

Section A (21 pts): (3 pts/question). Circle the letter corresponding to the *best* answer.

1. One ligand binds more tightly to a protein than another ligand if:
 - a) its K_A (K_{EQ}) is lower than the other ligand.
 - b) its K_D is lower than the other ligand.**
 - c) its Hill coefficient is >1 .
 - d) its Hill coefficient is <1 .

2. A ligand that binds more tightly to a protein than another ligand probably has a
 - a) a slower kinetic on-rate
 - b) a faster kinetic on-rate. (+ 2pts)
 - c) a faster kinetic off-rate.
 - d) a slower kinetic-off rate.**

3. If protein binds two ligands in a *non-cooperative* manner, then:
 - a) the dissociation constant is $[L]$ when $Y=0.5/2$
 - b) the dissociation constant is $[L]$ when $Y=0.5$ This is always the case!**
 - c) the dissociation constant is $[L]$ when $Y=0.5*2$
 - d) the dissociation constant cannot be determined from a binding curve in this case.

4. The oxygen bound to hemoglobin or myoglobin is directly attached to the
 - a) helix-F in the protein.
 - b) the proximal histidine.
 - c) the iron atom.**
 - d) the heme group. (+ 2pts)

5. Enzymes increase the rate of chemical reactions by
 - a) providing suitable catalytic groups.
 - b) increasing the population of the transition state.
 - c) decreasing the free energy difference ΔG° of the transition state.
 - d) all of the above.**

6. HIV protease and Chymotrypsin are similar in that:
 - a) both use Serine as the nucleophile.
 - b) both are monomeric proteins.
 - c) both cleave hydrophobic containing peptides.**
 - d) both use Aspartate to activate the nucleophile.

7. SDS gel electrophoresis gives the _____ molecular weight and Gel filtration provides the _____ molecular weight.
 - a) native, denatured.
 - b) denatured, native.**
 - c) native, native.
 - d) denatured, denatured.

B1. Please answer **three** of the following **six** questions (15 pts total)

1. BPG (bisphosphoglycerate) is involved in the adjustment of Oxygen delivery at high altitude. *Briefly* explain how this works.
 - BPG is a heterotropic allosteric inhibitor of Hb, binding in a positively charged pocket distant from the heme.
 - Its concentration is increased at high altitudes.
 - It reduces Oxygen affinity, shifting the binding curve to the right.
 - Although the hemoglobin has a lower affinity, it is capable of increased delivery at high altitudes where the O_2 is low.
2. What is the purpose of a Scatchard plot? When is it appropriate to use it and when is it not?
 - To linearize the hyperbolic binding curve to obtain a more accurate measurement of K_D (or K_A) from the slope.
 - It is appropriate to use it for non-cooperative binding, regardless of the number of ligands.
 - It cannot be used for cooperative binding, since the equation is no longer linear.
3. What is the main purpose of a Hill Plot? What two parameters can be obtained from a Hill Plot?
 - To detect cooperative binding.
 - The K_D is the ligand concentration that gives $\log(Y/(1-Y))=0$ ($Y=1/2$). This is the x-intercept.
 - n_h is the slope of the line at $Y=0.5$.
 - $n_h = 1$ non-cooperative binding
 - n_h not equal 1 - cooperative binding.
4. What is the difference between microscopic and macroscopic binding constants. Use the example of a protein that binds two ligands in your answer.
 - The microscopic binding constant is the equilibrium constant for a ligand binding to a single site on a macromolecule. It is always k_1/k_{-1} and is directly related to the thermodynamics of binding.
 - The macroscopic binding constant considers the overall equilibrium when binding to two sites. It contains statistical factors that are related to the number of ways the ligand can bind or become unbound. In the case of two sites $K_1=2k_1/k_{-1}$.
5. If the ΔH° for the binding of a ligand is negative, will the binding constant ($K_A = K_{EQ}$) increase or decrease as the temperature is raised? Briefly justify your answer.
 - The van't Hoff relationship is $\ln K_{eq} = -\Delta H/RT + \Delta S/R$. If the enthalpy is negative then the equation becomes:
 - $\ln K_{EQ} = |\Delta H|/RT + \Delta S/R$. As the temperature is increased, $1/T$ decreases, therefore $\ln K_{EQ}$ decreases as does K_{EQ} .
6. How does an antibody differ from an enzyme? How are they similar?
 - Both bind other molecules with high specificity and generally high affinity. An enzyme is capable of catalyzing a reaction while an antibody cannot.

B2. Answer **one** of the following **two** questions (8 pts)**Choice A:**

i) Calculate the fractional saturation given the following equilibrium dialysis data (show your work) (4 pts)

1. Total concentration of protein (macromolecule) inside the dialysis bag: 1 μM
2. Ligand concentration outside the bag: 5 μM
3. Total ligand concentration inside the bag: 6 μM

$$Y = \frac{[\text{ML}]}{[\text{M}] + [\text{ML}]}$$

$$[\text{ML}] = L_{\text{INSIDE}} - L_{\text{OUTSIDE}} = 6 - 5 = 1 \mu\text{M}$$

$$[\text{M}] + [\text{ML}] = 1 \mu\text{M} \text{ (this is always the amount of macromolecule in the dialysis bag)}$$

$$Y = 1/1 = 1, \text{ i.e. the macromolecule is fully saturated.}$$

ii) Is this information sufficient to obtain K_D ? Briefly Justify your answer. (4 pts)

NO: This simply means that the $[\text{L}] \gg K_D$, or in other words $K_D \ll 5 \mu\text{M}$. In order to obtain K_D it is necessary to have Y values < 1.0 , preferably in the range of $Y = 0.5$.

Choice B:

What is the *highest* possible value of the Hill coefficient for hemoglobin? Why? (4 pts) If hemoglobin actually had such a Hill coefficient, do you expect to find any partially liganded species when oxygen is present (e.g. $[\text{Hb-O}_2]$, $[\text{Hb-(O}_2)_2]$, $[\text{Hb-(O}_2)_3]$)? Justify your answer. (4 pts)

The Hill coefficient cannot exceed the number of binding sites, which is 4 in this case. If the Hill coefficient was equal to four, you would never see any intermediate species. Initially, all the sites on the tetramer would be low affinity, however once one O_2 bound, the remaining three sites would become high affinity and immediately bind oxygen.

B3. Briefly describe how allosteric effects can be used to control biological processes. Your answer should include a discussion of tense (T) and relaxed (R) states. Give either a *specific* or a *general* example of how allosteric effects can modify the biological function of a protein (12 pts)

An allosteric protein exists in two states:

Tense (T) state of low activity (enzyme) or binding affinity.

Relaxed (R) state of high activity or binding affinity.

These states *differ* in conformation and these conformational differences are responsible for the different activities.

These states are also in equilibrium with each other, and the position of that equilibrium depends on the presence of the allosteric compound:

Allosteric activators - stabilize R state.

Allosteric inhibitors - stabilize T state.

Allosteric compounds that influence their own binding, such as O_2 to hemoglobin, are homotropic.

Allosteric compounds that influence the binding of another ligand, such as BPG or H^+ are heterotropic.

Examples include non-competitive enzyme inhibitors, and the above mentioned regulators of hemoglobin function.

B4: The figure to the right shows the active site region of the serine protease trypsin. (14 pts).

Part A: Select **three** of the following **four** residues and briefly discuss its role in the overall catalytic mechanism of the enzyme (12 pts).

i) Asp102

This stabilizes the positive charge that develops on His57 during activation of Ser.

ii) His57

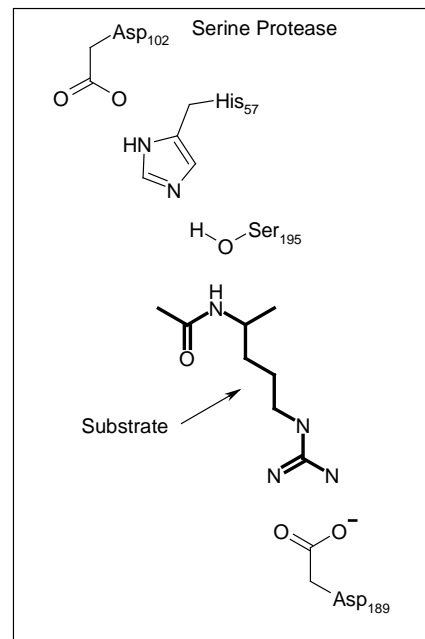
Activates both Ser and H₂O as the nucleophile in the reaction by acting as a base and abstracting the proton for both. It also donates the proton to the newly formed amino terminus.

iii) Ser195

This is the first nucleophile in the catalytic mechanism. After activation by His, it attacks the electro-positive carbonyl carbon, leading to peptide bond cleavage. The 2nd half of the product remains attached as an acyl-enzyme.

iv) Asp189

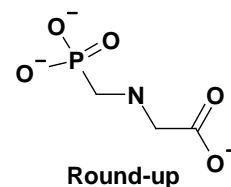
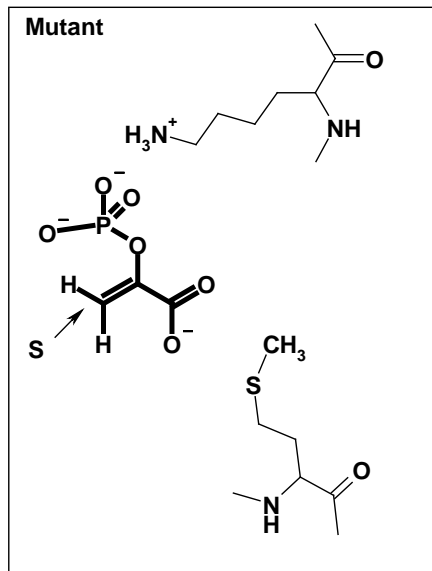
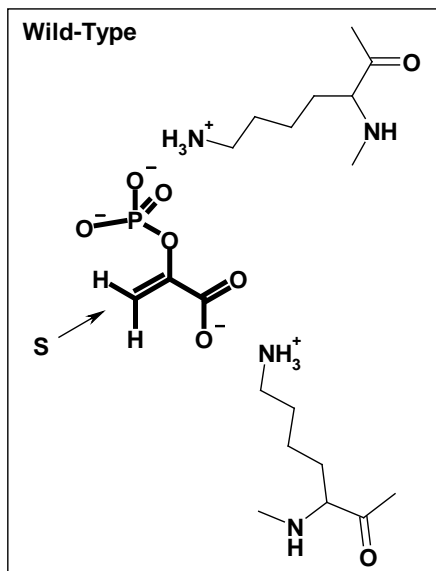
This defines the specificity of Trypsin, interacting with a favourable electrostatic interaction with the Lys or Arg sidechain of the bound substrate.



Part B: Which one of the above four residues performs the same role as the deprotonated Aspartate 25 in HIV protease? (2 pts).

The Asp residue in HIV protease activates water to be an efficient nucleophile, leading to bond cleavage. Therefore it plays the same role as His57

B5: (20 pts) The left panel shows a substrate bound in the active site of the enzyme that is responsible for the synthesis of phenylalanine in plants. This enzyme is readily inhibited by 'Roundup', a widely used herbicide. The bound substrate is shown in thick lines and labeled with 'S' and residues that belong to the enzyme are shown in thin lines. The right panel shows a mutant enzyme that has been isolated from plants that are resistant to 'Roundup'. The structure of Roundup is shown to the far right.



i) How does the mutant enzyme differ from the wild-type enzyme? (2 pt).

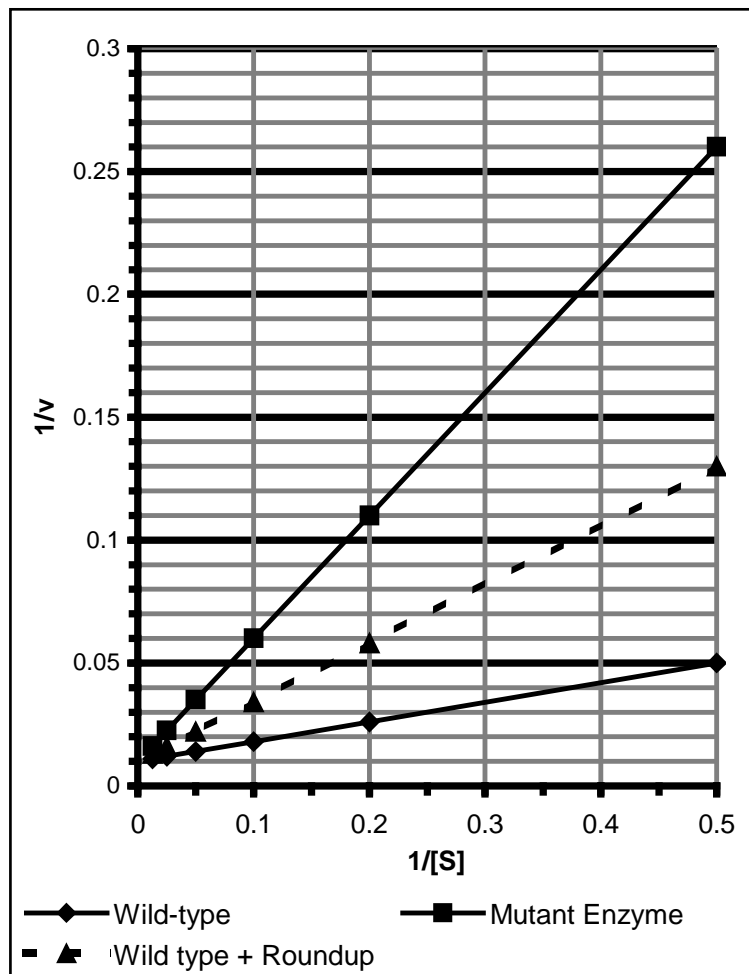
The polar and charged Lysine sidechain has been replaced by the non-polar sidechain of Methionine

ii) The enzyme velocity data of the wild-type and mutant enzyme have been plotted on a double reciprocal plot (solid lines) shown to the right. Both of these plots are in the absence of the inhibitor. (The dotted line is the wild-type enzyme in the presence of the inhibitor.)

Has the mutation affected K_M or V_{MAX} of the enzyme? (It is not necessary to obtain numerical values, but explain how you arrive at your answer.(4 pts)

The slope of the line in a double reciprocal plot is K_M / V_{MAX} . The slopes of the solid lines are clearly different, so either K_M or V_{MAX} have changed.

However, since the Y-intercept is $1/V_{MAX}$, and the Y-intercepts are the same for both, only the K_M values are different. The mutant shows an increased slope, so the K_M is larger.



iii) Based on your answer to *part ii)* does the mutation affect the ability of the enzyme to bind the substrate or the enzyme's ability to catalyze the chemical reaction? Justify your answer (3 pts).

- The K_M is largely a measure of the substrate binding affinity, and is similar to K_D for ligand binding. A lower K_M generally means that the substrate binds more tightly. In this case, the K_M for the mutant is higher, therefore the substrate does not bind as well.
- The catalytic efficiency is unaffected, since $V_{MAX} = E_t k_{CAT}$, and both enzymes have the same V_{MAX} .

iv) What type of inhibitor is Roundup - a competitive one or a non-competitive inhibitor? Justify your answer with reference to the *chemical structure* of Roundup *as well* as the curve plotted on the figure on the previous page. (4 pts)

It is a competitive inhibitor for the following two reasons:

1. It is structurally very similar to the true substrate, with a negatively charged phosphate at one end and a carboxylic acid group at the other.
2. The V_{MAX} is unchanged for the reaction, the dotted curve intersects the y-axis at the same location as the wild-type enzyme. This is a hallmark of a competitive inhibitor - at high substrate its inhibition can be completely removed.

v) Determine either K_I or K_I and K_I' (depending on the type of inhibitor) from the data presented in the graph on the previous page. The concentration of inhibitor was $10 \mu\text{M}$ in the experiment (3 pts)

These parameters are found from the following general equation: $\alpha = 1 + [I]/K_I$. α is obtained from the ratio of the slopes and α' is obtained from the ratio of the y-intercepts.

The data should be taken from the lower solid curve (wild-type enzyme) and the dotted line (wild-type with $10 \mu\text{M}$ inhibitor). The upper solid line is the uninhibited data for the mutant enzyme. If you used the upper curve, you would get a negative K_I , which is not physically possible.

$$\text{Slope of inhibited reaction: } (0.13 - 0.01) / 0.5 = 0.24$$

$$\text{Slope of uninhibited reaction: } (0.05 - 0.01) / 0.05 = 0.08$$

$$\alpha = 0.24 / 0.08 = 3$$

$$K_I = [I] / (\alpha - 1) = 10 \mu\text{M} / (3 - 1) = 5 \mu\text{M}$$

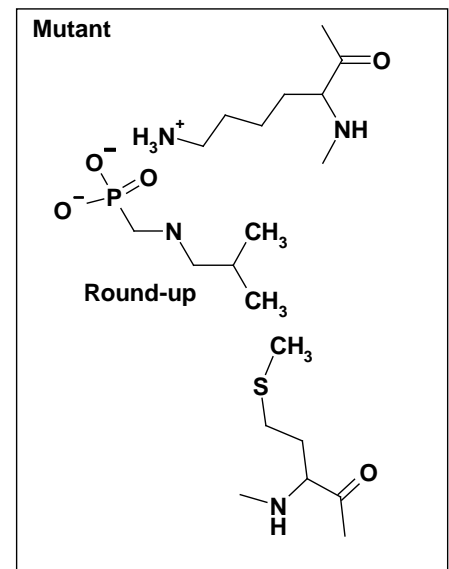
Both curves have the same y-intercept, therefore $\alpha' = 1$, as expected for a competitive inhibitor.

vi) Roundup binds poorly to the mutant enzyme, How might you modify Roundup such that it will become a more effective inhibitor of the mutant enzyme and therefore be able to kill the plant? If you are successful in the redesign of Roundup, how will the K_I values change? Will they increase or decrease? Why? (4 pts)

The reason Roundup binds poorly to the mutant enzyme is the unfavorable interaction between the polar/charged carboxylic acid group and the Methionine side-chain. If this was changed to a non-polar group, this would result in favourable van der Waals interaction as well as a favorable hydrophobic effect. One possibility is shown to the right.

(Note: Methionine does NOT form disulfide bonds, only Cysteine can.)

B6 (10 pts): Do **one** of the following **three** choices:

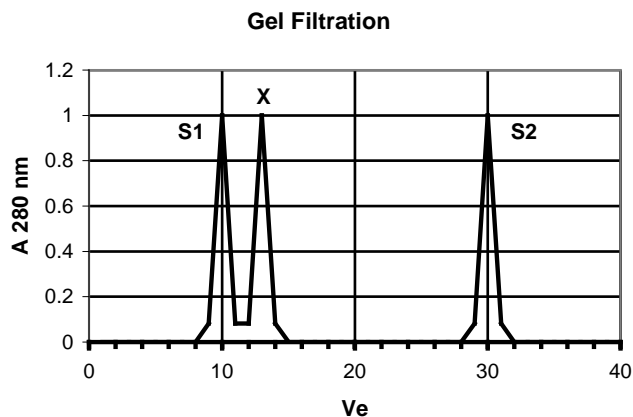


Choice A: Biochem Bob is trying to purify a single protein from a complex mixture of proteins. He knows the protein that he is trying to purify has a large number of Aspartic and Glutamic acid residues, and no Lysine, Arginine, or Histidine residues. He loads the mixture onto a anion exchange column at pH 7.0. Much to his dismay, his protein cannot be washed off (eluted) from the column. He asks you what to do, and you say add salt or acid and your protein will come off the column..

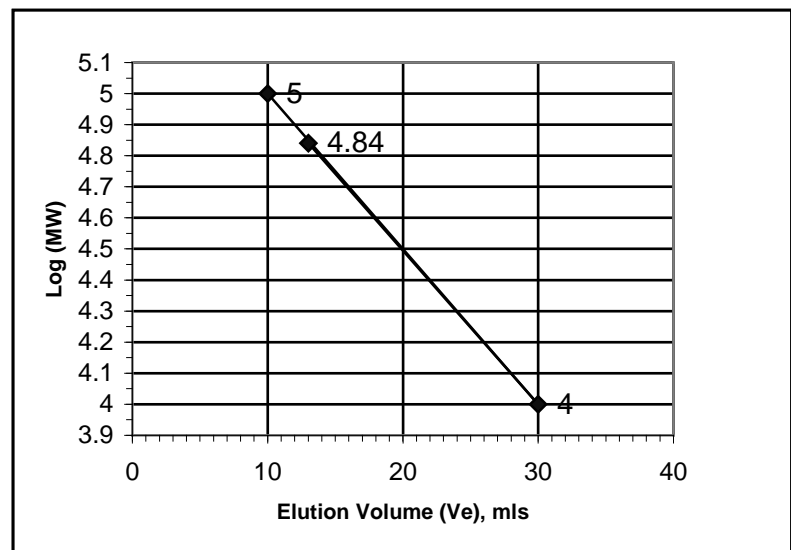
Fill in the three blanks (3 pts), explain why Bob's protein remains bound to the column at pH 7.0 (3 pts), and discuss how *each* of your suggestions will release his protein from the column (4 pts).

Bob's protein has a negative charge at pH=7.0, because this pH is much higher than the pKa of the Asp and Glu side chains. Therefore it sticks tightly to the positive charge on the anion exchange column. Adding salt will interfere with the electrostatic interaction by screening the charges on the protein. Lowering the pH to below the pKa of the groups will reduce the negative charge on the protein, reducing the strength of interaction with the resin.

Choice B: You are trying to determine the quaternary structure of protein X. You run the protein on a gel filtration column and SDS-page Gel electrophoresis. The SDS-page gel electrophoresis shows a *single* band from your protein, giving a molecular weight of 10 KDa (10,000 Da). The elution profile of the gel filtration column is shown below. The peaks labeled S1 and S2 correspond to 100 KDa and 10 KDa standards, respectively. A table of logs can be found on the face page. What is the quaternary structure of this protein? Briefly explain your approach (6 pts) and label the axis of any plots that you use (2 pts).



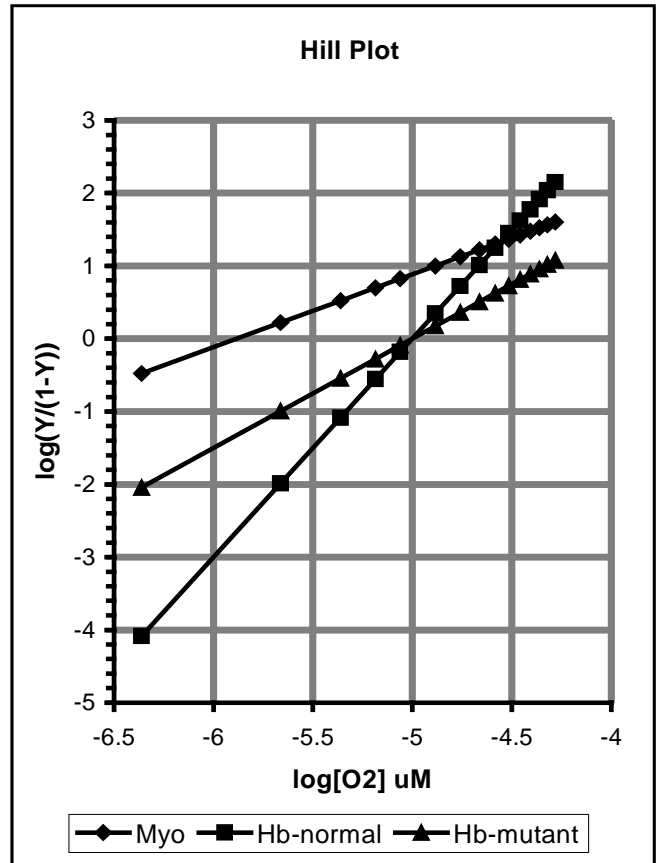
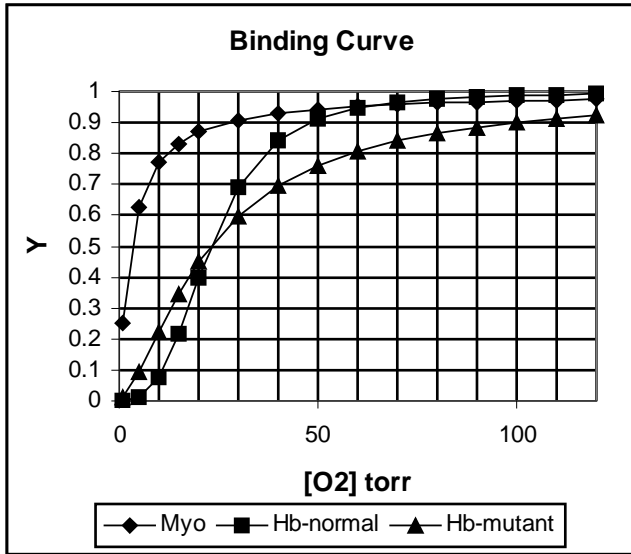
The standards, S1 and S2, are used to determine the calibration curve. Plotting logMW versus V_e . The 100,000 Da ($\log MW=5$) protein elutes first, at an elution volume of 10 ml while the 10,000 Da protein ($\log MW=4$) elutes at 30 ml. The unknown protein, X, elutes at 13 ml, corresponding to a $\log MW=4.84$, which corresponds to a molecular weight of 70,000Da. Since the SDS-PAGE gel indicated that each chain was 10,000 Da, this protein is likely a septamer (7) of identical chains (a homo-septamer). However, it may actually contain more chains, see below.



Can you say anything about the presence or absence of disulfide binds in protein X (2 pts)? Why?

There is no indication that beta-mercaptoethanol, which cleaves disulfide bonds, was present during electrophoresis. Therefore disulfide bonds could still exist and each 10,000 Da subunit could consist of two (or more) chains that are still held together by disulfide bonds, i.e. $(\alpha\beta)_7$.

Choice C: The binding curves and corresponding Hill plot shows data for the binding of oxygen to Myoglobin, normal Hemoglobin, and a mutant Hemoglobin.



i) Obtain the Hill coefficient, n_h , for the mutant hemoglobin. Please show your work (2 pts).

The Hill coefficient is the slope of the line when it crosses the x-axis (corresponding to a fractional saturation, $Y=0.5$). Taking the curve from $x = -5.5$ to $x = -4.5$:
 $n_h = \text{slope} = 0.7 - (-0.7) / 1 = 1.4$

ii) Is the mutant hemoglobin more or less cooperative than normal hemoglobin? (Normal hemoglobin has a Hill coefficient of 3.0.) Justify your answer, with reference to either the Hill coefficients or the binding curves.(3 pts)

The mutant hemoglobin is less cooperative than the wild-type. This is evident from the Hill coefficient (the closer n_h is to the number of binding sites, the more cooperative the system is. The closer n_h is to 1, the less cooperative the system is.

From the binding curve, the mutant shows a less sigmoidal shape, the curve rises more slowly and is almost hyperbolic in shape. The closer the shape is to hyperbolic, the less cooperative the system is.

iii) Discuss how this change in cooperativity would effect oxygen delivery to the tissues by the mutant enzyme. You may find it useful to refer to the binding curves. Oxygen levels in the lungs are 100 torr, while those in the tissue are about 20 torr. (5 pts)

The most straightforward way of answering this question is to simply calculate the amount of oxygen delivered by calculating the difference in fractional saturation at 100 torr and 20 torr:

Normal Hb: $Y_{100} - Y_{20} = 0.97 - 0.40 = 0.57$
 Mutant Hb: $Y_{100} - Y_{20} = 0.90 - 0.43 = 0.47$

The mutant Hb is less efficient at delivering oxygen to the tissues. It binds less in the lungs and releases less in the tissues.