Problem Set 1

BMB178
Fall, 2016

Due 10/14/2016, noon
Office Hour: 7-9pm 10/12/2016, 121 Braun

Problem 1:
Consider a general unimolecular reaction, where a substrate (S) is converted to a product (P):

At 25°C, the equilibrium constant (Keq) is 250 and the rate constant of the spontaneous reaction, k_{uncat}, is 1.2×10^{-4} s^{-1}. The reaction can be catalyzed by an enzyme, E, which provides a 10 kJ/mol stabilization of the transition state without forming detectable intermediates. (25 points)

(a) Calculate ΔG° and ΔG‡ for both the uncatalyzed and enzyme-catalyzed reactions. (8 points)

Uncatalyzed:

$$\Delta G° = -\mathrm{RT}\ln K_{eq} = -8.314\,J\cdot mol^{-1}\cdot K^{-1}\times 298\,K\times \ln(250) = -13.7\,kJ\cdot mol^{-1}$$

$$\Delta G^{\ddagger} = -\mathrm{RT}\ln\left(\frac{k_{uncat}h}{k_B T}\right)$$

$$= -8.314 J\cdot mol^{-1}\cdot K^{-1}\times 298\,K\times \ln\left(\frac{1.2\times 10^{-4}s^{-1}\times 6.626\times 10^{-34}Js}{1.38\times 10^{-23}J\cdot K^{-1}\times 298K}\right)$$

$$= 95.4\,kJ\cdot mol^{-1}$$

Catalyzed:

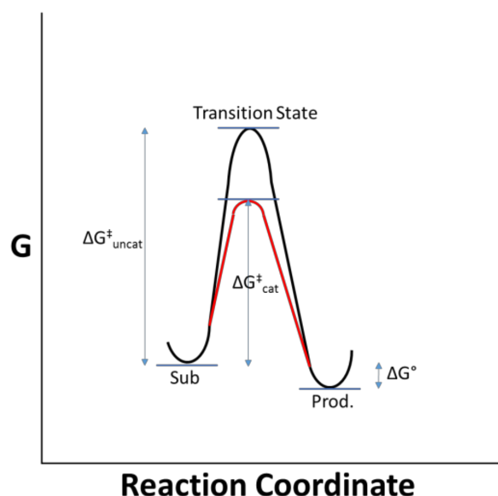
$$\Delta G° = -\mathrm{RT}\ln K_{eq} = -8.314\,J\cdot mol^{-1}\cdot K^{-1}\times 298\,K\times \ln(250) = -13.7\,kJ\cdot mol^{-1}$$

$$\Delta G^{\ddagger} = \Delta G^{\ddagger}_{uncat} - 10\,kJ\cdot mol^{-1} = 85.4\,kJ\cdot mol^{-1}$$

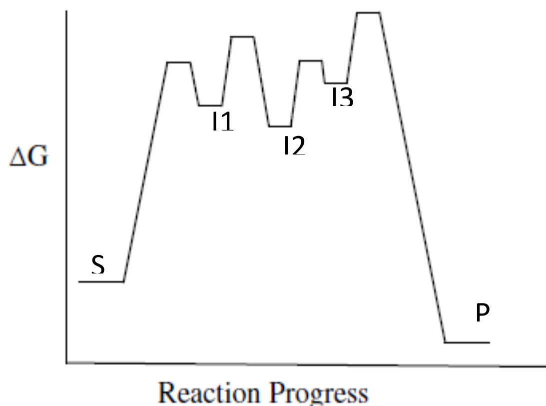
(b) Calculate the rate enhancement k_{cat}/k_{uncat} of the enzyme. (5 points)

$$\frac{k_{cat}}{k_{uncat}} = \exp\left(\frac{\Delta G^{\ddagger}_{uncat} - \Delta G^{\ddagger}_{cat}}{RT}\right) = \exp\left(\frac{10^4 J}{8.314\,J\cdot K^{-1}\times 298\,K}\right) = 56.6$$

(c) Draw a free energy diagram to scale showing both the uncatalyzed and catalyzed reactions. Label the substrate, transition states, and product appropriately. Show and label ΔG° and ΔG‡ for both reactions. (5 points)



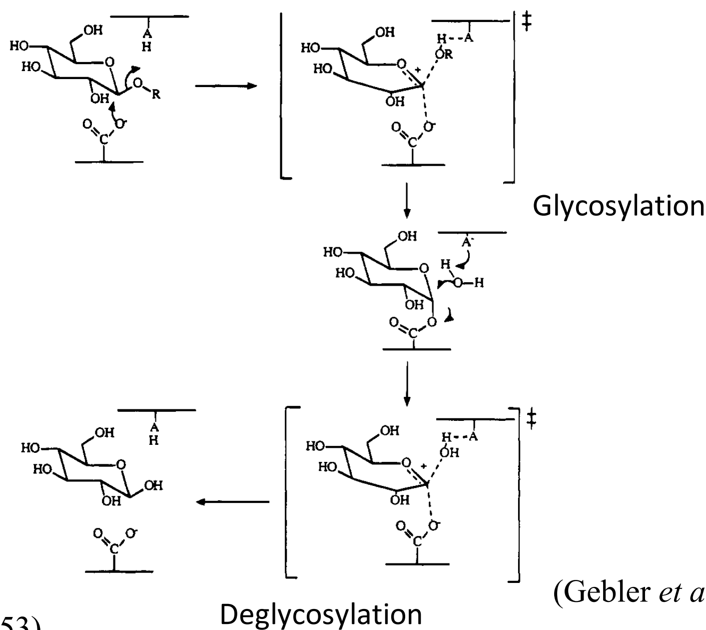
(d) Consider the following free energy diagram for a different enzyme catalyzed reaction. Label S, P and the three intermediates on this diagram. (2 points)



(e) The reaction progresses through three intermediates. Which intermediate would be the best structural mimic for designing an inhibitor of the enzyme? Explain your answer. (5 points)
The third intermediate in the reaction is the least stable intermediate. According to the Hammond postulate, its structure most closely resembles the structure of the transition state of the reaction. Since the enzyme binds most tightly to the transition state, the third intermediate is the best choice.

Problem 2:
The work by Wang *et al* (*Biochemistry*, 1995) focused on the analysis of catalytic strategies taken by β-Glucosidase. (25 points)

(a) Draw the step-wise electron-pushing mechanism of a β-glucoside hydrolysis catalyzed by β-Glucosidase. The enzyme can be simplified to two amino acids at the active site in the reaction scheme. (3 points)



(Gebler *et al. Biochemistry* **1995,** *34* (44), 14547-53)

(b) What are the three evidences showing that Glu170 is a general acid-base catalyst? (12 points)

i. For E170G mutant, the rate-limiting step is glycosylation with PNPG as the substrate and deglycosylation with DNPG. The E170G mutation decreased the glycosylation rate with PNPG by 10600-fold, whereas with DNPG only 2.4 fold, implying that E170 is a general acid catalyst. Similarly, the deglycosylation rate with DNPG decreased by 1760-fold, indicating E170 a general base catalyst.

ii. The k_{cat} of E170G did not show pH dependence compared to wild-type enzyme.

iii. The k_{cat} for DNPG hydrolysis by E170G increased significantly upon addition of nucleophiles.

(c) Why did sodium azide rescue the activity of the enzyme mutant? (4 points)

The rate-limiting step for DNPG is deglycosylation. For E170G, there is no longer carboxylate group to help generate hydroxide to attack the glycosyl-enzyme intermediate. The azide here serves as a strong nucleophile to compete with water to remove the glycosyl group from the enzyme.

(d) Considering Figure 6a, why are the two Brønsted slopes different? (3 points)

Without azide, the rate-limiting step is deglycosylation, which is independent of leaving group; in the presence of azide, the deglycosylation rate is enhanced, so now the rate-limiting step is glycosylation, which depends on the leaving group ability.

(e) In Figure 7, why isn't the rate of PNPG affected by azide concentration? (3 points)

The rate-limiting step for PNPG is glycosylation, which is not affected by addition of nucleophiles.

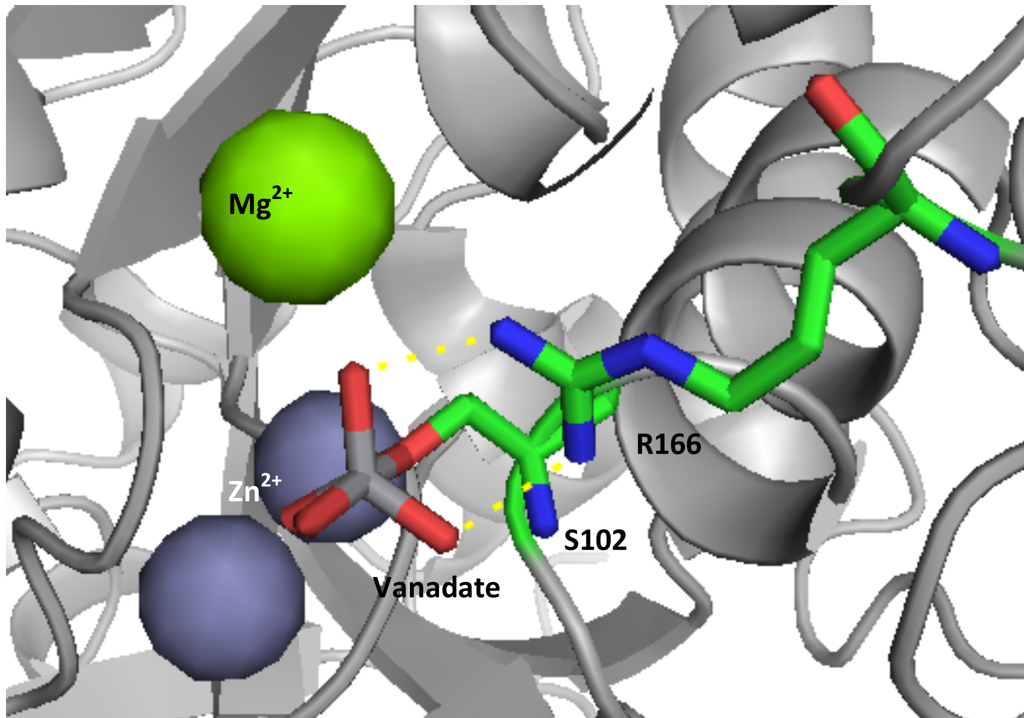
Problem 3:

Alkaline phosphatase (AP) of *E. coli* is a very efficient enzyme catalyzing the hydrolysis of phosphate monoesters more than 10^{20} fold. (25 points)

(a) Download the structure 1B8J from PDB. This is an E. coli AP structure in complex with vanadate. What is the role of vanadate here? Is the structure more similar to the ground state or transition state? (5 points)

Vanadate is an inhibitor, which mimics the structure of the transition state of the phosphorous being attacked by water before the –OR group leaves. The enzyme structure here is thus more similar to the transition state.

(b) Use Pymol to highlight the active site, and show the contacts between the vanadate and the enzyme. (3 points)



The vanadate is covalently bonded with S102, and contacts R166 with hydrogen bonds. The oxygens on the vanadate coordinate to the two zinc ions.

(c) According to the structure, what might be the catalytic role of R166? (2 points)
R166 catalyzes the reaction by stabilizing the transition state.

Please refer to Andrews *et al*. (PLos Biol, 2013) for the following questions.

(d) According to Table 1, what is the catalytic role of S102? Please explain based on the data. (4 points)
By mutating S102 to Glycine or Alanine, the affinity of the enzyme to phosphate increased at least 1000 fold, meaning that S102 destabilizes the E·S ground state and reduces the energy barrier for the reaction.

(e) Why did the authors measure the affinity of Pi to the enzyme instead of phosphate monoester in order to determine the ground state stability? What assumption did they make? (3 points)
They couldn't use phosphate monoester because trace contamination of Pi in the stock dominates the binding with S102 mutants. In addition, trace contamination of phosphatase activity in S102 mutants can generate Pi from phosphate monoester substrates. They measured the binding affinity of Pi to determine ground state stability based on the assumption that the structure of E·S is similar to E·P. Therefore, destabilization of E·P is equivalent to destabilization of E·S.

(f) Why did the authors mutate R166? (3 points)
If S102 is mutated in the wild type AP, the binding to Pi was extremely tight so it could not be used in any kinetic assay. Therefore, mutations on R166 were introduced to reduce binding affinity to reasonable range for the assay.

(g) In Figure 4a, why does the phosphate affinity of R166S show a bell-shaped pH dependence? How do the authors verify their prediction for the acidic pH region? (5 points)
pH dependence of Pi binding can provide information about which phosphate species is binding to the enzyme and/or protonation events on enzyme that may affect interaction. The bell shaped curve indicates that two protonation events, one in acidic region the other in basic region, are changing the binding pattern. The authors predict that the acidic region is caused by change in phosphate species. pKa of H2PO4- is 6.9,which corresponds to the peak of bell-shaped pH dependence curve. The authors verify this by using an alternative substrate, HWO4-, which does not have a pKa around 6.9. The linear curve in acidic region is absent in this case of HWO4-, which confirms that the pH dependence in acidic region is indeed caused by pKa of phosphate species.

Problem 4:
Please refer to the paper by Lassila *et al*. (PNAS, 2010). The authors analyzed the catalytic contributions of a *de novo* designed enzyme. (25 points)

(a) How were the retroaldolases designed? Please briefly describe the logic. (6 points)
First the catalytic mechanism needs to be defined in order to determine the residues to generate the active site, which is then optimized to achieve high affinity with the transition state. Secondly, the protein scaffold needs to be chosen to accommodate the composite active site. The neighboring residues are then optimized to ensure proper catalytic geometry and substrate positioning.

(b) What are the three elements that were intentionally designed to contribute to the catalysis? According to the analysis in this study, how much does each of them contribute? (6 points)
   i. A hydrophobic pocket was designed to lower the pKa of the catalytic lysine. The shift of the lysine pKa from 10.6 to 6.8 in enzyme RA61 contributes to 10-fold rate enhancement.
   ii. A hydrogen network was designed to stabilize a water molecule to facilitate proton transfer. However, in the enzyme RA61, the hydrogen-bond interactions of Tyr78 and Ser87 with the water molecule did not contribute to the rate enhancement.
   iii. The interaction of the enzyme with the hydrophobic surface of the substrate was designed to stabilize the transition state. It provides about 500-fold rate enhancement.

(c) Why did the author compare the rate constants of enzyme-catalyzed reactions with that of free lysine-catalyzed reaction, instead of k_{uncat}? (3 points)
The retroaldol reaction in solution, without additional catalyst, is specific-base catalyzed and the mechanism is different from the enzyme-catalyzed reaction. It is thus more reasonable to compare the enzyme with free lysine.

(d) In Figure 4b, why does the rate show maximum at pKa = 7.5? (4 points)
In a buffered reaction, the amine is more nucleophilic with higher pKa, but the concentration of active neutral species is lower. The combined effect of reactivity and concentration results in the maximum at the pH of the reaction condition.

(e) What were the functions of Tyr78 and Ser87? Did they work as expected? Why or why not? (6 points)
Tyr78 and Ser87 were intended to stabilize a water molecule in order to facilitate proton transfer, but mutation of these two residues did not decrease the enzyme activity. In fact, the

rate was enhanced. One possible reason is that the active site is already open enough for the bulk solvent to help proton transfer, and mutation of the residues makes the active site even more accessible.

(f) **(Bonus)** What are other possible elements that can accelerate the reaction rate? What experiments can be done to verify the assumption? (3 points)