BMB170c
Problem set 1
Regulated proteolysis

1.) Kenniston et al. (2003) studied the mechanistic details of protein degradation by bacterial ClpXP. Read the paper carefully and answer the following questions.

A.) State the authors' main hypotheses of the paper. (3)

The authors hypothesized that protein substrates that have different stabilities will be degraded by ClpXP at different rates mainly due to their differences in the energy required for denaturation, especially the stability close to the ssrA tag. They want to investigate the rate-limiting step, which could be denaturation or translocation, of the process.

B.) A hypothetical protein composed of 120 amino acids (comparable length to Titin I27) has ΔG_u of 7 kcal/mole. When an ssrA tag is attached to the C-terminal end of this protein, ClpXP degrades it at an overall rate of $k_{deg} = 0.7\ min^{-1}$. Based on the results in this paper, estimate, for this hypothetical substrate, the binding affinity to ClpXP, the rate of translocation k_{trans}, the rate of denaturation k_{den}, the number of ATP hydrolyzed per molecule degraded, and the ATPase rate. Show your work/reasoning. (10)

For WT: $K_d \sim 1\text{-}3\ \mu M$ (ssrA tag; see next question).
$k_{trans} \sim 4\ min^{-1}$ since it is about the same size as titin, and k_{trans} is susceptible to the length of the substrate (Fig 3C). So the translocation rate of this protein should be comparable to that of titin-I27.
$k_{den} \sim 0.85\ min^{-1}$ according to the equation $1/ k_{deg} = 1/ k_{trans} + 1/ k_{den}$
ATP used = 270 molecules according to the equation #ATP $= 144/ k_{den} + 100$ (which is a best fit of empirical data).
ATPase rate is then 189 min^{-1} because the overall time of degradation = 1/0.7 = 1.43 min, which is the time that 270 molecules of ATP are used up.

C.) Based on the results in this paper, do you think different protein substrates will bind to ClpX with similar or different affinity? Why or why not? (4)

They probably bind to ClpX with similar affinities since 1.) the different proteins used in this paper have the same K_m for this set of substrates and 2.) the ssrA tag is shown to be the primary recognition module for ClpXP. In fact, GFP-ssrA also binds to ClpXP with similar affinity (3 uM; Singh, 2000). Moreover, ssr peptide binds to ClpXP with the same affinity as tagged proteins. Therefore, primary recognition is due to the peptide signal that is attached to the substrate rather than the characteristics of the substrate itself. Therefore, different substrates are expected to bind to ClpX with similar affinities.

D.) From the results in the paper, which step(s) provide the main difference in the ATP usage for degradation of different substrates? Which step(s) is the rate-determining step in degradation of native protein substrates by ClpXP? (3)

The denaturation step, according to the function they have derived in the paper. Rate-determining steps are either denaturation and/or translocation, depending on how difficult it is to unfold substrate.

2.) Kisselev et al. (2006) reported the extent of inhibition of the protease activity in the proteasome core.

A.) What is the main conclusion of the paper? (7)

Not one single protease site contributes exclusively to the degradation by the proteasome. Depending on the types of the substrate, the extents of inhibition of individual active sites vary.

B.) What could be potential problems of targeting protease active sites? (7)

First, you can't inhibit just one active site by one kind of inhibitor; the paper shows that it doesn't work very well. Second, degradation might not be the rate-limiting step (c.f. Kenniston et al.), and inhibition of that might not be maximally useful.

C.) From the results of this paper, and others that you have read, where would you target if you want to design a novel inhibitor of the proteasome? Why do you think your new inhibitors could be more superior than these existing ones in the paper? (8)

This is an open question. I will consider anything sensible, such as interaction surface between 19S and 20S, activation loops, the AAA^{+} ATPases, or multivalent inhibitors that target multiple proteolytic sites simultaneously.

3.) Wang et al. (2008) solved the structure of ClpS bound to the N-end rule substrate and elucidated the mechanism by which the substrate is specifically recognized by ClpS.

A.) Look at the structure (PDB: 3DNJ) and zoom in around the N-end recognition moiety. Show in a structural snap shot (similar to Fig 4A) and briefly explain how the structure rationalizes the results in Figure 4D. When you create the snap shots using the mutagenesis function of your favorite PDB-rendering program, report % occurrence of the rotamer you pick. Discuss if their results in Figure 4D are obvious to you considering your *in silico* mutagenesis experiments, and propose plausible explanation for any discrepancy. (12)

Most of the work is just to create snap shots of M53-Y1 pair (in vdw spheres to maximally show steric) using "mutagenesis" function in PyMol (or equivalent function in others). One set of answers involves mutations

on the N-end rule peptide (Y1) to other amino acids in Figure 4D. The other set involves mutation from Met to Ala on ClpS protein with various N-end rule substrates. You can show all of them, or representatives of each group. They will be accompanied by brief explanation, mostly about steric clash or lack thereof. Some pictures might not be obvious, depending on the rotamer they pick. One plausible explanation might be that the peptide might readjust the conformation since it is small and it needs to fit into the hydrophobic groove and other residues following the N-end might readjust themselves also. Especially important for Thr, which contains O that might not be favorable to fit in hydrophobic groove in Fig 2D, even though the M53A mutation might allow the b-branch sidechain. It might be less likely that ClpS residues readjust immensely due to restrictions from other parts of proteins.

B.) Propose biochemical experiments that would test the model in Figure 5, especially addressing the movements of substrate-ClpS-N domain of ClpA toward the ClpA chamber or the specific interaction that is replaced afterward. (8)
Will take anything sensible, including crystal structure of ClpS with the whole ClpA showing the replaced interaction, cross-linking, FRET, mutations on both regions and activity tests, etc.

4.) In 2000, the structure of a heterologous proteasome complex consisting of the 11S regulatory particle and the 20S proteasome was solved by x-ray crystallography (Whitby et al., 2000). The structure provided structural basis of the activation of the 20S particle, specifically the pore opening that presumably let substrates in or products out.

A.) Where do most of the conformational changes in the 20S occur? (3)
N-terminal parts of alpha-subunits, esp. 2,3,4,5.

B.) Describe the mechanism of 20S activation proposed by the authors, based on the structural changes they observed. (3)
The activation loops from the regulatory subunits bind to the N-termini of the alpha subunits at the gate of the 20S proteasome core. Upon binding, the N-termini undergo conformational changes to reciprocate the exact 7-fold symmetry of the regulatory particle. This results in the opening of the pore.

C.) Propose a biochemical experiment to test this model that the author proposed. In other words, how do you test whether the movement of the N-terminus of the alpha subunits is important to the activation of proteasome. (7)
Construct a mutant subunits in which the N-termini is deleted or truncated, and assemble mutant 20S particles. These particles will likely have a more open pore and exhibit enhanced rate of peptide degradation

compared to the wildtype 20S particle. Further, the 11S regulatory particle should have a smaller effect on the peptide degradation rate of the mutant than the wildtype particle.

D.) Based on your answer in C, predict what the results of the experiment in each case would be if the substrates were small peptides (<4 amino acids long), denatured proteins, or folded proteins with degradation tags? (7)

If the substrates were short peptides, both WT and mutant (lacking N-termini) proteasome should be able to degrade it at the same rates.

If the substrates were denatured proteins, the proteasome without N-termini of alpha subunits should be able to degrade the substrates while the WT should not (since the pore is closed).

However, for the folded proteins, the 20S (WT or mutant) + 11S cannot degrade it as the 11S is not an ATPase that has been shown to unfold proteins.

5.) Based on the results and the conclusions in Thrower et al. (2000), predict how the kinetic parameters (K_m and k_{cat}) would change (higher, lower, unaffected) in each scenario given below. Provide a brief explanation for each of your answer. (3 each)

The experimental setup would be like that in Figure 2B, and, for comparison, K_m and k_{cat} values are 50 nM and 0.05 min^{-1} as reported by the authors. If the scenario does not involve change in substrate, take Ub_5DHFR as the substrate in that reaction.

A.) If Uba1 is omitted.

According to this paper, proteasome can degrade substrates without deubiquitination, and the author predicts that deubiquitination will be competing with degradation. Therefore, omitting Uba1 will likely make K_m higher ('real' concentration of the substrate, Ub_5DHFR, would be lower due to deubiquitination and apparent Km should be higher). k_{cat} should not change as kcat is limited by unfolding of substrate, which should be the same with or without Uba1.

B.) If the substrate is Ub_9DHFR

Lower K_m (more Ub = binds better to proteasome; Figure 2E)

Same k_{cat} (unfolding the same protein; Figure 2E)

C.) If the substrate is Ub_3DHFR

Higher K_m (less Ub = binds worse)

Same k_{cat} (unfolding the same protein)

D.) If the reaction contains high concentration of MTX

MTX stabilizes folded conformation of DHFR, possibly making it harder to be unfolded by the proteasome. Figure 4B shows that it is a noncompetitive inhibitor of the reaction, which, by definition, leaves K_m unaffected and k_{cat} lower.

Same K_m (same amount of Ub)

Lower k_{cat} (harder to unfold)

E.) If the substrate is Ub_5DHFR*, where DHFR* denotes a DHFR mutant that has lower stability.

Same K_m (same amount of Ub)

Higher k_{cat} (easier to unfold)

F.) If the substrate is Ub_5*DHFR, where Ub_5* denotes chain 11 from Table IIB of the paper.

Chain 11 binds 3 times less tightly than WT Ub5, according to Table IIB, so K_m should be higher, but k_{cat} should be unaffected.

G.) If a hypothetical "extraproteasomal chaperone" is present (mentioned in the discussion part of the paper; assume this extraproteasomal chaperone has been identified and purified; Assume that the purified 26S proteasomes used in the experiments are intact and completely active and that each experiment is done side by side with the standard (like that in Figure 2E) to minimize the variation.)

K_m—possibly unaffected as there is the same number of Ub chain. However, this elusive "extraproteasomal chaperone" might have an extra function in recruiting Ub-substrate to the proteasome, resulting in lower Km. There is no correct answer; I will take anything as long as the explanation is plausible.

Higher k_{cat}—that's basically what they are trying to reconcile between the slow rate of degradation by purified proteasome and the fast rate of degradation by lysate. If they find this "extraproteasomal chaperone," it must help accelerate the rate by definition.