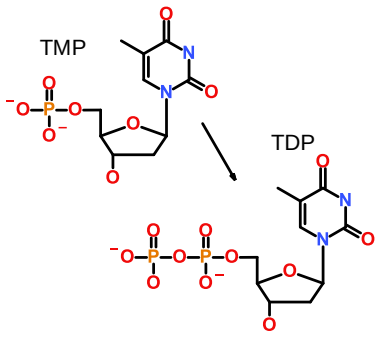
Instructions: this exam consists of 11 questions on 6 pages, for a total of 100 points. On questions with choices, all of your attempts will be graded and you will be awarded the highest grade. Please use the space provided or the back of the preceding page. **Note Q10 on page 5 is worth 18 points.**

1. (10 pts) Thymidylate kinases are enzymes that add a phosphate group to the nucleobase TMP (thymidine monophosphate) to generate TDP (thymine diphosphate). You want to measure the binding of TMP to a thymidylate kinase. Please answer the following questions for **one** of the following choices:



- **i)** What is the fractional saturation (5 pts)?
- **ii)** Briefly discuss how you would obtain K_D from a series of measurements at different TMP concentrations (4 pts)
- **iii)** Although you have measured K_D, what other parameter is your measurement similar to? K_M, K_I, or K_I'? (1 pt)

Choice A: You place 10 uM of the enzyme inside a dialysis bag and add TMP on the outside. After equilibrium is reached you find the concentration of TMP outside the bag is 100 uM and the concentration inside the bag is 109 uM.

Choice B: The UV absorption of a 10uM solution of enzyme is 0.1 in the absence of TMP and 0.4 when saturated with TMP. You measure the absorbance when the TMP concentration is 100 uM and the measured absorbance is 0.37.

i)

Choice A: $[M_{TOT}] = 10$ uM. $[ML] = [L]_{IN}-[L]_{OUT} = 109 - 100 = 9$ uM. $Y = 9/10 = 0.9$

Choice B: $Y = (A - A_M)/(A_{ML} - A_M) = (0.37 - 0.1)/(0.4-0.1) = (0.27/0.3) = 0.9$

ii)

- You could plot Y versus [L] to get a binding curve, K_D is the TMP concentration to give Y=0.5.
- You could also plot log (Y/(1-Y)) versus logTMP (Hill plot), the intersection on the x-axis is $\log K_D$.

iii) The K_M, since this is related to substrate binding, and TMP is a substrate for the enzyme.

2. (10 pts) Discuss the general framework for allosteric effects on ligand binding (e.g. T and R states). Use any **one** feature of oxygen transport (by hemoglobin) to illustrate your answer (e.g. oxygen binding, altitude adaptation).

A protein exists in two states:

T (tense) inactive form

R (relaxed) active form

The two states are in equilibrium and the equilibrium can be shifted by inhibitors (more T) and activators (more R).

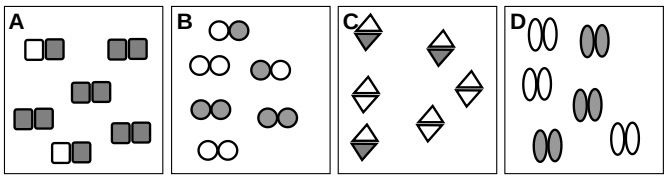
Hemoglobin:

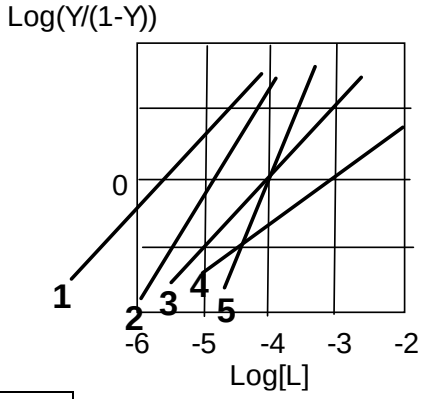
Oxygen binding to one subunit is propagated to others by changes in the shape of the heme when oxygen binds. This causes the other subunits to preferentially take the R form, increasing affinity as more oxygen binds.

BPG. This only binds to the T-form in a positively charged pocket between two beta chains, so it increases the concentration of the T-form. This reduces oxygen affinity – more oxygen is required to reach the same amount of binding. The increase in cooperativity of oxygen binding ultimately leads to higher delivery.

Points on Page:_____

3. (14 pts) You are measuring the binding of a ligand to four different dimeric proteins. You have a magic camera that allows you to take a snapshot of the distribution of bound ligands at equilibrium. In all cases the ligand concentration is **10^{-4} Molar**. Free ligand is not shown and subunits with ligand bound are shaded. You will find it useful to determine Y for these four cases, a column has been provided on the table to enter these values if you like.

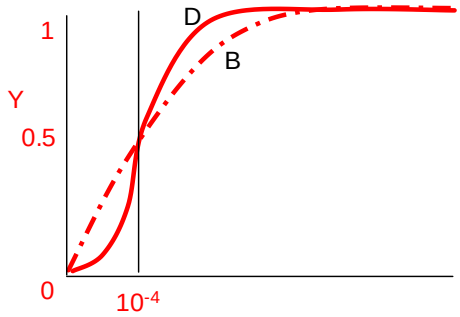




i) Match the distribution of bound ligands to the curve on the Hill plot and indicate the correct match in the table below. Note that there are five curves on the Hill plot. *You must justify your answer with a discussion of K_D and cooperativity for each protein (10 pts).*

Protein	Hill Curve (1-5)	Y	
A	1 or 2	0.83	Higher affinity than others (Y is higher). Difficult to determine cooperativity at high ligand.
B	3	0.50	Same affinity as D, but non-cooperative.
C	4	0.25	Lowest affinity, neg cooperative
D	5	0.50	Same affinity as B, but highly cooperative (inf pos.)

ii) Sketch the binding curve (Y versus [L]) that you would expect to see for proteins B and D on the graph on the right. Be sure to label the axis and provide a scale (4 pts). *Briefly justify your answer.*



B is non-cooperative – hyperbolic curve with $K_D = 10^{-4}$ M
D is positive cooperativity – S-shaped curve, but with the same K_D

4. (8 pts) Why do enzymes increase the rates of reaction? Discuss the key feature that is applicable to <u>all</u> enzymes.

They increase the concentration of the high-energy transition state by lowering its energy. This is accomplished by pre-ordering of functional groups prior to forming the transition state (all enzymes) (6 pts).

The rate of reaction is proportional to the amount of transition state, if the energy of the transition state is lowered, there is more of it, so the rate is faster. (2 pts).

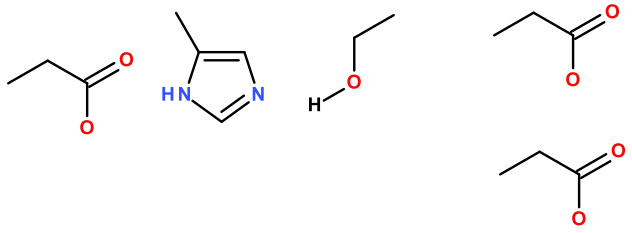
Partial Credit: Direct enthalpic interactions with only the transition state (some enzymes, e.g. serine proteases) (4 pts)

Points on Page:_____

5. (8 pts) Please do **one** of the following choices:

Choice A: Select either serine proteases or HIV protease and briefly discuss the role of active site residues in the mechanism. The structures on the right may be helpful.

Choice B: Why is trypsin specific for Lys and Arg substrates while Chymotrypsin is not?



Choice A:

Serine proteases: His activates Ser, Ser becomes the nucleophile and attacks C=O, breaking the peptide bond. Asp stabilizes the pos charge on His. His donates proton to new amino terminus.

HIV protease: One asp (more acidic) is deprotonated. It activates a water molecule that acts as a nucleophile. The OH- attacks the C=O carbon, and breaks the peptide bond. The other Asp (protonated) provides a proton to the new amino group.

Choice B:

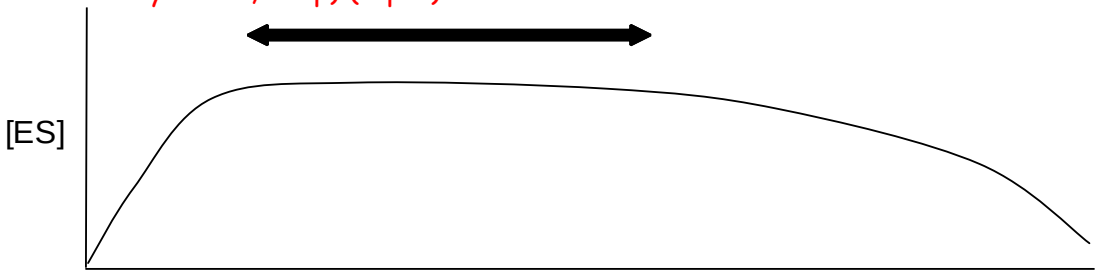
Trypsin has a negatively charged Asp in its specificity pocket. Lys/Arg containing substrates will have low off-rates because of the favorable electrostatic interactions. This allows time for cleavage of the peptide bond to occur.

6. (6 pts)

i) Sketch a curve of [ES] versus time, beginning at t=0 when enzyme is mixed with substrate.

ii) What time period on your plot would be suitable for determining the velocity of the reaction? Why?

i) The concentration of [ES] starts at zero, rises to a constant value (steady state), and then slowly drops as substrate is consumed, it was necessary to show all three phases (rise, steady-state, drop) (3 pts)



ii) The time period where d[ES]/dt = 0, where there is no change in [ES]. At steady state the analysis of the velocity is simpler: $v = V_{max}[S]/(K_M + [S])$

Points on Page:_____

7. (10 pts) Compare and contrast competitive and mixed-type inhibitors. How are they similar and how do they differ. Your answer should explain their effect on kinetic parameters k_{CAT} (V_{MAX}) and K_M.

Competitive inhibitors bind in the active site and therefore look like substrate, however they cannot undergo a reaction.

They will increase the observed K_M because more substrate will be required to ½ saturate the enzyme.

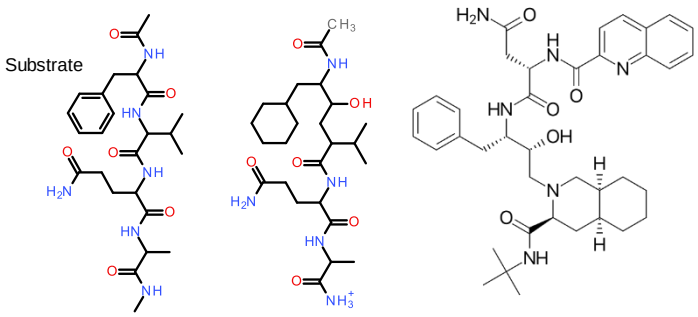
At high substrate the inhibitor will be displaced, so there is no effect on V_{MAX}.

Mixed-type inhibitors do not bind in the active site. Therefore they are unlikely to be similar to the substrate. They change the structure of the active site.

The structural change will reduce V_{MAX}.

The observed substrate binding can stay the same, get better (lower K_M) or worse (higher K_M).

8. (2 pts) The structure of the normal substrate for HIV protease, and the structure of two HIV protease inhibitors, are shown to the right. Why are both of these compounds inhibitors, what do they have in common?

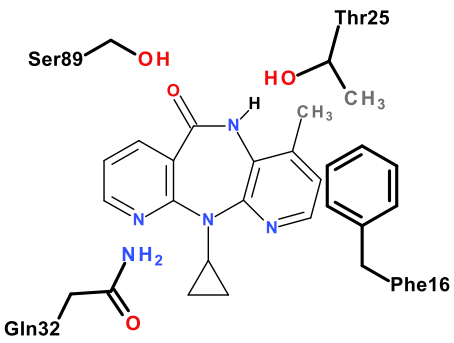


- Both are similar to the substrate (and therefore competitive inhibitors),
- both have a modification of the peptide bond that is cleaved in the substrate, so they cannot be converted to product.

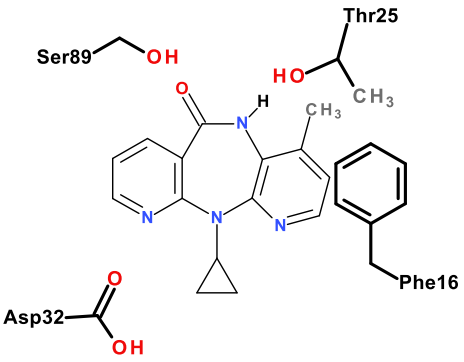
Points on Page:_____

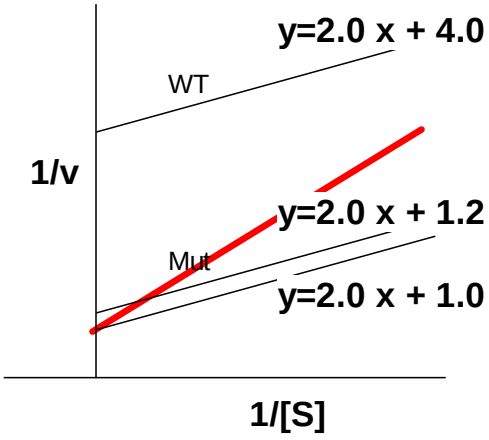
9. (18 pts) You have determined the structure of nevirapine bound to wild-type and a mutant HIV reverse transcriptase. The structure of the two complexes is shown on the right. The drug (thin lines) interacts with Gln32, Ser89, Thr25, and Phe16 in the wild-type enzyme. You also measure enzyme kinetic data using a concentration of **1 nM of nevirapine** with both enzyme and the resultant double reciprocal plots are shown on the right. The activity of the mutant and wild-type in the absence of the inhibitor was the same.

Wild-type Enzyme-Drug Complex



Mutant Enzyme-Drug Complex





i) Nevirapin is a mixed-type inhibitor. How would the double reciprocal plots change if it was a competitive inhibitor (you can use the plot on the right to illustrate your answer) (2 pts)?

The y-intercept would be the same, since Vmax is not affected by a competitive inhibitor.

ii) Determine the affinity of nevirapine to the wild-type and mutant enzymes from the double reciprocal plots, i.e. obtain K_I and K_I' (6 pts).

Dissociation from the EI complex (EI ↔ E + I): $K_I = [I]/(\alpha-1)$

Alpha is obtained from the ratio of the slopes, but the slopes are all the same so K_I is infinite, the inhibitor does not bind to the free enzyme.

Dissociation from the ESI complex (ESI ↔ ES + I): $K_I' = [I]/(\alpha'-1)$

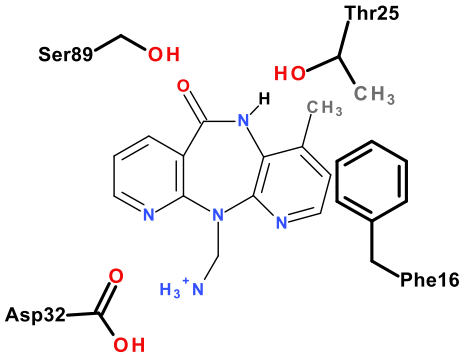
Alpha' is obtained from the ratio of the y-intercepts:

WT: $\alpha' = 4/1 = 4$. $K_I' = [I]/(\alpha'-1) = 1\ nM/(4-1) = 0.33\ nM$

Mut: $\alpha' = 1.2/1 = 1.2$. $K_I' = [I]/(\alpha'-1) = 1\ nM/(0.2) = 5\ nM$

iii) Explain the change in affinity with reference to the interactions between the drug and the enzymes and the values you obtained in *part iii.* (4 pts).

Mutant Enzyme-**MODIFIED** Drug Complex



The binding to the mutant enzyme has decreased because of loss of a hydrogen bond when Gln32 is changed to Asp.

iv) Suggest a modification to the drug that might restore affinity to the mutant enzyme (4 pts).

Change the cyclopropane ring to an amide with a positive charge, the Asp will have a negative charge.

v) Based on your K_I and K_I' values that you obtained in part ii, is Nevirapin able to bind to the free reverse transcriptase (2 pts)? Why or why not?

No, because K_I is infinite. K_I is the dissociation constant for the EI complex: EI ↔ E + I

Points on Page:_____

10. (4 pts) Typically, patients infected with HIV are treated with both nevirapin and a HIV protease inhibitor.

i) Why is this approach more effective at reducing replication of the virus than treating the patient with just one drug?

ii) Which drug would be more effective at higher substrate concentrations, nevirapine, or an HIV protease inhibitor, why?

i) There is a high mutation rate of the virus, due to the error prone reverse transcriptase, leading to drug resistance. If the patient is treated with two different drugs, it is less likely for mutations to arise that would confer resistance to both drugs at the same time. (1 pt)

ii) Nevirapine, because it is a mixed type inhibitor and not displaced by high substrate levels. HIV proteases inhibitors are competitive and would be displaced by high levels of substrate. (3 pts)

Bonus (2 pt): Nevirapine is rapidly removed from the patient by the kidneys. In what general way could you modify nevirapine to increase its lifetime in the patient?

Convert it into a prodrug whose properties prevent it from being removed by the kidneys, the prodrug will circulate longer and will be converted to the active drug over a longer time period.

11. (10 pts) You are trying to purify thymidine kinase from a mixture of other kinases.

i) Suggest purification step(s) that will purify thymidylate kinase. *Briefly describe how the separation method works for any chromatography method that you use (8 pts).*

ii) What will happen to your specific activity after performing these steps (2 pts).

	[Ammonium Sulfate] that precipitates 50% of protein*	# Residues (Mol Wt)	#Asp (pKa=4)	#Lys (pKa=9)
Thymidylate kinase(TK)	1.0 M	120 (13,200 Da)	6	4
Adenosine kinase(AK)	1.5 M	120 (13,200 Da)	6	6
Pyruvate kinase(PK)	4.0 M	120 (13,200 Da)	8	5
Hexose kinase(HK)	6.0 M	240 (26,400 Da)	10	12
*Concentration 1M above will precipitate 100% of the protein, 1 M below will precipitate none of the protein.				

i)

Step one: add ammonium sulfate to 2 M, the Thymidylate and adenosine kinase will precipitate, separating them from the other two kinases.

Step two: At pH=7, the charge on thymidylate kinase is -6 + 4 = -2, so it would bind to the positively charged beads in an anion exchange column. The charge on adenosine kinase is 0, so it would not bind and would be washed off of the column. The thymidylate kinase can be released by changing the pH or adding salt.

Single Step! Use an affinity column that is specific for thymidylate.

ii) The specific activity (=target/total protein) should increase because the amount of target protein stays almost constant (hopefully!), but the total amount of protein decreases as impurities are removed.

Points on Page:_____