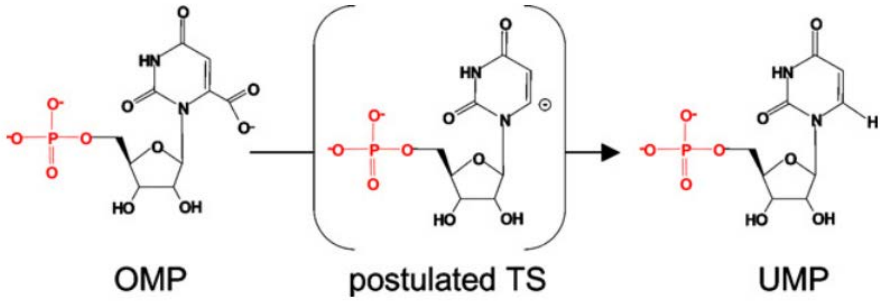
**Problem Set 2**

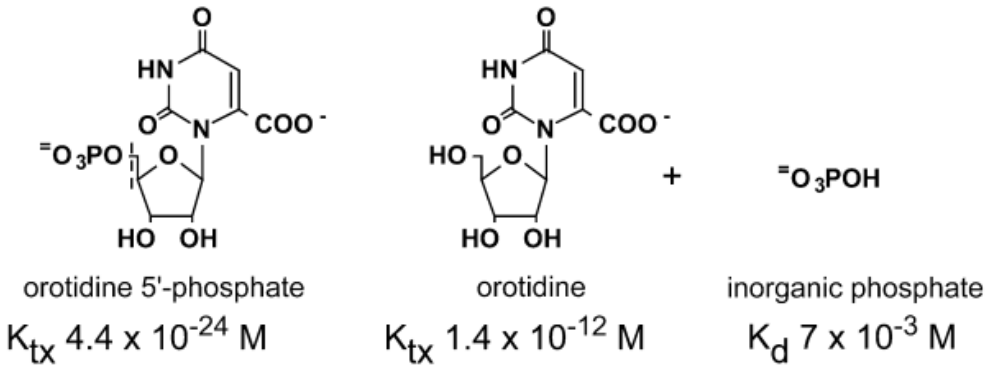
**BMB178**
**Fall, 2016**

**Due 11/02/2016, noon**
**Office Hour: 7-9pm 11/01/2016, 121 Braun**

**Problem 1 (25 points):**
Orotidine 5'-phosphate decarboxylase (OMP decarboxylase) catalyzes the following reaction:




(a) The estimated $K_{tx}$ (maximal dissociation constant) for the transition state of the reaction is $4.4 \times 10^{-24}$ M. Dividing the substrate into two pieces substantially weakens binding to the enzyme. For example,




What is the effective concentration achieved by the OMP decarboxylase active site with OMP in the above case? Using a standard state of 1M, calculate the free energy change at 298K for the effect of dividing the substrate into the two pieces shown above. (6 points)

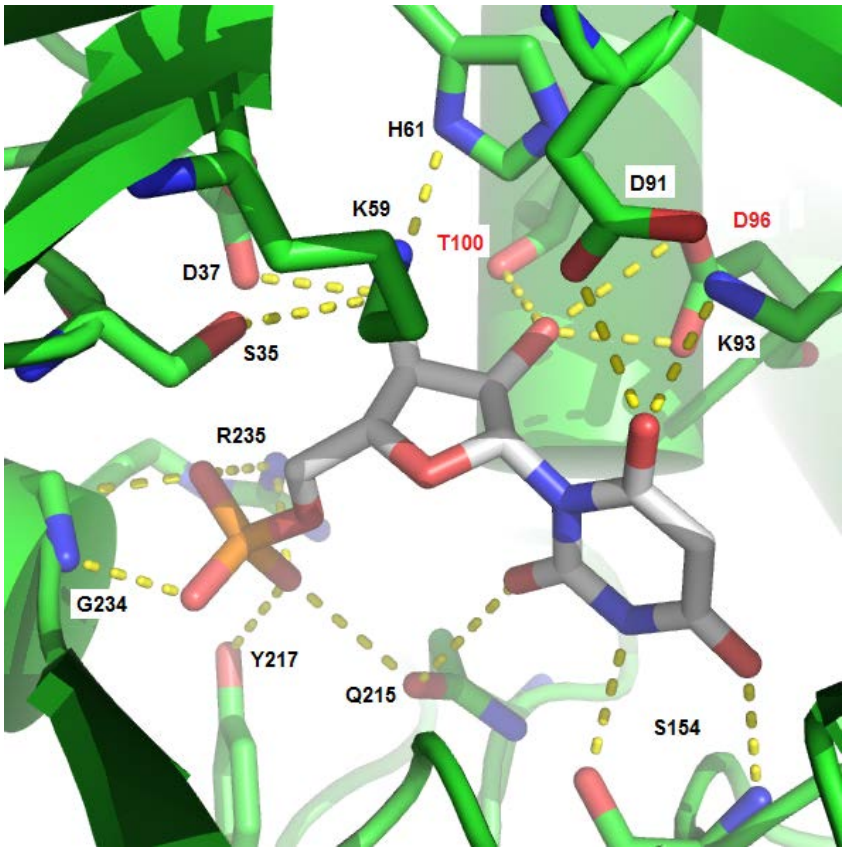
To evaluate the chelate effect, we can calculate the effective molarity (EM):

$$EM = K_A K_B / K_{AB}$$
$$= (1.4 \times 10^{-12}\ M)(7 \times 10^{-3}\ M) / (4.4 \times 10^{-24}\ M)$$
$$= \mathbf{2.2 \times 10^{9}\ M}$$

We can then calculate the free energy change ($\Delta G_{conn}$):

$$\Delta G_{conn} = -RT \ln(EM)$$
$$= -(8.314\ J\ K^{-1}\ mol^{-1})(298\ K) \ln(2.2 \times 10^{9})$$
$$= -53\ kJ/mol = -13\ kcal/mol\ (\rightarrow \mathbf{13\ kcal/mol})$$

(b) Using PyMOL, create a figure of OMP decarboxylase (pdb: 1DQX) that illustrates the non-covalent binding interactions between OMP decarboxylase and an inhibitor, 6-hydroxyuridine 5'-phosphate (BMP). Using the figure and the discussions in lecture, explain how large rate enhancement can be achieved by OMP decarboxylase with OMP as a whole but not fragmented. (6 points)



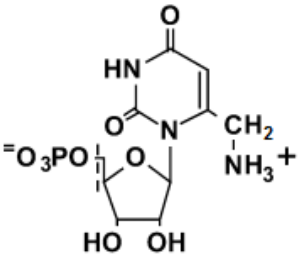

OMP decarboxylase forms an extensive network of non-covalent interactions to correctly orient its substrate for catalysis. Ten different residues help position the orotidine moiety of the substrate (S35, D37, K59, H61, D91, K93, S154, Q215, D96, T100), while four residues help position the phosphate (Q215, Y217, G234, R235). Connecting these two pieces greatly reduces the possible conformations of the substrate and provides more available binding interactions within one molecule, effectively reducing the entropy. In other words, when the enzyme binds part of the molecule, it already paid for a large fraction of the entropic cost for localizing the other parts of the substrate to the active site so that the instrinsic binding energy for binding other components of the substrate is fully realized.

(c) What type of inhibitor is BMP? Would you expect to see the same effect observed with dividing OMP for BMP? Why or why not? (5 points)

BMP mimics the structure of the transition state of the substrate, so it is a transition state analog inhibitor. You would also expect to see the same entropic effect for dividing OMP for BMP, since the entropic effect is general for the binding of substrates, products, and their analog inhibitors. However, entropic effects are generally much larger for transition state

analogs than for substrates due to the significantly more precise alignment required for the transition state at the active site.

(d) Based on the crystal structure, suggest a possible role for residues 91 and 96 in the rate enhancement achieved by OMP decarboxylase. In light of this possible role, what differences would be expected between using OMP as a substrate versus the substrate illustrated on the right? (8 points)




Residues 91 and 96 are both aspartic acid residues that have negatively charged side chains pointed towards the negatively charged carboxylate group on OMP. Charge repulsion would occur between these residues and the substrate, and therefore one could suggest that these residues may have a role in destabilizing the ground state of the reaction to accelerate catalysis. If this was the case, reversing the charge (as in the substrate above) should increase the affinity between the substrate and the enzyme. However, this was ultimately not the case (see Callahan BP & Wolfenden R, *JACS*, 2004).

**Problem 2 (21 points):**

To test how T7-DNA polymerase handles mismatches that might occur during DNA replication, the following experiment was carried out. DNA polymerase was allowed to synthesize DNA using the labeled primer/template complex:

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5′ – GCCTCGCAGCCGTCCAACCAACTCA  (labeled primer)
     CGGAGCGTCGGCAGGTTGGTTGAGTAGGTCTTGTTT – 5′  (template)
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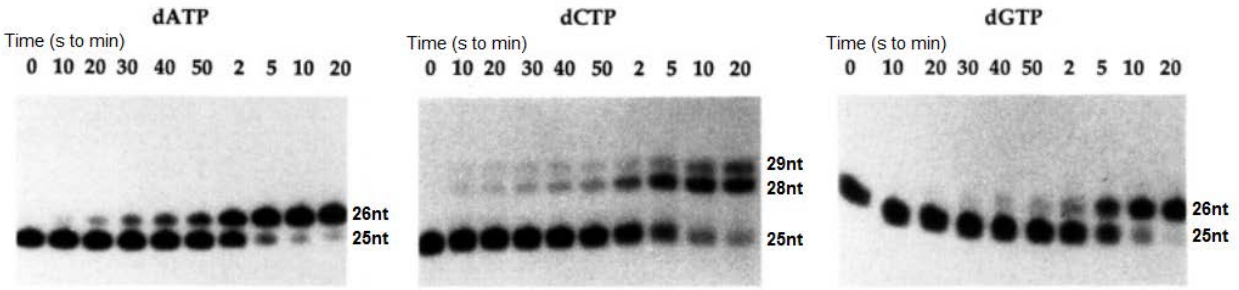
Replication reactions were carried out in the presence of only one dNTP and quenched at various time points. The DNA present at each time point was resolved via electrophoresis:

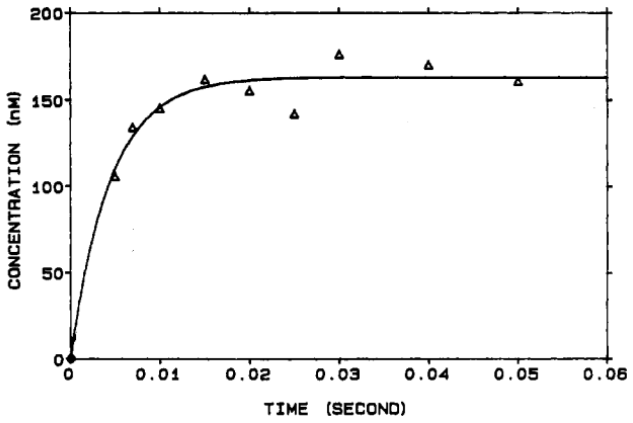



(a) What reaction(s) happened with dATP and dGTP? What reaction(s) happened with dCTP and why? Based on the data, estimate the half-time (the time where 50% of substrate is converted to product) for the reactions with dATP and dGTP. (6 points)

For dATP and dGTP, one nucleotide was added to the primer by the polymerase, which indicates one mismatched nucleotide was incorporated during replication. However, for dCTP, we can see either 3 or 4 nucleotides were added to the primer by the polymerase. Addition of 3 nucleotides occurs due to one mismatch followed by two correct incorporations of dCTP, while addition of 4 nucleotides indicates incorporation of a second mismatch to the

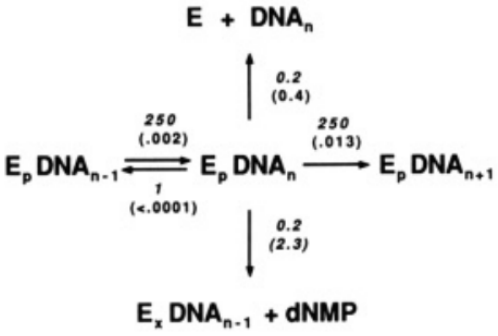
primer. The half-time for the dATP reaction is ~2 min, while the half-time for the dGTP reaction is ~5 min.

(b) In a separate experiment, the time course on the right was observed for the reaction using dTTP. Estimate the half-time for this reaction. What can you conclude about DNA synthesis with matched versus mismatched nucleotides? (6 points)




The half-time for the dTTP reaction is ~ 3 ms, which is much faster than the dATP and dGTP reactions. Therefore, matched nucleotides are much more efficiently incorporated compared to incorporating mismatched nucleotides.

(c) In addition to the previous data, subsequent experiments were performed to build the model shown below. In this model, the left to center reaction represents incorporation of either a correct or incorrect nucleotide; the center to the right reaction indicates incorporation of an additional nucleotide after the first; the top reaction represents dissociation of the product from enzyme; and the bottom reaction represents transfer of the product to alternative sites for destruction. The rate constants for the matched nucleotide are shown in *italics*, and the rate constants for the mismatched nucleotide are shown in parentheses.

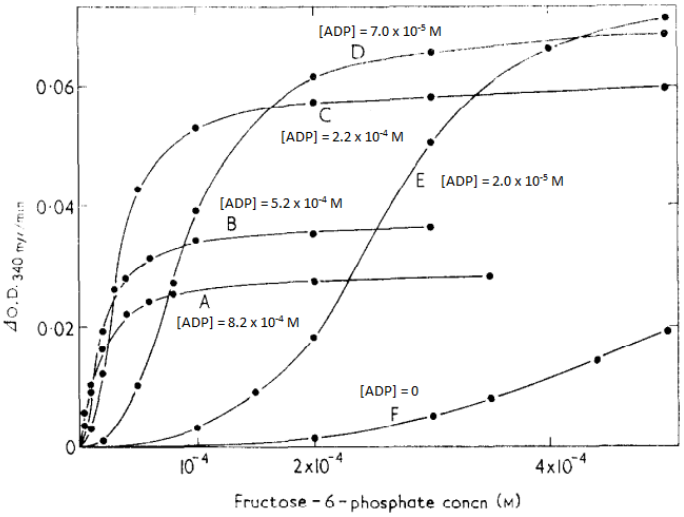



Based on the model, describe three different mechanisms DNA polymerase uses to ensure fidelity of replication. (9 points)

1. Differential binding: The DNA polymerase/template complex has about 10-fold higher affinity for matched nucleotides versus mismatched nucleotides.
2. Induced fit: Incorporating a matched nucleotide will induce the polymerase to a conformation that will allow for rapid incorporation of a second matched nucleotide. Incorporating a mismatched nucleotide will not induce a conformational change, and thus the rate of incorporating a subsequent nucleotide is much slower.
3. Kinetic proofreading: Following incorporation of a mismatched nucleotide, alternative pathways are more kinetically favorable compared to incorporating a second nucleotide.

**Problem 3 (30 points):**

Phosphofructokinase (PFK) catalyzes the phosphorylation of fructose-6-phosphate to form fructose-1,6-diphosphate using ATP. PFK shows allosteric effect towards one of its substrates, fructose-6-phosphate (F-6-P), but not towards ATP. Monitoring the initial rates of F-6-P phosphorylation by PFK under different conditions yields the figure on the right. In this figure, the X-axis shows the concentration of substrate, i.e. F-6-P, and the Y-axis represents the reaction rate. From curve A to F, the ATP concentration is kept constant, but the ADP concentration gradually decreases from ~$10^{-3}$ M to 0. Also note that ADP acts as a competitive inhibitor of ATP.



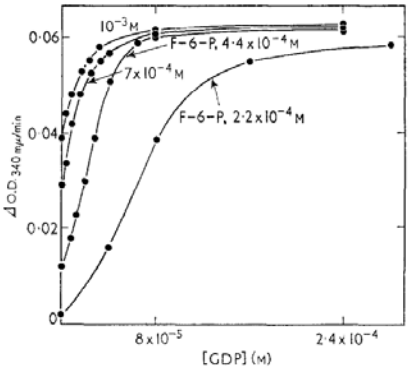

a) Judging from the shape of each curve, which ADP concentration gives the most allosteric effect? Which ADP concentration gives the least allosteric effect? Please describe a qualitative trend of the Hill coefficient from curve A to F. (6 points)

Curve A (high ADP concentration) has the least cooperativity. Curve F (low ADP concentration) has the most cooperativity. From A to F, the curves turn from standard Michaelis-Menten curves to cooperative ones. At lower substrate concentration, the initial rate is decreased from A to F, while at higher substrate concentration, the initial rate is increased. Therefore, the Hill coefficient increases from A to F.

b) Propose an explanation to the phenomena above based on the two-state allosteric model. Why does the allosteric effect change with different ADP concentrations? Why does the maximum reaction rate decrease at high ADP concentrations? (6 points)

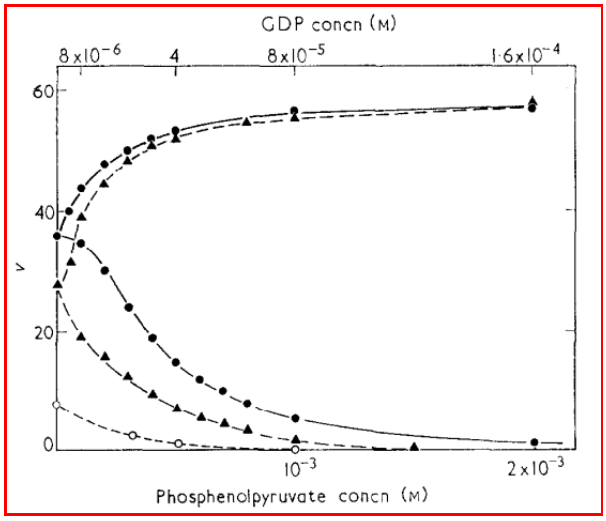
Model: PFK has two conformational states (R and T) whose equilibrium is shifted by the binding of ATP or ADP. The R state binds F-6-P stronger than the T state. In the absence of ATP or ADP, PFK is primarily in the T state. ADP or ATP also binds more strongly to the R than the T state. Therefore addition of ADP shifts PFK to the R state, so that substrate binding becomes better and the reaction rate becomes faster. However, ADP also acts as a competitive inhibitor for ATP and therefore high concentrations of ADP decreases the maximal reaction rate.

c) GDP serves as an allosteric regulator of PFK analogous to ADP. The influence of GDP or ADP on the initial velocity of the reaction can also be cooperative, and the stimulatory effect of GDP is influenced by substrate (F-6-P) concentration (see figure). Based on the two-state allosteric model, explain the effect of substrate on both the shape and magnitude of the stimulatory effect of GDP. (6 points)



Since F-6-P binds more strongly to the R than the T state, increasing F-6-P also shifts PFK towards the R state, to which GDP binds better. So the allosteric effect between F-6-P and GDP is reciprocal. At high F-6-P concentrations, PFK is primarily in the R state, so GDP binds tighter and the binding exhibits simple Michaelis-Menten behavior. At low F-6-P concentrations, the enzyme is largely in the T-state and GDP binding is required to shift PFK to the R state. This gives rise to more cooperative binding curves.

d) Phosphoenolpyruvate (PEP) serves as an allosteric inhibitor for PFK. What is the effect of increasing PEP concentration on PFK activity? Draw a figure to illustrate how the rate of reaction varies as a function of PEP concentration. How will the PEP inhibition curves differ at different concentrations of F-6-P? (8 points)
See the bottom three curves of the figure. The effect of PEP is just the opposite of GDP. At low F-6-P concentrations, the enzyme is predominantly in the T-state to which PEP binds strongly; under these conditions, reaction rate is slow but binding of this inhibitor is tight and shows no cooperativity. As F-6-P concentration increases, the enzyme transitions to the R state to which PEP does not bind well. Therefore, inhibition by PEP is weaker and exhibits cooperativity, as binding of the first molecules of PEP is required to induce PFK to the T state.



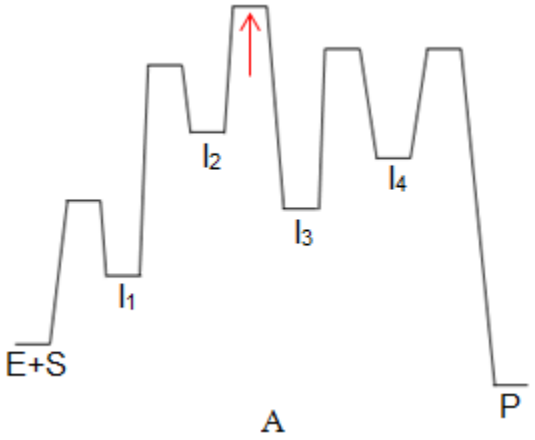

e) In the context of the glycolysis pathway, what is the importance of the fact that ADP stimulates PFK while PEP (a product of the glycolysis pathway) inhibits PFK? (4 points)
When ADP starts to accumulate in the cell, it will stimulate the glycolysis pathway to produce more ATP. When PEP, the product of glycolysis, accumulates, it will inhibit the glycolysis pathway. This kind of feedback control of glycolysis allows the cell to maintain a balance of glucose (the chemical storage of energy) and ATP (the currency of energy).

**Problem 4 (24 points):**

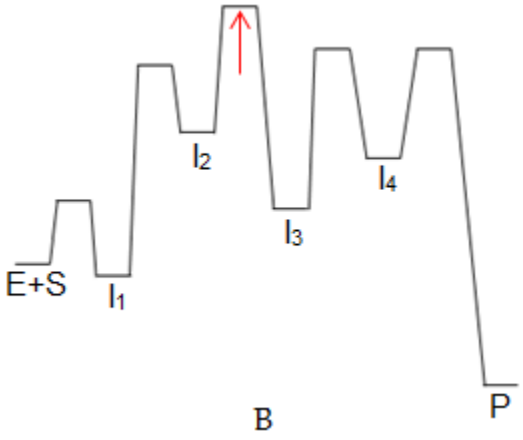
a) Consider the following reaction sequence, where substrate S is converted to product P by the enzyme E through several intermediates.

$$E + S \rightleftharpoons I_1 \rightleftharpoons I_2 \rightleftharpoons I_3 \rightleftharpoons I_4 \rightleftharpoons P$$

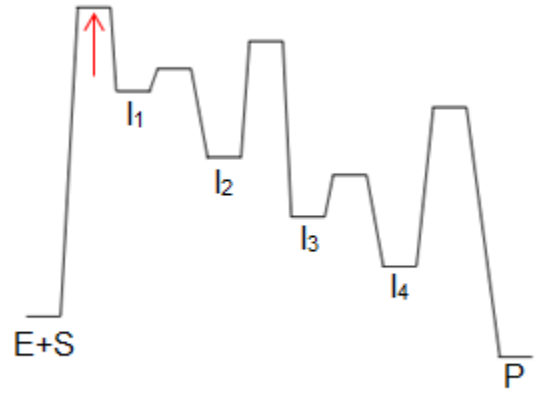
Identify the rate-limiting step in each of the following cases (A to F). Briefly explain your choices. (6 points)



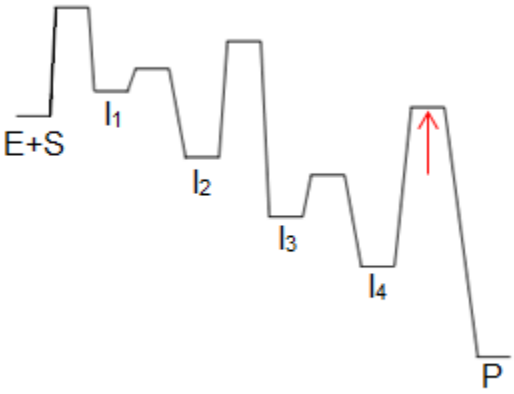

A



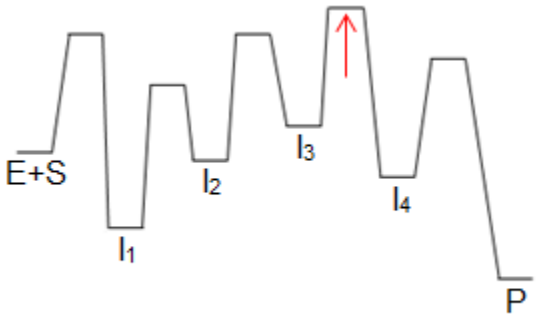

B



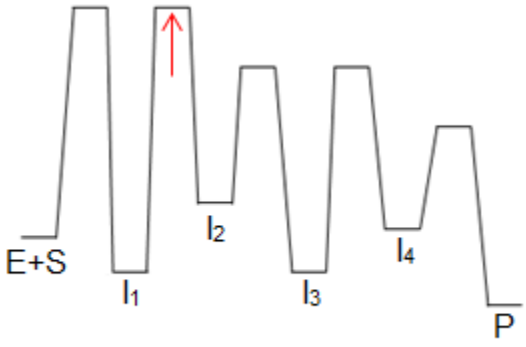

C




D




E




F

A: Step 3 is the rate determining step. The most abundant species is E+S, and step 3 has the highest energy transition state in the escape.

B: Step 3 is the rate determining step. The most abundant species is $I_1$, and step 3 has the highest energy transition state in the escape.

C: Step 1 is the rate determining step. The most abundant species is E+S, and step 1 has the highest energy transition state in the escape.

D: Step 5 is the rate determining step. The most abundant species is $I_4$, and step 5 is the only step between $I_4$ and P.

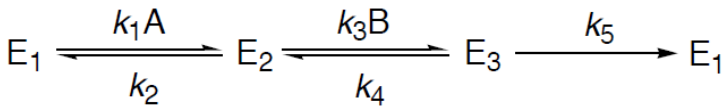
E: Step 4 is the rate determining step. The most abundant species is $I_1$, and step 4 has the highest energy transition state in the escape.

F: Step 2 is the rate determining step. The most abundant species is $I_1$, and step 2 has the highest energy transition state in the escape.

b) Scenarios A and C above can be separated from scenarios B and D into two groups based on their starting reaction conditions. What distinguishes these two groups and how can a difference in starting conditions affect the rate-limiting step? Which of the two groups would scenarios E and F fall under? (6 points)

Substrate is sub-saturating at the start of the reaction for scenarios A and C, while substrate is saturating for scenarios B and D. Under sub-saturating conditions, E+S is always the most abundant species, and therefore, the rate-determining step is the highest point on the free energy profile. Under saturating conditions, intermediates can become the most abundant species, and the rate-determining step can change to the one that has the highest energy barrier after the most abundant species along the reaction pathway. Scenarios E and F represent reactions performed under saturating conditions.

c) Consider the following reaction where two substrates, A and B, bind to the enzyme E.




Using the method developed by Cleland (Cleland, W.W., *Biochemistry*, **1975**, *14*, 3220), derive the overall observed rate constant for this reaction. Simplify the final answer (no fractions in the numerator or denominator). (6 points)

Using Cleland's method, the net rate constants for the three steps of the reaction are (in reverse order):

$$k_5' = k_5$$

$$k_3' = \frac{k_3[B]k_5}{k_4 + k_5}$$

$$k_1' = \frac{k_1[A]\frac{k_3[B]k_5}{k_4 + k_5}}{k_2 + \frac{k_3[B]k_5}{k_4 + k_5}}$$

The overall observed rate constant for the reaction (k) is equal to the reciprocal of the sum of the reciprocals of the net rate constants. Therefore:

$$k = \frac{1}{\frac{1}{k_5'} + \frac{1}{k_3'} + \frac{1}{k_1'}} = \frac{1}{\frac{1}{k_5} + \frac{1}{\frac{k_3[B]k_5}{k_4 + k_5}} + \frac{1}{\frac{k_1[A]\frac{k_3[B]k_5}{k_4 + k_5}}{k_2 + \frac{k_3[B]k_5}{k_4 + k_5}}}}$$

$$k = \frac{k_1 k_3 k_5 [A][B]}{k_1 k_3 [A][B] + k_1 k_4 [A] + k_1 k_5 [A] + k_3 k_5 [B] + k_2 k_4 + k_2 k_5}$$

d) For the same reaction, assume that [A] is constant and saturating. Derive the apparent first and second order rate constants with respect to B (i.e. $k_{cat}$ and $(k_{cat}/K_m)^B$). Explain the significance of these two rate constants. (6 points)

Since [A] is saturating, the observed rate constant reduces to:

$$k_{app} = \lim_{[A]\to\infty} k = \lim_{[A]\to\infty} \frac{k_1 k_3 k_5 [A][B]}{k_1 k_3 [A][B] + k_1 k_4 [A] + k_1 k_5 [A] + k_3 k_5 [B] + k_2 k_4 + k_2 k_5}$$

$$= \lim_{[A]\to\infty} \frac{k_1 k_3 k_5 [B]}{k_1 k_3 [B] + k_1 k_4 + k_1 k_5 + \frac{k_3 k_5 [B] + k_2 k_4 + k_2 k_5}{[A]}} = \frac{k_1 k_3 k_5 [B]}{k_1 k_3 [B] + k_1 k_4 + k_1 k_5}$$

$$k_{app} = \frac{k_3 k_5 [B]}{k_3 [B] + k_4 + k_5}$$

The apparent first-order rate constant is:

$$k_{cat} = \lim_{[B]\to\infty} (k_{app}) = \lim_{[B]\to\infty} \left(\frac{k_3 k_5 [B]}{k_3 [B] + k_4 + k_5}\right) = \frac{k_3 k_5 [B]}{k_3 [B]}$$

$$k_{cat} = k_5$$

The apparent first-order rate constant is the rate constant of the reaction from the Enzyme-A-B complex to the product.

The apparent second-order rate constant is:

$$\left(\frac{k_{cat}}{K_m}\right)^B = \lim_{[B]\to 0} (k_{app}) = \lim_{[B]\to 0} \left(\frac{k_3 k_5 [B]}{k_3 [B] + k_4 + k_5}\right) = \frac{k_3 k_5 [B]}{k_4 + k_5}$$

$$\left(\frac{k_{cat}}{K_m}\right)^B = \frac{k_3 k_5 [B]}{k_4 + k_5}$$

The apparent second-order rate constant for B is the rate constant of the reaction between the Enzyme-A complex and substrate B.