Instructions:

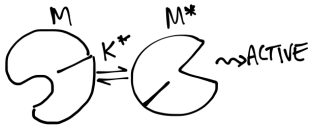
- Turn in your results in some kind of clear form (handwritten, typed, pdf).
- Feel free to work in groups.
- I have tried to be consistent in my language in my prompts if I want something specific.
  - *Sketch:* Hand draw a plot. Axes should be labeled conceptually, with key features indicated and explained.
  - *Generate a plot:* Plot using software. Label axes, use appropriate significant figures, etc.
  - *Calculate:* Actually calculate numbers using math. Report units and error bars, as appropriate.
  - *Describe/Argue:* Use any combination of writing, sketching, plotting, and calculation to argue for an interpretation.
  - Finally, I will sometimes specify the sort of explanation I want.
    - *Molecular*: Describe what the atoms and molecules are doing in space and time. Depending on the context of the question, this might also involve explaining the result in terms of atomic properties like hydrophobicity.
    - *Energetic*: Describe in energetic terms (entropy, free energy, statistics).
    - *Mathematical*: Answer in terms of how the functions behave. For example, if I asked "mathematically, why does $Kx/(1+Kx)$ saturate with increasing x" the answer would be: "because as $x \to \infty$, $Kx \gg 1$ and the function tends to 1."
- Some of the questions may require you to play with math and/or ideas we did not explicitly discuss in class. This is intentional. Many times in science you will be faced with a paper that uses an approach you are not familiar with. Learning how to gather enough information to critically evaluate their findings, as well as understand any mathematical models employed, is important.
- Some questions are listed as **GRAD STUDENT** questions. Undergrads are free to do those questions, but it is not required.

3 Equilibria, linked and otherwise

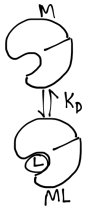
The following exercise explores how allostery can arise from linked equilibria. We're going to analyze the behavior of an allosterically regulated enzyme that has a non-competitive inhibitor (L), which binds at a different site on the protein than the active site.

1. What fraction of the enzyme is in its active form, and how is this influenced by $[L]$? To answer this question, we need something like θ for binding, but for active versus inactive enzyme. Let's call this θ^*. We're going to derive an expression describing it that will allow us to see how linked equilibria can lead to allostery. We're going to assume that the total concentration of macromolecule, $[M]_{tot}$, is much less than the K_D.

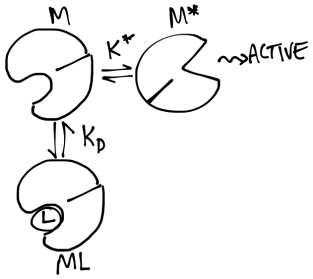
   (a) First, we start by thinking about an enzyme in equilibrium between an inactive form (M) and an active form (M^*). **Given the following scheme, write out the definition of K^*, and then solve for $[M^*]$.**

   (b) Next, we can think about binding of ligand L. It can *only* bind to the inactive form of the protein M. **Given the following scheme, write out the definition of K_D and then solve for $[ML]$.**

   (c) Now we're going to put the schema together. The different forms of the protein in this scheme are: $[M]$, $[M^*]$, and $[ML]$. **Write an expression for θ^*, the fraction of the molecules that are in the active, M^* form.**

   (d) Substitute your definitions from 1a and 1b into your equation from 1c and then simplify. There you go. You've now derived an expression modeling allostery. Now we can play with it to understand interesting behavior.

2. Next, we'll explore what kinds of K^* values make a good allosteric switch. Using your expression from 1:

   (a) What happens to θ^* as $K^* \to \infty$?

      i. Would such an enzyme be active or inactive?

      ii. Could it be allosterically regulated by L?

   (b) What happens to θ^* as $K^* \to 0$?

      i. Would such an enzyme be active or inactive?

      ii. Would it be allosterically regulated by L?

(c) What happens to θ^* when $K^* = 1$?

i. Would such an enzyme be active or inactive?

ii. For $K^* = 1$ and a $K_D = 1\ nM$ calculate the following and sketch a graph of $[L]$ vs. θ^*:

$[L]$ (nM)	$K_D/[L]$	θ^*
0.01		
0.1		
1		
10		
100		

iii. As you increase $[L]$, what happens to θ^*?

iv. For this K^*, can enzyme activity be regulated by $[L]$?

(d) Now, follow up your analysis of K^* by looking at two more possible values: 0.01 and 100.

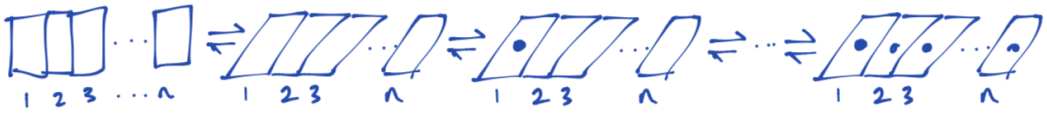
i. Using the same approach as in 2(c)ii, populate the following table for $K^* = 0.01$ and $K^* = 100$.

$[L]$ (nM)	θ^*, $K^* = 0.01$	θ^*, $K^* = 1$	θ^*, $K^* = 100$
0.01			
0.1			
1			
10			
100			
1,000			
10,000			

ii. Which value of K^* makes a good allosteric switch? Why or why not?

iii. If you followed activity vs. $[L]$ and extracted a "K_D" from the curve, how would it differ from the K_D for binding to M? Why does it differ?

3. In class, we studied the following scheme for cooperativity:

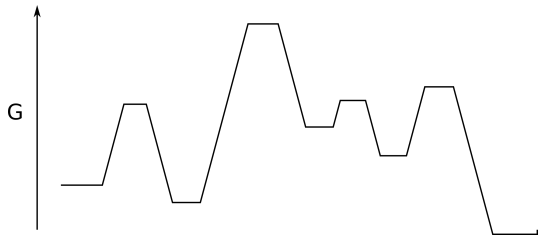


$$\theta = \left(\frac{n[X]}{K_D}\right) \frac{K^*(1 + [X]/K_D)^{n-1}}{1 + K^*(1 + [X]/K_D)^n}$$

What range of values for K^* give you cooperativity for $n = 4$? What does this tell you about the mechanism of cooperativity?

4 Kinetics

1. The energy diagram for a multi-intermediate enzyme mechanism is shown below:

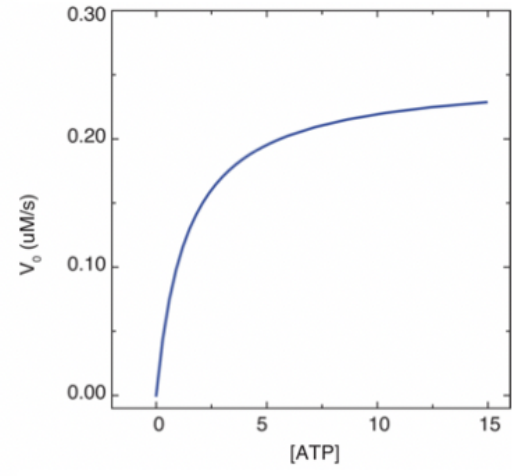


   (a) You determine that a mutation of the enzyme causes a 250-fold decrease in k_{obs} (rate of product accumulation). What is the change in the energy of the transition state for the mutant compared to the wt enzyme at 300 K?

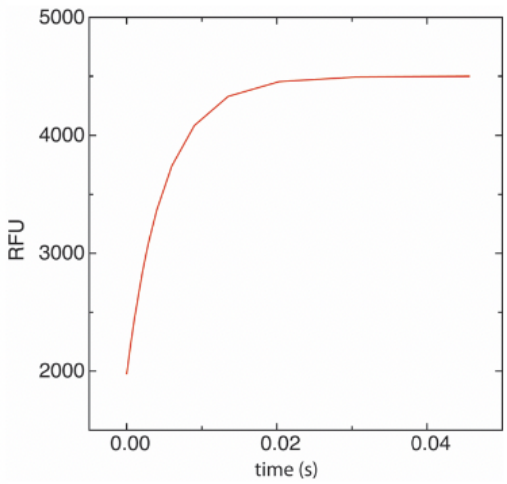
   (b) Can you determine which elementary step(s) have been affected by the mutation based on your experiments? Why or why not?

2. Myosin function and nucleotide binding: You wish to determine the rate-limiting step for the ATPase cycle for a Type II myosin.

   (a) You set up a series of reactions in which you measure the initial rate of ATP hydrolysis in the presence of 25 nM myosin and a range of concentration of ATP. Shown below is the plot of the initial velocity versus the initial ATP concentration. What is the k_{cat} for the hydrolysis of ATP by myosin? (Note that the units for [ATP] are μM and $V_{max} = 0.25\ \mu M \cdot s^{-1}$).



   (b) In a separate experiment, you preincubate 25 nM myosin with 4 μM ADP, add 500 μM mant-ADP (a fluorescent analogue of ADP), and then measure the fluorescence signal versus time. Shown below is the data. Write an equation that expresses the fluorescent signal as a function of time, expressing RFU (relative fluorescent units) as a function of time, k_{obs}, RFU_{max} and RFU_{min}.



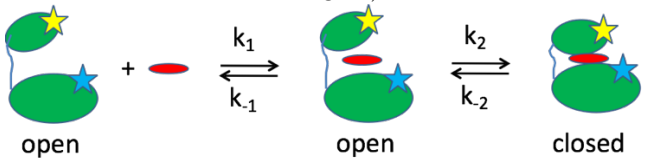
(c) The k_{obs} value you calculate for this experiment is $200 \cdot s^{-1}$. If you repeat the experiment with 50 nM myosin, will you expect the value of k_{obs} to increase, decrease, or stay the same? Explain your answer.

(d) To measure the interaction of ADP with myosin, you set up a reaction in which you rapidly mix a range of concentrations of ADP with10 nM myosin and measure the increase in fluorescence. $[ADP] \gg [myosin]$ in these experiments. A fit of k_{obs} versus $[ADP]$ for each reaction yields a straight line with a slope of 10 $\mu M^{-1} \cdot s^{-1}$. Based on this experiment and information presented earlier in this problem, calculate the binding affinity of ADP for myosin.

(e) For the reaction conditions in (d), calculate the concentration of myosin ADP when $t = 4\ ms$ and $[ADP]_0 = 15\ \mu M$.

(f) Based on the information in this problem, is ADP release the rate-limiting step for ATP hydrolysis when ATP is saturating? Explain your answer.

3. Single molecule assays to measure rate constants for a conformational change: During the catalytic cycle of a protein kinase, the nucleotide binding cleft undergoes a round of opening, closing, and then reopening. Without ATP, the open conformation is favored. ATP binding causes the nucleotide cleft to close. You attach two fluorophores to the kinase to measure the opening and closing of the cleft using FRET (cleft closure increases the FRET signal).



(a) You add a very high concentration of ATP to the reaction so that the rate of ATP association is much greater (many orders of magnitude) than the rate of cleft closure. You measure the FRET signal as a function of time in a bulk assay. Write an equation that expresses the FRET signal as a function of time and k_{obs}.

(b) Write an equation that expresses k_{obs} as a function of the relevant microscopic rate constants shown in the reaction scheme above.

(c) Under these conditions, you measure k_{obs} to be 58 s^{-1}. If the equilibrium ratio of closed to open kinase with ATP bound is 28:1, what are the values of the microscopic rate constants k_2 and k_{-2}?

(d) You repeat the bulk binding experiment at multiple concentrations of ATP. In each case, the concentration of ATP is much greater than the kinase, so $[ATP]_0 \approx [ATP]_{final}$. However, you

observe that kobs changes as a function of $[ATP]_0$. Draw on the plot showing the expected relationship between k_{obs} and $[ATP]_0$ and explain the shape of your plot.

(e) You wish to monitor opening and closing of single molecules of the kinase. You attach the labeled protein to a cover slip and measure FRET of individual molecules of the kinase using TIRF microscopy under saturating concentrations of ATP. Assume that the conformation of the kinase is two state. That is, it is either in the open or the closed conformation; intermediate states are not populated. Draw a graph showing the FRET efficiency versus time you expect to observe as you monitor a single molecule of the kinase. Assume that you can measure the fret efficiency fast enough that you can clearly observe the two states experimentally. Label the "open" and "closed" states.

(f) You observe many single molecule open and closing events and measure the lifetimes of the closed or open state for each event. Based on your answer to c, what is the average lifetime of the closed state?

(g) What would the plot of FRET efficiency versus time look like for a bulk assay in which you monitor the FRET of a 250 μL solution of a 10 nM kinase equilibrated with a saturating concentration of ATP? Draw your expected results.

(h) What would the plot of FRET efficiency versus time look like for a bulk assay in which you monitor the FRET of a 250 uL solution of a 10 nM kinase equilibrated without ATP? Draw your expected results.