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.

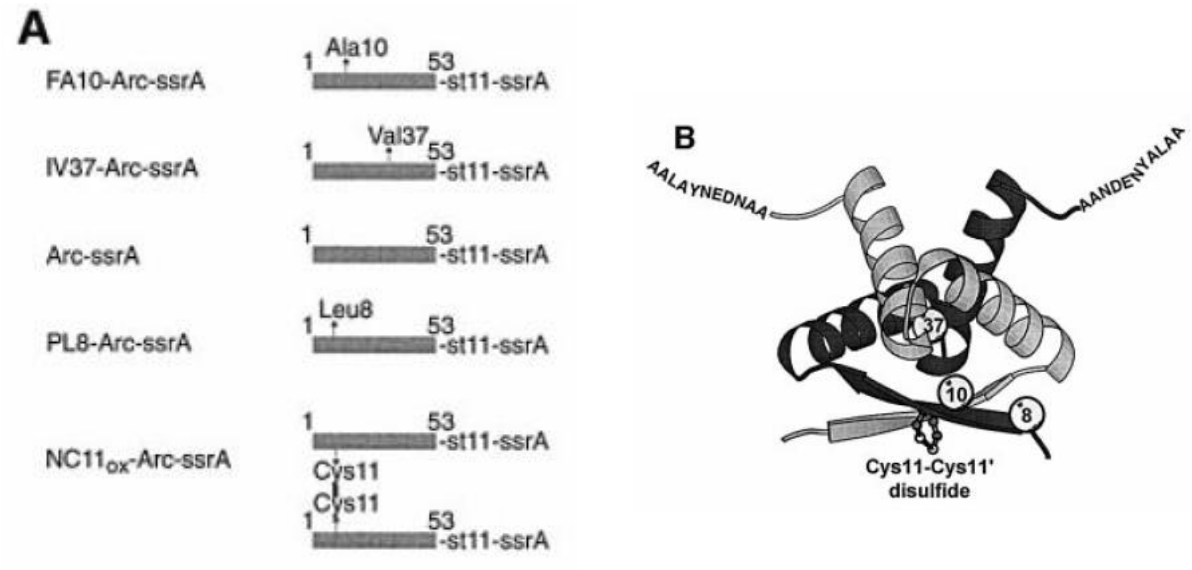

Figure 1. (A) The Arc mutants. The abbreviation "st11" stands for a short linker comprised of eleven amino acids, His_6KNQHE, that is between the Arc sequence and the ssrA tag. This linker contains a His_6 tag that was used for Ni-NTA affinity purification. (B) Structural depiction of the Arc-ssrA homodimer.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

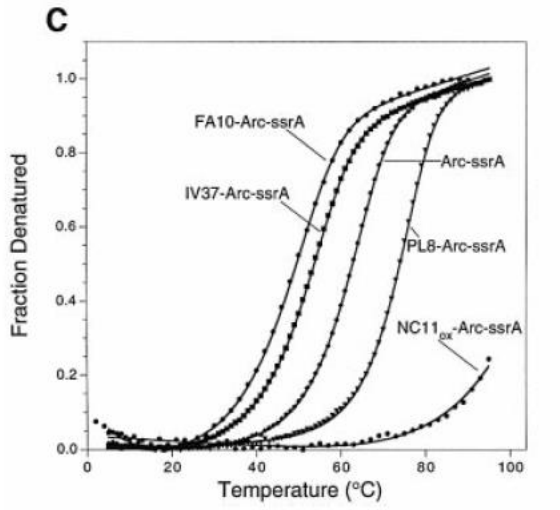

Figure 2. Thermal denaturation studies of the Arc mutants.

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.

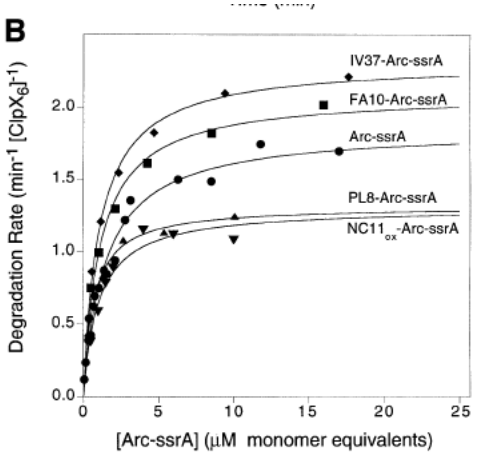

Figure 3. Degradation studies of the Arc mutants by ClpXP.

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.

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

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.

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

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?

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?

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)?

5.08J Biological Chemistry II

Spring 2016