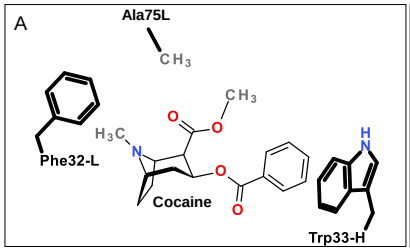
Instructions: this exam consists of 11 questions on 5 pages, for a total of 100 points. On questions with choices, all of your attempts will be graded and you will be awarded the highest grade. Please use the space provided or the back of the preceding page.

1. (10 pts) The binding of cocaine to two different Fab fragments (from antibodies) was measured using equilibrium dialysis and absorption spectroscopy. The data for one concentration of cocaine is shown for both of these Fabs in the table on the right. Binding curves are also shown for these two Fab fragments.



	$[L]_{Free}=[L]_{OUT}$	$[L]_{IN}$	A_{280}
A	10 uM	11.25	0.25
B	10 uM	12.27	0.37

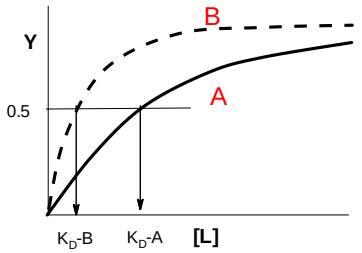
i) (5 pts) Determine the fractional saturation, Y for **both** antibodies for [L]=10 uM, using **either** method. *You **must** show your work for full credit*. The following information is required to solve this problem. [Fab]=2.5 uM, A_M=0.1, A_{ML}=0.4.

Equilbrium Dialysis: $Y=([L]_{IN}-[L]_{out})/[Mtot]$

A: Y = 1.25/2.5 = 0.5 B: Y = 2.27/2.5 = 0.91

Absorbance: $Y = (A-A_M)/(A_{ML} - A_M)$ = change/total change

A: Y = .15/0.3 = 0.5 B: Y = .27/0.3 = 0.91



ii) (2 pt) Indicate which binding curve corresponds to which antibody, based on your answer to part i and with reference to the structures above.

FAB B has the higher affinity because it has a higher fractional saturation than FAB A at the same ligand concentration.

There are more favorable interactions with the bound cocaine in FAB B (H-bond, hydrophobic effect)

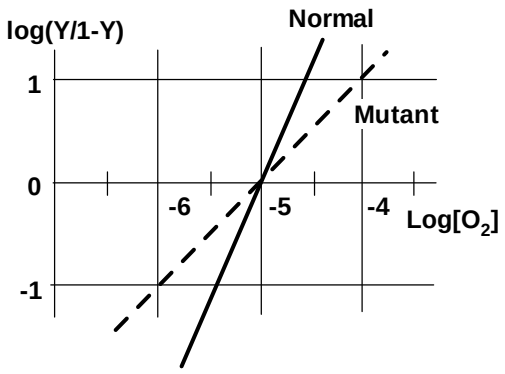
Dotted curve = B

Solid curve = A

iii) (3 pts) Indicate how you would obtain the K_D for both antibodies from the binding curves. It is not necessary to give numerical values.

Simply the ligand concentration to give Y=0.5

2. (16 pts) The binding of oxygen to two different hemoglobins is being studied. The Hill plots for these two proteins are shown on the right. One hemoglobin is normal hemoglobin (solid line) while the second is a mutation (dashed line), where the distal histidine has been replaced by glycine (glycine lacks a sidechain). (*Part of this question is on pg 2*).



i) What are the K_D values for each of these proteins (2 pts)?

Log(K_D) is the intercept with the x-axis, which is -5.

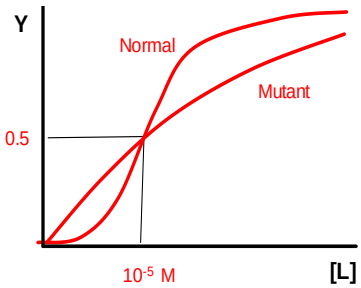
Therefore $K_D=10^{-5}$ M

ii) Assess the cooperativity of each of these proteins, using the Hill plot, i.e. is the binding cooperative, and if cooperative, weakly or strongly (4 pts).

The slope of the normal hemoglobin is ~3, so moderately strong positive cooperativity since the Hill coefficient is fairly close to 4, the number of binding sites.

The slope of the mutant hemoglobin is 1, so non-cooperative.

iii) Sketch the binding curve for these two proteins in the graph on the right. Take care to indicate accurate K_D values (3 pts).



The curve for normal Hb will be S-shaped, with Y=0.5 at $[O_2] = 10^{-5}$ M

The curve for the mutant will be hyperbolic, and will have Y=0.5 at the same point.

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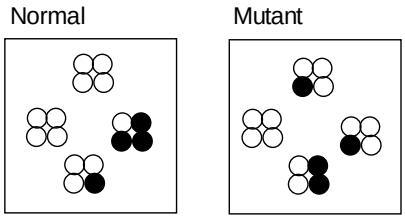
Question 2, continued

iv) Individuals that have the **mutant** hemoglobin suffer from poor oxygen delivery. Explain why this is the case (5 pts).

The binding of oxygen is non-cooperative, so it is difficult to saturate in the lungs, and only small amounts will be released by the time the mutant Hb gets to the tissues.

v) Indicate the distribution of bound oxygen on the normal and mutant hemoglobins using the diagrams by shading a circle if oxygen is bound. Assume Y=0.25, i.e. ¼ of the binding sites are occupied. Briefly justify your answer (2 pts).



There are 16 possible binding sites, if Y=0.25, then four will have O_2 bound.

Normal – since the binding is pos coop, once one binds, the empty sites will have higher affinity, it is not infinitely positive cooperative, so the will be some intermediates (-1/2 if all were shaded on one tetramer).

Mutant – the distribution would be random, one possibility is shown (one ligand bound to all tetramers would be possible as well).

3. (10 pts) Please do **one** of the following choices:

Choice A: Discuss how BPG (bisphosphoglycerate) is used to increase oxygen delivery at high altitudes.

Choice B: Briefly discuss the general framework of allosteric effects, your answer should compare and contrast tense and relaxed states, homotropic and heterotropic compounds.

Choice A:

BPG changes the affinity and cooperativity of the oxygen binding curve, even though the binding is weaker, the cooperativity is larger, so Hb releases more oxygen in the tissues; the fractional saturation drops faster at higher BPG levels.

Choice B:

The tense (T, inactive) form is in equilibrium with the relaxed (R, active) form.

Activators and inhibitors change the equilibrium position, activators stabilize R and inhibitors stabilize T.

Homotropic compounds affect the binding of the same ligand (e.g. oxygen)

Heterotropic compounds affect the binding of other ligands

4. (12 pts) Briefly describe why enzymes increase the rate of reactions. Feel free to illustrate your answer with a diagram. Your answer should indicate factor(s) that enhance the rate of all enzymes and factor(s) that are only found with some enzymes.

- The rate of the reaction depends on the transition state concentration.
- The transition state concentration depends on the height of the activation energy barrier
- Enzymes lower the barrier, making more [EX], therefore the rate is faster.
- The barrier is lowered in all enzymes by pre-ordering of the active site, so there is no unfavorable decrease of entropy.
- Some enzymes form favorable enthalpic interactions with just the transition state, for example the oxyanion hole in serine proteases.

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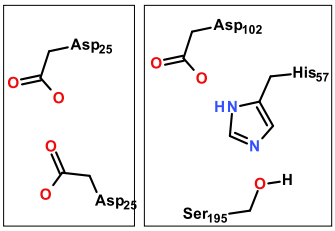
5. (12 pts) Amino acid residues in the active site of enzymes are involved in substrate binding or catalysis. Pick any **one** of the enzymes that we have discussed in the class thus far (trypsin, chymotrypsin, elastase, HIV protease) and answer all of the following questions. You need not use the same enzyme for parts i and ii. The chemical structures on the right may be helpful.



THIS WAS CHANGED TO DO 2 of 3 – 6 points for each.

i) (5 pts) Discuss how the amino acid sidechains are responsible for peptide bond hydrolysis. You need not give the complete mechanism for serine proteases.

Serine proteases : Serine is the nucleophile that is activated by the His (deprotonation). The positive charge on the His is stabilized by the Asp. The nucleophile attacks the C=O, ultimately causing the bond to break.

HIV Protease: One Asp is protonated, the other is deprotonated. The deprotonated Asp activates a water molecule by deprotonating it, the OH- attacks the C=O, breaking the peptide bond. The protonated Asp is required to provide a hydrogen for the new amino terminus.

ii) (5 pts) Discuss how residues in the active site define substrate specificity. Your answer should discuss **molecular complementarity** (e.g. molecular details of the interaction between S and E) and how this affects K_M.

Trypsin: Lys and arg substrates have low Km values because of a favorable electrostatic interaction with the Asp(-) in the specificity pocket.

Chymotrypsin & HIV protease: Large non-polar substrates will have low Km values because the specificity pocket is large and non-polar, the bound substrate is stabilized by the hydrophobic effect and vdw.

Elastase: Smaller non-polar substrates (e.g. Leu) have low Km, the non-polar specificity pocket is smaller and the bound substrate is stabilized by the hydrophobic effect and van der waals.

iii) (2 pts) For **either** part i **or** part ii, briefly discuss how pH would affect **either** k_{CAT} (part i) **or** K_M (part ii).

Kcat:

Serine proteases: Low pH will inhibit the enzyme because the His needs to be deprotonated to function.

HIV protease: The enzyme is only active at pH values where one Asp is protonated, the second is deprotonated, i.e. a bell shaped curve.

KM:

Trypsin: The Asp in the specificity pocket needs to be deprotonated, so pH values >> pKa will give low Km.

Chymotrypsin, Elastase, HIV protease: no pH effect since there are no ionizable groups involved in binding the substrate.

6. (5 pts) Please do **one** of the following choices:

Choice A: What is the "steady-state assumption" and what is its importance in the measurement of enzyme kinetic parameters, such as K_M and V_{MAX}?

Choice B: Explain why it is preferable to experimentally obtain the velocity of enzyme catalyzed reactions as soon as possible after substrate is added.

Choice A:

Steady-state means [ES] is constant during the measurement (4 pts).

It allows a very simple solution for the velocity as a function of [S] (and [I]). (1 pt)

Choice B:

The [S] while the measurement is being made is close to the known starting amount (little has been converted to product) (3 pts)

[P]~0, so there is little product inhibition (2 pts)

7. (4 pts) Compare and contrast competitive and suicide inhibitors. You answer should discuss the following: i) the location of the binding site on the enzyme, ii) whether the binding is reversible.

- Both bind at the active site, the suicide inhibitor forms an irreversible covalent bond with the enzyme (typically after activation to a reactive compound by the enzyme).
- A competitive inhibitor binds reversibly.

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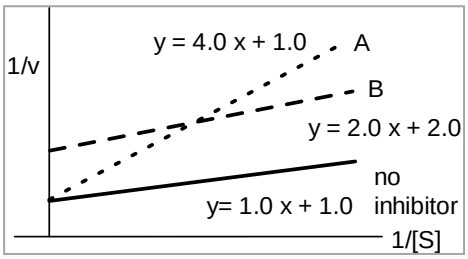
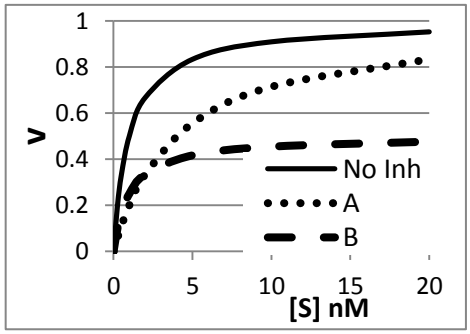
8. (16 pts) A new class of HIV protease inhibitors has been invented. You perform enzyme kinetic measurement using 1 nM of the new inhibitor to evaluate its effectiveness. For comparison purposes you also collect data using the existing drug (also at 1nM). These data are plotted on a double reciprocal plot, (A=existing drug, B=new drug). The equations of the lines are also given. The data are also shown in a plot of v versus [S]. Please answer all of the following questions.





i) Which drug is a competitive inhibitor and which is a mixed inhibitor? *Justify your answer* (4 pts).

A is a competitive inhibitor because it has the same Vmax (same Y-intercept), competitive inhibitors are displaced from the active site at high [S]

B is a mixed type, because V max changes. Mixed type inhibitors cause allosteric changes that decrease Vmax.

ii) Which drug binds more tightly to the **free enzyme**? You answer should be based on a calculation of K_I or K_I', whichever is appropriate (5 pts).

You need to calculate K_I, since that is the dissociation constant for binding to the free enzyme, for both types of inhibitor: (EI) ↔ (E) + (I).

$K_I=[I]/(\alpha-1)$, and α is the ratio of the slopes:

Inhibitor A: $\alpha = 4/1 = 4$, $K_I = 1\ nM / 3 = 0.333\ nM$

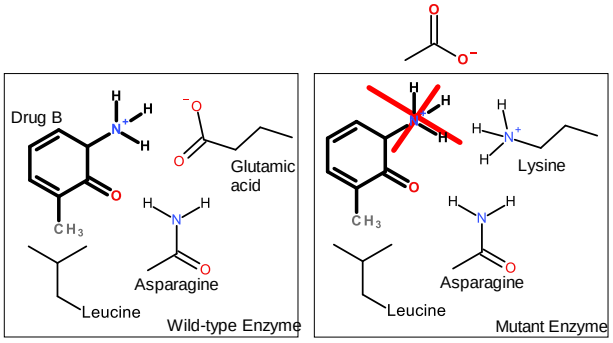
Inhibitor B: $\alpha = 2/1 = 2$, $K_I = 1\ nM / 1 = 1\ nM$

Therefore inhibitor A binds better since it has the lower K_I.

iii) Which is the better drug when the substrate concentration is 10 nM, A or B? Why? Your answer should consider the type of inhibitor (comp/mixed) and how the type of inhibitor affects its sensitivity to the substrate concentration (3 pts).

Drug B shows a lower velocity when [S]=10 nM. The velocity is less than 0.5, while drug A the velocity is higher (~0.7).
Drug B is better because it is a mixed type inhibitor that cannot be displaced by the substrate.

iv) The new drug is initially quite successful, but then the drug becomes ineffective due to mutations in the HIV protease. The structures of the enzyme-drug complex for the wild-type (left) and mutant enzyme (right) are shown. The binding pocket for drug consists of a Leu, Asp, and Glu residue. The mutation is Glu to Lys. Indicate how you would modify the inhibitor to increase its affinity for the mutant protease. Cross out the group(s) on the drug on the right panel and indicate their replacement. *Briefly explain your approach (use the back of the prev. page if necessary)* (4 pts).



The drug binds poorly to the mutant enzyme because there is an unfavorable electrostatic interaction between the (+) amino group on the drug and the (+) charge on the new lysine residue. Replacing the amino group on the drug with a (-) carboxylate will restore binding.

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Question 8 continues:

v) (Bonus 2 pts) What aspect of the HIV life-cycle is responsible for the generation of mutations in the protease?
Use the back of the preceding page for your answer.

HIV Reverse transcriptase, which copies the genetic information from the viral RNA to DNA is error prone, the errors result in mutation in the HIV protease.

9. (6 pts) Pick any **one** of the following purification techniques and describe how it works to separate proteins. Your answer should include a description of the resin (beads), how proteins interact with the resin, and how the proteins are removed (eluted) from the resin:

Choice A: Size exclusion (gel filtration): The beads have small pores in them, small proteins enter the beads and spend time in the column, so they will elute according to their molecular weight – smaller proteins later. Elution is just by washing the resin, since the proteins don't stick to the beads.

Choice B: Anion exchange: The beads have (+) charges, so negatively charged proteins stick to the beads. They will elute in the order of charge, the more negatively charged ones will elute later. It may be necessary to add NaCl to disrupt the electrostatic interaction between the protein and the beads, or to change the pH to change the charge on the protein.

Choice C: Cation exchange: The beads have (-) charges, so positively charged proteins stick to the beads. They will elute in the order of charge, the more positively charged ones will elute later. It may be necessary to add NaCl to disrupt the electrostatic interaction between the protein and the beads, or to change the pH to change the charge on the protein.

Choice D: Affinity chromatography: There is something attached to the bead (ligand, antibody, Ni) that has a specific interaction with the target protein, so only it binds to the resin and all of the other impurities can be washed out. The protein is eluted by disrupting the interaction (free ligand, change in pH to affect antibody binding, free Histiine).

10. (4 pts) You are performing a purification scheme and measure the specific activity after each step. How should you modify your purification scheme? Suggest **one** step that you should change? *Justify your answer*.

Sample	Specific Activity
Lysate	13.5 units/mg
After step 1	12.0 units/mg
After step 2	50.0 units/mg
After step 3	50.0 units/mg

The specific activity should increase after each step.
It does not after step 3, so step 3 should be altered in some way.

11. (5 pts) You are purifying a glucose binding protein from an E. coli lysate. The target protein has six histidine residues at its amino-terminus. Describe a purification scheme that will separate the target protein from the other four proteins. The properties of all of the proteins are shown in the table. You can assume pKa values of 4.0 and 9.0. Values of ammonium sulfate 1M below the listed solubility will not precipitate any protein, values 1 M above will precipitate all of the protein). Points: One step=6 pts (1 point bonus), Two steps=5 pts, Three steps=3 pts

Protein	Sol. Amm. Sulfate	Molecular Wt	# Asp Residues	# Lys Residues
Target	**2.0**	**15,000**	**5**	**5**
A	2.0	25,000	10	15
B	2.5	15,000	5	10
C	6.0	15,000	15	5
D	6.0	30,000	20	10

You should ignore any charges from the Histidine residues, to keep charge calculations simple.

One-step:

- The easiest one step scheme is to use affinity chromatography – with either glucose or Ni ions on the resin. The protein will bind to the resin because of its affinity for glucose or the his-tag on the amino-terminus. The his-tag will bind to the Ni ions.
- you would then elute the protein with free glucose or free histidine.

Points on Page:_____

Two Step:
You were asked to ignore the His residues in your charge calculations:
At pH = 6, the Asp residues have a charge of -1, the Lys +1, so the charges are:

Target:	0
A	+5
B	+5
C	-10
D	-10

The target protein will not bind to either an anion or cation exchange column, so the mixture can be passed over a cation column, removing A and B, and then an anion, removing C and D.

Other possible two steps:

3 M ammonium sulfate gives Target + A + B
Cation exchange at pH 7, A and B bind, target does not and will flow through

Size exclusion: -> target + B + C
Do cation exchange at pH=4 (pKa of Asp)
Target = +2.5
B = +7.5
C = -2.5
C flows through, target elutes first, B next.

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