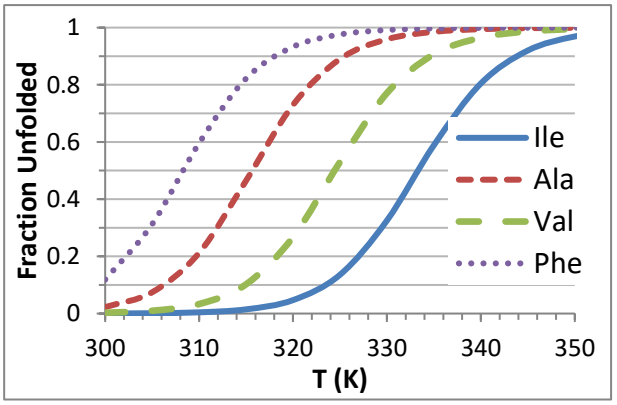
Due Sunday September 29, 2019 52 pts Total time required: ~ 95 min.

1. (10 pts, 20 min) CSP (cold shock protein) is produced in bacteria after a sudden drop in temperature. The wild-type protein contains an Ile at position 18 in the chain. You can view the structure of the wild-type and mutant proteins on the Jmol page associated with this problem set. Three different mutant proteins are studied, with the Ile replaced by Phe, Val, or Ala. The thermodynamic parameters for unfolding of the wild-type protein are: ΔH^o = +200 kJ/mol, ΔS^o= +600 J/mol-deg. The denaturation curves for all four proteins are shown on the right.



i) Estimate the ΔS^o for unfolding of all three mutant proteins (Ala, Val, Phe) using the data (entropy change due to transfer to water) given in lecture 8 and the ΔS^o observed for the wild-type protein. The units on the y-axis of the plot are J/mol-deg (4 pts). You can assume the entropy for the transfer of the sidechains to water is:

Ile = -2.5 J/mol-K
Val = -1.5 J/mol-K
Phe = -2.5 J/mol-K
Ala = -0.5 J/mol-K

The figure gives the entropy change for different amino acids when they are transferred to water (i.e. unfolding). The difference between Ala and Ile is 2 J/mol-deg. Therefore, the entropy change for unfolding the Ala protein should be +2 J/mol-deg larger (less water is ordered when the Ala unfolds), giving a total entropy of +602 J/mol-deg. In the case of valine, the entropy change in transferring valine to water is -1.5, a difference of 1 from Ile, so the observed entropy will be +601 J-mol/K. Ile and Phe have about the same transfer entropy, so the overall entropy for denaturation will remain the same.

ii) Using your estimate from *part i)* calculate the ΔH^0 for the unfolding for all three mutant proteins [Hint: No van't Hoff analysis is required, remember that $\Delta H^o = T_M \Delta S^o$]. Use the following values for T_M: Ile=333.3, Ala=315.6, Val=324.5, Phe=308.3 (3 pts)

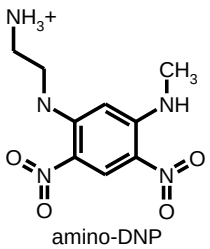
	ΔS_{H2O}/Res 18 (J/mol-K)	ΔH (kJ/mol)	ΔS_{OBS} (J/mol-K)	T_M (K)
Ile	-2.5	200	600	333.3
Ala	-1.5	190	602	315.6
Val	-2.5	195	601	324.5
Phe	-0.5	185	600	308.3

iii) Explain the differences in enthalpy and entropy between the wild-type and all three mutants in terms of the structural differences. (3 pts)

The entropy differences are explained above (part i).

Enthalpy effects: In the case of Ala and Val, which are both smaller than Ile, the decrease in enthalpy is just due to the loss of contact area between the sidechain and the rest of the core, reducing van der Waals. Phenylalanine is larger and in order to fit into the same location as the original Ile it would be necessary to disrupt the packing in the core, which would reduce van der Waals overall.

2. (6 points, 10 min) The Jmol page for this question contains structures for the complex between two different Fv fragments (A & B) bound to the same dinitrophenyl ligand (amino-DNP). The chemical structure of this hapten is shown on the right. In answering the following questions you should focus on the two nitro groups (NO_2) as well as the aromatic ring on the DNP, the ethylamine group ($-CH_2-CH_2-NH_3^+$) is not important for binding.



amino-DNP

i) Give the residue name and number (position in sequence) of the tryptophan

residues that contact the hapten, also indicate whether they are on the heavy or light chain. (2 pts)

Trp90 on the light chain, Trp100 on the heavy chain.

ii) What are the **two** energetically favorable interactions between these Trp residues and the hapten that would stabilize its bound state? (2 pts)

Van der Waals: There is good contact between the trp sidechains and the DNP, giving good van der Waals interactions.

Hydrophobic effect, both Trps and the hapten are somewhat non-polar and would release ordered water molecules when the complex is formed, increasing the entropy of the system.

No H-Bonds! Although the Trp residues have potential H-bond donors (N-H), the geometry is not optimal for a stable H-bond between Trp and the Hapten. The three atoms should lie in a straight line.

iii) Antibody A binds the hapten more tightly than B, why? [Hint: Are there any additional interactions between the hapten and A versus B?] (2 pts).

Antibody A has a Gln at the base of the pocket that can donate a hydrogen bond to the electronegative oxygen on the N=O group on the hapten. This interaction is missing in B, because the Gln has been replaced by an alanine.

3. (10 pts, 20 min)

i) You are using equilibrium dialysis to measure the binding constant of DNP to both F_v fragments (A and B). The concentration of the F_V inside the dialysis bag is 5 μM (μM=10^{-6} M). Use the "dry-lab" associated with this problem to obtain the amount of ligand in the dialysis bag. From these data obtain the fractional saturation for both proteins (4 pts).

The fractional saturation at each ligand concentration (L_{OUT}) is just $Y = (L_{IN}-L_{OUT})/M_T = (L_{IN}-L_{OUT})/5$. This is because $Y=[ML]/[M_T]$ and [ML] is the additional ligand in the bag that is bound to the macromolcule. $L_{out}=L_{Free}$.

ii) Use these values to plot a binding curve (Y versus [L]). Estimate the value of the K_D from your binding curve. You will need these values to fit the binding curve using the Solver routine in Excel. You can download an Excel sheet that is set up for Solver:

http://www.andrew.cmu.edu/user/rule/bc_oli/Pset/PS04/binding_Solver_DNP.xlsx (2 pts). Additional information on using Solver is available on the Jmol page for this problem set.

K_D is 5 uM for Fv-A and 20 uM for Fv-B. Your numbers may have been slightly different due to errors in the experimental data.

iii) Calculate the standard energy of binding for each K_D value (T=300K). Based on the K_D values and the ΔG^o values, which Fv fragment binds the DNP with higher affinity, Fv-A or Fv-B? (2 pts)

$\Delta G^o = -RT \ln(K_{EQ})$: Fv-A: $-8.31 \times 300 \times \ln(1/(5\times10^{-6})) = -30.4$ kJ/mol. Fv-B: = -27.0 kJ/mol

Fv-A has the larger, more negative ΔG^o (i.e. more energy is released on binding), so the binding is better to Fv-A.

iv) Relate the difference in binding energy to the difference in the interaction of the two Fv fragments and the bound DNP ligand (2 pts).

The only difference between how the two proteins interact with the hapten is the presence of a hydrogen bond in A. This is lost in B because the Gln has been replaced by Ala. The energy difference, $\Delta\Delta G^o$ = -3.4 kJ/mol would represent the energy due to the hydrogen bond in the DNP-FvA complex. The remaining energy, -27 kJ/mol would represent the energy associated with vdW and the hydrophobic effect due to the close contact between the DNP ring and the Trp residues.

4. (12 pts, 20 min) This problem investigates the effects of changing the dissociation constants (i.e. ligand-protein binding energy) on the degree of cooperativity for a protein that binds two identical ligands. You should download the EXCEL spreadsheet for this problem:

www.andrew.cmu.edu/user/rule/bc_oli/Pset/PS05/bindingcurve_sim.xlsx.

Change the values of K_{D1} and K_{D2} (cells F2 and G2 on spreadsheet, highlighted yellow) to the values shown in the table on the right to determine the empty cells in the table:

a) n_h (Hill coefficient),
b) K_D
c) fraction of the system in the (ML) form at Y = 0.5 (f_{ML}).

	K^{μ}_{D1}	K^{μ}_{D2}	n_h	K_D	f_{ML}
A	5	20	0.75	10	0.533
B	10	10	1.00	10	0.250
C	50	2	1.52	10	0.015
D	200	0.5	1.84	10	0.000
E	1000	0.1	1.96	10	0.000

You should get n_h and K_D from the Hill plot, f_{ml} is the cell in the table highlighted green. Before you begin, think whether you expect the binding to be cooperative or non-cooperative, based on the differences between the two K_D values. Note that the units are μM for the binding curve and log(M) for the Hill plot. The K_D values entered for the various questions are in μM, i.e. entering 10 in cell F2 is really 10 μM.

i) Is the Hill coefficient consistent with the values of the individual dissociation constants, K_{D1} and K_{D2} for each pair of values? Justify your answer by comparing the binding constants to the Hill coefficient for the five proteins? (2 pts)

Yes, the values are consistent:

Protein A shows negative cooperativity because the second K_D is higher than the first. Therefore, binding of the first makes it more difficult to bind the second. As expected the Hill coefficient is less than one.

Protein B is non-cooperative, the second K_D is the same as the first, the binding of the first ligand has no effect on the binding energy of the second. As expected the Hill coefficient is one.

Proteins C through E show increasingly more positive cooperativity since the second K_D is increasingly smaller than the first. The binding of the first ligand makes it easier for the second to bind. Accordingly, the Hill coefficient is above one for all, and increases from C to E.

ii) Sketch the Hill plot, on the same graph, for all five of the above conditions. What is the difference between the five Hill plots? The slope or the x-intercept? (4 pts)

The plots all differ in the slope – because the Hill coefficient is changing. The x-intercept is the same, showing a $K_{D\text{-}OBS}$ of 10 uM. Your plot should be a series of five lines, all intercepting the x-axis at -5, with slopes of 0.75 (A), 1.00(B), 1.52 (C), 1.84 (D), 1.96 (E). **Case E, with a Hill coefficient of almost 2 is the closest to infinite positive cooperativity. For two binding sites, the maximum value of the Hill coefficient is 2.**

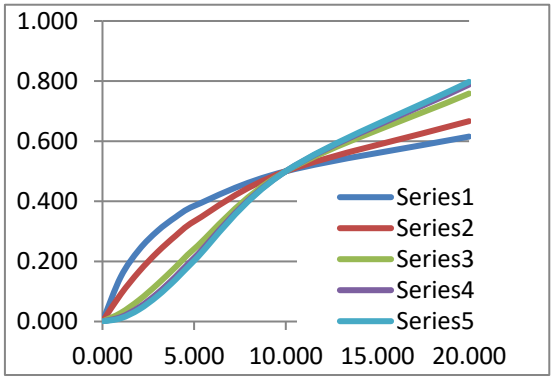
iii) How does the observed K_D, as determined from the Hill plot, change for the different values of K_{D1} and K_{D2} (2 pts)?

The $K_{D\text{-}OBS}$ is the same in all cases because for two binding sites $K_{D\text{-}OBS}$ is simply the square root of the product of the K_D values. The [L] that gives ½ saturation = $K_{D\text{-}OBS} = \sqrt[2]{K_{D1} \times K_{D2}}$.

iv) How does the fraction of the system in the (ML) form at Y=0.5 change as the cooperativity changes? Which of these five scenarios (A-E) more closely represents infinite positive cooperativity? Justify your statement based on the Hill coefficient (2 pts).

The fraction of the protein in the intermediate state (ML) is higher for the negatively cooperative system because it is harder for a ligand to bind to the remaining empty site to form (ML_2). The non-cooperative system shows random behavior. As the + cooperativity increases the amount of the intermediate decreases; as the first site is filled, the second binds ligand more readily, increasing [ML_2] and decreasing [ML]. In case D & E, the cooperativity is so high that there is essentially no intermediate, once one ligand binds, the second site is occupied due to its high affinity.

v) How does the shape of the binding curve change for each set of values? What is a more reliable measure of the cooperativity? The shape of the binding curve (hyperbolic for non-coop, sigmoidal for positively cooperative, or flattened hyperbolic for neg-cooperativity) or the Hill coefficient (2 pts)?

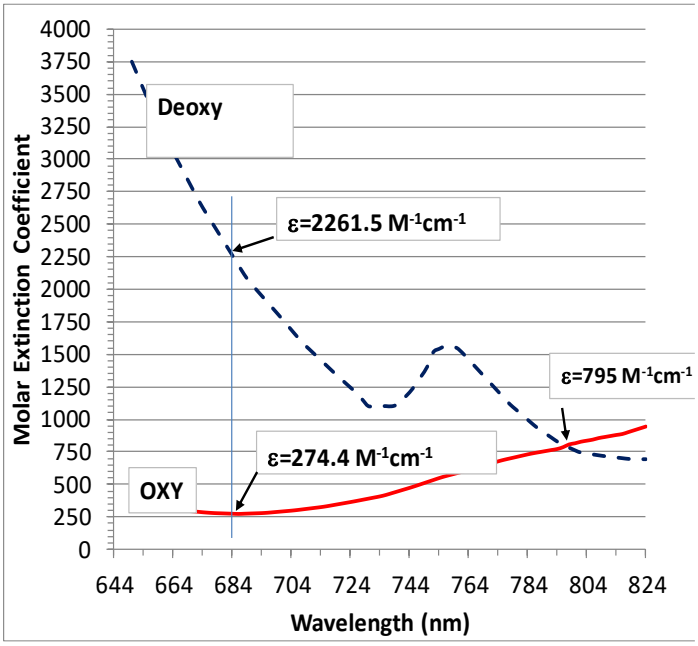


The binding curve appears to be hyperbolic for cases A and B, indicating that it is difficult to distinguish negative cooperativity from non-cooperative binding by curve shape alone. Curves C, D and E, even though these all have different levels of cooperativity, show almost the same sigmoidal shape. **The Hill coefficient is a much better (and quantitative) measure of the degree of cooperativity.**

5. (14 pts, 25 min) Fetal hemoglobin, although also tetramer, is composed of different chains than adult hemoglobin which give it different oxygen binding characteristics. The fractional saturation of fetal hemoglobin at a number of different oxygen concentrations is given in the table on the right. The units of oxygen are μM. One data point is missing, at $[O_2]$= 0.5 uM, which you will obtain from optical absorption.

$[O_2]$ (μM)	Y
0.2	0.008
0.5	**0.125**
1.0	0.510
2	0.890
5	0.990

The absorption spectra for deoxy (blue, dotted) and oxy-(red, solid) hemoglobin are shown on the right, along with the molar extinction coefficients of the deoxy (Y=0) and oxy form (Y=1) of hemoglobin at 684 nm (ε = 2261.5 $M^{-1}cm^{-1}$ (deoxy) and 274.4 $M^{-1}cm^{-1}$ (oxy)) and 796 nm (ε=795 $M^{-1}cm^{-1}$). You can assume the concentration of hemoglobin in solution is 1 x 10^{-4}M.



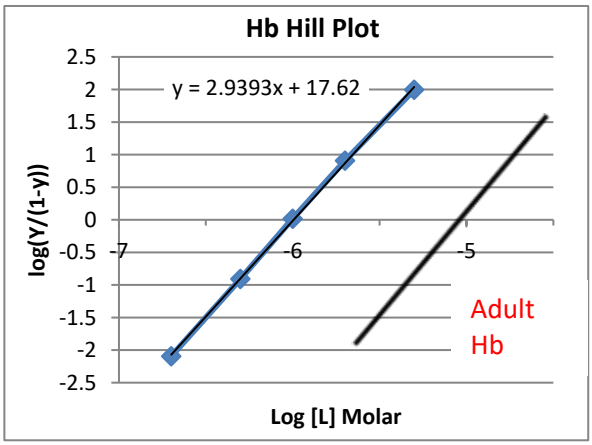
i) Calculate the expected absorption of the solution of fetal hemoglobin at 684 nm for both the oxy and deoxy forms, this gives you A_{ML} and A_M, respectively (2 pts).

Deoxy (Y=0) = 10^{-4} M X 2261.5 = 0.2262
Oxy (Y=1) = 10^{-4} M X 274.4 = 0.0274

ii) The absorbance of a solution with $[O_2]$=0.5 μM was 0.201. What is the fractional saturation at this oxygen concentration? (2 pts)

Y = $(A - A_M)/(A_{ML} - A_M)$ = (0.201 - 0.226)/(0.0274 - 0.2262) = -0.025/-0.1988 = **0.125**

iii) Using the values of Y in the above table, plot a Hill plot. Convert the oxygen concentration to molar before doing your plot. Determine the observed K_D value and the Hill coefficient from your data (4 pts).



The Hill Plot is shown on the right. The slope at Y=0.5 (log (Y/(1-Y))=0) is 2.93. Therefore the Hill coefficient is: n_h=2.93. The Hill plot intersects the x-axis at -6, therefore log[L]=-6, or [L]=10^{-6} M.

iv) Using the same graph, sketch the central portion of a Hill plot for normal adult hemoglobin (K_D = 10 μM, n_h =3) (2 pts).

Also shown on the right, the Hill plot for adult Hb

intersects the x-axis at -5 (=log10^{-5}), with a slope of 3.

v) Compare your data (K_D and n_h) for normal hemoglobin to that obtained for fetal hemoglobin. What are the significant differences and similarities? (2 pts)

The cooperativity is about the same, however the fetal hemoglobin binds oxygen more tightly, since its K_D is lower than the value of the maternal hemoglobin (~10 μM), by a factor of 10.

vi) Why are these differences and similarities in K_D and n_h important for the *biological* function of fetal hemoglobin? (2 pts)

- The fetal hemoglobin has to obtain its oxygen from the maternal hemoglobin across the placenta, therefore it must have higher affinity so that it can effectively become saturated at the relatively low oxygen concentration at the placenta.
- The cooperativity of the two proteins should be similar so that there is effective oxygen transport to the fetal tissues.