Problem Set 1

BMB / Ch 178
Fall 2017

Due: 10/13/2017 (Friday)
Office Hours: 10/11/2017
7-9pm 121 Braun

1. In the study by Matouschek et al. (Nature 340: 122-126, 1989), the authors mapped the structure of the transition state of the unfolding reaction of barnase. (20 Points)

a) The approach involved careful selection of single residues for mutagenesis. In selecting these residues, what did the authors look for, and why was this important for the study? (6 Points)
The authors aimed to (1) use equilibrium measurements to determine the contribution of different parts of the protein to the equilibrium stability of the folded protein (relative to the unfolded state); and (2) use kinetic measurements to establish what fraction of the stabilizing interaction in the folded state (measured in part 1) is realized in the transition state of folding. To this end, mutations were placed at different locations on the protein to report on the folding status of the various structural elements. In addition, the authors made very conservative mutations to disrupt a small interaction in discrete regions of the structure because more radical mutations would disrupt multiple interactions, potentially introduce steric effects, and change interactions with the solvent. This would makes the interpretation of the result more difficult.

b) The authors concluded that the transition state for unfolding of barnase is compact and retains considerable secondary structure. State specific evidence that allowed them to draw this conclusion. (6 Points)
Refer to Table 1 in the paper and examine the Φ values for different mutations. Φ values close to 1 indicate significant disruption of the structure in the transition state, whereas Φ values close to zero suggest that the region under question is largely intact. Since conservative point mutations had only local effect on the structure and negligible change in solvent interactions, the values allowed the authors to map the structure of the transition state. Low Φ values resulting from mutation in the hydrophobic core show that the core of the structure is mostly intact in the transition state. Also, mutation of His18, which interacts with the C-terminus of the alpha helix, results in a Φ value close to 0.2. The low Φ value shows that this interaction is preserved in the transition state, suggesting that the helix is largely folded in the transition state.

c) In a similar study, the authors studied the unfolding of a small protein, p20. The free energy of unfolding is linearly dependent on urea concentration according to:

$$\Delta G = \Delta G_U^{H_2O} - m_U[Urea] \qquad (1)$$

where m is a proportionality constant that measures the degree of solvent exposure. A similar equation relates the urea concentration and the activation energy of unfolding:

$$\Delta G_U^{\ddagger} = (\Delta G_U^{H_2O})^{\ddagger} - m_U^{\ddagger}[Urea] \qquad (2)$$

The value of m_U from equilibrium unfolding experiments is 3.2. The data for the kinetics of unfolding are shown below, where k_U is the rate constant of unfolding. Calculate the fractional increase in solvent exposure ($m_U^{\ddagger}/m_U$) for the protein in the transition state of unfolding. What can you conclude about the structure of the transition state? (8 points)

[Urea] (M)	k_U (s^{-1})
3	3.16
4	5.01
7	12.0
8	15.8

Using the following formula, it is possible to calculate $\Delta G_U^{\ddagger}$ from k_U:

$$\Delta G_U^{\ddagger} = -RT \ln(\frac{k_u h}{k_B T})$$

We can thus convert the values of k_U into $\Delta G_U^{\ddagger}$ values at different urea concentrations :

[Urea] (M)	ku (s-1)	$\Delta GU^{\ddagger}$ (kJ/mol)
3	3.16	70.1
4	5.01	69.0
7	12.0	66.8
8	15.8	66.1

Plot $\Delta G_U^{\ddagger}$ as a function of [urea] and fit to equation 2. This gives an $m_U^{\ddagger}$ value of 0.78.

The fractional increase in solvent exposure in the transition state is therefore

$$\frac{m_U^{\ddagger}}{m_U} = \frac{0.78}{3.2} = 0.24$$

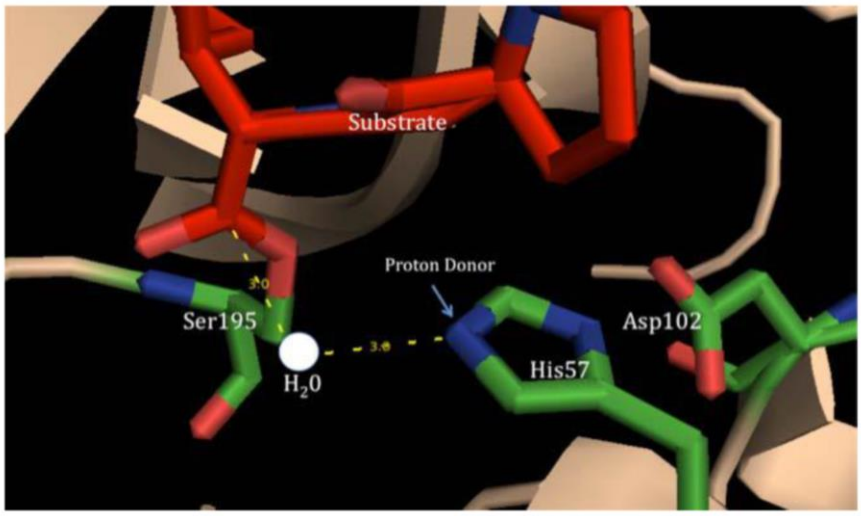
The small fractional increase in solvent exposure in the transition state compared to the unfolded structure suggests that the transition state of folding/unfolding remains globally folded, in which most of the protein is largely protected from solvent.

**Because I did not specify the units for $m_U^{\ddagger}$, if you used different units and got a different number, you still received full credit.

2. The catalytic center of serine proteases is universally conserved. A crucial step in the reaction mechanism for serine proteases is the formation of an acyl-enzyme intermediate, during which the substrate becomes covalently linked to a catalytic serine in the enzyme active site. (30 points)

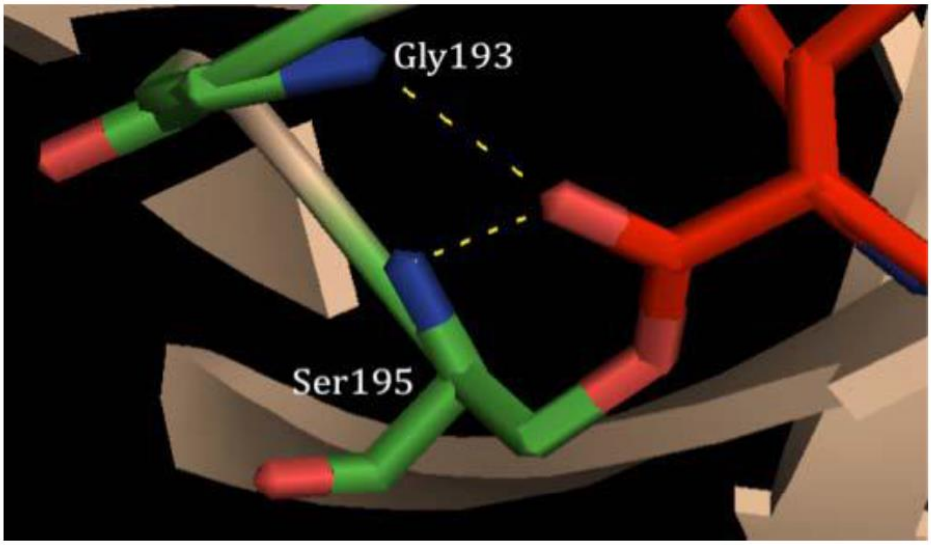
a) Download the crystal structure of porcine pancreatic elastase complexed with human beta-casomorphin-7 (PDB: 1QIX). Create an image using PyMOL that shows the active site interactions that assist in the *breakdown* of the acyl-enzyme intermediate. Start by

showing and labeling all the residues in the catalytic triad (Ser195, His57, Asp102) and the substrate (VEPI). Label the nucleophilic water and measure the distance between: (1) the nucleophilic water and the electrophilic carbonyl group in the substrate; and (2) the nucleophilic water and the general base that facilitate proton transfer. Lastly, suggest and label a reasonable proton donor that can help regenerate the hydroxyl group on Ser195 during the breakdown of the acylenzyme intermediate. (8 points)



His57 extracts a proton from the water molecule to generate the nucleophilic hydroxide ion. His57 is then poised to donate it back to S195 for regeneration.

b) The breakdown of the acyl-enzyme intermediate is energetically taxing due to charge accumulation on the reactive carbonyl of the substrate (forming an oxyanion). Elastase and other serine proteases circumvent this issue by placing <u>two</u> "stabilizing" residues in close proximity. Locate these residues using PyMOL and create an image showing the interactions. Briefly explain how the interactions aid in stabilization. (5 points)



Stabilization occurs because Gly193 and Ser195 use their main chain amido nitrogen groups to hydrogen bond with the oxyanion. The distances are approximately 2.7-2.9 Å.

c) Steady-state rate constants for pancreatic elastase and mutants are as follows:

Protein	k_{cat} (s^{-1})	K_m (μM)
wild type	$(2.0 \pm 0.1) \times 10^{1}$	400 ± 25
D102V	$(5.5 \pm 0.3) \times 10^{-2}$	1250 ± 25
H57V	$(5.5 \pm 0.2) \times 10^{-4}$	900 ± 25
S195V	$(2.3 \pm 0.1) \times 10^{-5}$	700 ± 25
D102E	$(5.7 \pm 0.2) \times 10^{-1}$	410 ± 35
S195T	$(2.5 \pm 0.1) \times 10^{-1}$	590 ± 44
G193A	??	??

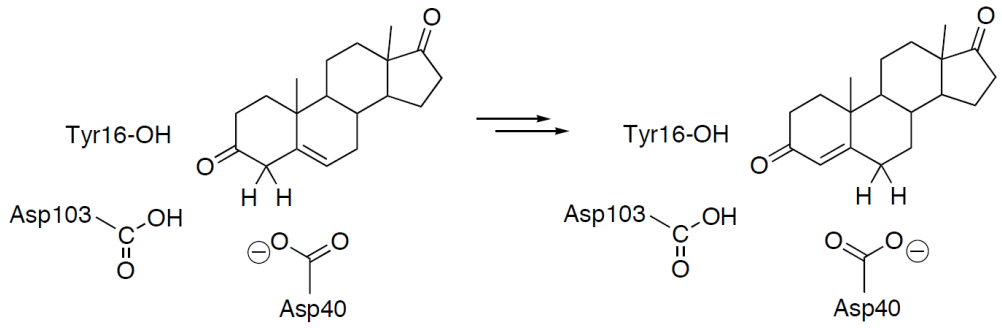
i) Consider the k_{cat} and K_m for the residues in the catalytic triad. What can you conclude about their roles in binding and catalysis? (5 points)
The values for Km, which relay information about substrate binding, vary by at most 4 fold amongst the mutants. The values for k_{cat}, which relay information about the chemistry step, are drastically decreased by the mutation. Therefore, the catalytic triad residues are not required for substrate binding and contribute mainly to catalysis.

ii) Consider the mutations D102E and S195T. Why do these mutations cause a modest defect compared to the corresponding D102V and S195V mutations*?* What is likely to be the primary source of defect associated with these mutations ?(6 points)

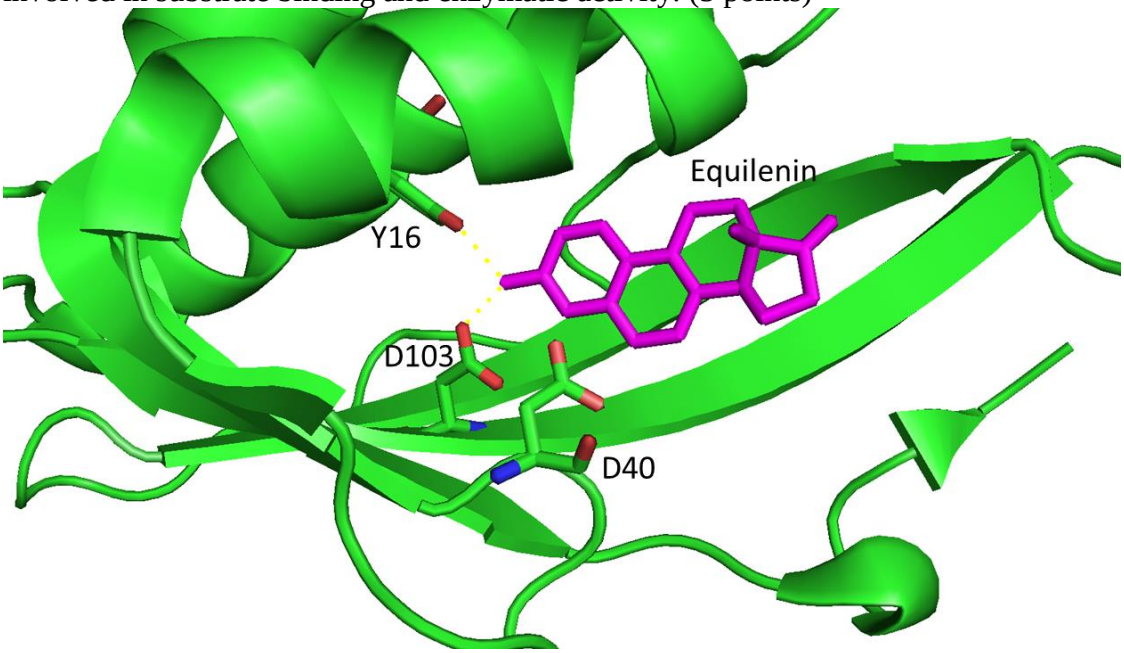
S195T: Ser195 does not participate in stabilizing interactions for substrate binding, so the value of K_m did not change. This mutation is less deleterious for catalysis because it preserves the nucleophilic nature of the hydroxyl group. The precise positioning of the nucleophile relative to the substrate is disrupted, however, resulting in a modest reduction in k_{cat}.
D102E: This is mutation is not very deleterious because it preserves the carboxylate group for hydrogen bonding with and stabilization of the imidazole in H57. The modest reduction in k_{cat} likely results from less precise positioning of the carboxylate group to H57 in the catalytic triad.

iii) Imagine that a new student wants to engineer a mutation to *destabilize* the oxyanion hole during breakdown of the covalent intermediate. The student suggests that G193 be mutated to an alanine. Is this a good suggestion? Explain why or why not. If not, suggest an alternate amino acid to which you would mutate G193. (6 points)
G193A is not a good mutation because G193 hydrogen bonds to the carbonyl oxygen in the intermediate using its backbone NH, which is unlikely to be unaffected by small side-chain mutations. A better mutation would be G193P, because the proline backbone does not provide a hydrogen bonding group.

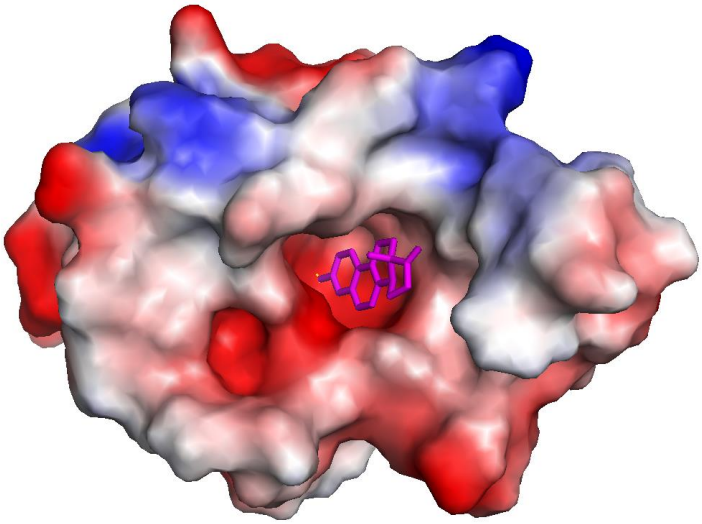
3. Ketosteroid isomerase catalyzes the following reaction using three key residues: Tyr16, Asp103, and Asp40. (30 points)



a) Download PDB: 1OH0. Generate a figure showing the substrate analogue, key residues involved in substrate binding and enzymatic activity. (5 points)



b) Generate electrostatic view of the enzyme showing the substrate in substrate binding pocket. What can you infer from this diagram? What kind of interactions are mainly involved for binding substrate? (3 points)



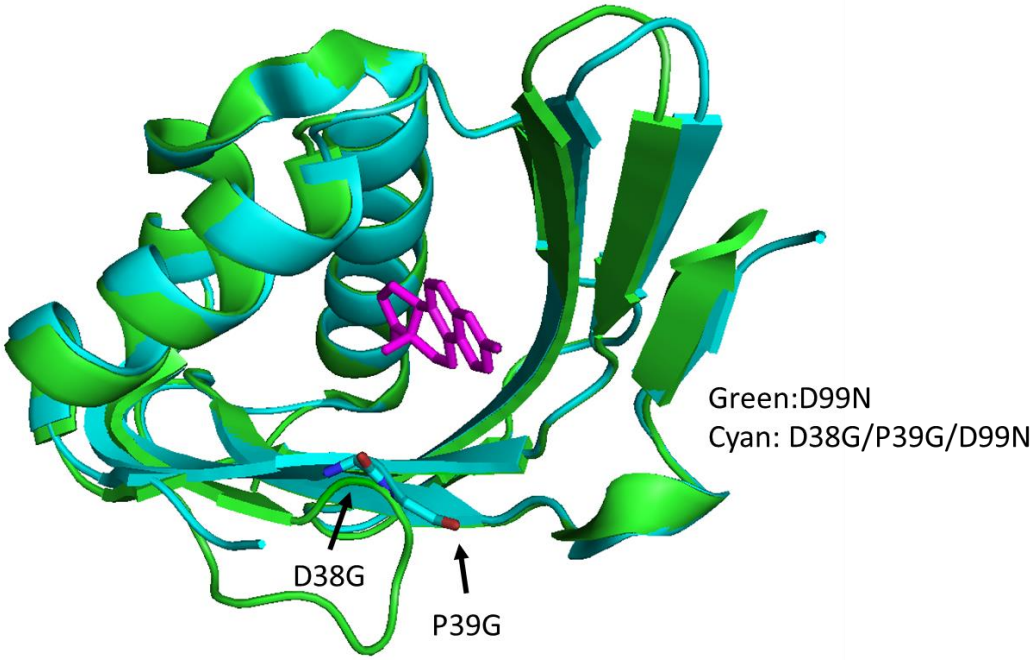
One side of the binding pocket is mainly hydrophobic interactions indicated by relatively white surface. This makes sense since the substrate is hydrophobic. The other side is highly negatively charged (indicated by red surface) and provides an electrostatic interaction surface with the oxyanion.

c) Describe what effective molarity means and how it relates to enzyme function. (2 points)
Effective molarity is described as the concentration of B that is required to make the rate of bi-molecular reaction between A and B match that of the first-order reaction with A-B. It describes the difference between intra-molecular reaction and inter-molecular reaction to understand the catalytic contribution from positioning and reduced entropic barrier.

d) Refer to Lamba V et al. (JACS 2016, 138, 9902-9909). What are the limitations of studying catalytic contribution from general base by using a mutant enzyme that can be rescued by exogenous general base? What did authors do to overcome this issue? (5 points)
Catalytic contributions can be over/underestimated due to steric effects and suboptimal positioning of the non-covalently bound base. The authors did more extensive mutations in the active site pocket to minimize steric effects. They also tested a series of exogenous base moieties that have similar basicity but different steric properties.

e) Authors determined k_{base} values across different mutants and different exogenous bases. What was the reason that they excluded some of the values for determining 'real' effective molarity? (5 points)
Some mutant shows steric effects evidenced by large differences across different exogenous bases. As the exogenous base became more bulky, the reaction rates decreased indicating steric effects are present for those reactions. Additionally, some show drastic increase of reaction rate as the exogenous base gets more bulky. This implies that there are additional binding interactions introduced by bulky groups that enhances the activity of the enzyme. Since the authors are interested in the pure catalytic contribution from the base, those showing steric/binding effects were excluded.

f) Download PDB file 5DRE. This structure has D38G/P39G/D99N Mutations. Align this mutant to the structure 3M8C and make a figure showing the mutations. (5 points)



g) How did the authors determine if the mutation did not cause change in the mechanism? What was the prediction and what did they observe? (5 points)
The authors wanted to confirm whether the mutations affected the oxyanion hole. They know that the D99N mutation would cause a specific amount of defect in the WT enzyme. If the mechanism utilizing oxyanions is not affected with the mutations, the mutants will show the same degree of defect with D99N mutation as the WT. This is what they observed experimentally, and they concluded that the mutations did not affect the reaction mechanism, at least regarding the catalytic interactions in the oxyanion hole.

4. 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. (20 points)

a) What are some of the strategies used by AP to provide its enormous rate enhancement based on previous studies? (4 points)
From structural studies, it is obvious that metal ions are involved in the enzymatic reaction. Zinc provides stabilization of negative charge build up on the leaving group oxygen. Zinc ion can also activate the nucleophilic Ser102, which provides moderate rate enhancement. In addition, positioning of substrate likely provides additional rate enhancements.

b) Compare the scenarios in Figure 2C and 2D. By destabilizing the ground state, the barrier for reaction from E•S to E•P is reduced to allow the reaction to proceed faster. What

additional effect does ground state destabilization have in enzyme activity? Why is this important? Think in terms of enzyme recycling. (6 points)

In Figure 2D, destabilizing the ES complex would also destabilize the EP complex. The likelihood of this depends on how similar ES is to EP. But assuming that this is the case, destabilizing the EP complex would reduce the barrier for product release and thus prevent product inhibition. Thus, the enzyme can be rapidly recycled for multiple turnover.

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 wild type AP, the binding to P_i was extremely tight so it could not be used in any kinetic assay. In order to reduce the binding of P_i to S102 mutants, it was necessary to introduce additional mutations on R166 to reduce binding affinity to a 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.

d) The authors tested the pH dependence of P_i binding to AP. Why does R166S AP show a bell shaped pH dependence? How do the authors verify their prediction for the acidic region of this pH dependence? (5 points)

The pH dependence of P_i binding can provide information about protonation events on the phosphate species or on the enzyme that may affect their interaction. The bell shaped curve indicates that two protonation events, one in acidic region and the other in the basic region, that affects the enzyme–substrate interaction. The authors hypothesized that the acidic region is caused by deprotonation of $H_2PO_4^-$, which has a pK_a of 6.9 that corresponds to the inflection point after the acidic limb of the pH-binding profile. The authors tested this by using an alternative substrate, HWO_4^-, which does not have a pK_a around 6.9. The acidic limb in the pH-dependence is absent with HWO_4^-, which confirms that the pH dependence in the acidic region is indeed caused by deprotonation of the phosphate species.