Heme Protein Engineering, Evolution, and Developmental Biology

Is a biophysical approach good enough or do I have to become a real biologist?

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The answer is no and yes!

Basic Respiration Physiology

Myoglobin (Mb)

Cytochrome oxidase

O2

HbO2
Hb + O₂ \rightleftharpoons \text{HbO}_2

Y_{\text{observed}} = \text{fractional absorbance change}

K_{\text{dissociation}} = \frac{[\text{Hb}][\text{O}_2]}{[\text{HbO}_2]}

[Hb] + [\text{HbO}_2] = \text{Hb}_{\text{total}} \text{ or } \text{Hb}_0

[HbO_2] = Y \cdot \text{Hb}_0; [\text{Hb}] = (1 - Y) \cdot \text{Hb}_0

K_{\text{dissociation}} = \frac{(1 - Y)[\text{O}_2]}{Y}

P_{50} = K_d = [\text{O}_2]_{Y=0.5}

\text{Expected curve}

Y = \frac{[\text{O}_2]}{K_d + [\text{O}_2]}

\text{Simple hyperbolic binding curve}

Y = \frac{[\text{O}_2]}{K_d + [\text{O}_2]}

\text{Spectrophotometer cuvette}

\text{Y, the fractional increase in red color or absorbance at 578 nm}

\text{Y definition} = \frac{[\text{HbO}_2]}{[\text{Hb}] + [\text{HbO}_2]}

\text{Oxyhemoglobin}

\text{Deoxyhemoglobin}

\text{Concentration of O}_2 \text{ in } \mu\text{M}

\text{Y or fractional amount of HbO}_2

0.00 0.20 0.40 0.60 0.80 1.00

0.00 0.25 0.50 0.75 1.00

480 500 520 540 560 580 600 620 640 660

\text{Wavelength (nm)}

\text{Relative absorbance}

10 15 0
Cooperative O₂ binding is observed for HbA

**Y or fractional amount of HbO₂**

\[ Y = \frac{[O₂]}{K_d + [O₂]} \]

Hb + O₂ $\xrightarrow{K_d}$ HbO₂

\[ Hb + nO₂ \xrightarrow{K_{Hill}} Hbₙ(O₂)_n \]

Adair's equation seems complex, but if $K_2 >> K_3$, $K_4$, or $K_1$, then it reduces to:

\[ Y = \frac{4K_1K_2K_3K_4[O₂]^4}{4(1 + K_1K_2K_3K_4[O₂]^4) + 1 + K_1K_2K_3K_4[O₂]^4} \]

This equation is similar to Hill's with $n = 4$

\[ Y = \frac{[O₂]^n}{(1/K_1K_2K_3K_4) + [O₂]^4} \]

Thus, $n_{Hill}$ is an empirical measure of cooperativity and can't be greater than the number of subunits or binding sites.
Cooperativity is expressed through the subunit interfaces and changes iron reactivity.

Clam, sea cucumber, and lamprey hemoglobins are dimers and \( n_{\text{max}} = 2.0 \).

\[
Hb_2 + O_2 \rightleftharpoons Hb_2(O_2) + O_2 \rightleftharpoons Hb_2(O_2)_2
\]

Scapharca inequivalvis  
Caudina arenicola  
Petromyzon marinus
Hemoglobin tetramers and larger can have n greater than 3.0

\[
\begin{align*}
K_1 & : H_b^4 + O_2 & \rightarrow & H_b^4(O_2) + O_2 \\
K_2 & : H_b^4(O_2) + O_2 & \rightarrow & H_b^4(O_2)_2 + O_2 \\
K_3 & : H_b^4(O_2)_2 + O_2 & \rightarrow & H_b^4(O_2)_3 + O_2 \\
K_4 & : H_b^4(O_2)_3 + O_2 & \rightarrow & H_b^4(O_2)_4 + \cdots
\end{align*}
\]

Urechis caupo Hb, n=1.0

Homo sapiens HbA, n=3.0

Giant earthworm Hb is a 12-mer of dodecamers

Lumbricus terrestris giant Hb, n=3.5

Importance of cooperativity in maximizing O\(_2\) transport (increasing n)

Amount of transport = ΔY*[Hb]_{total}
Mutant hemoglobins with low cooperativity or too high O\textsubscript{2} affinity cause polycythemia (too high a hematocrit, ≥ 55%), which can lead to bleeding, heart attacks, and death.
Human developmental biology

\[ \text{HbEmbryonic} = \alpha_2\epsilon_2 \quad \text{HbF} = \alpha_2\gamma_2 \quad \text{HbA} = \alpha_2\beta_2 \]

Chromosome 16
- \(\zeta\)  
- \(\alpha_1\)  
- \(\alpha_2\)

Chromosome 11
- \(\epsilon\)  
- \(\gamma_\text{A}\)  
- \(\gamma_\text{G}\)  
- \(\delta\)  
- \(\beta\)  
- \(\text{HS 1-6}\)

\[ \text{HbEmbryonic} = \alpha_2\epsilon_2 \quad \text{HbF} = \alpha_2\gamma_2 \quad \text{HbA} = \alpha_2\beta_2 \]

\(\alpha_2\epsilon_2\) Hb embryonic  
(High \(O_2\) affinity)

\(\alpha_2\gamma_2\) HbF  
(moderate \(O_2\) affinity)

\(\alpha_2\beta_2\) HbA  
(low \(O_2\) affinity)
Acid, base, and isopropanol tests for hemoglobin stability

Embryonic and Fetal Hbs are much more stable
Red blood cells containing HbS with β Glu6 to Val replacements

http://www.sicklecelldisease.org/research/index.phtml

Normal red blood cells with no deformations

Irreversibly sickled cells

Sickling cell

Echinocyte

O₂ transport through the placenta or uterine wall

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AP Biology Lecture 3

6/27/06
The β E6V mutation dramatically decreases the solubility of hemoglobin and causes long fibers to form that sickle the red cells. This was the first genetic disease that was identified at the amino acid level.

Hb AS is benign (only shortens red cell life time to ~80 days)

Hb SS leads to severe anemia and early death

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The sickle cell trait is maintained by strong selective pressure because AS individuals are resistance to malaria. It is a classic case of natural selection.

The Plasmodium parasite must remain in the red blood cells for more than 90 days to be able to change its cell coat and avoid antibody responses

Anopheles gambiae,

Infection with Plasmodium falciparum

HbAS cells only last ~80 day and release immature parasite which is destroyed by the immune system.

The Plasmodium parasite must remain in the red blood cells for more than 90 days to be able to change its cell coat and avoid antibody responses

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Lysis of irreversibly sickled cells releases free hemoglobin, which then scavenges NO and causes severe vasoconstriction, much like the problem with first generation blood substitutes.

Pathology of sickle cell crises

Smooth muscle (sGC NO signalling and vasodilation)

eNOS (NO synthesis)

(Regulated by NO and eNOS)
A secondary function of HbO₂ and MbO₂ is to remove excess NO

Red Cells

Lumen of arteriole
Direction of flow with cells in the center of vessel

Endothelial Cells with NO Synthase (NOS)

Smooth muscle cells With guanylyl cyclase (GC)

Myoglobin in aerobic skeletal muscle

Prevents NO inhibition of mitochondrial respiration

NO is potentially very toxic

1. NO can inactivate aconitase at levels ≤ 200 nM and shuts down TCA cycle.
2. NO can inhibit cytochrome oxidases at levels ≤ 1 µM.
3. NO synthesis inhibits respiration in endothelial and smooth muscle cells.

2Hb(FeII)O₂ + 2NO → 2NO₃⁻ + 2Hb(FeIII) (non-toxic)

O₂
cytochrome b₅ or flavoprotein(FAD)
(reductase)

NAD
NADH

NO dioxygenase (NOD) activity detoxifies NO
NO dioxygenation occurs rapidly in Hb and Mb

Efficiency depends on the rate of reduction of the Fe(III) atom

Effects of NO inhalation on metHb, HbNO, and nitrate levels

(Efforts of NO inhalation on metHb, HbNO, and nitrate levels

(Gladwin et al., 2000 PNAS 97, 9943-48)

Concentration in µM

Time (hr)

NO inhalation by healthy human subjects

nitrate (~80 µM increase)

metHb (~80 µM increase)

80 ppm NO HbNO (~3 µM)

HbNO (~2 µM)
Strategies for Protein Engineering

- 1. **Random mutagenesis** - Vary amino acids randomly to obtain new combinations and then select or screen for better gene products (like nature - "directed evolution")

- 2. **Comparative design** - Examine animal and plant hemoglobins and myoglobins for more optimal properties and unusual active sites ("natural products" and bioinformatics).

- 3. **Rational design** - Use chemical mechanisms to design new active sites and more stable proteins (use of knowledge and "intelligent design").

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Can We Make Recombinant Hemoglobin As the Starting Material for All Hb-based Blood Substitutes?

Cellular Engineering of bacteria

Protein Engineering of Hb

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