

BCHM 461  
Problem set #2

**Problem 1.**

- a. A protein has binding affinity for its ligand (a peptide) of  $K_a = 2 \cdot 10^5 \text{ M}^{-1}$  at pH 5.0 and 25°C. At what concentration of the ligand is half of the protein bound? (A:)
- b. What fraction of the protein is bound at ligand concentration of 1.25  $\mu\text{M}$  (a reminder: 1  $\mu\text{M} = 10^{-6} \text{ M}$ )?
- c. At what ligand concentration will be 80% of the protein bound?
- d. When the pH was raised to 6.5, the  $K_d$  increased to 20  $\mu\text{M}$ . Is the binding tighter or weaker at this pH compared to pH5.0? Explain why.
- e. What functional groups/residues are most likely responsible for this change in the binding affinity with pH?

**Problem 2.**

You study binding of two ligands, A and B (separately), to a protein. The total conc. of protein was 10  $\mu\text{M}$ . You perform two separate assays:

1: In the assay for ligand A, you found that when ligand concentration was 50  $\mu\text{M}$ , the concentration of free protein dropped to 7.5  $\mu\text{M}$ .

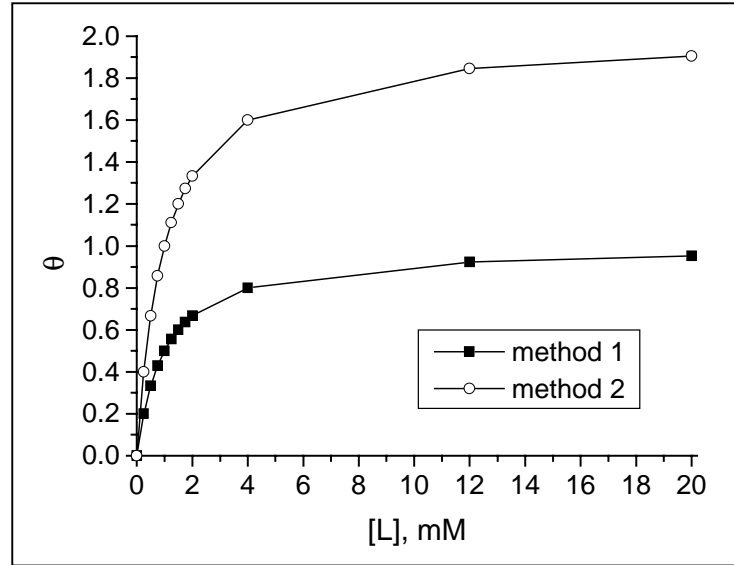
2: In the assay for ligand B, you start with 20  $\mu\text{M}$  of protein and ligand concentration of 100  $\mu\text{M}$  and you find that the concentration of free protein is 15  $\mu\text{M}$ .

- a. Which of the two ligands binds tighter to your protein?
- b. What would be your answer in the assay 2 if the concentration of bound protein was 10  $\mu\text{M}$ ?
- c. Assuming that the binding affinity for ligand B is the same as for A, predict the concentration of the free protein in assay 2.

**Problem 3.**

Ligand binding studies were performed by adding ligand to a certain amount of protein X. The fraction  $\theta$  of protein bound to ligand was assessed using two different methods: (1) by measuring the concentration of the protein bound to ligand and (2) by measuring the concentration of ligand bound to protein. In both cases  $\theta$  was derived as  $\theta = [PL]/[P]_{\text{total}}$ , where  $[PL]$  represents the results of these measurements and  $[P]_{\text{total}}$  is the total concentration of the protein present in solution. The data are shown in the table below and in the Figure (the data points are connected by lines just to guide the eye, these lines do not represent any fitting curves).

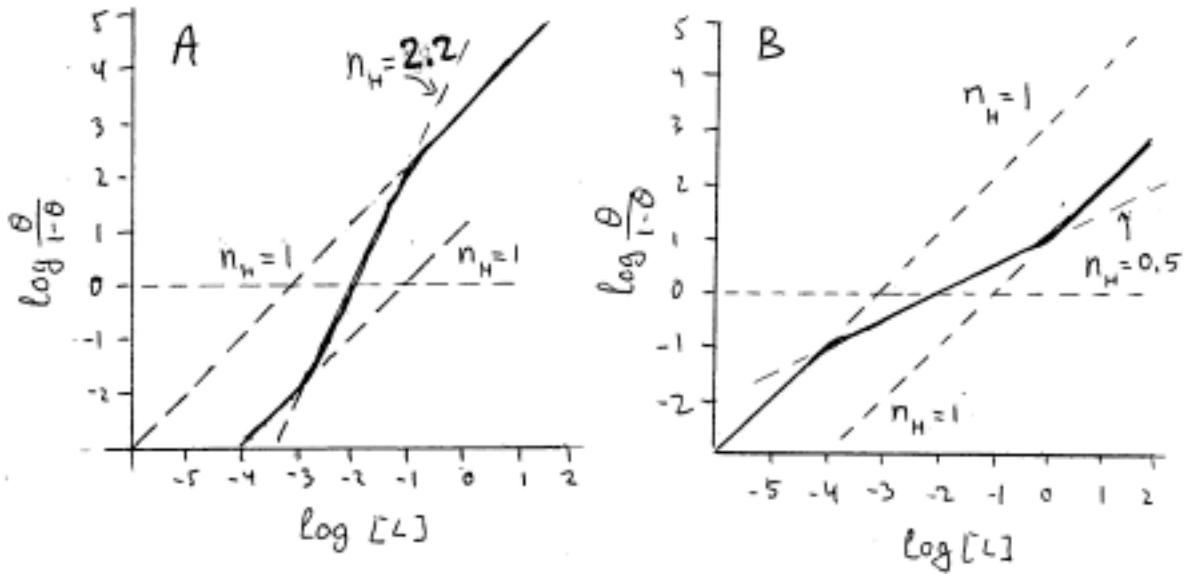
| 1            | 2                    | 3                    |
|--------------|----------------------|----------------------|
| [L]<br>in mM | $\theta$<br>method 1 | $\theta$<br>method 2 |
| 0            | 0                    | 0                    |
| 0.25         | 0.200                | 0.400                |
| 0.50         | 0.333                | 0.667                |
| 0.75         | 0.429                | 0.857                |
| 1.00         | 0.500                | 1.000                |
| 1.25         | 0.556                | 1.111                |
| 1.50         | 0.600                | 1.200                |
| 1.75         | 0.636                | 1.273                |
| 2.00         | 0.667                | 1.333                |
| 4.00         | 0.800                | 1.600                |
| 12.00        | 0.923                | 1.846                |
| 20.00        | 0.952                | 1.905                |



- Explain the difference between the results of these two measurements.
- How many ligand binding sites are on the protein molecule?
- What is the affinity of protein X for the ligand? Explain your reasoning
- What could you assume about possible cooperativity of the ligand binding from these data? Suggest additional data analysis that could help verify your assumption.

**Problem 4.**

Ligand binding to proteins A and B is characterized by Hill plots shown below.



- a. What conclusions about possible cooperativity of the binding can you draw from these plots? Explain the different shape of the binding curves.
- b. Based on these plots, what is the minimal number of ligand binding sites on protein molecule for each of the proteins? Explain.
- c. Which of the two proteins binds ligand tighter at the midpoint of the binding curve (i.e. where  $\theta = 0.5$ )? Explain.
- d. For each of the proteins determine and compare their binding affinities in their high- and low- affinity states.
- e. For each of the proteins draw schematically their ligand binding curves in the coordinates  $\theta$  versus  $[L]$ .

**Problem 5.**

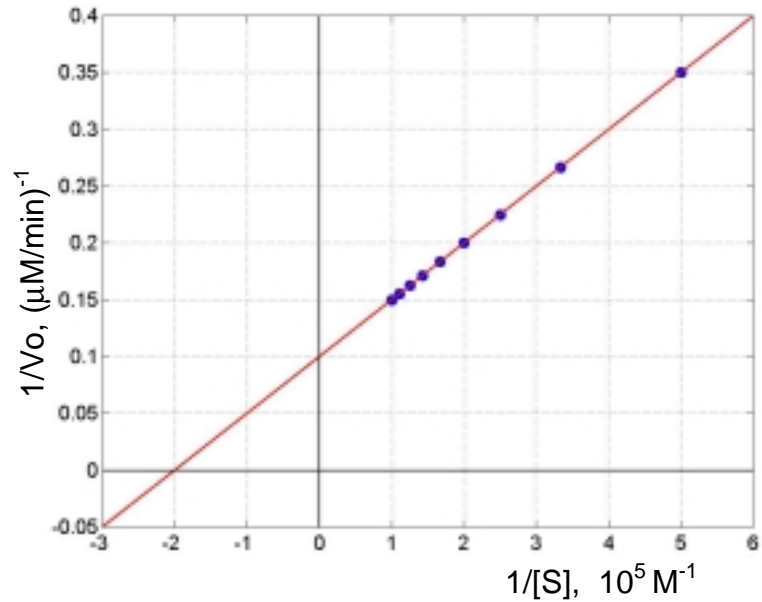
A particular chemical reaction,  $A \leftrightarrow B$ , is characterized by the difference in the free energy  $\Delta G_{A \rightarrow B} = -20$  kJ/mol. The uncatalyzed reaction is, however, slow, because of the high activation barrier,  $\Delta G^\ddagger = 50$  kJ/mol, therefore, you need an enzyme to speed up the reaction. What should be the activation barrier for the catalyzed reaction,  $\Delta G^\ddagger_{\text{cat}}$ , in order to achieve a 1000 fold increase in the reaction rate at standard conditions? How will this affect the rate of the backward reaction,  $B \rightarrow A$ ?

Is the required change in the  $\Delta G$  a big or a small number? Compare it with the typical energies associated with a hydrogen bond or a covalent bond.

### **Problem 6.**

You measure the kinetics of an enzyme E as a function of substrate concentration and plot the data using the double-reciprocal (Lineweaver-Burk) plot (Figure below). The enzyme concentration is maintained constant at a level of  $1 \mu\text{M}$  ( $=10^{-6} \text{ M}$ )

| [S],<br>$\mu\text{M}$ | $V_o$ ,<br>$\mu\text{M}/\text{min}$ |
|-----------------------|-------------------------------------|
| 2                     | 2.9                                 |
| 3                     | 3.8                                 |
| 4                     | 4.4                                 |
| 5                     | 5.0                                 |
| 6                     | 5.4                                 |
| 7                     | 5.8                                 |
| 8                     | 6.2                                 |
| 9                     | 6.4                                 |
| 10                    | 6.7                                 |



From these data, determine  $V_{\max}$ ,  $K_M$ ,  $k_{\text{cat}}$ , and the turnover number for the enzyme.

Determine these parameters by two different methods: (1) from the double-reciprocal plot and (2) from the table (or you can plot the hyperbolic curve). Compare the results. What is the reason for the difference between the values you obtained by these two methods? Which method is more accurate?

### Some answers to some of the problems

#### Problem 1

- $[L] = K_d = 1/K_a = 5 \cdot 10^{-6} \text{ M}$
- $\theta = \frac{[L]}{[L] + K_d} = \frac{1.25 \mu\text{M}}{1.25 \mu\text{M} + 5 \mu\text{M}} = \frac{1}{5} = 20\%$
- $[L] = \frac{0.8 K_d}{1 - 0.8} = 4 K_d = 20 \mu\text{M}$
- Since  $K_a = 1/K_d$ , higher  $K_d$ , indicates lower affinity. The binding is weaker at pH 6.5.
- Histidine. Its  $pK_a$  is 6.0. No other group in proteins (under normal conditions) has its  $pK_a$  in the pH range between 5.0 and 6.5.

#### Problem 3

- A. The two methods measure the same quantity,  $[PL]$ , but by monitoring the concentration of the two partners in the protein-ligand complex. Stoichiometry of ligand binding is important when determining  $[PL]$  by measuring the concentration of bound ligand. The fact that  $q$  (method 2) is 2-fold greater than  $q$  (method 1) suggests that the stoichiometry (ligand : protein) is 2:1.
- B. 2 (see pr.A)
- C. From the table:  $K_d = [L]_{0.5} = 1 \text{ mM}$ ;  $K_a = 1/K_d = 10^3 \text{ M}^{-1}$
- D. The binding curves look as hyperbolic (by eye), suggesting no cooperativity. To verify this, the data should be analyzed using Hill plot.

#### Problem 4.

- a.** Both proteins show cooperative ligand binding:  
A shows positive cooperativity ( $n_H > 1$ ) while B has negative cooperativity ( $n_H < 1$ ).  
This determines the shape of the binding curve:  
A: a transition from low-affinity to a high-affinity binding;  
B: a transition from high-affinity to low-affinity binding.
- b.** The number of binding sites is equal or greater than  $n_H$ . For protein A,  $n_H = 2.2$ , so the minimal integer number  $\geq n_H$  is 3. For protein B ( $n_H = 0.5$ ), the minimal number of ligand binding sites is 2. The minimal integer  $\geq n_H$  is 1, but at least two binding sites are required for cooperativity.
- c.** Hill plot:  $\log(\theta/(1-\theta)) = n_H \log[L] - \log K_d$ . At  $\theta = 0.5$ , the left-hand side is 0, hence  $\log K_d = n_H \log([L]_{0.5})$ . This gives  $K_d = ([L]_{0.5})^{n_H}$ .  
For both proteins  $[L]_{0.5} = 10^{-2} \text{ M}$ . For protein A:  $K_d = (10^{-2})^{2.2} = 10^{-4.4}$  while for protein B  $K_d$  is significantly greater:  $K_d = (10^{-2})^{0.5} = 10^{-1}$ . Therefore we can conclude that protein A binds ligand tighter at the midpoint of the binding curve.  
Same conclusions can be obtained from the intercept of the middle-part (or its extension) of the graph with the  $\log[L]=0$  axis: according to Hill equation, the ordinate of this point gives  $-\log K_d$ . We thus get for  $-\log K_d$  the values of approx. 4 (A) and 1 (B).

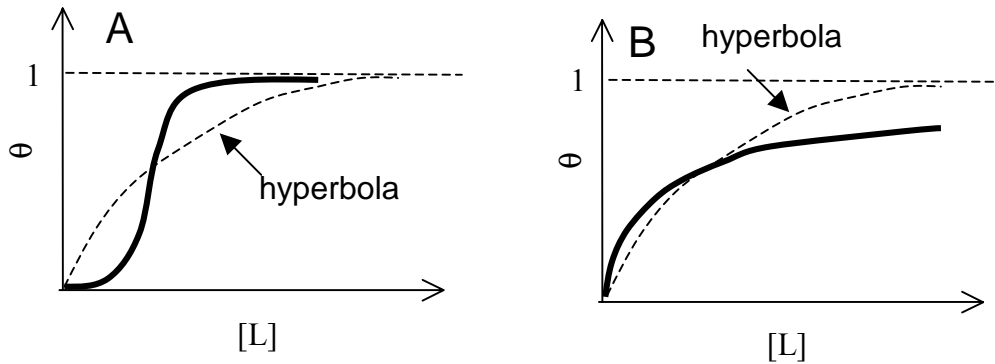
Note: just the fact that the slope ( $n_H$ ) in the Hill plot is greater for A than for B means higher cooperativity, not necessarily higher binding affinity.

**d.** The  $K_d$ 's for these two states can be determined directly from the Hill plots as the values of  $[L]$  at  $\theta = 0.5$ , i.e. where the corresponding lines intercept the  $\log(\theta/(1-\theta)) = 0$  axis. Since for both high- and low-affinity states  $n_H=1$ , we can use the relationship:  $K_d = [L]_{0.5}$ .

From the plots (the results are same for both A and B):

High-affinity state:  $K_d = 10^{-3}$  M; low-affinity state:  $K_d = 10^{-1}$  M, so the high-affinity state has a 100-fold greater  $K_a$  than the low-affinity state.

**e.**



**Problem 5.**

Since the rate of the reaction (all other conditions being equal) is proportional to  $e^{-\Delta G^\ddagger / RT}$ , the difference in the  $\Delta G$ 's can be written as  $\Delta\Delta G^\ddagger = \Delta G^\ddagger_{\text{cat}} - \Delta G^\ddagger = -RT * \ln 1000 = -17.1$  kJ/mol. Therefore,  $\Delta G^\ddagger_{\text{cat}} = 32.9$  kJ/mol.

The rate of the backward reaction will also increase 1000 fold.

The  $\Delta\Delta G^\ddagger$  is about or slightly higher than the energy of one hydrogen bond (-12 to -30 kJ/mol), and is a small fraction of the energy of a typical covalent bond.

**Problem 6.**

From the intercepts in the plot:

$$V_{\text{max}} = 10 \mu\text{M}/\text{min};$$

$$K_m = 5 \cdot 10^{-6} \text{ M} = 5 \mu\text{M};$$

$$\text{The turnover number, } k_{\text{cat}} = V_{\text{max}}/[E]_t = 10 \text{ min}^{-1}.$$

From the table, assuming that  $V_{\text{max}} \approx 6.7 \mu\text{M}/\text{min}$ , we obtain  $K_m = [S]$  (at  $V_o = V_{\text{max}}/2$ )  $\approx 2.5$  mM. The problem with this method is that  $V_o$  is not yet saturated at  $[S]=10 \mu\text{M}$ .