Discovering Oxygen Binding to Hemoglobin

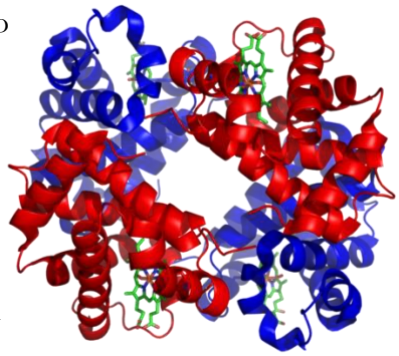
Organize your code, graphs, and answers to the questions clearly in the .Rmd file, using comments and chunks corresponding to each part of the lab.

This is an individual assignment, but you are allowed to work together in groups and discuss coding and answers. That said, you are responsible for all of the material in this laboratory assignment. DO NOT COPY from anyone that you work with. You are NOT allowed to share code. You need to write the code and answer the questions yourself. Try coding yourself first before seeking help.

***Continue working on the .Rmd file from your pre-lab homework assignment**. Rename the file to indicate that this is the lab assignment and be sure to include your name in the file name as follows: lastname_firstname_labday.Rmd. Also, type your full name as the first comment in your .Rmd file.*

Total Points: 20 points
Due: In one week – before the start of your lab during week 8. Upload 1) .Rmd and 2) knitted html files to Canvas.

Overview



Most of the necessities of life can be accumulated to some extent during good times to allow survival through lean times. A healthy human can survive for days to weeks without water and for up to two months without food. Oxygen, though critical to life, is not so easily stored. We do well to swim underwater for a minute or two before surfacing, while diving mammals can function for an hour at most. Many ectothermic and hibernating animals can reduce their metabolism to limit their need for oxygen, but they must have a ready supply to be active. Even the best chemical carrier of oxygen, **hemoglobin**, requires more than 500g of hemoglobin to store just one gram of oxygen. A day's supply of oxygen for a UChicago student studying in the Reg would require on the order of 160 kg (350 lb) of hemoglobin, or over 300 gallons of blood! Given this constraint, any high level of sustained activity by an animal must depend on immediate uptake of oxygen from the environment. For nearly all vertebrates and many invertebrates, this ability depends on the oxygen-binding properties of hemoglobin.

The goal of this lab is to explore how hemoglobin functions to acquire oxygen from the environment and release it where needed by metabolically active tissues. The **affinity** of hemoglobin for oxygen determines at what **partial pressures** of oxygen ($\mathbf{p_{O2}}$) the hemoglobin picks up oxygen from the environment and at what p_{O2} it releases it to tissues. This affinity differs among species and may vary within an animal depending on the stage of development and the physical and chemical conditions in the blood.

In this **lab we will explore mathematically** the association and dissociation kinetics of O_2 for hemoglobin and consider the consequences of these kinetics on the cells in our body.

The **oxygen-binding properties** of hemoglobin can best be visualized by an **oxygen dissociation curve** (Figure 1). For hemoglobin to function most effectively as an oxygen carrier, it should become nearly 100% saturated with oxygen at the p_{O2} found at the site of gas exchange with the environment (i.e., **oxygen loading** in the lungs, gills, etc.), and it should release oxygen at the p_{O2} found in actively metabolizing tissue (i.e., **oxygen unloading**).

The steep portion of the oxygen dissociation curve should ideally lie between the loading and unloading regions for blood that circulates to active tissues, especially for animals engaging in strenuous long-endurance aerobic activities. On this steep part of the curve, a very small drop in p_{O2} results in a large release of oxygen into solution.

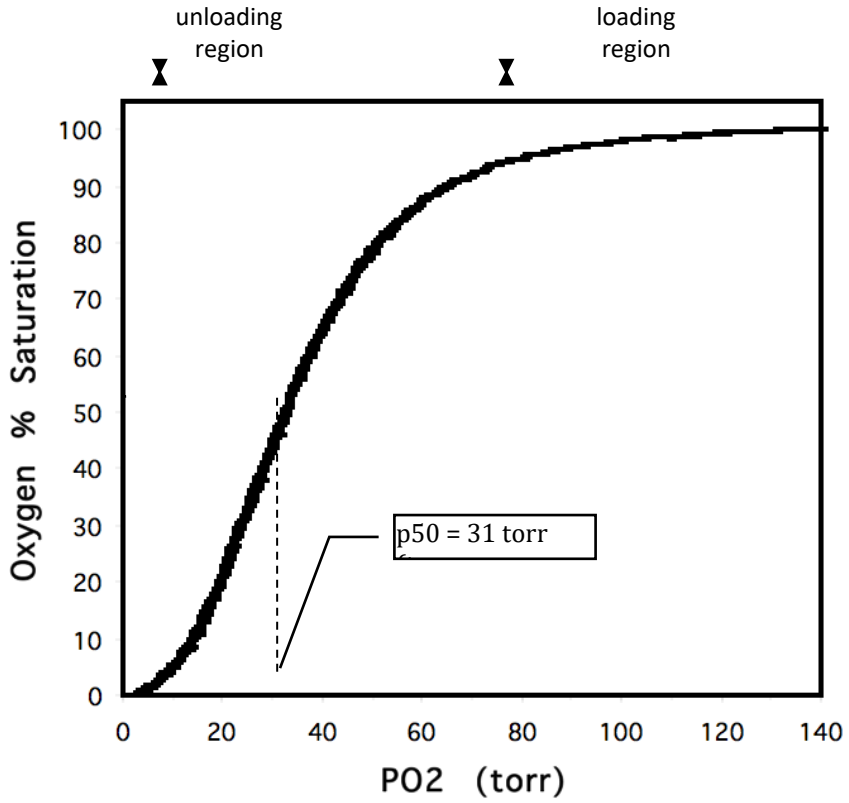


Figure 1. Oxygen dissociation curve for dog blood. Note that torr = mmHg.

The shape of the oxygen dissociation curve can be summarized with two parameters, p50 and the Hill coefficient. The **p50** is the p_{O2} at which the hemoglobin is 50% saturated with oxygen (Figure 1). Physiologists perversely call p50 the **affinity** for oxygen despite the inverse relationship; a *high* p50 is associated with a *low* affinity, and a *low* p50 is associated with *high* affinity for oxygen. The second parameter, the **Hill coefficient**, indicates the degree to which the curve is sigmoid. A sigmoid shape occurs in polymeric respiratory pigments (like tetrameric human hemoglobin) due to **cooperativity** among the subunits. This cooperativity means that the binding of the first O_2 to one of the four hemoglobin subunits facilitates binding of oxygen to the second subunit, and so on. In a monomeric pigment, such as myoglobin or lamprey hemoglobin, there is no cooperativity, so the curve is a rectangular hyperbola.

There is a remarkable diversity of oxygen dissociation curves among different animals, among developmental stages within animal species, and even within a single red blood cell as it makes a circuit through the circulatory system. In some cases, the differences in p_{50} or Hill coefficient are due to genetically determined features of the hemoglobin. In other cases, they result from physical or chemical differences in the immediate environment of the hemoglobin, including pH, temperature, CO_2, CO, and binding of molecules that allosterically influence its oxygen-carrying properties (e.g., 2, 3-BPG).

You may be wondering by now what's the point of all these mechanisms for variation in O_2 dissociation curves when we all breathe the same air with the same 20.93% oxygen? Think carefully about animals whose habitats might not be so similar to ours in oxygen availability. A mammal native to high mountains experiences an atmosphere of 20.93% O_2, but the atmospheric pressure is much lower, making the p_{O2} proportionately lower. A fish relies on oxygen dissolved in water, which can vary substantially in its oxygen

content. A bird flying long distances may need a higher gradient of O_2 between blood and tissues to improve oxygen delivery. How might that affect the optimum p_{50}? What happens if this bird is flying at high altitudes? How do the gas exchange surfaces of a bird embryo or a mammalian fetus compare with those of an adult of the same species? As you consider each of these cases, think of both the loading AND unloading regions of the curve, since a shift in p50 affects both. A higher affinity for oxygen (lower p50) makes it easier to load oxygen, but the tradeoff is a greater difficulty unloading at the other end of the circuit (the hemoglobin is more likely to hang on to its oxygen as well as more likely to pick it up!). What about effects of pH and temperature that might differ between loading and unloading regions, allowing different p_{50}'s in each? Consider the Hill coefficient. What does the degree of sigmoid shape do to the functionality of the curve?

Introduction to Hemoglobin

Hemoglobin can bind up to 4 O_2 molecules at a time. If we assume each of these O_2 molecules bind sequentially, then we can write down the following set of reactions:

$$Hb + O_2 \leftrightarrow HbO_2$$
$$HbO_2 + O_2 \leftrightarrow Hb(O_2)_2$$
$$Hb(O_2)_2 + O_2 \leftrightarrow Hb(O_2)_3$$
$$Hb(O_2)_3 + O_2 \leftrightarrow Hb(O_2)_4$$

Each with their own equilibrium constant:

$$K_{eq1} = \frac{HbO_2}{Hb \cdot pO_2}$$

$$K_{eq2} = \frac{HbO_{2\_2}}{HbO_2 \cdot pO_2}$$

$$K_{eq3} = \frac{HbO_{2\_3}}{HbO_{2\_2} \cdot pO_2}$$

$$K_{eq4} = \frac{HbO_{2\_4}}{HbO_{2\_3} \cdot pO_2}$$

Each having units 1/torr.

To generate the expression for **Y – the fraction of total hemoglobin that is oxygenated** – we need to keep in mind that Y=100% means that all 4 O_2 binding sites on a single hemoglobin tetramer are occupied. Thus, the species HbO_2 is $1/4^{th}$ saturated, HbO_{2_2} is $2/4^{th}$ saturated, and so on.

For accounting purposes, it's important to **keep track of the distinct ways that O_2 can bind to the hemoglobin tetramer**. For simplicity, assume that the K_{eq} values for all subunits are identical. That assumption implies that K_{eq} depends only on the number of O_2 molecules already bound to the tetramer.

Expression for Y for hemoglobin:

$$Y = \frac{\left[4 \cdot \frac{1}{4} \cdot \left(HbO_2\right)\right] + \left[6 \cdot \frac{2}{4} \cdot \left(HbO_{2\_2}\right)\right] + \left[4 \cdot \frac{3}{4} \cdot \left(HbO_{2\_3}\right)\right] + \left[1 \cdot \frac{4}{4} \cdot \left(HbO_{2\_4}\right)\right]}{[1 \cdot (Hb)] + \left[4 \cdot \left(HbO_2\right)\right] + \left[6 \cdot \left(HbO_{2\_2}\right)\right] + \left[4 \cdot \left(HbO_{2\_3}\right)\right] + \left[1 \cdot \left(HbO_{2\_4}\right)\right]}$$

We can rearrange our K_{eq} statements to reduce expression for Y.

$$HbO_2 = Hb \cdot pO_2 \cdot K_{eq1}$$
$$HbO_{2\_2} = HbO_2 \cdot pO_2 \cdot K_{eq2}$$
$$HbO_{2\_3} = HbO_{2\_2} \cdot pO_2 \cdot K_{eq3}$$
$$HbO_{2\_4} = HbO_{2\_3} \cdot pO_2 \cdot K_{eq4}$$

Y can be reduced to:

$$Y = \frac{(pO2 \cdot Keq1) + 3\left(pO2^2 \cdot Keq1 \cdot Keq2\right) + 3\left(pO2^3 \cdot Keq1 \cdot Keq2 \cdot Keq3\right) + \left(pO2^4 \cdot Keq1 \cdot Keq2 \cdot Keq3 \cdot Keq4\right)}{1 + 4(pO2 \cdot Keq1) + 6\left(pO2^2 \cdot Keq1 \cdot Keq2\right) + 4\left(pO2^3 \cdot Keq1 \cdot Keq2 \cdot Keq3\right) + \left(pO2^4 \cdot Keq1 \cdot Keq2 \cdot Keq3 \cdot Kec\right.}$$

Knowing 4 K_{eq} values, and given the partial pressure of oxygen p_{O2}, we can calculate the fraction of hemoglobin that will be oxygen-bound (Y). This is called the Adair equation (Adair, G.S. (1925) Journal of Biological Chemistry 63:529-545).

Since we do not know the 4 K_{eq}s, and instead of guessing, let's make some assumptions in order to simplify.

1. Binding of oxygen is **independent**: binding of O_2 does not depend on hemoglobin saturation. All K_{eq}s would be equivalent. $K_{eq1}=K_{eq2}=K_{eq3}=K_{eq4}$

2. **Fully cooperative binding**. All 4 O_2 molecules bind simultaneously to the hemoglobin. Either no O_2 bound or all 4 would be bound. K_{eq} values would progressively get larger going from K_{eq1} to K_{eq4}. Simultaneous binding also implies the ratio of K_{eq2}/K_{eq1} is very large. $K_{eq1}<<K_{eq2}\sim K_{eq3}\sim K_{eq4}$

For hemoglobin, neither extreme is correct. Reality lies between #1 and #2. There is cooperative binding of O_2, but not to the point that all 4 O_2s bind simultaneously.

PART III: Independent Binding

If we assume independent binding (first O_2 molecule binds just as easily as second O_2 molecule, and so on), all the K_{eq}s are equal. We can replace all the K_{eq}s with one single variable K (1/torr):

$K=K_{eq1}=K_{eq2}=K_{eq3}=K_{eq4}$
This reduces Y:

$$Y = \frac{(pO2 \cdot K) + 3\left(pO2^2 \cdot K^2\right) + 3\left(pO2^3 \cdot K^3\right) + \left(pO2^4 \cdot K^4\right)}{1 + 4(pO2 \cdot K) + 6\left(pO2^2 \cdot K^2\right) + 4\left(pO2^3 \cdot K^3\right) + \left(pO2^4 \cdot K^4\right)}$$

The only free parameter is K. We can choose a value for K and try to fit it to the experimental data in order to try and prove **independent binding**.

Assign K=1/12 torr^{-1}:
Assign p_{O2} to contain a range of values from 0 → 110 with a step size of 0.01. In the pre-lab, you played with 3 different ways to access the data that you read into the R environment. You can choose whichever of the 3 ways you like the best to use in the whole lab.

1. Plot experimental data and simulation run assuming independent binding on the same plot (include axes labels, title, units, legend, and set axis range). What do you see? How do the 2 curves compare?

2. Play with K until you get the "best" fit (show at least 3 different K's that you have chosen). Graph the experimental data and all the different K's that you choose (all on one graph – include axes labels, title, units, legend, and set axis range). Plot a horizontal line at 50% in order to assess the effects of changing K. more accurately.

3. What is the "best" K? Does our model of independent binding accurately display the characteristics of the experimental data? Do you think O_2 binding in hemoglobin is independent? Why/why not? What is the shape of your plot with the "best" fit K? Is it sigmoidal or hyperbolic?

PART IV: Fully Cooperative (Simultaneous Binding)

If O_2 binding is fully cooperative, that means all 4 O_2 molecules bind simultaneously to hemoglobin.

$$K_{eq1} << K_{eq2} \sim K_{eq3} \sim K_{eq4}$$

At equilibrium, only the concentrations of unbound (Hb) and fully bound (HbO_{2_4}) will be non-zero. Either no O_2 is bound or all 4 O_2s are bound.
We can reduce Y:

$$Y = \frac{\left[1 \cdot \frac{4}{4} \cdot (HbO_{2\_4})\right]}{[1 \cdot (Hb)] + \left[1 \cdot (HbO_{2\_4})\right]}$$

We know:

$$HbO_2 = (Hb) \times pO_2 \times K_{eq1}$$
$$HbO_{2\_2} = (HbO_2) \times pO_2 \times K_{eq2}$$
$$HbO_{2\_3} = (HbO_{2\_2}) \times pO_2 \times K_{eq3}$$
$$HbO_{2\_4} = (HbO_{2\_3}) \times pO_2 \times K_{eq4}$$

Which allows us to express Y in terms of pO2 and K_{eqs}:

$$Y = \frac{pO2^4 \cdot Keq1 \cdot Keq2 \cdot Keq3 \cdot Keq4}{1 + pO2^4 \cdot Keq1 \cdot Keq2 \cdot Keq3 \cdot Keq4}$$

This is a major simplification, but there are still 4 parameters—K_{eq1}, K_{eq2}, K_{eq3}, and K_{eq4}—that we don't really have values for. Contrast this with our previous independent binding assumption, which yielded only a single parameter K. With only one parameter, we can poke around and explore its effect. But with four parameters, exploring that large of a parameter space would be a lot of work.
For convenience, let us introduce a new parameter—**P50—which we'll define as the p_{O2} value that yields 50% saturation**. P50 has units of torr. You can see that the **K from above was the same as 1/P50**. In simultaneous binding:

$$\left(\frac{1}{P50}\right)^4 = Keq1 \cdot Keq2 \cdot Keq3 \cdot Keq4$$

Then you can further simplify the expression for Y:

$$Y = \frac{pO2^4}{pO2^4 + P50^4}$$

Assign a value for P50 (in torr). Think of what value you found above for K. **Solve for Y**.

4. Plot experimental data and simulation run assuming simultaneous binding on the same plot (include axes labels, title, units, legend, and set axis range). What do you see? How do the 2 curves compare?

5. Play with P50 until you get the "best" fit (show at least 3 different P50's that you have chosen). Graph the experimental data and all the different P50's (all on one graph - include axes labels, title, units, legend, and set axis range). What is the "best" P50? Plot a horizontal line at 50% in order to assess the effects of changing K. more accurately

6. What is the shape of your plot with the "best" fit P50? Is it sigmoidal or hyperbolic?
   Do you think O_2 binding in hemoglobin is simultaneous? Why/why not?

PART V: Cooperativity and The Hill Equation

Oxygen binding to hemoglobin lies in between 2 extremes. It is cooperative, but not FULLY cooperative. This suggests that O_2 binding is sequential; with each O_2 binding, subsequent bindings are easier.
Fully cooperative binding:

$$Y = \frac{pO2^4}{pO2^4 + P50^4}$$

Let's introduce a new parameter to define not FULL cooperativity, n (describes cooperativity of O_2 binding) (Hill Equation)

$$Y = \frac{pO2^n}{pO2^n + P50^n}$$

This expression, first described by Archibald V. Hill, is known as the Hill equation. The new parameter, n, is known as the Hill coefficient. Archibald V. Hill shared the Nobel Prize in Physiology or Medicine in 1922. In this Part, we'll use the Hill equation to obtain a better fit to the experimental hemoglobin saturation data.

Assign a value **n=4**, to keep with full cooperativity. We will change this value in a minute to demonstrate Hill's model.
Solve for Y.

7. Plot experimental and simulation data assuming cooperative binding on the same plot (include axes labels, title, units, legend, and set axis range). What do you see? How do the 2 curves compare?

8. Play with n until you get the "best" fit (show at least 3 different n's that you have chosen). Graph the experimental data and all the different n's that you choose (all on one graph - include axes labels, title, units, legend, and set axis range). What is the "best" n (± 0.1)?

9. What is the shape of your plot with the "best" fit n? Is it sigmoidal or hyperbolic? Do you think O_2 binding in hemoglobin is cooperative? Why/why not?

PART VI: Temperature-Sensitivity and the Hill Equation

We will now investigate temperature effects on O_2 saturation with hemoglobin. You have already read in the experimental file corresponding to 7 different temperatures and the O_2 saturation curves.

10. Use the Hill equation (part V) to solve EACH temperature-sensitive curve for hemoglobin O_2 saturation. Play with p50 and hill coefficient "n" in order to match your simulation curves with each of the experimental curves (i.e., 32oC, then 35oC, etc.). You can change the hill coefficient and p50 simultaneously until you get a good fit. Plot each temperature on its own graph (you will have 2 curves per temperature – 1) simulation curve with the best "n" and p50 and 2) experimental curve for that temperature). Include axes labels, title, units, legend, and set axis range. You will have 7 independent graphs (with 2 curves) – each corresponding to the 7 temperatures.

11. Create 3 new vectors 1) to contain 7 values for p50, 2) 7 values for "n", and 3) 7 distinct temperatures.

12. Graph 1) the p50 vs. temperature and 2) the "n" vs. temperature.

13. Perform a linear fit for both the p50 and "n" data independently. What is the slope? Intercept? Correlation coefficient? What does it say about p50 and "n" if they are linear in temperature?

14. On the same graph 1) plot the p50 vs. temperature (as points) and your linear fit line (as a line) and 2) plot the "n" vs. temperature (as points) and your linear fit line (as a line).

Quick R reminders:
For linear least squares regression use *fit <- lm(y ~ x)*

You can see the intercept, slope, and R^2 values.
intercept<-fit$coefficients[[1]]
slope<-fit$coefficients[[2]]
R2<-summary(fit)$r.squared

in order to add the straight line calculated by the linear least squares regression
abline(fit)

PART VII: The Bohr Effect

Given the physiological importance of hemoglobin, it's not surprising that **numerous regulators affect hemoglobin O_2 binding**. One of the most important regulators is H +, which increases the P50 of hemoglobin. This is known as the Bohr effect, discovered by Christian Bohr (father of the atomic physicist Niels Bohr). The Bohr effect is important in the "loading" and "unloading" of O_2 from hemoglobin. **Metabolically active tissues produce H+, lowering the pH of nearby blood, and enhancing the "unloading" of O2 from hemoglobin for use in the tissue.** Conversely, removal of CO_2 from the blood at the lungs consumes H+ via the following reaction:

$$H+ + HCO3- \rightleftarrows H2CO3 \rightleftarrows H2O + CO2$$

This raises the pH of blood in the lungs, enhancing "loading" of O_2 onto hemoglobin to be transported elsewhere. In this Part, you will use the following experimental data to construct a model of the Bohr effect. The data are from human hemoglobin, with the **P50** determined at a variety of pH values [Dahms, *et al.* (1972) *Journal of Physiology* 223(Suppl):29P-31P].

pH = (7.61, 7.36, 7.15, 6.92) p50 = (19.1, 23.4, 30.9, 40.7) in torr

15. Create a graph of experimental data – plot p50 vs. pH; just plot points, not a line.

You should notice that the above plot of **data_P50** vs. **data_pH** forms a fairly straight line. This suggests that we can predict **P50** at arbitrary pH values by fitting a straight line to the data. Let's do that now (see above section for instructions on finding the slope, intercept, and correlation coefficient for a straight line).

16. Graph experimental data (circles) and your fit line (as a line) on the same graph. What is the intercept, slope, and correlation coefficient? Is this a good fit?

*Your goal here is to use these data to create a function of hemoglobin saturation (**Y**) at any arbitrary pO_2 value and arbitrary pH value. That is, you will show how **Y** is simultaneously a function of both pO_2 and pH.*

Procedure: To do this, you will use the Hill Equation (you can call the function you already created). The Hill equation has two parameters:

1. The Hill coefficient, **n.** We will assume this to be **2.8** and that it is NOT affected by pH. This value of **2.8** should be close to what you determined in Part IV.
2. The half-saturation pO2 value—**P50.** This you will infer from the experimental data above, eventually creating a function, **P50(pH),** that will ***accept pH as an input and return a P50 value as an output***.

OK, now we're going to use our global function of the Hill equation and the equation of a straight line (using the slope and intercept from the above fitted straight line to experimental data) to solve for p50 based on any pH value you choose.

1) Define a pH value, 2) Solve for p50 based on the expression for a straight-line and the slope and intercept calculated from above, and 3) Call hill() feeding in the newly calculated p50 based on the linear correlation with pH.

17. Solve the Hill equation for each pH (4 values). Increase the range of p_{O2} from 0 → 160. Arterial p_{O2} is on the order of 100torr, so we will need to extend the range to see what happens to hemoglobin in the lungs (venous blood is ~ 40 torr).

18. Plot all 4 curves on the same graph corresponding to each pH. You will plot $\%O_2$ saturation vs. p_{O2} (include axes labels, title, units, legend, and set axis range).

19. Write a few sentences about what you see in your graph. How does pH appear to affect hemoglobin's O2 saturation curve (Y)? Think about the p_{O2} value that produces 50% saturation; how does this change with pH? Think about the steepness of the saturation curve; how does this change with pH?

20. In your graph above, take a look at the saturation curves at the presumed p_{O2} values of the venous and arterial blood (40torr and 100torr respectively). Draw in a vertical line at 40 Torr and 100 Torr (use abline(v=?)).

21. Does pH have the same effect on Y in both locations? What do you think this means with respect to pH effects on hemoglobin "loading" and "unloading" of O_2? Use the data below to help you answer the question.

Y(40torr ~ pH=7.6) - 90.616%	Y(40torr ~ pH=6.8) - 44.882%
Y(100torr ~ pH=7.6) - 99.21%	Y(100torr ~ pH=6.8) - 91.374%

22. Now plot the myoglobin curve (p50=35.6 mmHg) on the same graph above. You will have the pH shift of hemoglobin (4 curves), the 2 vertical lines indicated for venous and arterial blood p_{O2} pressures, and the curve for myoglobin.

23. Considering that venous and arterial blood carry 40 and 100 mmHg respectively of pO_2 pressure, explain the role of myoglobin and hemoglobin in delivering O_2 to active muscle cells. Be sure to explain the shape of the curves and correlate the shape with molecular function of hemoglobin and myoglobin.

If you have further questions, please email me: Esmael Hadaddian: haddadian@uchicago.edu or contact your TAs.

The structural change of hemoglobin upon addition of oxygen (cooperativity) is shown below
https://en.wikibooks.org/wiki/Structural_Biochemistry/Hemoglobin