Biochemistry I, Spring Term 2000 - Second Exam Solution Key:

Section A (28 pts): (2 pts/question). Circle the letter corresponding to the **best** answer.

1 - d

2 - d (partial credit (1pt) given for a or c)

3 - c

4 - a (partial credit (1/2 pt) given for b)

5 - d (partial credit (1pt) given form a or c)

6 – d

7 - c

8 - c

9 - a

10 - c

11 - a (partial credit (1/2 pt) given for c)

12 - a

13 -d

14 -b (partial credit (1 pt) given for c or d)

Section B:

B1 (18 pts) : A drug that inhibits the HIV protease has the following structure:

i) Circle the bond that would be cleaved by the HIV protease in the normal substrate (1 pt).

 It is the bond between the carbonyl carbon and the carbon bearing the OH (marked with an arrow). In other words, the NH group has been replaced by a C-OH group. This problem also tested your knowledge of the structure of normal peptides.
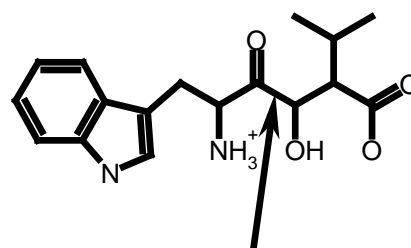
ii) Based on the structure of this inhibitor, briefly describe two of the forces, or energetic components, that might stabilize the drug-protein complex. This problem tested your knowledge of the thermodynamic forces behind ligand binding – they are the same as in protein folding.

 Any two of the following were acceptable:
 Hydrophobic - Trp and Valine side chain
 Van der Waals - Trp and Val side chain
 Hydrogen bonds - Trp side chain nitrogen, C=O, OH, etc.
 Electrostatic - amide and COO-

The binding of this drug to the HIV protease has been measured by equilibrium dialysis. The amount of protein *and* ligand were quantified by UV absorption. The HIV protease dimer contains 4 Trp residues, while the drug contains one. You can assume that the extinction co-efficient (ε) of Trp is 10,000 $M^{-1}cm^{-1}$. Two different drug concentrations were used, giving the following data. The concentration of the HIV protease dimer was 10μM, you should also assume a path length of 1cm.

(**Hint**: The UV absorption inside the dialysis bag includes absorption from the protein as well as the ligand. So, calculate the UV absorption from the protein first, and subtract that from the total absorption to give the UV absorption due to the ligand)

UV absorption outside of the dialysis bag	UV absorption inside the dialysis bag
0.1	0.55
1.0	1.49

iii) What wavelength of light would you have used to measure the absorption of UV light? (1 pt)
 280 nm - the absorption maximum for Trp.

iv) Using the 1st set of data, calculate the dissociation constant, K_D. Don't forget the units! (5 pts)
 This problem tested your use of Beer's law as well as the experimental analysis of binding data.

The steps are:

i) Calculate the absorption due to the protein inside the bag: $A=\varepsilon Cl=4\times10,000\times10\times10^{-6}=0.4$ absorption units.

ii) Calculate the concentration of the ligand outside the bag: $C=A/\varepsilon l=0.1/10^4=10\mu M$

iii) Calculate the concentration (bound and free) of ligand inside the bag: A=0.55-0.40=0.15. $C=0.15/10^4=15\mu M$. Since the concentration of free ligand is $10\mu M$, the concentration of bound ligand is $5\mu M$

iv) Calculate the fractional saturation, $Y=[PL]/([PL]+[P])=5\mu M/(10\mu M)=0.5$. Since Y=1/2, the $K_D=10\mu M$.

Comments: A number of people gave/used an incorrect formula for K_D:

Incorrect: $K_D=([P]+[L])/[PL]$

Correct: $=([P]\times[L])/[PL]$

I think K_D was being confused with Y.

2 pts were given for the proper use of Beer's law

v) What is the free energy of binding of this drug to the protease (assume T=300K)? (2 pts)

$\Delta G=-RTlnK_{EQ} = -RT\ ln(1/K_D) = -2.5\ ln\ 10^5 = -29\ kJ/mol$

vi) Using the 2nd set of data, determine if the binding of the drug is cooperative or non-cooperative. Support your argument with a calculation. (3 pts)

i) Calculate the free ligand concentration: Since the absorption is 10X higher, the concentration is 100 μM.

ii) Calculate the amount of ligand in the bag: A=1.49-0.4=1.09, Therefore the concentration is $109\mu M$:

$[L]=100\mu M$

$[PL]=9\mu M$

$[P]=1\ \mu M$

$Y=[PL]/([P]+[PL])=0.9$

Since an increase in the ligand concentration of 10 fold only increased Y to 0.9, the binding is non-cooperative.

Many people did not have time to complete this calculation. However, a simple sentence stating the expectation was worth a point or two. i.e. "I would calculate the binding constant at high ligand and compare it to the value at the lower ligand. If equal, non-cooperative, if greater, positively cooperative, if less, negatively cooperative.

vii) *Calculate* how large of an effect the replacement of the Trp sidechain in the above drug with a phenyl group will have on the affinity (i.e. K_{EQ}) of the drug to the enzyme. Assume that both the Trp and Phe sidechain will bind in a hydrophobic pocket on the enzyme. The free energy of transfer of Trp from a non-polar environment to water is +12 kJ/mol while that of Phe is +10kJ/mol. (2 pts)

This problem tested your ability to apply thermodynamic data from one area of study (protein folding) to another (ligand binding affinities).

If the Trp side-chain was replaced by Phe, the binding of the drug would be worse because it is less favorable to transfer Phe to a hydrophobic pocket than Trp. Since the difference in free energy of transfer is 2 kJ/mol, the ΔG of binding would decrease to -26 kJ/mol, giving a binding constant of $3.2\times10^4\ M^{-1}$. It was acceptable to just calculate the expected change in binding affinity if you were not able to obtain a free energy from the earlier part of the question.

B2:(12 pts) A Hill plot for the binding of oxygen to Hemoglobin is shown below. This binding data was obtained in the presence of 10 µM bis-phosphoglycerate.

i) On the basis of this plot, what is the approximate K_D for oxygen binding. (2 pts) (**Hint:** The oxygen concentration is in given in µM).

> The curve intersects at 1.2, however since the oxygen concentration was in µM, the actual intersection point is -6+1.2 or -4.8. This gives a K_D of 1.6×10^{-5} M
>
> (Full credits were given for 1.6×10^5, i.e. errors in converting the log scale were acceptable here, but not if you work for NASA)
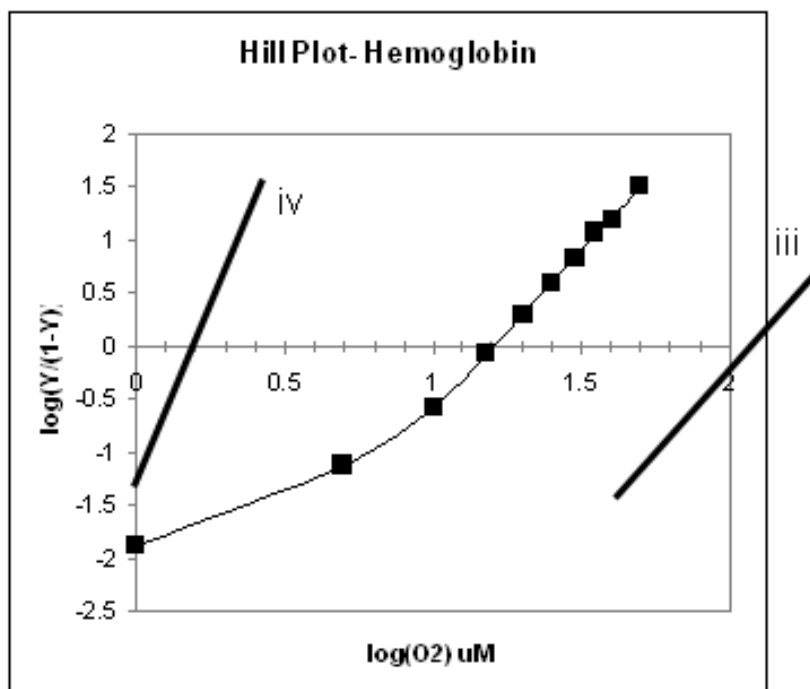
ii) What is the Hill coefficient? Briefly explain how you obtained your answer (2 pts)
> The hill coefficient is just the slope where the line intersects the $\log(Y/(1-Y))$ axis: $\Delta y/\Delta x=(1-(-0.5))/(1.5-1)=3$

iii) The binding of protons to hemoglobin decreases its O_2 affinity 10 fold, but does not change its cooperativity. Sketch, on the same plot to the right, the Hill plot that would be obtained in the presence of protons. Label the curve with "iii" (2 pts)

> The 10 fold decrease in affinity would cause the curve to be displaced 1 log unit to the right. However, since the Hill coefficient is the same, the slope at y=0 would be the same in both graphs.

iv) A reduction in the concentration of bis-phosphoglycerate to 1 µM increases the affinity by 10 fold and changes the Hill coefficient to a value of 3.5. Sketch, using a dashed line, the Hill plot under these conditions. Label the curve with "iv" (3 pts)

> The 10 fold increase in affinity would shift the curve 1 log unit to the left. The increase in the Hill coefficient causes an increase in slope.

v) Answer **ONE** of the following:

a) Briefly explain the molecular nature of cooperative oxygen binding in hemoglobin. Why is this effect important in normal oxygen transport?(3 pts).

> Oxygen binding causes a conformational change in one subunit (proximal His moves, Helix F moves, etc.), causing a change in the configuration of the unliganded subunits to increase their affinity for oxygen binding.

The positive coperativity is required for efficient unloading of oxygen from hemoglobin to myoglobin in the tissues.

b) Explain the molecular nature of the effect of bis-phosphoglycerate on the binding of oxygen to hemoglobin. Why is this effect important in the regulation of oxygen transport. (3 pts)

> The bisPG binds to the deoxy form, therefore decreasing the affinity for oxygen. The binding pocket for bisPG is open in the deoxy form - allowing the bisPG to bind. In the oxy form this site is closed, preventing bisPG from binding.

> This allosteric control provides a mechanism to control the oxygen affinity, based on the available amount of oxygen.

B3:(16 pts) The enzyme, succinate dehydrogenase, performs the following chemical transformation:

A series of kinetic measurement was performed for the inhibited and un-inhibited reaction. These data were plotted on the following Lineweaver-Burk (double reciprocal) plot:
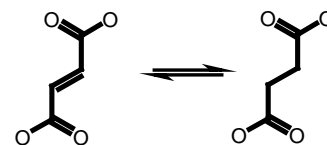
The substrate concentrations are in μM and the enzyme activity is in units of nM product produced/second.

i) Clearly label the curve that represents the data obtained in the absence of the inhibitor (-I) and the data obtained in the presence of the inhibitor (+I) (2 pts).

> See graph.

ii) What kind of inhibitor is this compound (ie. competitive, non-competitive)? Briefly Justify your answer? (2 pts)

> Competitive, since Vmax (1/y-intercept) is unchanged.

iii) Calculate V_{MAX} for the uninhibited reaction.(2 pts)

Vmax is found from 1/y-intercept.

> Vmax=1/0.1=10 nM/sec; the nM refers to product being formed. The units were important, generally 1/2 a point was taken off the entire question if units were left off.

iv) Estimate, using the graph above, the K_M for the substrate.(3 pts)

> 1/Km is the intercept on the x-axis. The approximate intersection point is -0.4, so Km was 2.5 μM

v) Given that the inhibitor concentration was 10μM, what is the dissociation constant for the inhibitor?(4 pts)
> There are two ways of doing this problem, either determine the ratio of the x-intercepts (i.e. comparing Km and αKm) or comparing the slopes of the plot (comparing Vmax/Km). Both of these are proportional to α.
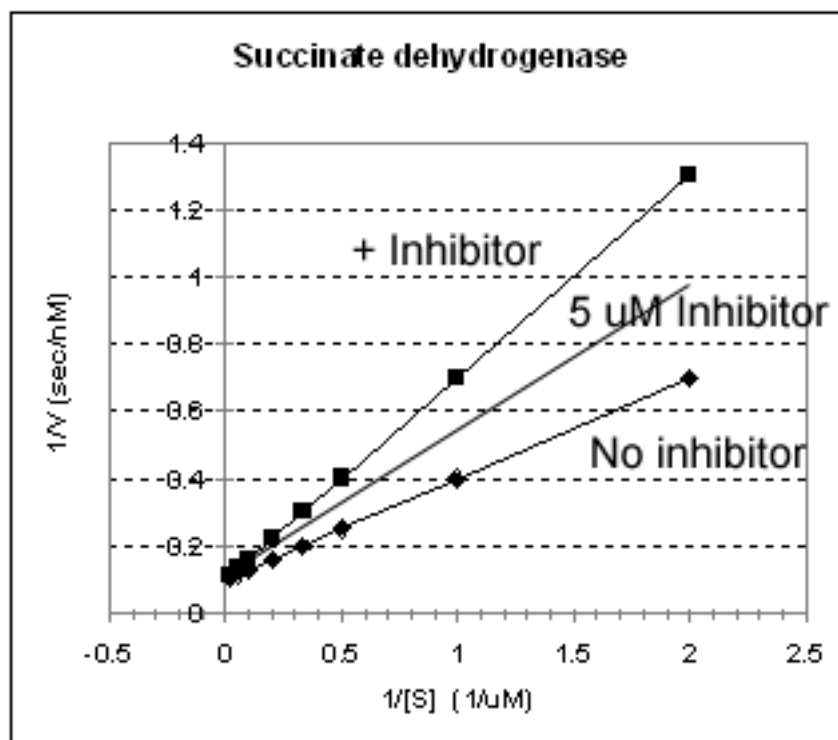
The x-intercept is about -0.1 for the inhibited reaction. Therefore, $\alpha=4$ on the basis of this number. A comparison of the slope is a little more accurate (actually a lot more). This clearly shows $\alpha=2$.

The next part of the question, which people appeared to have difficulty, even on the problem sets, was to calculate the dissociation constant from α:

$$\alpha=1+([I]/K_I)$$
$$K_I=[I]/(\alpha-1)$$

For the case of $\alpha=2$, $K_I=10$ μM

vi) Draw a line on the above graph that would represent the Lineweaver-Burk plot for reactions performed in the presence of 5 μM inhibitor(2 pts).

Since the concentration of the inhibitor is reduced by 2, $\alpha=1+(5/10)=1.5$. In other words, the line should be exactly between the two lines. (See drawing). Any reasonable line was acceptable. (i.e. you didn't have to have accurate K_I values to complete this question.)

A number of people thought the slope of this line should be even higher, but remember that as the inhibitor concentration decreases the line for the inhibited reaction should approach the line without inhbitor.

vii) On the basis of the type of inhibition, draw the chemical structure of a plausible inhibitor(1 pt).

Since the inhibitor is competitive, any chemically similar structure would do, for example:
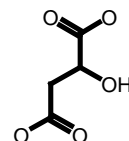
B4:(4 pts) The Scatchard plot for the binding of dinitrophenyl to the Fab fragment of an immunoglobulin is shown below (the ligand concentration is in μM);

i) Estimate the binding affinity, K_{EQ}, from this graph? Briefly describe how you arrived at your answer (2 pts).

The slope of the Scatchard plot is $-Keq$. This line has a slope of -1, so $Keq=1$ μM.

ii) How would the above plot change if this experiment was performed on an intact antibody, instead of the Fab fragment? Re-label the x and y axis and, if necessary, re-plot the data (2 pts).

The intact antibody would contain two independent binding sites. The plot would look the same with the following exceptions: ν would replace Y, and both axis would go from 0 to 2 instead of 0 to 1.
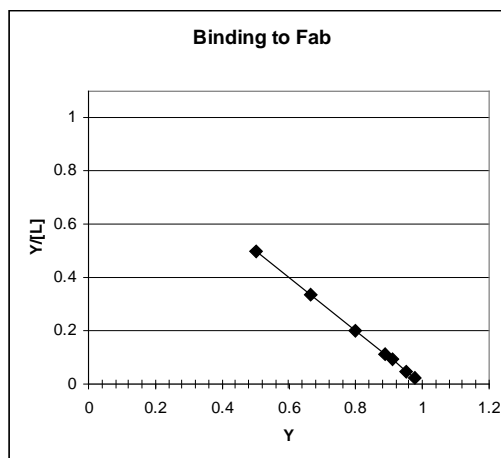
B5:(10 pts) The color handout shows the active site of Trypsin, a serine protease. The bound substrate is drawn with thin lines while residues from the protein are drawn with thicker sticks. The residue that is responsible for the substrate specificity (Asp189) is labeled. The other three residues, labeled A, B, and C, comprise the catalytic triad.

i) How would the specificity of Trypsin change if Asp189 was replaced by Methionine (if you don't remember the structure of Methionine, draw what you think the structure is and then proceed with the question.)(2 pts)

Met is a large hydrophobic residue. Therefore the sidechain of the normal substrate (Lys or Arg) would not interact favorably with the Met. Therefore, the specificity would change such that amino

acids with small hydrophobic sidechains would interact favorably with the Met. For example, Ala or Val would probably be good substrates.

ii) The effect of replacing Asp189 with Met would have the largest effect on the K_M or on V_{MAX}? Briefly explain your reasoning.(2 pts)

> Km, since the changed residue effects the binding of the substrates, but not the catalytic triad. Remember that Vmax is defined as the velocity of the reaction when all of the enzyme is in the ES complex, it doesn't matter what the value of Km is (unless it exceeds to solubility of the substrate).

iii) Which residue (A, B, or C) is the nucleophile in this reaction?(2 pts)

> Serine – labeled 'A'

iv) Briefly explain how the other two residues in the catalytic triad increase the reactivity of the nucleophile.(4 pts).

> Both function to aid in proton removal from the Ser (1st part of the reaction) or water (hydrolysis of the acyl enzyme). The His residue is primarily responsible for abstraction of the proton, but it is aided by the Asp residue.

B6 (5 pts). The activity of the HIV protease is highest at pH 5. Explain this observation with specific reference to the ionization properties of the active site residues in this enzyme.

> The HIV protease has two Asp residues in the active site. The catalytic mechanism requires that one is protonated while the other is not. The pKa of one is about 3, the other about 6. The optimal concentration of protonated and deprotonated Asp residues occurs about pH 5.0

B7 (7pts) : To the right is an image of an SDS-PAGE gel (turned sideways). The top of the gel is on the left, the bottom on the right. A total of four bands were observed on this gel (labeled A, B, C, and D). The elution profile for the same mixture of proteins from a gel filtration (size exclusion) column is shown underneath the gel. The same protein has the same label in both experiments.
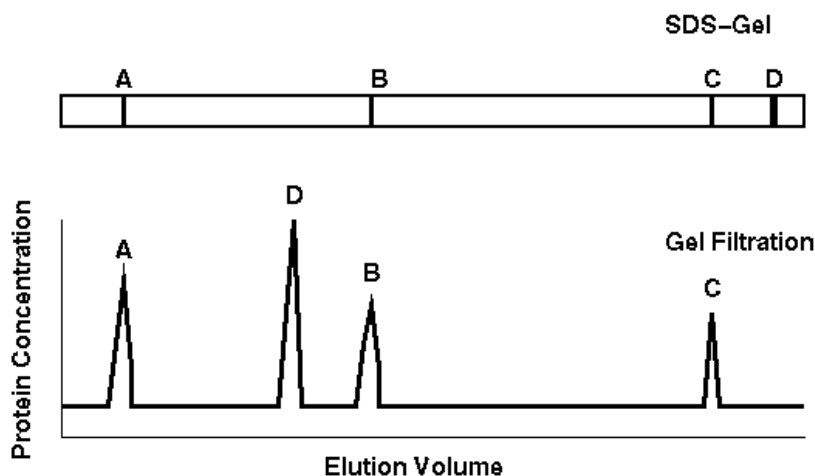
i) Why do most of the bands in the SDS-PAGE gel "line-up" with most of the peaks from the gel filtration column (i.e. A matches A, B matches B, etc.) (3 pts)

> Both techniques separate by molecular weight. In fact, both have the same Log dependence. It is also important to realize that A, B, and C are monomeric.

ii) Provide a reason why the protein labeled 'D' does not follow this pattern. That is, its migration distance on the SDS-PAGE gel is apparently unrelated to its elution volume on the gel-filtration column.(2 pts)

> Protein D must be dimeric or a higher oligomer. The subunits must not be linked by covalent bonds such that denaturation by SDS causes each subunit to run as a much smaller protein.

iii)Assume that you know the molecular weight of proteins A and C, briefly describe, for **either** gel filtration or SDS-PAGE, how you would obtain the molecular weight of the other proteins.(2 pts)

> Plot of logMW versus distance(SDS gel) or Ve/Vo (gel filtration) will give a straight line. Using the known molecular weights of A and C to define the position of the line. Then use the distance migrated (or Ve/Vo) of the unknown to determine log(MW).