent connections between the new \( \text{cp}\beta \)-termini and the G-H loop in the \( \alpha \)-subunit to create single-chain \( \alpha\text{cp}\beta \) (sc-\( \alpha\text{cp}\beta \)). Expression yields of this construct were greatly decreased compared to \( \alpha\text{cp}\beta \), implying decreased stability (6). Three mutations in HbA (\( \beta \) G16A, \( \beta \) H116I, and \( \alpha \) G15A) were discovered by Graves *et al.* to increase stability of apohemoglobin, add resistance to denaturation, and enhance expression levels in *E. coli* (6). In addition, the so-called “Providence” mutation, \( \beta \) K82D, was shown by Weickert *et al.* to enhance stability and expression yields in recombinant Hb (17). These four stabilizing mutations (“4sm”) have been incorporated into the \( \alpha\text{cp}\beta \) construct,
resulting in increased expression yields. Subsequently, these mutations were integrated into sc-αcpβ (sc-αcpβ 4sm). Analytical HPSEC data supports again that these Hbs exist as a mixture of heterotetramers and heterodimers at µM concentrations (data not shown). To stabilize the tetramer structure, a glycine linker was integrated between the two α-globins (diaα-cpβ). Based on equilibrium O₂ binding data, all of these constructs appear to be high affinity (R-like) and all have similar ligand binding affinities (Figure 16). This implies that the novel connections in sc-αcpβ and diaα-cpβ are well tolerated, but do not improve O₂ binding parameters for the permuted Hbs.

**Figure 16.** Equilibrium binding data was collected in 100 mM potassium phosphate buffer at pH 7.0, 1.5 mM EDTA. All samples were 30 μM in terms of heme.

One-dimensional NMR studies show folding around the heme pocket for sc-αcpβ variants remains relatively unperturbed compared to wild-type (Figure 17).
Figure 17. 1-D $^1$H NMR of wild-type and permuted Hbs. A: Oxygenated isoforms. B: Cyanomet isoforms. All samples obtained at 21°C in 20 mM potassium phosphate buffer, pH 7.0, 10% D$_2$O. Data was collected at 500 MHz with a WATERGATE pulse sequence. Oxygenated samples show minimal perturbation of the heme pocket and $\alpha_1\beta_1$ subunit interfaces. Unpublished data by Janette Myers and Jesse Gortner.
Based on the encouraging results obtained with previous permuted Hb constructs, our lab has generated the first reported scHb. As outlined in Figure 13, the sc-Hb gene was constructed by linking two consecutive sc-αcpβ 4sm genes with a single glycine codon. Preliminary ligand binding and structural studies have been performed to assess the consequences of covalently linking the four subunits of Hb. This information will help to further the knowledge in the field of RBC substitutes, and scHb will potentially serve as a starting material or scaffold for a next generation poly-Hb HBOC (Figure 18).

Figure 18. Schematic of a poly-Hb HBOC derived from scHb.
Materials and Methods

*Computationally designed circularly permuted myoglobin*

*Gene design.* The three computationally designed myoglobins were designed by Ashutosh Jogalekar and Brian Kuhlman using RosettaDesign software. The genes were synthesized and sent to us by GenScript (Piscataway, NJ) in pET-28a plasmids.

*Protein expression.* Plasmids were transformed into *E. coli* BL 21 cells and grown at 37° C in Terrific Broth (TB) medium (12 g/L tryptone, 24 g/L yeast extract, 4 mL glycerol, 2.31 g/L KH₂PO₄, and 12.54 g/L K₂HPO₄) with 30 μg/mL kanamycin. Using shake flasks, cells were grown to an optical density of 0.8-1.0 at 600 nm followed by induction with 1 mM isopropyl β-thiogalactopyranoside (IPTG) at 30 °C for 6 hours. Cultures were harvested by centrifugation at 5,000 x g for 10 minutes. The supernatant was discarded, and the cell pellet was stored at -80°C.

*Inclusion body preparation.* All steps were performed on ice. Cells from 1-L growths were rapidly thawed and resuspended in 35 mL of Buffer A (50 mM Tris-HCl, 1 mM EDTA, pH 7.5 ) with the addition of 42 μL beta-mercaptoethanol. 10 mg lysozyme per gram of cells was added to the cell suspension, immediately followed by the addition of one cOmplete Protease Inhibitor Cocktail tablet (Roche Applied Sciences) dissolved in 2 mL ddH₂O. The solution was mixed with a glass rod every 5 minutes to avoid metal contaminants, and allowed to incubate for 30 minutes. Once the mixture had a thicker consistency, MgCl₂ was added to a final concentration of 5 mM, immediately followed by the addition of 300 μL of 1 mg/mL DNase. The cell suspension was again mixed on ice for 30 minutes. The resulting lysate was centrifuged at 30,000 x g for 15 minutes. The supernatant was discarded and the pellet was resuspended in 35mL of Buffer A. The suspension was sonicated on ice for 3 minutes, in 1 minute increments with 1 minute intervals between each round, using a Branson...
Instruments, Inc. Sonifier (model 450) equipped with a 1.8 cm diameter horn using a 50% duty cycle and a power output level of 5. This sonication and resuspension process was repeated two more times, each time discarding the supernatant. The final pellet was resuspended in 35 ml of Buffer A and 2.1 g of urea. This suspension was incubated for 30 minutes, followed by centrifugation at 30,000 x g for 15 minutes. The supernatant was discarded and the pellet was retained and stored at -80 °C.

The inclusion body pellet was thawed and resuspended in 30 mL of Buffer B (10 mM dithiothreitol (DTT), 0.1% trifluoroacetic acid) and sonicated on ice for 5 minutes, in 1 minute increments with 1 minute intervals between each round, with a 50% duty cycle and a power output level of 7. The suspension was centrifuged at 30,000 x g for 15 minutes. The supernatant was saved and acetonitrile added to 10% (w/v). The supernatant was filtered through a 0.45 μm syringe cassette filter (Millipore Durapore) with a fiber glass prefilter.

The filtered solution was applied to a preparative, 22 x 250 mm, PROTO 300 (Higgins Analytical) C4 reversed-phase high performance liquid chromatography column at a flow rate of 2 mL/min. The initial mobile phase was 10% MeCN/H₂O. All mobile phase buffers contained 0.1% TFA. The sample was eluted at a flow rate of 10 mL/min, with a gradient up to 70% MeCN/H₂O over the course of 30 minutes. The absorbance was monitored at 280 nm and peaks were collected in separate fractions. These fractions were lyophilized to remove solvent and stored at -80 °C.

**Protein reconstitution.** Each step of protein reconstitution was carried out at 4 °C. Lyophilized protein was solubilized with 5 mL of sodium acetate buffer (10 mM, pH 6.0) containing 6 M urea. A heme dicyanide solution was prepared to provide a 1.2 molar heme excess over apomyoglobin. Hemin (98% +, Alfa Aesar), was dissolved in 10 mL of 10 mM sodium hydroxide solution. 1 mL of heme sodium hydroxide solution was added to 25 ml of potassium phosphate
buffer (100 mM, pH 8.0) containing 100 mM KCN, and allowed to mix for 20 minutes. A rapid 10-fold dilution was carried out by the addition of potassium phosphate buffer (100 mM, pH 8.0), followed by immediate addition of the heme dicyanide solution. This solution was allowed to stir for approximately 40 min.

Concentrated holoprotein samples were applied to an Amersham Pharmacia Biotech Superdex 75 10/300 size exclusion column (SEC) equilibrated in 100 mM potassium phosphate buffer, pH 8.0, with a flow rate of 0.8 mL/min. The absorbance at 423 nm was monitored and fractions corresponding to peaks at this absorbance were collected.

15% SDS-PAGE. Samples were prepared by the addition of 2x SDS-PAGE protein loading buffer (50mM Tris-HCL, 2% SDS, 0.2 % bromophenol blue, 10% glycerol, pH 6.8) and 1 μL of 1 M DTT, and incubated for five minutes at 100 °C. Once polymerization of the 15% (w/v) SDS-PAGE resolving layer was complete, the 10 % (w/v) SDS-PAGE stacking layer was added. The protein samples along with a protein ladder of known molecular weight standards were loaded and placed in the electrophoresis apparatus with Tris-glycine reservoir buffer (25 mM Tris, 250 mM glycine, 0.1% (w/v) SDS, pH 8.3). The gel was then subjected to 60 V through the stacking layer and increased to 120 V at the beginning of the resolving layer until the tracking dye reached the bottom of the gel. After electrophoresis, gels were stained overnight with Coomassie Brilliant Blue solution (50% (v/v) methanol, 10% (v/v) acetic acid, 0.1% (w/v) Coomassie Brilliant Blue R250). After staining was completed, the gels were destained with a 1:3:4 glacial acetic acid:methanol:water solution.

UV-Visible spectroscopy. Data was collected on a Hewlett Packard 8452A diode array spectrophotometer. Samples were placed in a 400 μL quartz cuvette with a 1 cm path length. Apo protein concentrations were estimated using $\varepsilon_{280}= 15,200 \text{ M}^{-1}\text{cm}^{-1}$. Holo cyanomet protein concentrations were estimated using $\varepsilon_{423}= 109.7 \text{ mM}^{-1}\text{cm}^{-1}$ (72).
**Electrospray Ionization Mass Spectrometry (ESI-MS).** Purified proteins were analyzed by electrospray ionization mass spectrometry (ESI-MS) with an Applied Biosystems 2000 API 2000 LC/MS/MS mass spectrometer. Samples were in either a 1:1 ratio of acetonitrile (0.1% formic acid) to water (0.1% formic acid) at a heme concentration between 10-20 µM. The total sample volume was 500 µL. Acetonitrile aided in the solubilization of the samples while the formic acid served as the proton source for ionization. Mass to charge ratios were obtained, averaged, and used to reconstruct the peptide masses.

**Single-chain hemoglobin**

*Polymerase chain reaction (PCR).* sc-αcpβ plasmids were isolated from BL21 (DE3) *E. coli* cells to be used for the cloning of scHb. Four sets of primers were ordered from Integrated DNA Technologies (San Diego, CA) in order to attempt the insertion of two distinct linkers between the termini of the α-globins. The first two sets of primers were designed to amplify the 5’ end of the scHb gene and the 3’ end of the scHb gene utilizing the KasI restriction site to insert a linker that codes for “Gly-Ala”:

```
TTTGGGATCCGAGCTGTTGACAATTAATCATCG
TTTGGCCGACCGGTATTTCGAAGTCAGAACGGTAG
```

```
TTTGGCGCGCTTCTGCTTCCGGCCGATAAAAACC
TTTAAGCTTCTATACGGAATTTCGAAGTCAGAAC
```

The second two sets of primers were designed to amplify the 5’ end and the 3’ end of the scHb gene utilizing the SalI restriction site to insert a linker that codes for “Gly-Ser-Thr”:

```
TTTGGGATCCGAGCTGTTGACAATTAATCATCG
TTTGGTCGACCCACGGTATTTCGAAGTCAGAACGGTAG
```

```
TTTGGCGCGCTTCTGCTTCCGGCCGATAAAAACC
TTTAAGCTTCTATACGGAATTTCGAAGTCAGAAC
```
TTTTGGGTGACGTTCTGTCCGGCGATTTTAC
TTTTAAAGCTTCATTAACGGTATTTCGAAGTCAGAAC

A Stratagene Robocycler equipped with a hot top was used to perform all PCR amplification reactions. Each reaction consisted of 1x Crimson Taq reaction buffer (12.5mM Tricine, 42.5 mM KCl, 1.5 mM MgCl$_2$, 6% Dextran, pH 8.5 at 25 °C, supplied by New England Biolabs), 50 pmol of each primer, 50 ng of template DNA, 10 pmol of each dNTP, and two units of Crimson Taq DNA Polymerase (New England Biolabs) in a final volume of 50 μL. The temperature program consisted of 35 cycles of melting at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds, followed by 90 seconds of primer extension at 68 °C.

**DNA visualization.** DNA was analyzed by gel electrophoresis on 1% (w/v) agarose gels containing 1X Gel Red Nucleic Acid Stain (Biotium, Inc.). Samples were electrophoresed at 200 V in a 1x sodium borate buffer, pH 8.2 for 45-60 minutes. Samples were combined with a 1x loading buffer (0.04% (w/v) bromophenol blue, 0.04% (w/v) xylene cyanol, 5% (v/v) glycerol) and run alongside 10 μL of Hi-Lo Marker (Minnesota Molecular) for approximate molecular weight determination. The DNA was then visualized using a UV transilluminator and documented using a digital camera.

**DNA purification.** DNA was purified from PCR reactions using a QIAGEN MiniElute PCR Purification Kit as per the manufacturer’s instructions. DNA from restriction digests was purified using a QIAGEN MiniElute Reaction Cleanup Kit. Plasmid DNA was purified from overnight cultures of *E. coli* using a QIAGEN QIAprap Spin Miniprep Kit.

**Restriction digestions.** All restriction digests were performed using restriction endonucleases supplied by New England Biolabs in the supplied buffer. Bovine serum albumin (BSA) was added to the reaction to a final concentration of 100 μg/mL when specified by the supplier. Single digests
were performed at 37 °C for 30 minutes. 20 units of each enzyme were used in each reaction. The total reaction volume for each digest was 20 μL.

**Ligation.** Ligation reactions were performed in 1X T4 DNA ligase buffer (50 mM Tris-HCl, 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM ATP, 50 μg/mL BSA, pH 7.8 at 25 °C, supplied by New England Biolabs). Relative concentrations of reactants were estimated by visualization of bands on agarose gels prior to ligation. The reactants were then added to the reaction mixture in an approximately 1:1 ratio. Four hundred units of T4 DNA ligase (supplied by New England Biolabs) were added and the reaction mixture was incubated for approximately 16 hours at 16 °C.

**Gene design.** Attempts to clone the scHb gene proved to be unsuccessful. A novel scHb gene was designed with a single glycine residue between the α-globins. This gene was synthesized, and subcloned into a custom recombinant Hb expression vector (described in ref. 58) by GenScript (Piscataway, NJ).

**Preparation of electrocompetent cells.** One L of LB broth (10 g/L Bacto-tryptone, 5 g/L yeast extract, 170 mM NaCl, pH 7.0) was inoculated with a 10 mL overnight culture of BL21 (DE3) cells (F-, ompT, hsdSb(rB-, mB-), dcm, gal, λ(DE3)) and incubated with 175-200 rpm shaking at 37 °C until OD₆₀₀ = 0.5-0.7 was reached. The cells were then harvested by centrifugation at 4,000 x g for 10 minutes. The supernatant was discarded and the cells were centrifuged again briefly to remove any remaining supernatant. The cells were gently resuspended in 1 L of ice-cold dd H₂O and centrifuged at 4,000 x g for ten minutes. The supernatant was discarded and the cells were resuspended in 0.5 L of ice-cold dd H₂O. The cells were again centrifuged at 4,000 x g for ten minutes and the supernatant discarded. Cells were then resuspended in 20 mL of sterile, ice-cold 10% (v/v) glycerol in dd H₂O and centrifuged at 4,000 x g. The supernatant was discarded and the cells were resuspended in 2-3 mL ice-cold 10% (v/v) glycerol and held on ice. The cells were then pipetted in 100 μL aliquots into pre-
chilled microcentrifuge tubes using pre-chilled pipette tips. Cells were flash-frozen on liquid N\textsubscript{2} and stored at -80 °C.

***Transformation by electroporation.*** E. coli cells were transformed using a Biorad Gene Pulser II set to a resistance of 200 Ohms, a potential of 1.25 kV/mm, and a capacitance of 25 μF. Fifty μL of electrocompetent BL21 (DE3) cells were added to 2 μL of either a ligation mixture or purified plasmid DNA (50-100 ng/μL) in a 1 mm gap cuvette and gently mixed. The mixture was then incubated on ice for 15 minutes. Cells were bathed in 1 mL of SOC medium (0.5% (w/v) yeast extract, 2% (w/v) Tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl\textsubscript{2}, 10 mM MgSO\textsubscript{4}, 20 mM glucose) immediately following electrical discharge, transferred to a culture tube, and incubated for one hour at 37 °C with ~175 rpm shaking. Several dilutions of the culture were then plated on LB-agar plates containing 25 μg/mL tetracycline and incubated at 37 °C for approximately 16 hours.

***Protein expression.*** scHb was expressed in 1-L bacterial cell cultures (BL21 (DE3)). Overnight cell cultures (incubated 14-16 hours at 37 °C) were used to inoculate 1-L of TB medium containing 5 μg/mL tetracycline in 2.8 L Fernbach flasks. The cultures were incubated at 37 °C with 200 rpm shaking in a Lab Line incubator-shaker. The cells were grown until an OD\textsubscript{600} of 2.0-2.2 and induced with 1 mM isopropylthiogalactoside (IPTG). Heme solution was prepared by dissolving 0.05 g of hemin per liter of culture in 5 mL (per 0.05 g) of 20 mM NaOH and then filtering with a sterile 0.2 μm disposable cellulose acetate membrane syringe filter (VWR International). 1 mL of the hemin solution was added to each culture every hour. The cultures incubated for 5 hours at 30 °C and harvested by centrifugation at 5,000 x g for 10 minutes. The cell pellet was resuspended in ~35 mL lysis buffer (17 mM NaCl, 50 mM Tris-HCl, pH 8.5) and stored at -80 °C.

***Protein purification.*** Resuspended cell pellets were thawed and then lysed by sonication for 90 seconds with a Branson Instruments, Inc. Sonifier (model 450) equipped with a 1.8 cm diameter
horn using a 50% duty cycle and a power output level of 6. The crude lysate was centrifuged at 21,000 x g for 20 minutes. The pH of the supernatant was adjusted to 8.0 - 8.5 and zinc acetate from a 0.5 M stock was added to a final concentration of 4 mM. The lysate was centrifuged again at 40,000 x g for 20 minutes and filtered in a Millipore cassette with a glass prefilter, then filtered with a 0.45 µM SFCA bottle top vacuum filter (Fischer Scientific).

**Immobilized metal ion affinity chromatography (IMAC).** The clarified cell lysates were IMAC purified using a 10 mL column of Amersham Pharmacia Biotech Chelating Sepharose Fast Flow resin. The flow rate was 3 mL/min. The column was prepared for loading of clarified lysate as follows: 4 column volumes (CV) of 0.2 M NaCl, 2 CV of 20 mM Zn(OAc)$_2$, and 6 CV of 0.2 M NaCl. The clarified lysate was then loaded and the column was washed as follows: 8 CV of 20 mM Tris-HCl, 0.5 M NaCl, pH 8.5; 2 CV of 250 mM Tris-HCl, pH 8.5; and 6 CV of 20 mM Tris-HCl, pH 8.5. The flow rate was then slowed to 2 mL/min and the protein eluted with 20 mM Tris-HCl, 15 mM EDTA, pH 8.5. A total of 10-15 mL of purified protein was collected and then buffer exchanged into 20 mM Tris-HCl and stored at 4 °C.

**Ion exchange chromatography.** Proteins were purified by quaternary amine anion exchange chromatography using an Amersham Biosciences Mono Q 10/100 GL column (8 mL volume). The flow rate was 2 mL/min. Protein samples were prepared for loading onto the column by filtering through a 0.22 µM Cellulose Acetate Spin-X centrifuge tube filter (Costar) at 13,000 rpm for 1 minute. The column was prepared as follows: 5 CV of dd H$_2$O; 5 CV of buffer A (20 mM Tris-HCl, pH 8.5); 5 CV of buffer B (20 mM Tris-HCl, 0.5 M NaCl, pH 8.5); and then a minimum of 5 CV of buffer A.
A volume of 500 µL of the IMAC-purified protein sample was then loaded and eluted with an 80 minute gradient to 100% buffer B at a flow-rate of 2 mL/min.

The absorbance of the eluent was monitored at 280 nm and fractions with significant absorbance were collected and analyzed by SDS-PAGE. Protein samples typically eluted when the buffer composition reached 25% B. Samples were then concentrated to a volume of 500 µL.

*High pressure size-exclusion chromatography (HPSEC).* Filtered protein samples post Mono Q were purified by HPSEC using an Amersham Pharmacia Biotech Superdex 75 size exclusion column (24 mL volume connected to an Äkta Purifier FPLC system (GE HealthSciences). The flow rate was 0.8 mL/min. The column was prepared for loading of protein by washing with 2-3 CV of buffer (for buffer composition, see the respective protein characterization experiments). All buffers were filtered before use with Millipore 0.45 µM filters. A sample volume of 300-500 µL was injected and the absorbance monitored at 280 nm. All peaks with significant absorbance at 280 nm were collected and concentrated as described above in the IMAC purification section.

*Protein characterization.* scHb was characterized by SDS-PAGE, UV-Visible spectroscopy, and electrospray ionization mass spectrometry (ESI-MS) to determine purity, concentration, and size. For methods, see sections described above for computationally designed myoglobins.

*Analytical HP-SEC.* Analytical size exclusion chromatography was performed using the same column as described in the HPSEC purification section connected to a Varian ProStar automated HPLC system. The retention times based on the absorbance at 414 nm were recorded by the manufacturer’s software package. Experiments were performed using a 20 mM potassium phosphate buffer, pH 7.0 at a flow rate of 0.5 mL/min. Protein samples were concentrated to 160 µM (by heme) and 30 µL sample volumes were injected.
Nuclear magnetic resonance. $^1$H NMR spectra of the scHb and rHb0.0 were obtained at 25°C for oxy isoforms. NMR samples consisted of unlabeled recombinant proteins between 0.5-1 mM in heme concentration in 20 mM potassium phosphate buffer at pH 7.0 in 10% D$_2$O. All data were collected on a Varian Inova Unity spectrometer operating at 11.75 T (500 MHz) field strength with a Varian high-field triple resonance 500 TR/PFG TRIAX probe. Water suppression was achieved with a Presat pulse sequence.

Ligand binding studies. Ligand binding studies were performed in the laboratory of Professor John Olson at Rice University. The HbA samples used in the studies were provided by Dr. Olson. The buffer used in all ligand binding experiments was 100 mM potassium phosphate, 1 mM EDTA, pH 7.0. Anaerobic buffer was prepared by putting 20-30 mL of buffer in a gas-tight syringe and dispensing any air bubbles. The syringe was stoppered and then bubbled with a steady stream of 100% CO or 5% (v/v) CO in N$_2$ for 30 minutes.

Laser flash photolysis. For laser flash photolysis of samples in 100% CO, stoppered, 1 mm path length quartz cuvettes containing a few grains of sodium dithionite were purged with CO for five to ten minutes. Samples were prepared to a final concentration of ~50 µM in heme in a final volume of 500 µL using gas-tight Hamilton syringes. The syringe was rinsed several times with water to remove any air bubbles and then rinsed with 100% CO buffer three times. Buffer was then dispensed into the stoppered cuvette, followed by the corresponding amount of protein. UV-Vis spectra were taken to determine the final heme concentrations. The 100% CO samples were incubated in a 20 °C water bath for approximately ten minutes and then kept on ice.

Photolysis experiments were conducted using the method described by Rohlfs et al. (57). Briefly, ligand was dissociated using a ~0.3 µsecond pulse from a Phase-R Model 2100B dye laser containing Rhodamine575. Transmittance changes were monitored at 436 nm using a
photomultiplier tube attached to an oscilloscope and the resulting signal was then digitized using an A/D converter and transferred to a personal computer, where it was then converted to absorbance data. For partial photolysis, the intensity of the laser pulse was attenuated by placing neutral density optical filters in front of the laser beam. The percent photolysis was then calculated by taking the amplitude in absorbance change for partial photolysis as a percentage of the amplitude in absorbance change for full photolysis. Single and double exponential decay fits were performed on the raw data for partial and full photolysis, respectively (with the data prior to t = 0 sec removed) using either Origin 8.5 or Igor Pro to obtain the observed rates for ligand recombination (k_{obs}).

Pseudo first order rate constants were calculated by dividing the observed rates by the ligand concentration (928 µM for CO). The rate constants for association to the R state, k'_{R} were calculated from both full and partial photolysis data, while the rate constants for association to the T state, k'_{T}, were determined from the full photolysis data.

**Stopped flow.** Stopped flow experiments were conducted at 20 °C using a Gibson-Dionex stopped flow apparatus with the OLIS model 3820 data collection system described by Rohlfs et al. (57). For the measurement of O₂ off-rates by displacement with CO, 4 µM Hb (in heme) samples were prepared in a final volume of 3 mL. Samples were rapidly mixed with an equal volume 100% CO buffer containing sodium dithionite to remove the displaced O₂ and the absorbance change at 424 nm was monitored. The final concentrations of CO and O₂ after mixing were 464 µM and 131 µM, respectively. Single exponential decay fits were performed on the raw data using Origin 8.5 to obtain r_{obs}, the observed rate for replacement of O₂ by CO.

For measurement of the rate of CO binding to deoxy HbA, αcβ, sc-αcβ, and scHb, samples were prepared to the same heme concentrations and final volumes as listed above in N₂-equilibrated buffer containing sodium dithionite. Samples were rapidly mixed with an equal volume
of buffer containing 5% (v/v) in N\textsubscript{2} to a final concentration of either 23 \( \mu \)M or 93 \( \mu \)M CO. The absorbance change at 436 nm was monitored and the raw data fit with a single exponential decay using Origin 8.5 to obtain \( k_{\text{obs}} \). The pseudo first order rate constant for CO binding to the T state, \( k'_{\text{T}} \), was then calculated by dividing \( k_{\text{obs}} \) by the final concentration of CO.

\textit{Oxygen equilibrium curves.} Oxygen equilibrium curves (OEC) were collected using a HEMOX apparatus. The absorbance at 430 nm was monitored at 20 °C as the partial pressure of oxygen was increased. The first derivatives of the OEC curves were then calculated using Origin 8.5. The OEC curve fitting was determined using an excel spreadsheet provided by Professor Olson. Based on the best-fit curves, \( n \) Hill and \( P_{50} \) values were calculated using the solver function in excel.
Results

Computationally designed circularly permuted myoglobin

Protein expression. The permuted model proteins were expressed in BL21 (DE3) *E. coli* cells. To determine whether or not the model proteins could be expressed in *E. coli*, several test expressions were performed to determine soluble and insoluble expression using SDS-PAGE. Over-expressed bands were present in the insoluble fractions for ML1 and ML2 (Figure 19). These expression studies indicated that the model proteins were likely forming inclusion bodies. Inclusion body yields for ML1 were determined post HPLC purification and were typically found to be 2.5-3 mgs of protein per liter of culture. After reconstitution and final purification over SEC, the final yield of ML1 was roughly 0.2-0.3 mgs per liter of culture with a purity of ≥95% (Figure 20A).

![Figure 19](image)

**Figure 19.** Analysis of test expression by 15% SDS-PAGE. Red circles indicate bands of the expected MW for the desired protein.

Protein purification and reconstitution. ML1 was purified using an inclusion body preparation, followed by a two-column purification procedure and reconstitution to the fully-folded cyanomet isoform. Compared to the HPLC elution profile of wild-type Mb and HGL16, ML1 had a slightly longer retention time on a C4 reversed-phase column due to the additional hydrophobic
residues in the permutein linker (data not shown). Unlike wild-type Mb and HGL16, ML1 eluted as two distinct peaks (peak 2A and peak 2B, Figure 20B). These peaks were collected and reconstituted separately. Analysis by SDS-PAGE revealed peaks 2A and 2B both appeared to have the expected molecular weight for ML1.

![Figure 20 A: 15% SDS-PAGE analysis of ML1 elution from C4 reversed-phase column (lanes 1 and 2), ladder (lane 3), and analysis of purity post SEC (lane 4). B: Typical HPLC elution profile of ML1 from a C4 reversed-phase column using an acetonitrile gradient. Peaks 2A and 2B are the desired permutein.](image)

Figure 20. A: 15% SDS-PAGE analysis of ML1 elution from C4 reversed-phase column (lanes 1 and 2), ladder (lane 3), and analysis of purity post SEC (lane 4). B: Typical HPLC elution profile of ML1 from a C4 reversed-phase column using an acetonitrile gradient. Peaks 2A and 2B are the desired permutein.

Optimized reconstitution conditions were determined based on the final percent recovery of folded protein. The conditions that resulted in the greatest protein recovery consisted of a rapid 10-fold dilution with a 100 mM potassium phosphate buffer pH 8.0 and an addition of 1.2 mol excess of cyanomet heme. Several other conditions were tested, but most caused substantial protein aggregation. Systematic variation of the three sets of conditions described below led to the eventual determination of optimal folding parameters. First, rapid 10-fold dilution with a 50 mM
potassium phosphate buffer of denatured protein dissolved in 6 M urea buffer followed by immediate addition of cyanide buffer with 1.2X mol excess hemin to protein (pH 7.5). Attempts at varying concentrations of cyanide, volumes added for dilution, and pH at 8.0 failed. Second, slow dilution of denatured protein in 6 M urea and cyanide buffer with 1.2X mol excess hemin using dialysis in 1-L dialysis buffer (50 mM potassium phosphate pH 7.5). Third, a wider scan of conditions was utilized to find optimal refolding conditions using several additives (the starting concentration of protein was 0.5 mg/mL). Each individual additive was incorporated in a slow drop-wise manner into 50 mM or 100 mM potassium phosphate buffer pH 8.0 with diluted protein. The additives included: 10% and 20% glycerol; 0.5 M, 1.0 M, and 1.5 M trimethylamine N-oxide dihydrate; 40 μM PEG 3350; 400 mM L-Arginine; and 10 mM DTT. Most of these approaches resulted in significant aggregation, except for 100 mM potassium phosphate in the absence of an additive, and 100 mM potassium phosphate with glycerol, PEG, or arginine. Proper folding of the permuted myoglobin under these conditions was assessed using UV-Vis spectrometry. The typical UV-Vis absorbance spectrum of a wild-type cyanomet Mb possesses a Soret band with a $\lambda_{\text{max}}$ at 423 nm and a smaller peak at 538 nm. This characteristic spectrum was used as a reference to determine whether ML1 was correctly folded around the heme pocket. Each of the samples obtained from refolding conditions that showed little to no aggregation had a $\lambda_{\text{max}}$ less than 423 nm. This indicated that the resulting proteins were not correctly folded around the heme pocket.

Several unsuccessful attempts were made to drive ML1 into soluble expression. The first effort varied the OD$_{600}$ at which cells were induced, from ~ 0.6 to ~ 1.5, as this OD$_{600}$ had been shown to yield greater expression of soluble permuted Hb in our lab. Insertion of the heme uptake gene (HUG) cassette into the ML1 expression plasmid also did not yield soluble protein. Coexpression of the HUG cassette has been shown to increase expression of soluble heme proteins.
(59), presumably due to increased heme transport from the growth medium into the cell, which should favor folding of the apo-protein vs. aggregation into inclusion bodies.

**Protein characterization.** The expected mass of the purified ML1 protein was verified by ESI-MS (Table 5). The two peaks from HPLC were analyzed by ESI-MS separately to determine whether there was a mass difference between them. The observed mass of peak 2A was consistent with the expected mass of ML1, but the mass of peak 2B was approximately 30 Da heavier. The observed mass difference for peak 2B may be a result of post translational modification by methylation (60). This observation can be supported by the protein’s C4 reversed-phase elution: the retention time of 2B is slightly longer than 2A, which would be consistent with the presence of additional methyl groups that increase hydrophobic interactions with the C4 column.

<table>
<thead>
<tr>
<th>Molecular weight (Da)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Expected mass</td>
<td>19,373</td>
</tr>
<tr>
<td>ML1 (Peak 2A)</td>
<td>19,377</td>
</tr>
<tr>
<td>ML1 (Peak 2B)</td>
<td>19,408</td>
</tr>
</tbody>
</table>

Using the previously described optimal reconstitution conditions, folded protein from peaks 2A and 2B both possessed a UV-Vis spectrum identical to that of cyanomet wild-type Mb and HGL16 in the Soret region (Figure 21), suggesting that both peaks were ML1. Subsequently, the peaks were collected and reconstituted together as a single sample. The increased absorbance at 280 nm for ML1 compared to wild-type Mb and HGL16 is consistent with the presence of additional aromatic residues in the ML1 sequence.
Figure 21. UV-Visible spectra of wild-type Mb, HGL16, and ML1 are identical in the Soret region, indicating ML1 has a similar fold around the heme pocket compared to wild-type Mb. Spectra of proteins were recorded in 100 mM potassium phosphate buffer, pH 8.0.

Single-chain hemoglobin

Gene construction. PCR-based cloning strategies were initially employed to construct the scHb gene by insertion of a linker sequence between the α-subunits of two sc-αcpβ genes. The linker must meet the following design constraints: it can only be one to three residues in length; it must code for small, polar residues; and the restriction sites must be unique in the gene to allow assembly of PCR-amplified fragments. The two linker sequences that meet each of these requirements include the following:

1. KasI (G^GCGC_C) **gly-ala**

2. SalI GG(G^TCGA_C)C **gly-ser-thr**

Each of these recognition sites codes for short sequences containing small, polar residues. On its own, the SalI restriction site codes for Val-Asp residues, so codons were inserted out of frame to create a more suitable linker sequence. PCR primers were designed with either the KasI or SalI on
the 5’ ends. These primers were used in PCR reactions to amplify the sc-αcpβ genes with the addition of the incorporated restriction sites to create products that could be ligated to form the scHb gene fragment. Once this gene fragment was formed, the scHb DNA would be ligated into the vector used previously to express αcpβ in *E. coli* (58, Figure 22).

![Diagram](image)

**Figure 22.** Scheme for cloning scHb gene fragment into an appropriate expression vector.

When this process was attempted with primers for the KasI restriction site, a small quantity of ligated product was observed after the digestion with KasI and ligation with T4 ligase. However, no amplification of the ligated scHb gene was observed (Figure 23). Amplification of the DNA fragments with the SalI primer set yielded only the 3’ half of the gene. Following several failed attempts to clone the scHb gene, a novel scHb gene was designed with a single glycine residue between the α-globins. This gene was synthesized by GenScript.
Figure 23. Lanes listed from left to right: HI-LO ladder (lane 1), PCR product from amplification of ligation (lane 2), ligation product (lane 3), PCR product amplifying 5' end of scHb gene (lane 4), and PCR product amplifying 3' end of scHb gene (lane 5).

Protein expression and purification. Single-chain Hb was expressed in BL21 (DE3) cells. The protein expressed in the soluble fraction and was purified to ≥ 95% homogeneity using a three-column protocol (Figure 24). Expression yields were determined post IMAC and typically ranged from 0.4-0.6 mgs per liter of culture. After the final SEC purification step, the percent recovery of pure protein from the IMAC pool was roughly 20-30%.

Figure 24. Samples from three purification steps analyzed by 15% SDS-PAGE.
Protein characterization. The expected mass of scHb was verified by ESI-MS (Table 6). The 32 Da increase in the observed mass may either be due to di-methylation (60) or oxidation of a cysteine residue to cysteic acid, or methionine to the sulfone. The cyanomet isoform of Hb has a characteristic spectrum in the Soret region with a $\lambda_{\text{max}}$ at 418 nm and an additional peak at 541 nm. The UV-Vis spectra for wild-type Hb and scHb (in their cyanomet isoforms) are identical in the Soret region and provide evidence that the heme pocket is unperturbed in (Figure 25).

<table>
<thead>
<tr>
<th>Table 6. Mass determination of scHb by ESI-MS.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight (Da)</td>
</tr>
<tr>
<td>Expected mass</td>
</tr>
<tr>
<td>Observed mass</td>
</tr>
</tbody>
</table>

Figure 25. Preliminary UV-Visible spectra of wild-type Hb and scHb. A wild-type spectrum must be obtained from a sample of increased purity for more conclusive evidence, however the high similarity between the Soret peaks at 418 and 541 nm indicates that the heme pocket of scHb has not been perturbed. Spectra of proteins were recorded in 20 mM potassium phosphate buffer, pH 7.0.
Based on data obtained by analytical HPSEC, scHb forms a tetramer in oxygenated conditions. Shown in Figure 26, the retention time of scHb (18.9 min) corresponds to that of wild-type Hb (19.1 min). Furthermore, the elution profile of wild-type Hb possesses a shoulder at approximately 21.2 min due to a small formation of $\alpha_1\beta_1$ dimers. There is no evidence of a shoulder in the elution profile of scHb, indicating that scHb does not dissociate into dimers.

![Figure 26](image)

**Figure 26.** Comparison of scHb (blue) and wild-type Hb (red) elution profiles off of analytical HPSEC monitored at 414 nm. Both samples were prepared in 20 mM potassium phosphate, pH 7.0.

*Nuclear magnetic resonance (NMR).* Analysis of oxy-scHb by $^1$H NMR indicates that the heme pocket environment and $\alpha_1\beta_1$ subunit interfaces of wild-type Hb and sc-Hb are similar (Figure 27). Both spectra show shifts at 12.1 and 12.9 ppm which arise from NeH resonances of $\alpha103$His and $\alpha122$His side chains (61,62). These two His side-chains are located at the $\alpha_1\beta_1$ interface and contact $\beta131$N and $\beta35$Y, respectively. The $\alpha_1\beta_2$ interface was monitored by a characteristic R-state marker for quaternary Hb at 10.7 ppm, which arises from the NeH of the $\beta37$W side chain (61). The $\beta37$W
side chain is located within the hinge region of the $\alpha_1\beta_2$ interface, and forms an H-bond with $\alpha94D$ early in the R-to-T transition (63). In the upfield region, there is a discernible peak in both spectra at -2.5 ppm, as expected from ring-current shifted $\alpha62V$ and $\beta67V$ methyl resonances. The distal valine side-chains are located within 4 Å of the bound $O_2$, and provide useful markers to track the heme-pocket environment (61). This evidence implies that the incorporated linkers and mutations do not significantly perturb the structure of scHb in its R conformation.

**Figure 27.** $^1H$ NMR spectra of oxy scHb (stacked on top for both images) and oxy wild-type Hb (stacked on bottom for both images). Top: Comparison of full spectra from 15 to -5 ppm. Bottom: Downfield and upfield spectra with a higher scaled image, highlighting shifted resonances previously described in the text. Data were collected in 20 mM potassium phosphate pH 7.0 at 25 °C.
**Ligand binding.** Full photolysis studies of CO-bound HbA and permuted Hb constructs developed in our lab were used to estimate the pseudo first-order rate constant, \( k' \), for ligand recombination to the low-affinity T conformation. Partial photolysis was employed on the same Hb constructs to determine the pseudo-first-order rate constant, \( k' \), for ligand recombination to the high affinity R conformation. According to a four-step ligand association process, \( k' \) is defined as the final step in ligand binding (i.e., Hb\(X_3 + X\)) (64). Binding isotherms for full and partial photolysis of CO from HbA and Hb constructs are shown in Figure 28. In full photolysis experiments, each protein displays biphasic kinetics. The fast phase coincides with ligand rebinding to the R state conformation, and the slow phase with rebinding of ligand to the T state. The apparent rates depend on ligand concentration, which is used to calculate bimolecular association rate constants R and T states (Table 7). In each of the permutants (αcpβ, sc- αcpβ, and scHb), ligand binding is dominated by the fast phase. In contrast, HbA possesses dominant amplitude in the slow phase. This disparity may be a result of either (1) a presence of higher concentration of heterodimers, or (2) a destabilized T conformation in the permutants.
Figure 28. Normalized time courses for full photolysis of 1 atm CO from HbA, αcpβ, sc-αcpβ, and scHb (panel A), and approximately 10% partial photolysis of 1 atm CO from HbA and scHb (panel C). Time courses for full photolysis of 92.8 μM CO from HbA, αcpβ, sc-αcpβ, and scHb (panel B) and about 10% photolysis of 92.8 μM CO from scHb and αcpβ 6 MT (panel D). No new data for HbA at 10% photolysis with 92.8 μM CO was obtained, but based on previous data, αcpβ and mutants have nearly identical time courses to HbA. By extrapolation, scHb likely has similar time courses to HbA under these conditions. All experiments were conducted in CO-saturated 0.1 M potassium phosphate and 1 mM EDTA, pH 7.0 at 20 °C.

In the first scenario, the newly formed deoxy heterodimers remain in a highly reactive R conformation and will likely rebind CO before the deoxy-heterodimers can associate into heterotetramers and switch to a T-state conformation. For HbA, the rate of CO binding is relatively quicker than the rate of heterodimer aggregation ($k'_{2,4} = 0.1 \mu M^{-1}s^{-1}$) (64). In the second scenario, a destabilized T conformation would result in a lower population of the T state. Therefore, the highly reactive R-state conformation would dominate the population of deoxy-Hb molecules and result in a dominant amplitude for the fast phase of ligand binding. It is possible that both of these scenarios
may have a role in the observation of a dominant fast phase. However, analytical SEC shows that scHb exists as a tetramer (Figure 26), so it is more likely that a dominant fast phase is a result of a destabilized T-state for this particular mutant.

**Table 7.** Second-order rate constants for the faster and slower phases of CO binding to HbA and Hb mutants.

<table>
<thead>
<tr>
<th>CO binding</th>
<th>(k'_{1(CO)} (\mu M^{-1}s^{-1}))</th>
<th>(k'_{R(CO)} (\mu M^{-1}s^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA*</td>
<td>0.12</td>
<td>6.0</td>
</tr>
<tr>
<td>HbA 100% photolysis 1 atm CO</td>
<td>0.19 (72)</td>
<td>9.12 (28)</td>
</tr>
<tr>
<td>HbA 100% photolysis 10% CO</td>
<td>0.061 (74)</td>
<td>3.11 (26)</td>
</tr>
<tr>
<td>HbA ~10% photolysis</td>
<td></td>
<td>4.79</td>
</tr>
<tr>
<td>deoxy HbA + 23 μM CO</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>deoxy HbA + 93 μM CO</td>
<td>0.075</td>
<td></td>
</tr>
<tr>
<td>acpβ 100% photolysis 1 atm CO</td>
<td>0.56 (25)</td>
<td>6.80 (75)</td>
</tr>
<tr>
<td>acpβ 100% photolysis 10% CO</td>
<td>0.21 (31)</td>
<td>3.91 (69)</td>
</tr>
<tr>
<td>acpβ ~10% photolysis</td>
<td></td>
<td>6.54</td>
</tr>
<tr>
<td>deoxy acpβ + 23 μM CO</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>deoxy acpβ + 93 μM CO</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>sc-acpβ 100% photolysis 1 atm CO</td>
<td>1.02 (22)</td>
<td>10.08 (78)</td>
</tr>
<tr>
<td>sc-acpβ 100% photolysis 10% CO</td>
<td>0.29 (27)</td>
<td>4.52 (73)</td>
</tr>
<tr>
<td>sc-acpβ ~10% photolysis</td>
<td></td>
<td>9.10</td>
</tr>
<tr>
<td>deoxy sc-acpβ + 23 μM CO</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>deoxy sc-acpβ + 93 μM CO</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>scHb 100% photolysis 1 atm CO</td>
<td>1.0 (35)</td>
<td>9.10 (65)</td>
</tr>
<tr>
<td>scHb 100% photolysis 10% CO</td>
<td>0.26 (46)</td>
<td>-</td>
</tr>
<tr>
<td>scHb ~10% photolysis</td>
<td></td>
<td>8.97</td>
</tr>
<tr>
<td>deoxy scHb + 23 μM CO</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>deoxy scHb + 93 μM CO</td>
<td>0.30</td>
<td></td>
</tr>
</tbody>
</table>

The amplitudes of fast R state and slow T state phases are given in parentheses. The CO binding values were calculated from full and partial photolysis, as well as stopped-flow experiments. *HbA values are from Olson et al. (64)

The published value for \(k'_{R}\) for CO binding to HbA (6.0 \(\mu M^{-1}s^{-1}\), 64) and measured value for scHb (8.97 \(\mu M^{-1}s^{-1}\)) are not significantly different (Table 7). This implies that the proteins have similar R-states and reactivities. Although we have not distinguished differences between α and β subunits, which react differently in both R and T states (65), the differences are small in recombinant Hb and
HbA (less than 2-fold) (64). Unzai et al. have argued that although the structural mechanisms for ligand binding in the two subunits are significantly different, they have evolved with similar rates and equilibrium constants in order to maximize observed cooperativity (65). Thus, simple parameters assuming subunit equivalence are sufficient to simulate $O_2$ transport properties of Hb. Because the time courses for partial photolysis are similar between HbA and permuted Hb constructs, neither subunit has been particularly disturbed by the added linkers. Otherwise, multiphasic time courses would have been observed for the mutants.

The apparent $O_2$ dissociation rates were determined using stopped-flow by mixing HbO$_2$ with solutions containing excess CO and measuring the displacement of $O_2$ (Figure 29). The apparent rates for HbA and αcpβ are essentially identical, and rates for sc-αcpβ and scHb are slightly lower, but not significantly different (Table 8). Therefore, $O_2$ dissociation is very similar between the permutants and HbA.

<table>
<thead>
<tr>
<th></th>
<th>Apparent $k_{R(O2)}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA</td>
<td>8.0</td>
</tr>
<tr>
<td>rHb 0.1</td>
<td>6.5</td>
</tr>
<tr>
<td>αcpβ</td>
<td>8.2</td>
</tr>
<tr>
<td>sc-αcpβ</td>
<td>4.9</td>
</tr>
<tr>
<td>sc-Hb</td>
<td>5.2</td>
</tr>
</tbody>
</table>

Table 8. Apparent dissociation rates for $O_2$ displacement of Hb by CO
Figure 29. Normalized time courses for stopped-flow measurement of CO binding to deoxyHbA, deoxy acpβ, deoxy sc-acpβ, and deoxy scHb (panels A and B). Panel A shows data collected in the presence of 23.2 μM CO and panel B shows data collected in the presence of 92.8 μM CO. Panel C shows normalized time courses for O₂ displacement of HbO₂ by excess CO. Samples were prepared in 0.1 M potassium phosphate and 1 mM EDTA, pH 7.0, and data collected at 20 °C.

Although the R state ligand binding parameters are similar between HbA and mutants, values of $k'$; for the slow phases of ligand rebinding to Hb vary significantly from HbA values. In full photolysis experiments, $k'$ values are 3-8 fold higher than for HbA (Table 7), indicating the iron in Hb mutants has higher reactivity in the deoxy state. Additionally, the amplitude for the slow T-state is much lower in Hb mutants compared to HbA (Table 7). These rates were confirmed by stopped-flow methods by mixing deoxy Hb with either 10% or 2.5% CO and monitoring absorbance at 436 nm to determine the rate of CO association (Figure 29). The normalized curve for HbA shows
acceleration at the start of the reaction with an overall bimolecular rate constant of \( \sim 0.10 \text{ μM}^{-1}\text{s}^{-1} \) for reaction with 2.5% CO and \( \sim 0.075 \text{ μM}^{-1}\text{s}^{-1} \) for reaction with 10% CO. In contrast, each of the mutants displays a simpler exponential process. The single rapid rate derived from reaction with scHb is \( \sim 0.47 \text{ μM}^{-1}\text{s}^{-1} \) and \( \sim 0.30 \text{ μM}^{-1}\text{s}^{-1} \) for reaction with 2.5% CO and 10% CO, respectively. For each of the mutants, a considerably smaller \( k'_T \) association rate constant is observed compared to rates measured after partial photolysis. This implies that the mutants form a T state tetramer, but one with higher affinity compared to HbA. This conclusion is supported by equilibrium oxygen binding curves shown in Figure 30. Each of the mutants (sc-αcpβ and scHb) display lower \( P_{50} \) values and significant less cooperativity compared to HbA (Table 9).

![Figure 30](image.png)

**Figure 30.** Fractional saturation with oxygen versus log of oxygen partial pressure (mm Hg) for HbA, fetal Hb (HbF), sc-αcpβ, and scHb. Samples were prepared in 0.1 M potassium phosphate and 1 mM EDTA, pH 7.0, and data was collected at 20 °C.
<table>
<thead>
<tr>
<th></th>
<th>n Hill</th>
<th>P50 (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbF</td>
<td>1.74</td>
<td>8.69</td>
</tr>
<tr>
<td>HbA</td>
<td>2.0-2.1</td>
<td>13.2-13.8</td>
</tr>
<tr>
<td>sc-αcpβ</td>
<td>1.1-1.2</td>
<td>10.8-11.9</td>
</tr>
<tr>
<td>scHb</td>
<td>1.13-1.14</td>
<td>11.4-11.5</td>
</tr>
</tbody>
</table>
Discussion

*Computationally designed circularly permuted myoglobin*

Computational protein design provides a potentially useful tool for the construction of novel sequences and interactions. Previous investigations by Hu *et al.* have determined that it is possible to design a 10-residue loop with high accuracy using the current Rosetta energy function (46). For the first time using similar methods on Rosetta software, three circularly permuted sperm whale Mb designs were created. Each of these designs was modeled to contain an α-helical linker between the native N- and C-termini designed to include novel hydrophobic interactions with the Mb framework.

Attempts to express these models resulted in the formation of inclusion bodies with no evidence of soluble expression. This result is in contrast to the previously characterized permutein, HGL16, which had isolated yields of 0.6-1.2 mg soluble protein/L of fermentation broth. The absence of soluble expression for the computationally designed permuteins is probably due to a decreased thermodynamic stability (66). For swMb and HbA, it has been shown that decreased thermodynamic stability correlates with a decreased expression yield (6,68). Additionally, reconstitution of ML1 inclusion bodies proved to be more challenging compared to reconstitution of HGL16 from inclusion bodies (67). ML1 was more prone to aggregation during the refolding process than HGL16 and had a decreased recovery yield of fully folded protein, supporting the notion that ML1 has a reduced thermodynamic stability compared to HGL16.

UV-visible spectra shown in Figure 21 indicate that ML1 has the correct fold around the heme pocket. Further characterization of the computationally designed permuteins must be completed to quantify the loss of thermodynamic stability. Given the data collected so far, it does not appear that the designed swMb permuteins will inform the design of an optimal linker in the
permuted β-globin of scHb. However, it would be interesting to determine how accurately the computational design predicted the fold of the reconstituted permutein in order to validate the computational design algorithm.

**Single-chain hemoglobin**

Prior to the generation of scHb according to the scheme illustrated in Figure 13, two Hb constructs had to be produced and characterized as a proof of concept that Hb can tolerate the permutations and novel covalent linkages required by the scheme. The first proof of concept came from αcpβ, which had a similar fold and function to HbA in the R conformation. A similar conclusion was reached with sc-αcpβ, although each mutant became progressively more destabilized. Prior results show αcpβ and sc-αcpβ have R-state ligand binding and dissociation properties that are very similar to the R-state properties of wild-type Hb. However, unlike HbA, the faster phase of binding is dominant over the slower phase. This is evidence that the R ↔ T equilibrium for both αcpβ and sc-αcpβ is perturbed to favor the R state. Two explanations for this observation are plausible: (1) the T-state has been destabilized as a consequence of the manipulation of the Hb backbone topology, and/or (2) the α1β2 interface between heterodimers has been significantly destabilized, promoting the dissociation of the α1β1 heterodimers, which revert to an R state conformation in the absence of interactions across the α1β2 interface. Both analytical HPSEC and analytical ultracentrifugation (75) data show strong evidence that each of these mutants favor dissociation into dimers in oxygenated conditions and at lower protein concentrations.

The weakening of the α1β2 interface in the permuted Hbs is an expected consequence of permuting the β subunit. In the deoxy conformation of HbA, the α-carboxylate of β His146 forms a salt bridge with the ε-amino group of α Lys40 (69). As part of the linker sequence connecting native
N- and C-termini in the cpβ-globin, the C-terminal α-carboxylate of β His146 is converted to an amide bond, thus removing the stabilizing salt bridge. To strengthen the tetramer, the Providence mutation (β K82D) was incorporated into the Hb mutants (6,17). In addition to the Providence mutation, the three mutations (β G16A, β H116I, and α G15A) were also integrated into the permutants to increase apoprotein stability (6). As shown in Figure 31, incorporation of the three stabilizing mutations into αcpβ results in improved ligand binding function. Expression yields for the Hb permutants also increased after the four stabilizing mutations were incorporated, as would be expected for a protein with increased stability (6,68). These improvements in expression yield and function demonstrated the desirability of generating a scHb that also contained the stabilizing mutations.

**Figure 31.** Normalized time courses for full photolysis of 1 atm CO from HbA, αcpβ K82D 3sm, and αcpβ K82D 0sm. All experiments were conducted in CO-saturated 0.1 M potassium phosphate and 1 mM EDTA, pH 7.0 at 20 °C.
Expression yields of scHb are approximately 5-fold lower than those for sc-αcpβ. Yet, even with the “tethers” that covalently link all four Hb subunits, there is evidence to suggest that the scHb folds to a structure that has the same packing of the subunits as is found in HbA. The UV-visible spectrum of sc-Hb (Figure 25) shows that the environment surrounding the heme retains a fold similar to wild-type Hb. 1H NMR provides evidence to support the same conclusion. Wild-type Hb and scHb have similar spectra in the upfield region, with analogous peaks corresponding to proton shifts (-2.5 ppm) from the heme porphyrin ring (Figure 27). However, the peak at -2.5 ppm is attenuated for scHb compared to wild-type Hb. This may be due to the orientation of the heme (70). Wild-type Hb strongly favors one heme orientation over the other, but non-specific heme orientation has been observed in our Hb and Mb constructs (71). The downfield region of the 1H NMR spectra contains peaks that correlate with the amide backbone and aromatic residues. There are similar resolvable peaks in this region between the two spectra that arise from NeH resonances of α103His and α122His side chains that span the α1β1 interface, which indicate that this interface remains relatively unperturbed in scHb.

Investigation of ligand binding showed that scHb has similar reactivity to HbA in the R-state, but an increased reactivity (i.e., faster on rates) in the T-state. This finding is consistent with ligand binding data obtained with αcpβ and sc-αcpβ. However, compared to αcpβ and sc-αcpβ, scHb has slightly increased amplitude in the T-state (Table 7). This observation is probably related to the quaternary state of the protein at the moment of photolysis. αcpβ and sc-αcpβ primarily exist as heterodimers at the concentrations used for photolysis studies, in contrast to scHb which exists as a covalent tetramer. In order for αcpβ and sc-αcpβ to transition into a T conformation, they must first associate to form a tetramer, which occurs at a slower rate than the recombination with ligand. Because scHb exists as an obligate tetramer at the moment of photolysis, it eliminates the transition step from dimer to tetramer, and therefore a higher percentage of scHb is likely to adopt the T
conformation before recombination of ligand to the R-state occurs. It is disappointing that this proximity effect does not promote greater stabilization of the T state in scHb. This suggests that even though scHb is an obligate tetramer, the T-state is not favored under deoxy conditions (as it is for HbA). Heterodimer dissociation has also been observed in kinetics studies with HbA. Antonini et al. determined that fully liganded HbA dissociates into heterodimers with an equilibrium constant of $K_{4,2} = 2\text{-}10 \, \mu\text{M}$ (72). In flash photolysis experiments with 5 \, \mu\text{M} HbCO, which includes a significant population of heterodimers at this concentration, the amplitude in the T conformation decreases compared to photolysis with 20 \, \mu\text{M} HbCO (64). Although the T-state amplitude of scHb is still significantly reduced compared to HbA, there is evidence to suggest that stable quaternary contacts made between the four subunits of scHb assist in the formation of the T-state. Current work in our lab is directed at increasing the stability of the T conformation in permuted Hbs.

Significant differences were observed between previous and current equilibrium oxygen binding studies of Hb mutants from our lab. Figure 16 shows equilibrium binding data collected in 2011 under similar conditions to equilibrium binding data collected in 2013 (Figure 29). The most significant differences between the data sets are the $P_{50}$ values for the permuted Hbs. The current data possess $P_{50}$ values that are markedly closer to HbA. These differences are likely due to the incorporation of the three stabilizing mutations in the current generation of Hb mutants in our lab. Figure 31 shows that $\alpha cp\beta K82D$ 0sm (i.e., this mutant does not include the three stabilizing mutations) has an even more dominant fast phase than $\alpha cp\beta K82D$ 3sm, implying that $\alpha cp\beta K82D$ 0sm has an increased ligand affinity, or more R-state character. This is reflected in the approximate 1.5-2 fold increase in $P_{50}$ values observed for the current generation of 3sm mutants compared to the 2011 (0sm) mutants.
Ideally, scHb should possess a physiologically appropriate O$_2$ affinity, cooperativity, limited scavenging of NO, and a minimal autooxidation rate. Maillett et al. investigated a series of mutations that modulate some of these traits (61). Two of the mutations of interest include β N108K and α V96W. The β N108K mutation (also known as the “Presbyterian” mutation) has the following effects in rHb: increased Bohr effect (i.e., increased H$^+$ binding), decreased O$_2$ affinity, and the ability to switch to the T conformation without changing ligation state (61). The α V96W mutation located in the α$_1$β$_2$ interface was found to increase P$_{50}$ without compromising cooperativity (61). Additionally, an rHb incorporating both N108K and V96W displayed synergistic effects compared to the single mutation variants (61). For these reasons, we incorporated these two mutations in αcpβ K82D 3sm to assess whether these effects would be manifested in our permuted Hb constructs (αcpβ N108K K82D 3sm, αcpβ V96W K82D 3sm, and αcpβ 6MT, where “6MT” = 3sm + K82D + N108K + V96W). Flash photolysis and stopped-flow studies shown in Figure 32 indicate that αcpβ N108K enhanced both the rate and amplitude for ligand binding to the T-state ($k^{-1}$), whereas αcpβ V96W and αcpβ 6MT increased $k'_{1}$, but displayed a reduced amplitude for this slower phase than αcpβ K82D 3sm.
Figure 32. Left: Normalized time courses for full photolysis of 1 atm CO from HbA, αcpβ, αcpβ N108K, αcpβ V96W, and αcpβ 6MT. All experiments were conducted in CO-saturated 0.1 M potassium phosphate and 1 mM EDTA, pH 7.0 at 20 °C. Right: Normalized time courses for stopped-flow measurement of 92.8 μM CO binding to deoxyHbA, deoxy αcpβ, deoxy αcpβ N108K, and deoxy αcpβ V96W (αcpβ 6MT not shown). Samples were prepared in 0.1 M potassium phosphate and 1 mM EDTA, pH 7.0, and data collected at 20 °C.

Flash photolysis studies measured a $k'_{T(CO)}$ for αcpβ, which was approximately 2.8-3.5 fold higher than $k'_{T(CO)}$ for HbA (Table 7), and a $k'_{T(CO)}$ for αcpβ N108K that was only ~2-fold higher than that of HbA. In contrast, αcpβ V96W and αcpβ 6MT had greater than 5.5-fold increase for $k'_{T(CO)}$. These findings were consistent with data obtained by stopped-flow. Furthermore, equilibrium oxygen binding data indicate that αcpβ N108K is more cooperative than αcpβ. Cooperativities of sc-αcpβ and scHb are nearly 2-fold less than HbA (Table 9). With the addition of N108K, the Hill constant for αcpβ increased to ~1.47, which is only 1.4-fold less than that for HbA (Figure 33). Based on these observations, it appears that the incorporation of N108K in scHb might confer improved functional properties, such as increased cooperativity, and marginally increased $P_{50}$. In contrast to its effect on HbA, the V96W mutation does not confer increased T-state stability to the permuted Hbs.
Figure 33. Fractional saturation with oxygen versus log of oxygen partial pressure (mm Hg) for HbA, scHb, and acpβ N108K. Samples were prepared in 0.1 M potassium phosphate and 1 mM EDTA, pH 7.0, and data was collected at 20 °C.

The loss of cooperativity for the permuted Hbs could also be explained by oxidation of β Cys93 or β Met55. Others have observed the following effects as a result of oxidizing β Cys93 or β Met55 residues: (1) retention of native structure; (2) significant increase of ligand affinity; (3) decreased cooperativity (n ≈ 1); and (4) destabilized T-state (73). These observations are similar to those made with the permuted Hbs studied in our lab. ESI-MS yields an observed mass ~32 Da greater than expected for many of the permuteins purified in our lab. In the past we believed this mass increase to be a consequence of the well-known post translational methylation of rHbs (60); however, addition of dioxygen to Met or Cys would also account for this mass increase. Identifying the exact nature of this post-translational modification is a top priority, which we will address by
contract analysis of our permuteins by a protein analytical lab, and/or Ala mutations at these sites. If S atom oxidation is an issue for these proteins, it is possible the functional characteristics that have been observed in our mutants (i.e. increased ligand affinity, decreased cooperativity, etc.) can be corrected by altering fermentation conditions to a less oxidizing environment prior to cell harvest.

For scHb to be considered as a scaffold for a therapeutically useful HBOC, it must first satisfy ideal functional and structural qualities described above. Inclusion of the β N108K mutation holds promise for stabilization of the T-state conformation as well as increasing cooperativity in scHb. Additional challenges include improving expression yields and apoprotein stability, and efficient, controlled, polymerization of scHb to yield higher order oligomeric states (illustrated schematically in Figure 18).

There are many potential advantages to creating a polymerized scHb. A recent study of ultrahigh molecular weight polymerized bovine Hbs (bHb) by Buehler et al. describes the in vivo effects of poly-Hb infusion on guinea pigs (35). In this study, bHbs polymerized in either the T-state or the R-state were fractionated to isolate molecular weights >500 kDa. The \( P_{50} \) of polymerized T-state bHbs was approximately 41 mm Hg and the \( P_{50} \) of R-state poly-bHb was approximately 0.66 mm Hg (35). The cooperativities of both poly bHbs were < 1 (35). The T-state poly-bHb autoxidized at a slower rate than R-state poly-bHbs in vivo, and also had a longer circulatory half-life (35). The significant findings are that both polymerized bHbs possessed high viscosities and low colloid osmotic pressures, prolonged circulatory half-life, and limited poly-bHb dissociation. In a similar study, Cabrales et al. reported the pressor response in hamsters after the introduction of T-state poly bHb both above and below 500 kDa (37). This study showed that poly-bHb >500 kDa attenuated mean arterial pressure compared to poly-bHb <500 kDa (Figure 34). Additionally, poly-bHb produced with a greater ratio of glutaraldehyde to bHb (i.e., molar ratios of 50:1 versus 20:1)
showed greater attenuation of mean arterial pressure (37). These outcomes are similar to those described by Marquardt et al. for a monodisperse ~260 kDa tetra-Hb, and reinforce the idea that higher order polymers of scHb are promising candidates for an HBOC (34).

![Figure 34](image-url)

**Figure 34.** Relative changes from baseline in arteriolar diameter after infusion of PolybHb at varying concentrations. A: PolybHb fractions with molecular mass <500 kDa. B: PolybHb fractions with molecular mass >500 kDa (37).

Ideally, polymerized scHb will be expressed by a single gene in *E. coli*. In that case the use of chemical crosslinkers will not be needed, resulting in reduced production costs, as well as yielding a monodisperse MW (i.e., the resulting poly-Hb will have a single optimized MW). A second approach to increase the size of an HBOC involving scHb is to form a covalent linkage to another protein such as superoxide dismutase. Again, this would ideally be expressed as a single protein in *E. coli*. Such a
protein complex with SOD would have the advantage of eliminating superoxide radicals, which are generated during the reperfusion of ischemic tissues using whole blood (74).

In conclusion, scHb represents a significant achievement in globin protein engineering—it is the first example of a single-chain Hb, and based on preliminary structural and functional data, scHb is a promising scaffold for the next generation of HBOCs developed in our lab.
References


75. Kokona, B. and Fairman, R., unpublished data.