

Problem 1. (27 points total)

a. (5 points) A protein has binding affinity for its ligand (a peptide) of $K_a = 2 \cdot 10^5 \text{ M}^{-1}$ at pH 5.0 and 25°C. At what concentration of the ligand is half of the protein bound?

$$[L] = K_d = 1/K_a = 5 \cdot 10^{-6} \text{ M} = 5 \mu\text{M}$$

b. (5 points) What fraction of the protein is bound at ligand concentration of 1.25 μM (a reminder: $1 \mu\text{M} = 10^{-6} \text{ M}$)?

$$\theta = \frac{[L]}{[L] + K_d} = \frac{1.25 \mu\text{M}}{1.25 \mu\text{M} + 5 \mu\text{M}} = \frac{1}{5} = 20\%$$

c. (6 points) At what ligand concentration will be 80% of the protein bound?

$$\theta = \frac{[L]}{[L] + K_d} = 0.8 \quad \text{hence} \quad [L] = \frac{0.8 K_d}{1 - 0.8} = 4 K_d = 20 \mu\text{M}$$

d. (5 points) When the pH was raised to 6.5, the K_d increased to 20 μM . Is the binding tighter or weaker at this pH compared to pH 5.0? Explain why.

Since $K_a = 1/K_d$, higher K_d , indicates lower affinity. The binding is weaker at pH 6.5.

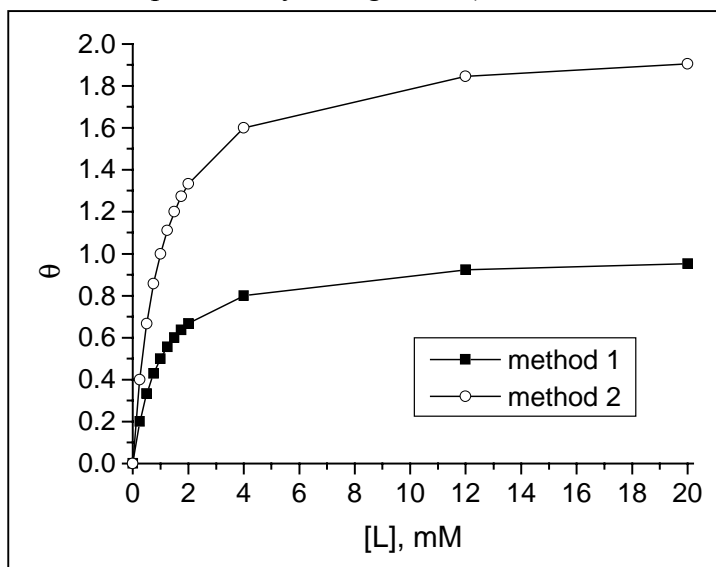
e. (6 points) What functional groups/residues are most likely responsible for this change in the binding affinity with pH?

Histidine. Its pKa is 6.0. No other group in proteins (under normal conditions) has its pKa in the pH range between 5.0 and 6.5.

Problem 2. (total 22 points)

Ligand binding studies were performed by adding ligand to a certain amount of protein X. The fraction θ of protein bound to ligand was assessed using two different methods: (1) by measuring the concentration of the protein bound to ligand and (2) by measuring the concentration of ligand bound to protein. In both cases θ was derived as $\theta = [PL]/[P]_{\text{total}}$, where $[PL]$ represents the results of these measurements and $[P]_{\text{total}}$ is the total concentration of the protein present in solution. The data are shown in the table below and in the Figure (the data points are connected by lines just to guide the eye, these lines do not represent any fitting curves).

1	2	3
[L] in mM	θ method 1	θ method 2
0	0	0
0.25	0.200	0.400
0.50	0.333	0.667
0.75	0.429	0.857
1.00	0.500	1.000
1.25	0.556	1.111
1.50	0.600	1.200
1.75	0.636	1.273
2.00	0.667	1.333
4.00	0.800	1.600
12.00	0.923	1.846
20.00	0.952	1.905



1. (6 points) Explain the difference between the results of these two measurements.

The two methods measure the same quantity, $[PL]$, but by monitoring the concentration of the two partners in the protein-ligand complex. Stoichiometry of ligand binding is important when determining $[PL]$ by measuring the concentration of bound ligand. The fact that q (method 2) is 2-fold greater than q (method 1) suggests that the stoichiometry (ligand : protein) is 2:1.

2. (5 points) How many ligand binding sites are on the protein molecule?

2 (see pr.1)

3. (6 points) What is the affinity of protein X for the ligand? Explain your reasoning

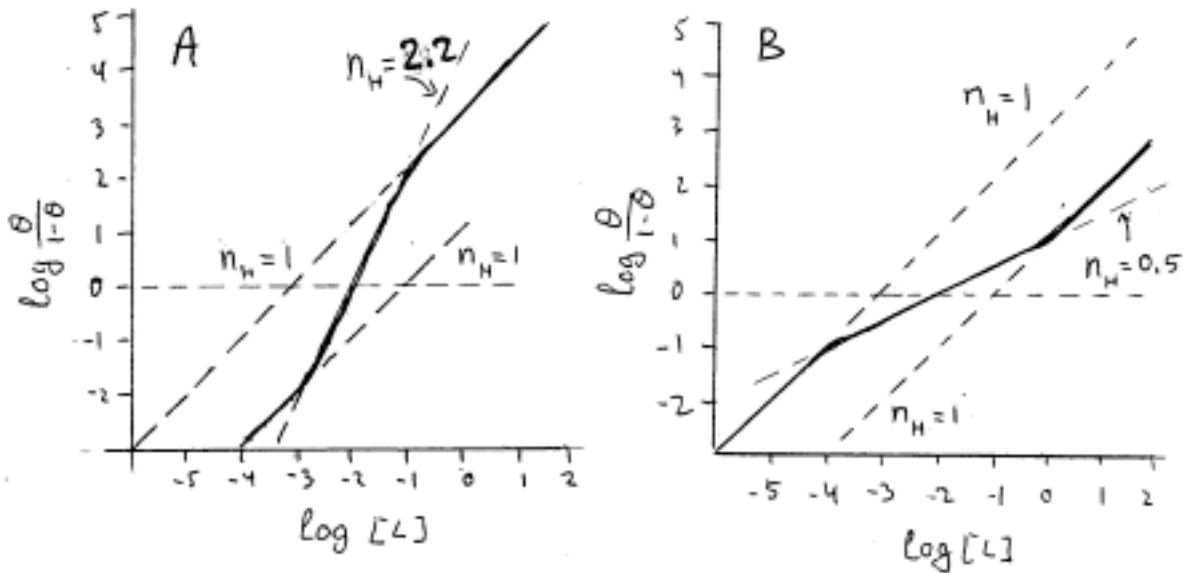
From the table: $K_d = [L]_{0.5} = 1 \text{ mM}$; $K_a = 1/K_d = 10^3 \text{ M}^{-1}$

4. (5 points) What could you assume about possible cooperativity of the ligand binding from these data? Suggest additional data analysis that could help verify your assumption.

The binding curves look as hyperbolic (by eye), suggesting no cooperativity. To verify this, the data should be analyzed using Hill plot.

Problem 3. (24 points total)

Ligand binding to proteins A and B is characterized by Hill plots shown below.



a. (6 points) What conclusions about possible cooperativity of the binding can you draw from these plots? Explain the different shape of the binding curves.

Both proteins show cooperative ligand binding:

A shows positive cooperativity ($n_H > 1$) while B has negative cooperativity ($n_H < 1$).

This determines the shape of the binding curve:

A: a transition from low-affinity to a high-affinity binding;

B: a transition from high-affinity to low-affinity binding.

b. (6 points) Based on these plots, what is the minimal number of ligand binding sites on protein molecule for each of the proteins? Explain.

The number of binding sites is equal or greater than n_H . For protein A, $n_H = 2.2$, so the minimal integer number $\geq n_H$ is 3.

For protein B ($n_H = 0.5$), the minimal number is of ligand binding sites is 2. The minimal integer $\geq n_H$ is 1, but at least two binding sites are required for cooperativity.

c. (4 points) Which of the two proteins binds ligand tighter at the midpoint of the binding curve (i.e. where $\theta = 0.5$)? Explain.

Hill plot: $\log(\theta/(1-\theta)) = n_H \log[L] - \log K_d$. At $\theta = 0.5$, the left-hand side is 0, hence

$$\log K_d = n_H \log([L]_{0.5}). \text{ This gives } K_d = ([L]_{0.5})^{n_H}.$$

For both proteins $[L]_{0.5} = 10^{-2}$ M. For protein A: $K_d = (10^{-2})^{2.2} = 10^{-4.4}$ while for protein B K_d is significantly greater: $K_d = (10^{-2})^{0.5} = 10^{-1}$. Therefore we can conclude that protein A binds ligand tighter at the midpoint of the binding curve.

Same conclusions can be obtained from the intercept of the middle-part (or its extension) of the graph with the $\log[L]=0$ axis: according to Hill equation, the ordinate of this point gives $-\log K_d$. We thus get for $-\log K_d$ the values of approx. 4 (A) and 1 (B).

Note: just the fact that the slope (n_H) in the Hill plot is greater for A than for B means higher cooperativity, *not* necessarily higher binding affinity.

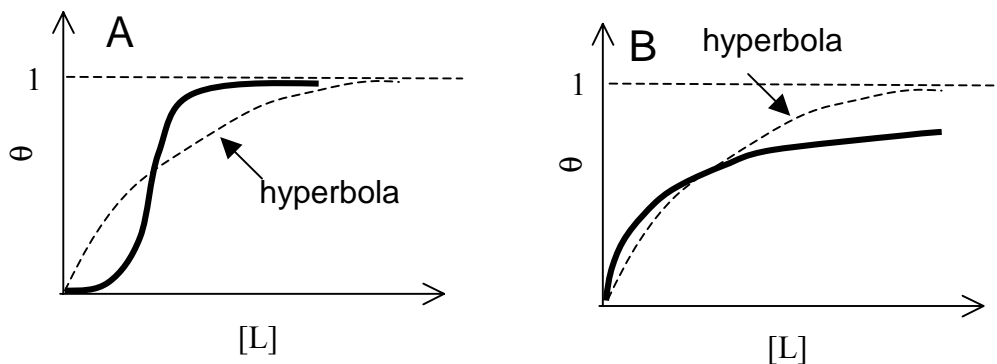
d. (4 points) For each of the proteins determine and compare their binding affinities in their high- and low- affinity states.

The K_d 's for these two states can be determined directly from the Hill plots as the values of $[L]$ at $\theta = 0.5$, i.e. where the corresponding lines intercept the $\log(\theta/(1-\theta)) = 0$ axis. Since for both high- and low-affinity states $n_H=1$, we can use the relationship: $K_d = [L]_{0.5}$.

From the plots (the results are same for both A and B):

High-affinity state: $K_d = 10^{-3}$ M; low-affinity state: $K_d = 10^{-1}$ M, so the high-affinity state has a 100-fold greater K_a than the low-affinity state.

d. (4 points) For each of the proteins draw schematically their ligand binding curves in the coordinates θ versus $[L]$.



Problem 4. (39 points total).

Both myoglobin and hemoglobin utilize heme to bind oxygen, the tertiary structure of myoglobin is very similar to that of the individual subunits in hemoglobin. And yet, the biological functions of the two molecules are very different.

a. (6 points) What is the principal difference in the character of oxygen binding to hemoglobin and to myoglobin?

Oxygen binding to hemoglobin is cooperative, the positive cooperativity is achieved via allosteric effect. There are four oxygen-binding sites per Hb molecule.

There is no cooperativity in oxygen binding to myoglobin. There is one oxygen binding site per Mb molecule.

b. (6 points) What are the principal structural differences between these two molecules that are responsible for the difference in their function?

Mb is a monomer, i.e. a single-chain protein. Whatever changes occur upon O₂ binding, they are not transferred to other Mb molecules, because there is no contact between them.

Hb is a tetramer: it consists of 4 chains, each folded as a separate subunit and each containing an O₂ binding site. The tight contact of quaternary packing of these subunits is responsible for two binding states of Hb: T and R. The role of Hb as O₂ carrier is due to cooperativity of O₂ binding – an allosteric transition from T to R state with the increase in the number of O₂ molecules bound to Hb. This relatively sharp transition allows Hb to tightly bind O₂ at lung pressure and to release substantial amount of O₂ in the tissues, where pO₂ is only ~ 3-4 times lower than in the lungs.

c. (10 points) Describe in structural terms the mechanism of allosteric effect of oxygen binding to hemoglobin, i.e. what changes (if any) in the secondary, tertiary, and/or quaternary structure occur upon O₂ binding.

Here is a very brief description.

In the deoxy conformation of Hb, heme has a dome shape. Oxygen binding to the heme pulls the iron into the heme plane, flattening the heme and causing strain in the neighboring parts of the structure of this subunit. This strain is relieved by the shift in the orientation of the proximal HisF8 and in ValFG5. This then involves helix F, and as a result, leads to the movement of the FG corner. This rearrangement is then communicated to the C helix of the neighboring subunit, due to its direct interaction (via side chains) with the FG corner.

d. (6 points) What could be thermodynamic explanation of the nature of positive cooperativity of O₂ binding to hemoglobin, i.e. why the binding affinity increases when one or more O₂ molecules are already bound.

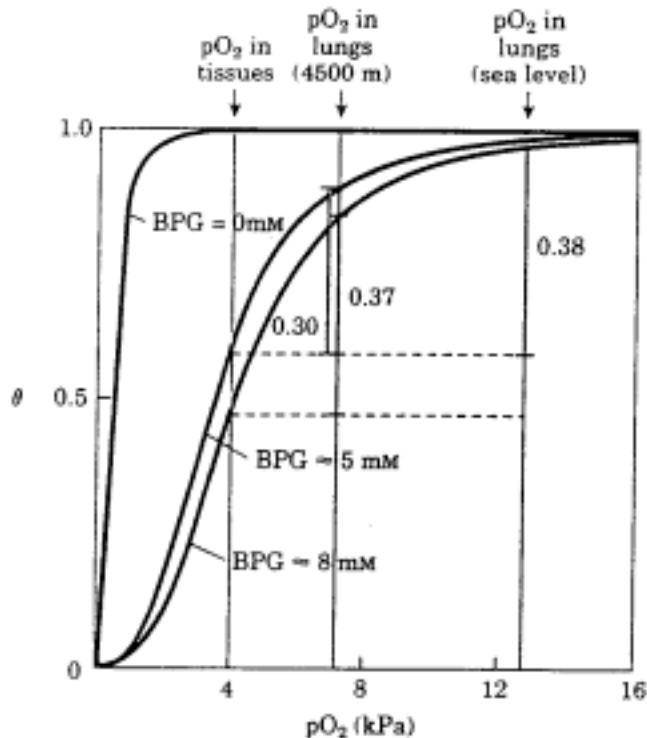
Binding of O₂ molecule to one of the subunits in Hb not only introduces changes in the tertiary structure of that subunit, but, due to interaction with its neighboring subunits, causes changes in the quaternary structure of the whole Hb molecule. Specifically, the $\alpha_1\beta_1$ and $\alpha_2\beta_2$ dimers rotate relative to each other. This rotation involves breakage of ionic bonds that link the subunits together. This energetically unfavorable process is coupled to the favorable free energy of oxygen binding. The absolute value of the resulting change in free energy will be reduced, hence lower binding affinity. In other words, binding of the first molecule involves paying additional energy price for the conformational transition in Hb. Once this price has been paid, binding of additional oxygen molecules is energetically more favorable, and will proceed with higher binding affinity.

e. (6 points) All following molecules when present in the blood impede the ability of hemoglobin to bind oxygen: CO, CO₂, H⁺, and 2,3-bisphosphoglycerate (BPG). What is the principal difference in the mechanism of how they affect oxygen binding?

CO bind directly to the heme – it competes with O₂ binding.

CO₂, H⁺, and BPG -- all bind at various sites different from the heme – they affect O₂ binding via allosteric regulation, by stabilizing the T state and therefore biasing the Hb conformation towards the T-state. These are heterotropic negative effectors.

f. (5 points) BPG is not only important for our adaptation to higher altitudes, but it is also critical for oxygen transport by the blood at the sea level. Based on the Figure below, explain why it is essential that a certain level of BPG is maintained in human blood. What would happen if BPG concentration drops to 1 mM?



BPG increases K_d (reduces affinity for oxygen) of oxygen binding to Hb to a level comparable with the O₂ concentration (partial pressure, pO₂) in tissues – to allow efficient release of oxygen. If BPG concentration drops to 1mM, the affinity for oxygen will be so strong that it will not be released at the pO₂ level in the tissues. This will be lethal.