ANSWER KEY
5.08 Biological Chemistry II (Spring 2016)
Problem Set #4

This problem set contains one problem and 4 pages.

Question 1:

The following experiments were performed in an effort to increase our understanding of ClpXP. A model substrate, the P22 Arc repressor (it is a homodimer), was engineered to contain a C-terminal ssrA tag. Additional Arc mutants were constructed, including one named $NC11_{ox}$-Arc-ssrA where the two Arc monomers were covalently linked with a disulfide (S—S) bond. This mutant was prepared by mutating an asparagine to cysteine, and the purified protein was allowed to oxidize such that the disulfide bond formed. The family of Arc mutants and the structure of the Arc dimer are shown in **Figure 1**. In panel B, one Arc monomer is black and the other is grey. Similar to the titin I27 domain mutants discussed in class, Arc mutants with a range of stabilities were characterized in previous studies.

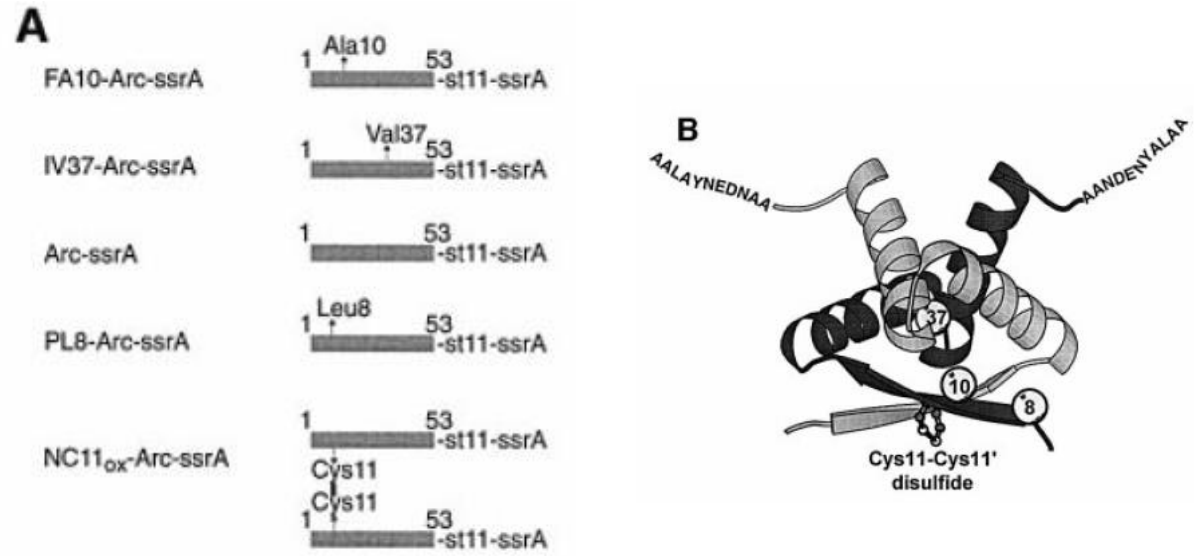


Figure 1. (A) The Arc mutants. The abbreviation "st11" stands for a short linker comprised of eleven amino acids, His_6KNQHE, that is between the Arc sequence and the ssrA tag. This linker contains a His_6 tag that was used for Ni-NTA affinity purification. (B) Structural depiction of the Arc-ssrA homodimer. The ssrA tag is AALAYNEDNAA.

In a first set of experiments, the stability of the protein fold of each mutant was examined by thermal denaturation, which is a method for monitoring protein unfolding. In this technique, the

circular dichroism (CD) spectrum at 220 nm was monitored over a range of temperatures for each protein. The results from this study are shown in **Figure 2.**

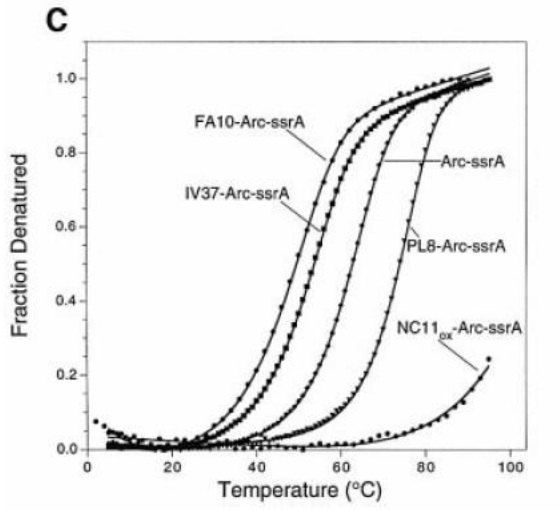


Figure 2. Thermal denaturation studies of the Arc mutants.

In a second set of experiments, the Arc mutants were [^{35}S]-labeled and the rates of degradation by ClpXP were monitored as described in class. The reactions were acid quenched at various time points, and these quench conditions precipitate the full-length [^{35}S]-labeled proteins whereas the short peptide fragments resulting from degradation remain soluble. The insoluble and soluble fractions were separated by centrifugation. These data are shown in **Figure 3** and summarized in **Table 1** below.

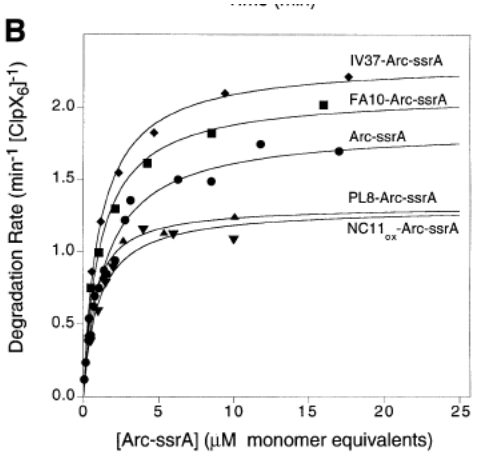


Figure 3. Degradation studies of the Arc mutants by ClpXP.

Table I. Stability parameters for Arc-ssrA variants and steady-state kinetic parameters for ClpXP degradation

Variant	ΔG_D at 1 μM (kcal/mol)[a]	Unfolding rate constant (/min)	ClpXP, K_M (μM)	ClpXP, k_{cat} (/min/[$ClpX_6$])
Arc-ssrA	1.3	8.4	1.5 ± 0.1	1.8 ± 0.1
PL8-Arc-ssrA	2.2	0.12	1.0 ± 0.2	1.3 ± 0.1
FA10-Arc-ssrA	–0.4	184	1.2 ± 0.1	2.1 ± 0.1
IV37-Arc-ssrA	0.2	44	1.1 ± 0.1	2.3 ± 0.1
$NC11_{ox}$-Arc-ssrA	14.6	4.8×10^{-6}	1.0 ± 0.2	1.3 ± 0.1

[a]Free energy changes of denaturation (ΔG_D) at 25°C and a standard-state concentration of 1 μM were calculated from K_u values reported in Milla *et al.* (1994), Milla and Sauer (1995), Schildbach *et al.* (1995) and Robinson and Sauer (2000). Values for the unfolding rate constants were taken from the same references and from Milla *et al.* (1995). The K_M and k_{cat} values for ClpXP degradation are from non-linear least squares fits of the data shown in Figure 2B.

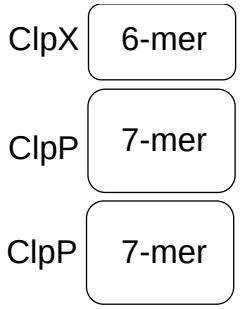
The ATP hydrolysis rates associated with degradation of the Arc-ssrA mutants were also determined. A spectrophotometric assay was employed and saturating concentrations of ATP and substrates were used. The results from these experiments are shown in **Table 2.**

Table II. Rates of ClpXP-catalyzed ATP hydrolysis stimulated by Arc-ssrA variants

Protein substrate	ATP turnover (/min)
None	170 ± 10
Arc-ssrA	340 ± 20
FA10-Arc-ssrA	380 ± 30
IV37-Arc-ssrA	370 ± 30
PL8-Arc-ssrA	240 ± 10
$NC11_{ox}$-Arc-ssrA	240 ± 10

Answer the following questions:

A) Draw ClpXP indicating oligomeric states.



Two back-to-back 7-mer rings for ClpP. ClpX is positioned on the top ring. In most studies, ClpXP is described as being in a 1:1 ClpX 6-mer / ClpP 14-mer stoichiometry.

B) What are two possibilities for how Arc-ssrA associates with ClpXP? For instance, what are possible Arc-ssrA:ClpXP stoichiometries?

The Arc-ssrA constructs are homodimers and each monomer unit has a C-terminal ssrA tag. Thus, it is possible that one ClpXP associates with one of the Arc-ssrA tags, providing a 1:1 complex. It is also possible that two ClpXP can associate with Arc at the same time such that one ClpXP is bound to each tag. Given what we learned about how ClpXP binds and releases substrates multiple times when trying to denature them, a dynamic mixture is another possibility.

C) In class, we described a five-step model put forth by Sauer and Baker for protein degradation by ClpXP. Describe these five steps and corresponding thermodynamic/kinetic parameters that we discussed in class. Note which steps are ATP dependent.

Step 1: Binding (K_d) The condemned protein is recognized by ClpXP via the ssrA tag. The adaptor protein SspB may facilitate this process.
Step 2: Denaturation (k_{den}). This step requires ATP by ClpX. The folded substrate must be denatured by the AAA+ ATPase.
Step 3: Translocation (k_{trans}). This step also requires ATP. The substrate must be translocated through ClpX and into the ClpP degradation chamber.
Step 4: Degradation (k_{deg}). The unfolded polypeptide is hydrolyzed. This step is fast (serine protease action).
Step 5: Peptide release. The short peptide fragments must be released from the chamber. We stated that this step is fast, but did not discuss it in detail from a kinetic standpoint.

D) Describe three control experiments to show that degradation of the Arc-ssrA derivatives require ClpXP and ATP.

Observation: Arc-ssrA + ClpXP + ATP → degradation

Control 1: Arc-ssrA + ClpP + ClpX → no degradation (no ATP control)
Control 2: Arc-ssrA + ClpX + ATP → no degradation (no ClpP control)
Control 3: Arc-ssrA + ClpP + ATP → no degradation (no ClpX control)

Another possible control is to perform the assay in the presence of a protease inhibitor (block ClpP action).

In each assay/control, the rate of degradation can be measured by using [^{35}S]-labeled Arc-ssrA as a substrate. Each reaction is quenched with acid at various time points, which results in precipitation of any remaining full-length [^{35}S]-labeled Arc-ssrA, and radioactivity in the pellet and supernatant can be measured.

E) What do the data in **Figure 2** tell you about the different Arc-ssrA constructs? Provide a rationale for the construction of the $NC11_{ox}$-Arc-ssrA mutant. In other words, what question(s) could be addressed by using this mutant?

The various Arc constructs either (i) denature more readily with increasing temperature (FA10, IV37) or (ii) denature less readily (PL8, $NC11_{ox}$) compared to wild-type Arc. These comparisons indicate that the different Arc constructs have different structural stability, and that changing the amino acid sequence can alter the stability of the Arc protein fold. The less stable constructs are more likely to be denatured/degraded faster by ClpXP (in analogy to what we learned with the titin I27 substrates in class).

The $NC11_{ox}$-Arc mutant is a very interesting construct. It was created to investigate the stability and degradation of a covalent dimer. Many questions can be probed with this mutant. For instance, does the presence of two ssrA tags aid in ClpXP recognition? Does a covalent dimer take longer to unfold (and more ATPs) than a non-covalent dimer? Comparing the covalent dimer to a non-covalent dimer, we can ask another question: when one monomer is denatured and translocated, will the other monomer (non-covalent or covalent) follow and be denatured/degraded consecutively?

Note: You can also imagine making an $NC11_{ox}$-Arc analog that is a heterodimer such that only one of the monomers has the ssrA tag. A comparison of the heterodimer and homodimer would provide insight into the effect of one vs. two ssrA tags.

F) What do the data in **Figure 3** tell you about the relationship between mutant Arc-ssrA and the rates of protein degradation? Does the data tell you anything about the basis of substrate selection? If so, how?

The data in Figure 3 show that ClpXP degrades the various Arc proteins at different rates and in the order IV37 > FA10 > wild-type > PL* ~ $NC11_{ox}$. This trend corresponds to the thermal denaturation studies: the mutants that display less thermostability are the mutants that are degraded more readily (faster) by ClpXP. (Note: FA10 and IV37 are reversed, but the values are pretty comparable. FA10 is slightly less thermostable than IV37, but FA10 degrades more slowly).

The K_M can provide some insight into the substrate selection (recall that K_M by itself does not tell us very much and is comprised of many rate constants). The K_M values for wild-type and mutant Arc are all similar, ranging between 1.0 ± 0.2 μM and 1.5 ± 0.1 μM. These values indicate that all four mutants have slightly higher affinity to ClpXP than the wild-type, but not by very much (do not over-interpret this data!). The K_M values of the mutants are all pretty similar and only vary from 1.0 ± 0.2 to 1.2 ± 0.1.

G) The role of ATP in proteasome machines is of major interest. From the data provided above, state the major conclusions that can be drawn from this study. Does a comparison of the data in **Figure 2** with the data in **Table 2** give you any additional insight about the role of ATP (hint: think about the experiments described in class for the titin I27 mutants)?

Recall that ClpX is an ATPase and ATP is needed for unfolding and translocation of a substrate. ATP hydrolysis by ClpXP converts chemical energy into mechanical energy to "tug" at the protein and denature it. ATP is also needed for pushing the peptide into the degradation chamber (translocation).

We observe higher rates of ATP turnover for Arc mutants that denature more readily (FA10, IV37). We can conclude that the stability of the protein influences how quickly ClpXP unfolds and translocates it, similar to what we learned about the titin I27 mutants. The less stable the protein, the faster ClpXP acts as measured kinetically by ATP hydrolysis.

5.08J Biological Chemistry II

Spring 2016