Problem Set 1

Problem 1

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

\[ K_{eq} \quad S \rightleftharpoons P \]

At 25°C, the equilibrium constant \( K_{eq} \) is 250 and the rate constant of the spontaneous reaction, \( k_{uncat} \), is \( 1.2 \times 10^{-4} \, s^{-1} \). The reaction can be catalyzed by an enzyme, E, which provides a \( 10^6 \)-fold rate enhancement without forming detectable intermediates. (20 points)

(a) Calculate the \( \Delta G^\circ \) for the spontaneous and enzyme-catalyzed reaction. (3 points)

Answer:
\[ \Delta G^\circ = -R \cdot T \cdot \ln K_{eq} = -8.314 \, J/mol \cdot K \times 298 \, K \times \ln(250) = -13.7 \, kJ/mol \]

(b) Calculate \( \Delta G^\dagger \) for both the uncatalyzed and enzyme-catalyzed reactions. (5 points)

Answer:
\[ \Delta G^\dagger_{uncat} = -R \cdot T \cdot \ln \left( \frac{k_{uncat} \cdot h}{k_B \cdot T} \right) \]
\[ = -8.314 \, J/mol \cdot K \times 298 \, K \times \ln \left( \frac{1.2 \times 10^{-4} \, s^{-1} \times 6.626 \times 10^{-34} \, Js}{1.38 \times 10^{-23} \, J/K \cdot 298 \, K} \right) = 95.4 \, kJ/mol \]
\[ \Delta G^\dagger_{cat} = -R \cdot T \cdot \ln \left( \frac{k_{cat} \cdot h}{k_B \cdot T} \right) \]
\[ = -8.314 \, J/mol \cdot K \times 298 \, K \times \ln \left( \frac{10^6 \times 1.2 \times 10^{-4} \, s^{-1} \times 6.626 \times 10^{-34} \, Js}{1.38 \times 10^{-23} \, J/K \cdot 298 \, K} \right) = 61.1 \, kJ/mol \]
(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 $\Delta G^\circ$ and $\Delta G^\dagger$ for both reactions. (5 points)

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

2. Refer to the study by Andrews et al. (PLoS Biol 11(7)). The authors studied interactions between Alkaline Phosphatase (AP) and substrates to elucidate the possible mechanism of enormous rate enhancement achieved by this enzyme. (25 points)

(a) What are some strategies that AP uses to provide such an enormous rate enhancement based on previous studies? (4 points)

From structural studies, it is obvious that metal ions are involved in the enzymatic reaction. Metal ion, Zinc, provides stabilization of negative charge build up on the leaving group oxygen. Zinc ion also provides activation of nucleophilic Ser102, which provides moderate rate enhancement. In addition, positioning of substrate likely provides additional rate enhancement.

(b) Compare scenarios in Figure 2C and 2D. By destabilizing the ground state, the barrier for reaction from E•S to E•P is decreased. This of course allows the reaction to proceed faster. What additional effect does this ground state destabilization have in enzyme activity? Why is this important? Think in terms of enzyme recycling. (6 points)

From the Figure 2D, destabilizing ES complex also destabilized EP complex. The likelihood of this depends on how similar ES is to EP. But assuming that this is the case, by destabilizing the EP complex, the barrier from EP to E+P is also decreased. This allows rapid release of the product from the enzyme, and prevent product inhibition. Thus, the enzyme can be rapidly recycled with faster turnover resulting in faster completion of the reaction.

(c) Why did the authors make mutations on R166? What is the function of R166 based on their structural analysis? (5 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. In order to reduce the binding of Pi to S102 mutants, it was necessary to introduce mutations on R166 to reduce binding affinity to reasonable range for the assay. R166 is known to interact with two of the phosphoryl oxygen atoms. As shown in Figure 3, without R166, the positioning of substrate is disrupted, which implies that this residue is
involved in proper positioning of the substrate for faster catalysis. Furthermore, previous studies showed that this residue is involved in stabilization of transition state.

(d) The authors test pH dependence of P<sub>i</sub> binding to AP. Why does R166S AP show a bell shaped pH dependence? How do the authors verify their prediction for acidic pH region? (5 points)

pH dependence of P<sub>i</sub> 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 H<sub>2</sub>PO<sub>4</sub><sup>-</sup> is 6.9, which corresponds to the peak of bell-shaped pH dependence curve. The authors verify this by using an alternative substrate, HWO<sub>4</sub><sup>-</sup>, which does not have a pKa around 6.9. The linear curve in acidic region is absent in this case of HWO<sub>4</sub><sup>-</sup>, which confirms that the pH dependence in acidic region is indeed caused by pKa of phosphate species.

(e) In the end, the authors estimate the ground state destabilization to be only 10<sup>3</sup>-fold. How do authors justify this result? What are some other possible contributions that help AP to achieve 10<sup>27</sup>-fold rate enhancement? (5 points)

As we discussed in class, enzyme does not use one strategy to reach the desired rate enhancement. Each mechanism provides moderate rate enhancement, and it is only in combination that the enzyme can reach enormous rate enhancement. Thus, authors say that 10<sup>3</sup>-fold rate enhancement is only small part of the mechanism. In addition to mechanisms discussed in (a), preferential transition state stabilization could be present. As implied by R166S mutation, proper positioning of substrate for catalysis is important for reaction. Slight difference in positioning between substrate and transition state would provide great rate enhancement. Additionally, other electrostatic repulsions (ground state destabilization) in substrate can lead to repositioning of the substrate for better catalysis.
3. The catalytic center of serine proteases is universally conserved. There are two major families of structures: trypsin-like serine proteases and subtilisin-like serine proteases. A typical catalytic center for subtilisin-like serine protease contains a ‘catalytic triad’ that includes a serine, a histidine, and an aspartic acid residue. (30 points)

(a) Download the crystal structure for subtilisin BPN’ serine protease (PDB: 1lw6, with inhibitor). This crystal structure contains a substrate analogue, CI2, at the active site. Make a figure showing catalytic center of this enzyme with substrate as well as residues in the ‘catalytic triad’ labeled accordingly. Assuming that CI2 is a “normal” substrate, i.e. it can be cleaved by serine protease, which peptide bond would be digested based on the crystal structure? Why? (6 points)

The peptide bond between M59 and E60 in CI2 will be cleaved. The reason is that S221, the catalytic center, is right pointing toward the carbonyl group of this peptide bond. This shows the first step of the hydrolysis reaction.

(b) Actually, CI2 acts as an inhibitor to serine proteases. It is proposed that the extensive hydrogen bonds from residue Thr58 to Arg67 provide a stable framework that prevents hydrolysis. Identify this hydrogen bonding network and make a figure. (5 points)
(c) According to the step-wise mechanism of serine proteases, which step or steps would be inhibited by CI2? Explain how each step may potentially be affected by this inhibitor. (5 points)

Serine proteases go through substrate binding, nucleophilic attach by Ser221 to form acyl-enzyme intermediate, recovery of enzyme, followed by product release. In the case of CI2, extensive hydrogen bonding network provides exceptionally stable substrate. The free energy required for the peptide bond to be broken and get released from the enzyme is high due to extensive hydrogen bonding network. In addition, even after formation of acyl-enzyme intermediate, the cleaved portion of the substrate stays bound due to hydrogen bonding network, which may allow re-ligation of the substrate from the acyl-enzyme intermediate. Thus, CI2 greatly stabilizes the ground state of the reaction, to the extent that reaction from acyl-enzyme intermediate to substrate is more favorable than to product.

(d) A series of mutations are introduced into wildtype serine protease, and their catalytic activity is determined as follows:

<table>
<thead>
<tr>
<th>Entry</th>
<th>Mutants</th>
<th>$k_{cat} \text{ (s}^{-1})$</th>
<th>$K_m \text{ (μM)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>wildtype</td>
<td>$(6.3\pm0.1)\times10^1$</td>
<td>440±30</td>
</tr>
<tr>
<td>2</td>
<td>S221A</td>
<td>$(5.4\pm0.3)\times10^{-5}$</td>
<td>650±90</td>
</tr>
<tr>
<td>3</td>
<td>H64A</td>
<td>$(1.9\pm0.1)\times10^{-4}$</td>
<td>1300±150</td>
</tr>
<tr>
<td>4</td>
<td>D32A</td>
<td>$(1.8\pm0.1)\times10^{-2}$</td>
<td>1400±120</td>
</tr>
<tr>
<td>5</td>
<td>D32A;H64A</td>
<td>$(1.8\pm0.1)\times10^{-3}$</td>
<td>460±40</td>
</tr>
<tr>
<td>6</td>
<td>H64A;S221A</td>
<td>$(5.2\pm0.2)\times10^{-5}$</td>
<td>480±60</td>
</tr>
<tr>
<td>7</td>
<td>D32A;S221A</td>
<td>$(5.9\pm0.3)\times10^{-5}$</td>
<td>460±80</td>
</tr>
<tr>
<td>8</td>
<td>D32A;H64A;S221A</td>
<td>$(7.8\pm0.3)\times10^{-5}$</td>
<td>730±70</td>
</tr>
</tbody>
</table>

(i) Consider the $K_m$ values. What can you conclude about the role of each residue in the catalytic triad in substrate binding? (5 points)

The $K_m$ values of wildtype serine protease and the mutants do not vary much from the point-mutations. The largest difference is only about 3-fold (entry 1 vs. 4). This suggests that S221, H64 and D32 residues may not contribute to the binding of the substrate.

(ii) Consider the $k_{cat}$ data. How much deleterious effect does the S221A mutation cause when this mutation is made in the wildtype protein? How much does the same mutation affect the reaction rate when D32 or H64 is already removed? Similarly, how much does the H64A mutation affect the reaction rate in the context of the wildtype enzyme vs. the D32A mutant? How do you explain this difference? Propose a model for the roles of each residue in this catalytic triad. (9 points)

S221A mutant will generally cause a $10^6$-fold decrease in $k_{cat}$. When D32 or H64 is removed from S221A mutant, the decrease is much smaller, 4-fold in the case of H64A and 300-fold in the case of D32A. H64A mutation reduces the reaction rate by $3\times10^5$-fold, but when D32A is
already removed this mutation has only a 10-fold effect. This indicates that S221 plays an indispensable role in the catalytic process, and further, that S221, H64 and D32 act in a cooperative network to facilitate the reaction. Without proton abstraction using H64 and D32 the S221 is a much less effective nucleophile.

Finally, H64 and D32 also work in concert with one another in a cooperative network. Without Asp32, H64 is much less effective as a general base. S221 acts as the covalent nucleophile. H64 is the general base that facilitates the deprotonation of Ser-OH in the transition state, and the negative charges on D32 help stabilize partial proton transfer to the imidazole ring of H64.

4. Ketosteroid isomerase catalyzes the following reaction using three key residues: Tyr16, Asp103, and Asp40. (25 points)

(a) Draw a possible electron-pushing mechanism of the isomerization reaction. What are the functions of the three key residues? (7 points)
Tyr16 and Asp103 provide hydrogen bonds with the enolate oxygen and thus stabilize the negative charge that is built up during the transition state and in the high-energy enolate intermediate. Asp40 acts as a general acid-base catalyst to facilitate proton transfer during the allyl isomerization.

(b) The ionized form of equilenin shown below serves as a competitive inhibitor of the enzyme. Based on the mechanism that you have proposed above, is equilenin a substrate-analog, a transition-stateanalog, or a product analog inhibitor of ketosteroid isomerase? Briefly explain. (5 points)

A transition-state-analog inhibitor. The planar structure of equilenin and the negative charge on the phenolate oxygen mimic the transition state of the isomerization reaction (see the mechanism above).

Surprisingly, a simple phenolate molecule can bind tightly to the active site of ketosteroid isomerase. Thus, researchers attempt to separate two different stabilization effects in the transition state: the electrostatic effect caused by the oxy-anion and the positioning effect caused by the rest of the steroid molecule.

(c) Consider the electrostatic effect. Mutation of Asp103 to Ala reduces the rate constant of the reaction (k_{cat}) 10^4-fold. What does this result suggest about the catalytic contribution of electrostatic effects to catalysis? How much is the contribution? (5 points)

The mutagenesis results suggest that the H bonding interaction from Asp103 contributes 22.8kJ/mol to preferential stabilization of the transition state and the high energy enolate intermediate.

\[ \Delta G = -RT \ln \left( \frac{k_1}{k_2} \right) \]

\[ = -8.314 \text{J/mol} \cdot \text{K} \times 298 \text{K} \times \ln(10^4) = -22.8 \text{kJ/mol} \]

(d) If electrostatic interactions with the substrate and the enolate-like transition state can be re-capitulated by binding of the phenolate molecule, what will fluorine substitutions on the phenolate ring do to the binding between the transition state and the enzyme? (8 points)
If this were true, then an electron-withdrawing group (like fluorine) that reduces the negative charge density on the phenolate oxygen will destabilize the binding of the phenolate to the enzyme.