**Third Generation Recombinant Hemoglobins As the Starting Material for All Hb-based Blood Substitutes.**

**Cellular Engineering of bacteria**

**Protein Engineering of Hb**

**John S. Olson**

*Department of Biochemistry and Cell Biology & W. M. Keck Center for Computational Biology, Rice University*

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**Not Blood Simple**

After decades of setbacks, compounds that act like blood to deliver oxygen are in the final stretch of clinical trials.

Scientists began working on artificial blood in the 1990s, trying to see whether iron-rich hemoglobin molecules extracted from red blood cells would be able to pick up oxygen molecules and deliver them to tissues when transfused into a patient. These molecules might cause other problems.” Other experts stress that more needs to be known about how...
Pressor responses to rHb1.1 and rHb Bethesda: no dependence on $O_2$ affinity

1. vasoconstriction
2. heart lesions
3. gastro-intestinal problems

Baxter Hemoglobin Therapeutics
First Strategy:

1. Make the hemoglobin molecules larger to prevent extravasation.

2. Non-specific polymerization of tetramers

3. Decoration with polyethylene glycol-like molecules or sugars.

Dependence of the Pressor effect on MW

Increasing the size of the Hb polymer reduces the blood pressure side effect.
Cross-linked Hemoglobin Polymers in Final Stages of Development

Biopure, Inc.
Hemopure™ - (phases II to III for U.S.A.)
(bovine hemoglobin-glutaraldehyde)
Approved for human use in South Africa, veterinary product approved in U.S.A.

Northfield Laboratories
Polyheme™ - (phases II to III)
(human hemoglobin-glutaraldehyde plus pyridoxalation)

Other Solutions to Rapid Extravasation

Conjugated Hemoglobins
Ajinomoto and Apex Bioscience - phase I (Human hemoglobin polyoxyethylene plus pyridoxalation)
Enzon, Inc. - phase I
(Bovine hemoglobin - polyethylene glycol or PEG)
Sangart Inc. - Phase I
(human hemoglobin + polyethylene glycol or PEG
+ Hemospan™)

Encapsulated Hemoglobins
1. Artificial membranes - liposomes
2. Biodegradable polymers - polylactide and polyglcolide (nanocapsules)
Second Strategy:

1. Re-engineer Hb to lower the rate of NO dioxygenation.

2. Then re-adjust the O$_2$ binding parameters for efficient transport.

3. Use the recombinant Hb expression system developed by Somatogen, Inc.
Mechanism of the dioxygenation of NO by \( \text{MbO}_2 \)

1. Entry of NO into the distal pocket and migration into the Xe sites.

2. Reaction with bound \( O_2 \), which "looks" like the superoxide anion radical.

\( \text{(Eich et al., 1996 Biochemistry 35, 6976-83)} \)

Mechanism of the dioxygenation of NO by \( \text{MbO}_2 \)

3. Isomerization of the peroxynitrite intermediate catalyzed by the Fe atom.

cis-peroxynitrite intermediate (pH >8.0)
Mostly high spin, EPR g = 6, unreactive, seen in rapid freezing EPR experiments and RSM stopped flow experiments.

trans-peroxynitrous acid intermediate, rapidly isomerizes (made immediately at pH 7)

4. Nitrate dissociation
Mechanism of the dioxygenation of NO by MbO₂

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   - cis-peroxynitrite intermediate (pH > 8.0)
     - Mostly high spin, EPR g = 6, unreactive, seen in rapid freezing EPR experiments and RSM stopped flow experiments.
   - trans-peroxynitrous acid intermediate, rapidly isomerizes (made immediately at pH 7)

4. Nitrate dissociation

At pH = 7.0, no intermediates

\[ \text{HbO}_2 + \text{NO} \rightarrow \text{methHb} + \text{NO}_3^- \]

\[ k'_{\text{NO}} = 70 \text{ µM}^{-1}\text{s}^{-1}, \text{ very fast (µs)} \]

Scott et al. 2001 J. Biol. Chem. 276, 5177-88
Tetracycline resistance

Synthetic hemoglobin Genes + lac promoter

Restriction enzyme site for cloning

Transformation into Escherichia coli

Chromosomal DNA

High copy plasmid

pET or Other Expression Vectors (ds DNA)

Grow on tetracycline to select for cells containing both plasmid DNAs and turn on rHb with IPTG.

1. Colonies that are resistant to tetracycline and express rHb
2. Red color is due to hemoglobin (HbO2) expression

Expression in Fermentor

1. 12-14 L growth
2. ~900 g wet cells
3. Automatic
Structure determination of recombinant Mb models and Hb prototypes
Reaction of 5 µM NO with 1 µM MbO$_2$

Simple second order bimolecular reaction

(Eich et al., 1996 Biochemistry 35, 6976-83)
Change to glutamine to weaken hydrogen bonding

Genetically crosslinked “Di-alpha” rHb is the background for all rHb-based oxygen carriers.
Summary of distal pocket mutants and the pressor effect

Elimination of TPR in rHb3011

TPR, % control

Time from administration

350 mg/kg
Top load doses
n = 6

Baxter Hemoglobin Therapeutics (Doherty et al., 1998 Nature Biotech. 16, 672-6 and Doyle, 2001)

Baxter Hemoglobin Therapeutics (Doyle & Lemon 2001, abstracts and unpublished)
**Oxygen delivery:** complete isovolemic exchange
O₂ consumption is maintained by both rHb1.1 and heme pocket variants

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![Graph showing oxygen delivery and control](image)

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**Key Requirements of a Hb-based O₂ Carrier (HBOC)**

1. **Moderate O₂ affinity** ($P_{50}$) and large dissociation rate constant for efficient transport.
2. Reduced rates of NO scavenging to prevent hypertension.
4. Low rates of heme dissociation to increase shelf-life, reduce oxidative stress, and inhibit bacterial infections.

All of these properties can be optimized by protein engineering.
BAXTER'S SALES GROW 11 PERCENT IN SECOND QUARTER

The Company Moves Forward with Initiatives to Improve Profitability and Return on Assets

Baxter to Discontinue Hemoglobin Therapeutic Development

DEERFIELD, Ill., July 17, 2003 -- Baxter International Inc. (NYSE: BAX) today reported its second quarter results, with sales increasing 11 percent and income from continuing operations declining primarily as a result of a previously announced restructuring charge.

Sales in the second quarter totaled $2.16 billion, an increase of 11 percent. Foreign exchange favorably impacted sales by 5 percent. Baxter's sales within the United States increased 4 percent to $997 million, while sales outside the United States grew 18 percent (including an 11 percent benefit from foreign exchange) to $1.17 billion. In the second quarter, Medication Delivery sales grew 17 percent to $938 million, Renal sales grew 11 percent to $452 million, and BioScience sales grew 5 percent to $773 million.

1. Human hemoglobin requires donors, and its availability is limited. Bovine hemoglobin is cheaper and more readily available.

2. Recombinant hemoglobin production is potentially unlimited, but it is costly, and the product is often contaminated with lipopolysaccharide antigens and degradation products.

Need 30-60 g per transfusion unit

Expression of rHb in E. coli is a key limiting factor in production yield.
Hemoglobin Synthesis in E. coli

Rate_{holoHb} = \frac{k_{\text{heme}}}{1 + K_{\text{UN}}} \frac{K_{\text{UN}}}{1 + K_{\text{UN}}} - \frac{k_{\text{degrade}}}{1 + K_{\text{UN}}}

Rate of heme synthesis or transport

Rate of precipitation or proteolysis

Additional variance due to different rates of proteolysis, precipitation, and translation.
Increase globin stability

- \( K_{\text{un}} = 1/K_{\text{ NI}} \) for human or wt pig apoMb
- \( K_{\text{un}} = 63,000 \) for wt SW apoMb and pig multiple mutant
- \( K_{\text{un}} = 1,300 \) for no holoMb expression

Pig Mb mutations to the five naturally occurring amino acids in sperm whale Mb
b. Comparison of human and sperm whale Hb β subunit sequences

a. Comparison of human and sperm whale Hb α subunit sequences
HbF = $\alpha_2\gamma_2$

HbA = $\alpha_2\beta_2$

Chromosome 16

Chromosome 11

Acid, base, and isopropanol tests for hemoglobin stability
Potential stabilizing HbF replacements in the $\alpha_1$-$\beta_1$ interface

C112T  H116I  P125E  Y130W  V133M  \(\beta\rightarrow\gamma\) chains

Mutations to stabilize the dimer interface based on Hb F (\(\gamma\) chains)

$\alpha_1$-$\beta_1$ interface

\(\beta\rightarrow\gamma\) replacements

- C112T  (G14)
- H116I  (G18)
- P125E  (H3)
- Y130W  (H8)
- V133M  (H11)
Comparison of the unfolding of WT and apo-Hb mutants at pH 7, 10°C.

**WT rH(0.0)**

α(G15A)(WT)

α(WT)β(G16A)

α(G15A)β(G16A)

α(WT)β(H116I)

α(G15A)

β(G16A/H116I)

α(WT)

β(G16A)

**Hemoglobin Synthesis in E. coli**

1. Increase globin stability

2. Add α subunit chaperone

3. Add heme transport genes

bacterial heme synthesis

heme transport genes

mRNA

Rb Protein

α1 β2 dimer

apo αβ2 dimer

holo αβ2 dimer

hemoglobin tetramer

α subunit chaperone

AHSP

β unfolded

α unfolded

Inner Membrane

Periplasm

bacterial heme synthesis
AHSP "mimics" the G and H helical surface of the β subunits at the α1β1 dimer interface

We think that the alpha hemoglobin stabilizing protein (AHSP) binds and stabilizes apo- and holo-α subunits. With Mitchell Wiess at Children's Hospital of Philadelphia, we want to co-express AHSP with rHb and look for improved expression yields.
Plesiomonas shigelloides
(Vibrio cholerae, Shigella dysenteriae, Yersinia pestis, Yersinia enterocolitica, and pathogenic E. coli) - heme transport genes under FUR control - Fe³⁺ binding transcription factor

Heme utilization genes (hug)

HugA

HugB

TonB

HugC

HugD

ExbD

ExbB

HugW,X,Z?

incorporation into apoHb

slow flipping, high affinity for membrane layers

H⁺ "active" uptake?

free Fe³⁺
heme degradation enzymes

Bloodletting explained. S. aureus bacteria obtain most of the iron (Fe) that they need for growth in mammalian hosts from an iron-containing porphyrin ring called heme. S. aureus produces hemolysins that lyse red blood cells containing heme in the form of hemoglobin. It is unclear how the bacteria break down the released hemoglobin to heme, but the bacterial enzymes HugA and HugB may be involved. The bacteria then import heme via transporter proteins encoded by the HugABC operon. The heme is then catalyzed by the heme oxygenase-like enzymes, HugC and HugD, with the release of iron and biliverdin (a breakdown product of the porphyrin ring). The free iron released from heme is used to fuel further bacterial growth. The practice of bloodletting in the pre-antibiotic era may have been an attempt to starve pathogenic bacteria of the iron that they need for growth.
Tetracycline resistance
Synthetic hemoglobin Genes + lac promoter
pET or Other Expression Vectors (ds DNA)

Restriction enzyme site for cloning

Transformation into Escherichia coli

High copy plasmid
Low copy plasmid
Chromosomal DNA

Growth on tetracycline and chloramphenicol plates to select for cells containing both plasmid DNAs and turn Hb on with IPTG and the heme transport genes with iron starvation

1. Colonies that are resistant to tetracycline and chloramphenicol and express both rHb and heme transport genes
2. Red color is due to hemoglobin (HbO₂) expression

Restriction enzyme site for cloning

Chloramphenicol resistance
pACYC vectors with helper genes

Engineering the E. coli bacterium

rHb0.0 production in BL21 cells with: Co-expression of pHUG21.1 (iron depletion with DIP = 2,2-dipyridine)/pSGE0.0-E4(rHb with IPTG)

Heme transport appears to double the yield of rHb0.0!
Currently technology:

- Genes for rHb blood substitute
- Helper genes (heme transport, chaperone, others)
- High copy plasmid
- Chromosomal DNA
- Low copy plasmid

Real Genetic Engineering

Put the helper genes and switches directly into the *E. coli* genome and create a bacterial cell designed to produce rHb.

Can we increase the production yield 2 to 3-fold and make recombinant Hb blood substitutes competitive?

Somatogen, Inc.

Biopure, Inc.