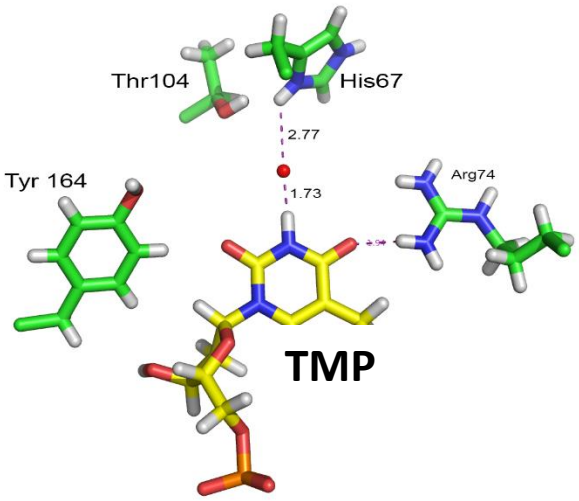
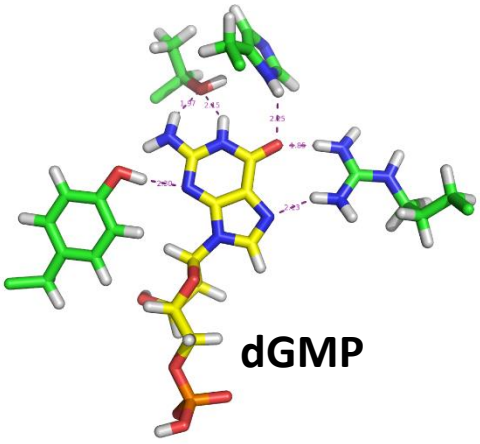
Instructions: this exam consists of 12 questions on 6 pages, for a total of 100 points. On questions with choices, all of your attempts will be graded and you will be awarded the highest grade. Please use the space provided or the back of the preceding page.

1. (4 pts) What do all of the following parameters all have in common? K_D, K_M, K_I, K_I'

They are all measures of ligand binding and will have smaller values if the affinity is high.
K_M = substrate binding
K_I = binding of inhibitors (both competitive and mixe-type) to free enzyme
K_I' = binding of mixed-type inhibitors to the ES complex.

2. (10 pts) Some thymidylate kinases can add a phosphate to either TMP or dGMP, using ATP as the phosphate donor. The interaction with TMP or dGMP is shown in the diagram, TMP is on the top, dGMP is on the middle. TMP and dGMP are colored yellow. Based on this diagram, please answer the following questions:
   i) What energetic factors are important for the binding of these substrates (2 pts)?
   ii) Which substrate would have the higher affinity, *and why*. (4 pts)
   iii) Mutation of a phenylalanine residue (Phe64) to Ala lowers the affinity by 10-fold. This Phe is stacked above the aromatic ring on the nucleobase, as shown in the lowest diagram. How is this phenylalanine stabilizing the bound TMP? What energetic interaction is involved? (4 pts)

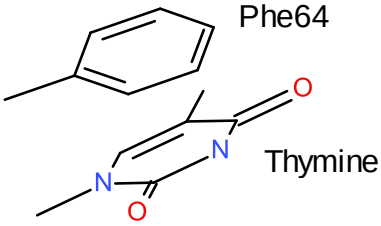




i) H-bonding

ii) dGMP has more H-bonds and would have higher affinity.

iii) van der Walls.



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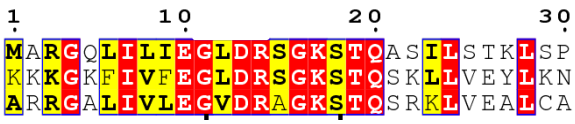
3. (6 pts) A portion of an amino acid alignment for thymidylate kinases from several species is shown on the right.



a) Why are Asp(D) at 13 and Glutamine (Q) at 20 good candidates to be part of the active site (2 pts)?

b) Briefly describe ***how*** you would identify if either Asp13 or Gln20 were important for catalysis. What parameter would you measure (k_{CAT} or K_M) (4 pts)?

a) They are conserved in all three protein sequences.

b) Asp 13 – the effect of pH on kCAT would show 50% activity at the pKa of Asp, around pH=4. The second residue, Gln, is not ionizable, so you could not use pH, but you could mutate it to Ala and see if the enzyme is active.

4. (8 pts) You want to measure the binding of TMP to the enzyme. Please do **one** of the following choices:

Choice A: You place 10 uM of 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. What is the fractional saturation?

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. What is the fractional saturation?

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

5. (12 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, pH effects). You should also discuss any molecular events that are important for the feature you have chosen to discuss.

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.

pH: At low pH the affinity for oxygen is decreased, enhancing the release of oxygen from Hb in active muscle tissue, which is more acidic due to the production of lactic acid.

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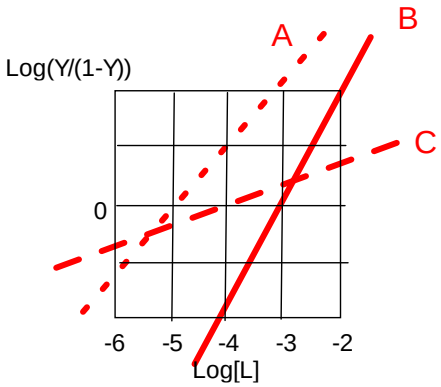
6. (8 pts) You have measured the K_D and Hill coefficient for three proteins and obtained the following values. All three proteins are dimers with two binding sites.

Protein	K_D	n_h
A	10^{-5} M	1.0
B	10^{-3} M	1.9
C	10^{-4} M	0.4



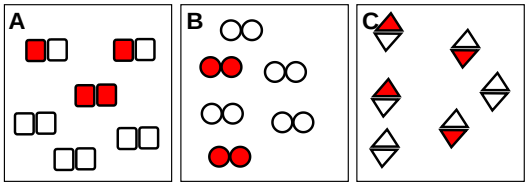
i) Describe, or sketch, the binding curves for these proteins (3 pts).

A – hyperbolic because it is non-cooperative.
B- S-shaped because it is positively cooperative (nh>1)
C- "flattened hyperbolic" because it is negatively cooperative, will rise fast, then more slowly.

ii) Use the graph on the right to draw the central portion of the Hill plot for each of the proteins. *Briefly justify your drawing using the space below (3 pts).*

The intercept on the x-axis is logKD,(A= -5, B = -3, C = -4). The slope of the line when it crosses the axis is equal to nh.

iii) Use the diagrams on the right to indicate the distribution of bound ligands for Y=0.33 for each of the proteins. *Briefly justify your answer (2 pts).*



Since Y = 1/3, four of the 12 sites will be occupied or shaped.
Protein A – random distribution.
Protein B – should be either fully bound or unbound since nh is close to 2, number of sites.
Protein C - should be mostly single ligand bound,

7. (10 pts) Why do enzymes increase the rates of reaction? Discuss features that are applicable to all enzymes and features that are found on only some enzymes, such as serine proteases.

They increase the concentration of the high-energy transition state by lowering its energy (+2 pts)

This is accomplished by either: (2 x 3 pts)
a) pre-ordering of functional groups prior to forming the transition state (all enzymes)
b) direct enthalpic interactions with only the transition state (some enzymes, e.g. serine proteases)

The rate of reaction is proportional to the amount of transition state (+2 pts)

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8. (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.

Choice B: Compare and contrast trypsin and elastase.

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.

Elastase has a small non-polar pocket which binds peptides with smaller non-polar sidechains (e.g. Leu).

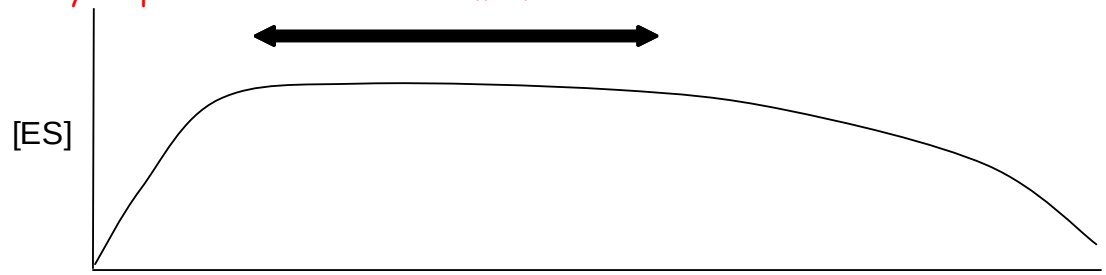
Both are serine proteases so both would have the same catalytic triad (+2 pts)

9. (6 pts) Please do **one** of the following choices:

Choice A:

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

The concentration of [ES] starts at zero, rises to a constant value (steady state), and then slowly drops as substrate is consumed.



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

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: $v = Vmax[S]/(K_M + [S])$

Choice B: Briefly describe how you would obtain V_{MAX} and K_M from experimental measurements of v versus [S] using SOLVER.

Use the formula that relates [S] to the observed velocity (v) to predict the velocities. Sum the differences between the observed and calculated and have SOLVER vary the two parameters in the equation (KM and VMAX) to find values that minimize the sum of the differences.

10. (6 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} (or 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).

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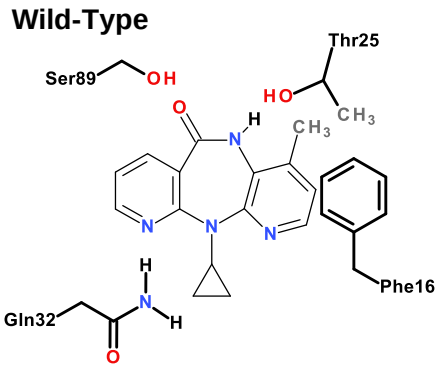
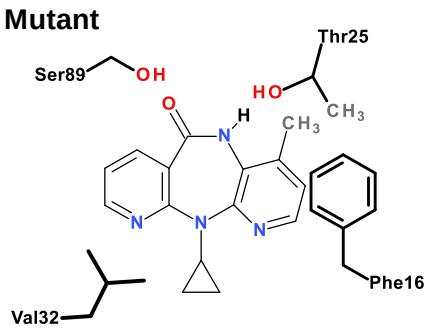
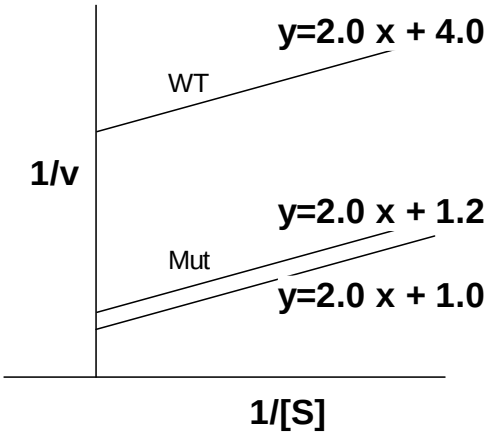
11. (14 pts) You have determined the structure of nevirapine bound to wild-type and a mutant (Gln32 to Val) HIV reverse transcriptase. The structure of the two complexes is shown on the right. The drug (thin lines) interacts with Gln32, Ser89, Thr25, and Phe16 in the wild-type enzyme. You also measure enzyme kinetic data using a concentration of **1 nM of nevirapine** with both enzymes 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 (lower line).







i) Determine the affinity of nevirapine to the wild-type and mutant **enzyme-substrate complex** from the double reciprocal plots (6 pts).

ii) 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).

iii) Suggest a modification to the drug that might restore affinity to the mutant enzyme (3 pts).

iv) Can Nevirapin bind to substrate-free reverse transcriptase (1 pt)?

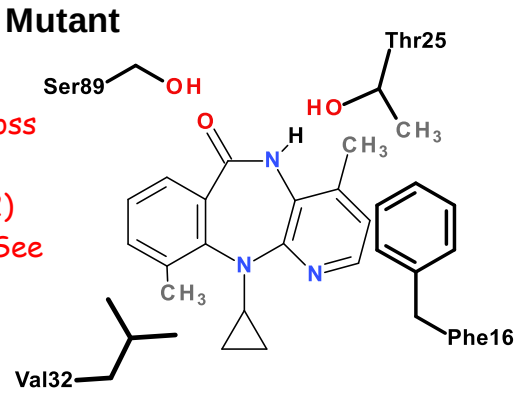
i)

Since the question asks for binding to the enzyme-substrate complex it must be an allosteric inhibitor and you need to calculate $K_I' = [I]/(\alpha'-1)$
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: $\alpha' = 1.2/1 = 1.2$. $K_I' = [I]/(\alpha'-1) = 1\ nM/(0.2) = 5\ nM$

iv) The binding to the mutant enzyme has decreased because of loss of a hydrogen bond when Gln32 is changed to Val

ii) Remove the N in the ring (acceptor for the H-bond from Gln32) and replace it with carbon, perhaps with an additional methyl. See diagram on the right.

vi) No, because K_I is infinite (the ratio of the slopes is 1).



Bonus (2 pts): Why is it advantageous to administer both nevirapin and a protease inhibitor to HIV patients?
This would reduce the chance of obtaining viruses that are drug resistant. To become resistant to both drugs, the virus would have to generate mutations in both binding sites at the same time – very unlikely.

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12. (8 pts) You are trying to purify thymidine kinase (see question 2 for some properties of this enzyme). After an ammonium sulfate precipitation you find that there are five major proteins left in your preparation and the properties of these are given below.

i) Suggest purification step(s) that will purify the thymidylate kinase from the other proteins. *Briefly describe how the separation method for each step works (6 pts).*

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

Protein	Size (kDa)	#Asp+Glu	#His	#Lys	Charge pH=6
Thymidylate kinase	50	10	6	8	-10 + 3 + 8 = +1
Adenosine kinase	50	16	8	4	-16 + 4 + 4 = -8
Pyruvate kinase	35	10	4	12	-10 +2 + 12 = +4
Hexose kinase	75	6	2	20	-6 +1 +20 = +15
Aspartyl kinase	50	10	4	8	-10 + 2 + 8 = 0

i)

One Step:

Use an affinity column with dGMP or TMP attached. None of the other kinases will bind. The bound thymidylate kinase can be eluted with high concentrations of dGMP.

or

Use a cation exchange column at pH 6. Thymidylate kinase would bind weakly to the negative charge on the beads and elute first.

Two Steps:

Size exclusion (small proteins are retarded because they enter pores in the beads) would separate thymidylate, adenosine, and aspartyl kinase from the other two. A cation exchange column could be used to complete the separation because thymidylate kinase will have a positive charge and the other two will have a negative charge (adenosine) or no charge (aspartyl).

ii) the specific activity should increase since impurities have been removed.

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