Long Assignment 3

NOTE: This introduction is largely copied and pasted from my hypothesis, as it is meant to contain the same information. I only did this for the purposes of the draft, and I am in the process of paraphrasing what is already here/cutting this section down for the final paper.

Proteins are integral to the existence of life; they are the movers and shakers of the biological world. From mechanical motion to metabolism, the actions of cells are governed by the interactions of proteins. By adopting extremely specific structures, proteins perform the multitude of tasks necessary to sustain the life of a single cell, breaking down each task into thousands of individual steps and relying on differently shaped proteins to execute each step. Before attaining their structure, proteins are simply a linear chain of amino acid building blocks. Somehow, this chemical sequence spontaneously folds, achieving the proper conformation to create extremely specifically structured proteins. How does the chemical composition of a protein relate to its structure and properties? —how does the environment of a protein change its structure and properties? —and how do the structure and properties of a protein relate to its function? These are the questions we tackled in PEBL this semester.

To study these overarching questions, we adopted a particular question regarding a model protein, SNase, investigating how the stability of the structure of SNase changes following a mutation to produce an ion pair on its surface. The specific mutant variant of SNase we investigated is K63E/E67K. This mutation is the inversion of an existing ion pair in SNase between lysine (K, or Lys) at position 63 and glutamic acid (E, or Glu) at position 67, allowing us to address the role of existing ion pairs/ion pairs in general on the stability of SNase. With the advent of protein engineering, for drug development and personalized medicine, addressing these types of questions regarding the basic science of protein structure and function is increasingly

important, for the difference between a life-saving drug and potent poison may be a difference of just one amino acid out of hundreds.

At the start of the semester, it was hypothesized that the K63E/E67K mutation will likely reduce the stability and function of SNase and alter its structure, since the Lys at position 63 is largely conserved across SNase-like genes from many organisms, suggesting it is important to the structure of SNase and that the mutation will disrupt SNase function (from the WebLogo provided in Lab 2), and because the length of the ionic interaction is 2.7 Å in the background SNase, while it is 3.6 Å in the mutant (using least strain rotamers), suggesting that the structure will more easily be disrupted due to this weakened (longer) Coulombic force. Furthermore, the mutant has one less hydrogen bond in the region of the mutation, further disrupting the structural integrity of SNase. PyMOL visualizations of the mutation site in the variant and background are included in Figure 1A and B.

Several experiments were performed to characterize the K63E/E67K mutant, broadly falling into three categories: structure-related, function-related, and stability-related. The structure of the variant was compared to the background protein via circular dichroism (CD) spectroscopy, which applies circularly polarized light to a sample to detect chiral structures, particularly protein secondary structures for the purposes of this experiment. CD spectroscopy was performed on two SNase solutions: mutant and wild-type, with two controls: unfolded and denatured (with GdnHCl) SNase, in four experimental conditions: pH 7, 0.1 M KCl; pH 7, 1 M KCl; pH_{mid} (≈ 2.25), 0.1 M KCl; and pH 1, 0.1 M KCl. The spectra for background SNase (with controls) and the variant (with controls) can be found in Figure 1C and D. Overall, they suggest that the mutation minimally impacted the secondary structure of SNase, since the shape of the mutant spectrum very closely resembles the characteristic "W" shape of the background at

standard conditions (pH 7, 0.1 M KCl). However, interestingly, they also suggest that the mutant is more stable in response to acid than the background, evidenced by the relatively larger amount of unfolded character in the background spectrum at pH_{mid}. Additionally, the variant appears to be more stabilized by salt than the background, evidenced by its larger MRE_{222}/MRE_{208} ratio (1.234 vs. 1.052) at the 1 M KCl condition. Nevertheless, comparing shapes of CD spectra is a qualitative measure, and the MRE_{222}/MRE_{208} ratio is only semi-quantitative, so this data cannot definitively comment on the stability of the mutant vs. the background. The biggest takeaway was the minimal structural change between mutant and background.

The function of background and variant SNase was assessed by blue-plate assay. The blue-plate assay produces a semi-quantitative measure of SNase activity using DNA (the substrate of SNase) in agar. Toluidine blue-O, the intercalating dye present in the matrix, inserts itself between nucleotides, producing a blue color, and is released following cleavage of the DNA by SNase, producing a pink color. By measuring the diameter of the resulting pink circle, we indirectly measure the activity of SNase. The blue-plate assay tested two SNase solutions: mutant SNase and wild-type SNase and three controls: unfolded SNase, folded/inactivated SNase, and water, in four conditions: 20 mM Ca++ at pH 9; EDTA at pH 9; 20 mM Mg++ at pH 9; and 20 mM Ca++ at pH 7. The data produced is included in Table 1. Overall, the data suggest that the function of the mutant is slightly impaired compared to the background. The mutant produced similar, yet consistently lower activity to the wild-type in all experimental conditions except for the EDTA condition, where all samples produced zero activity. The mutant always outperformed the unfolded and folded/inactive controls except for the Mg++ condition, which seems to be an outlier. The folded/inactive sample had zero activity (which is expected, since the active site is destroyed) in every trial except for the Mg++ condition, in which it had the highest

activity. Likely, the background sample was loaded by mistake into the folded/inactive dish. Additionally, the pink regions of activity were not perfectly circular, and were measured at different time points by different experimenters, likely contributing error due to differing measurement techniques/determinations of the diameter. Thus, this data is limited in quantifying the activity of the protein besides providing rough estimates of relational activity, such as the general trend that the variant underperforms the mutant, which was the key takeaway from this experiment.

Finally, the stability of the mutant was assessed and compared to the background using several experiments, including acid titration (pH stability), temperature denaturation (thermal stability), and chemical denaturation (thermodynamic stability/ΔG). The temperature and chemical denaturation experiments additionally measured the stability of mutant and background in high salt (1 M KCl) conditions to determine the impact of salt on stability. The state of protein folding in each of these denaturation experiments was determined by measuring Trp fluorescence. SNase has one Trp residue that is buried in the protein interior in the folded state (producing high energy fluorescent photons) and exposed to solvent in the unfolded state (producing low energy photons due to mechanical/vibrational energy losses to solvent molecules).

The fluorescence plots for the acid titration and temperature denaturation are included in Figures 2A and B, respectively. Figure 2C and D depict the ΔG data from the chemical denaturation for 0.1 M and 1 M KCl, respectively. The acid titration suggests similar, but lower acid stability for the variant, evidenced by its higher pH_{mid} (2.27 vs. 2.20), which is interesting because this counteracts the CD spectra data (which suggested higher pH stability of the mutant). However, since the CD spectra are more qualitative, this data provides better evidence and likely

indicates noise/error in the collection of the spectra or the interpretation of the shape. The temperature denaturation continued the trend, with similar, but lower T_m values for the variant for both salt conditions (see Fig. 2B), indicating slightly lower temperature stability. Finally, the ΔG (of unfolding) values at both salt conditions were lower for the variant, indicating reduced thermodynamic stability (see Fig. 2C and D). The slopes of the transition regions (m-values) were similar between variant and mutant in every experiment, indicating similar cooperativities of unfolding. It is important to note that there may be variability in the calculated ΔG values. The value is determined from fitting a line to the calculated ΔG values in the transition region, but the points chosen to constitute the transition region can vary. The two pairs of experimenters chose different fits for the low salt condition, so the reported ΔG value is actually 9.9 ± 0.2 kcal/mol. Interestingly, the ΔT_m (change in T_m between low and high salt conditions) and $\Delta\Delta G$ (change in ΔG between low and high salt conditions) were higher for the mutant than the background (ΔT_m: 5.0 vs. 4.3 and $\Delta\Delta G$: 2.8 vs. 2.0), suggesting that salt stabilizes the mutant more than the background. The key takeaway from the stability experiments was that the variant is slightly less stable than the mutant.

Overall, the data generally supports the initial hypothesis, though not to the extent expected. The structure was predicted to be altered by the mutation, however the CD spectra suggested minimal changes. The function was reduced, as predicted, however the maximum difference in blue-plate assay diameter between mutant and background was 6%, whereas the modeled Coulombic interaction length at the mutation site is stretched by 33%, corresponding to a 44% reduction in strength (using Coulomb's law and the least strain rotamer). Finally, the stability was reduced as well, but marginally in every experiment. The interaction is likely not weakened sufficiently to significantly impact the overall protein structure, and thus does not

significantly reduce the function or stability. The most interesting observation was the increased stability of the mutant in salt compared to the background. Two mechanisms were proposed to explain this. First, the mutant has more steric strain around the ion pair, which may prevent access of the salt ions to screen this favorable, stabilizing interaction, increasing the net stabilizing effect of the salt. Additionally, the mutation may produce destabilizing ion pairs not present in the background, which are screened by salt, increasing the net stabilizing effect.

The other five mutations produced by our cohort of experimenters were concerned with various methods of removing the ion pair entirely, to investigate the importance of this particular ion pair to the structure of SNase. The results for the other variants largely resembled our own, indicating that the removal of the ion pair produced minimal changes in the structure, and slight decreases in the function, and stability of the protein. Thus, the first impression is that this ion pair does stabilize the protein, but minimally, likely because it is distant from the active site and unimportant to the overall structure of SNase. We can compare the stabilizing effect of our ion pair to the original ion pair by determining the change in ΔG due to its presence. First, we can use the ΔG value for a completely absent ion pair (K63Q/E67Q) and find the ΔΔG between our variant and this one. But we must also subtract the effects from substituting the first Q for E (ΔΔG of K63E/E67Q) and the second Q for E (ΔΔG of K63Q/E67K) to determine the net effect of just the ion pair. The calculation becomes:

$$\Delta\Delta G_{Ion\ Pair} = \Delta\Delta G_{\frac{K63E}{E67K}} - \Delta\Delta G_{\frac{K63E}{E67Q}} - \Delta\Delta G_{\frac{K63Q}{E67K}}$$

$$= (9.9 - 10.0) - (10.4 - 10.0) - (8.5 - 10.0)$$

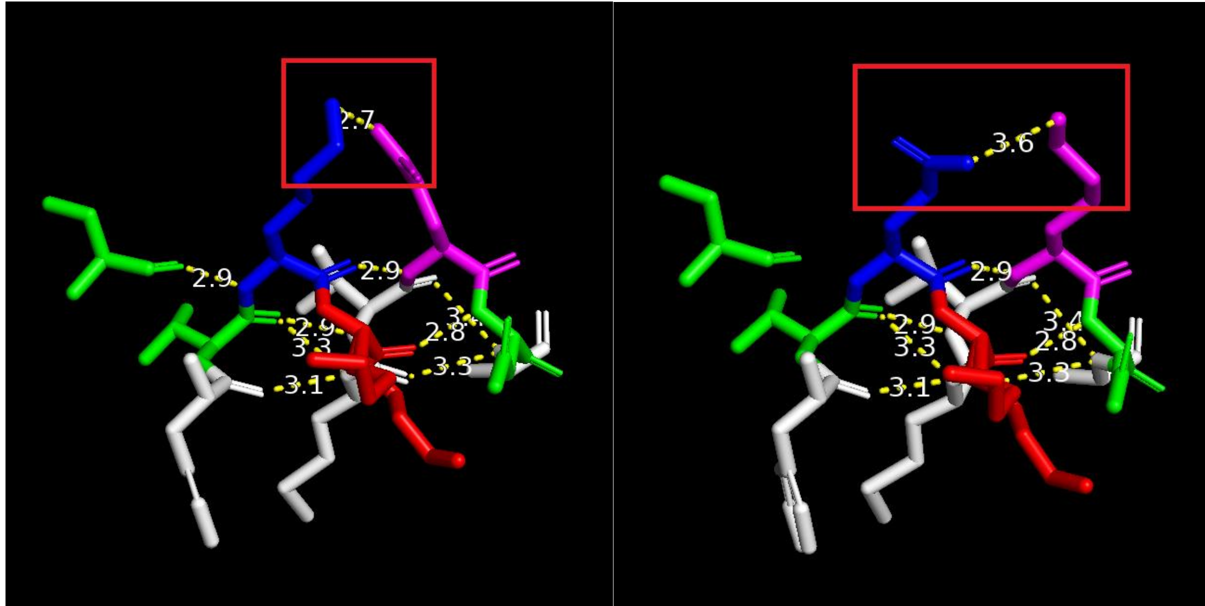
$$= 1.0 \text{ kcal/mol}$$

Since this value is positive, our ion pair had a net stabilizing effect on SNase. We can compare this to the background ion pair. The same train of calculations produces 2.2 kcal/mol. Thus, the original ion pair has a substantial stabilizing effect on SNase, and our inverted ion pair has a stabilizing effect, though not as significant. Likely, the reason the other mutations did not significantly change the structure/function/stability of SNase is because the amino acids used still had favorable interactions, particularly hydrogen bonding (Q to Q) and ion-dipole interactions (E to Q and K to Q), which served much the same role as the original ion pair. Through these comparisons, however, we see that the ion pair does play a larger role in stabilizing the protein than the data from individual variants would suggest, as 2.0 kcal/mol is a substantial value. Furthermore, we see that our mutation was successful in producing a net stabilizing effect in SNase, though not as strong as the background.

Through the experiments in PEBL this semester, we elucidated the impact of the K63/E67 (background) and E63/K67 (variant) ion pairs on the stability of SNase. Through the process, we addressed the broader questions of the course, demonstrating that noncovalent interactions (particularly Coulombic in these experiments) dictate the structure of the protein and minor alterations to this specific structure can reduce the function and stability of proteins. Furthermore, by assessing salt effects, we were able to show that the environment of a protein can alter its structure and stability. In the end, we achieved the goal of producing a stabilizing ion pair, but not as stabilizing as the one present in background SNase. The most valuable takeaways from this work are not the specific results, but rather the methods of analyzing proteins, which have relevance to the wider study of proteins and applications in industry. For example, perhaps the introduction of stabilizing ion pairs may allow a drug to survive adverse conditions on its way to delivery.

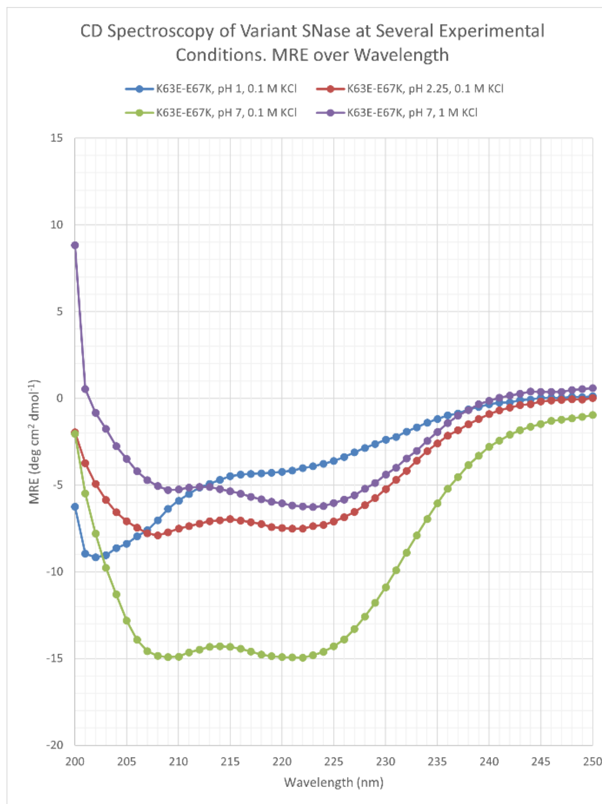
Figures:

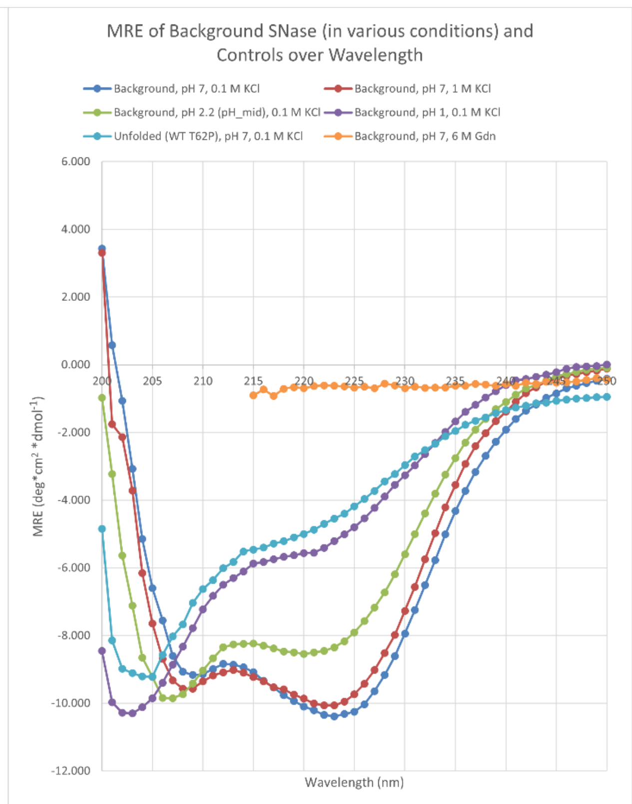
Figure 1:



1A, Left: Wild-Type SNase, with Lys63 in blue and Glu67 in magenta, also showing resiudes within H-bonding distance of both positions, with H-bonds shown as yellow dashes and distances indicated by number labels. The red box contains the Coulombic interaction. Polar residues are shown in green, nonpolar in white, and basic in red.

1B, Right: Mutant SNase, with Glu63 in blue and Lys67 in magenta. Same coloring as wild-type.

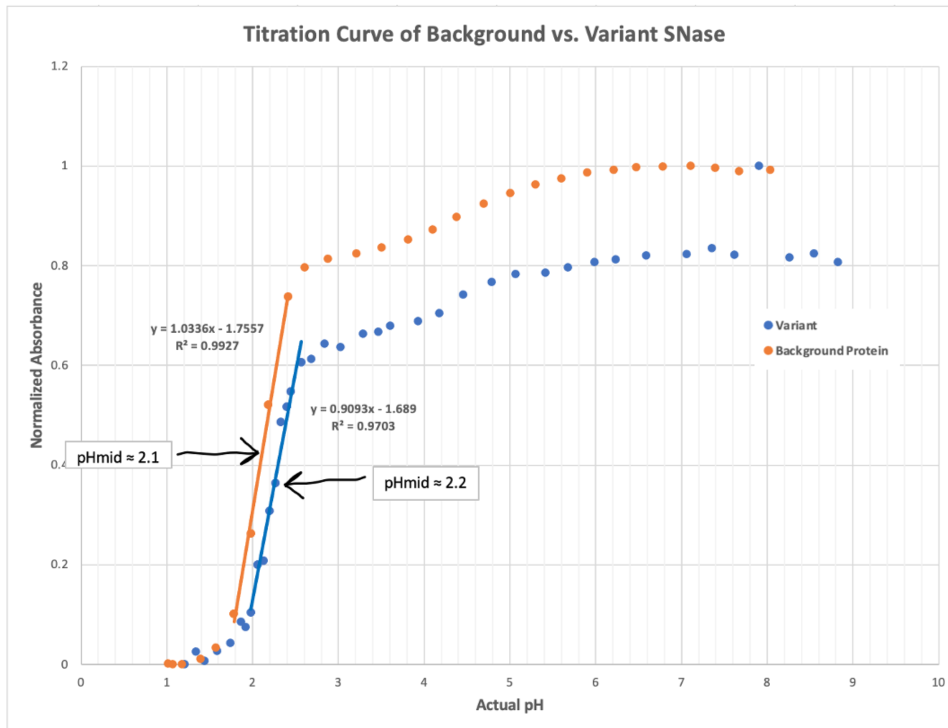


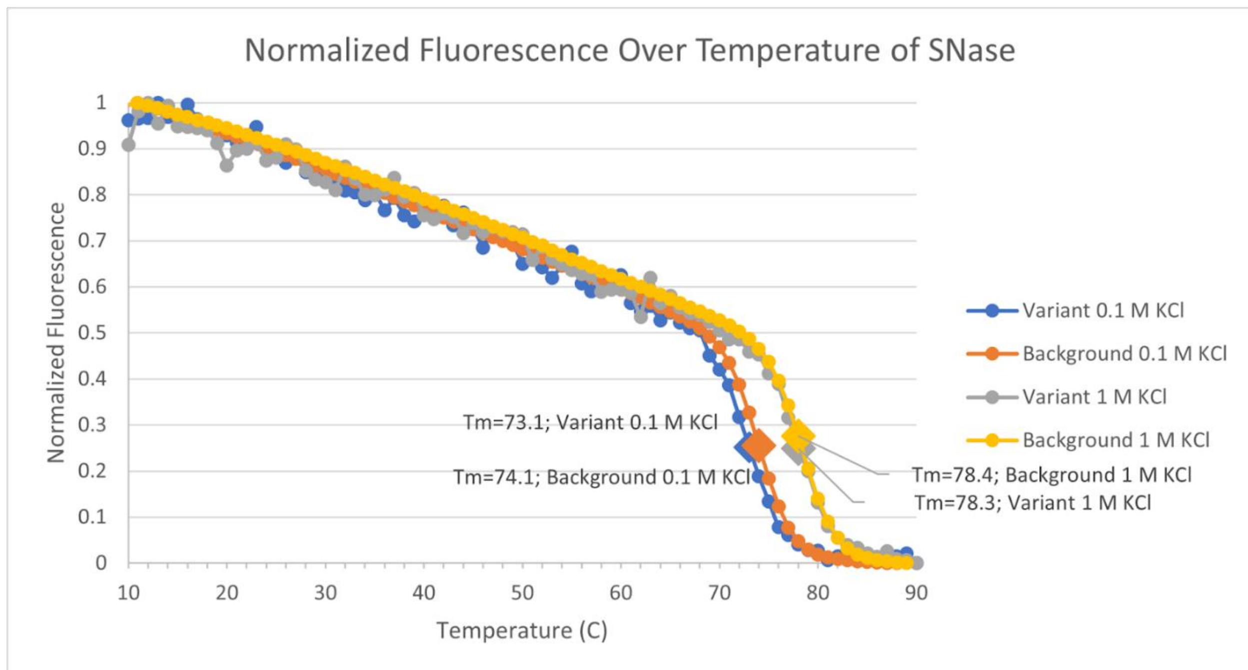


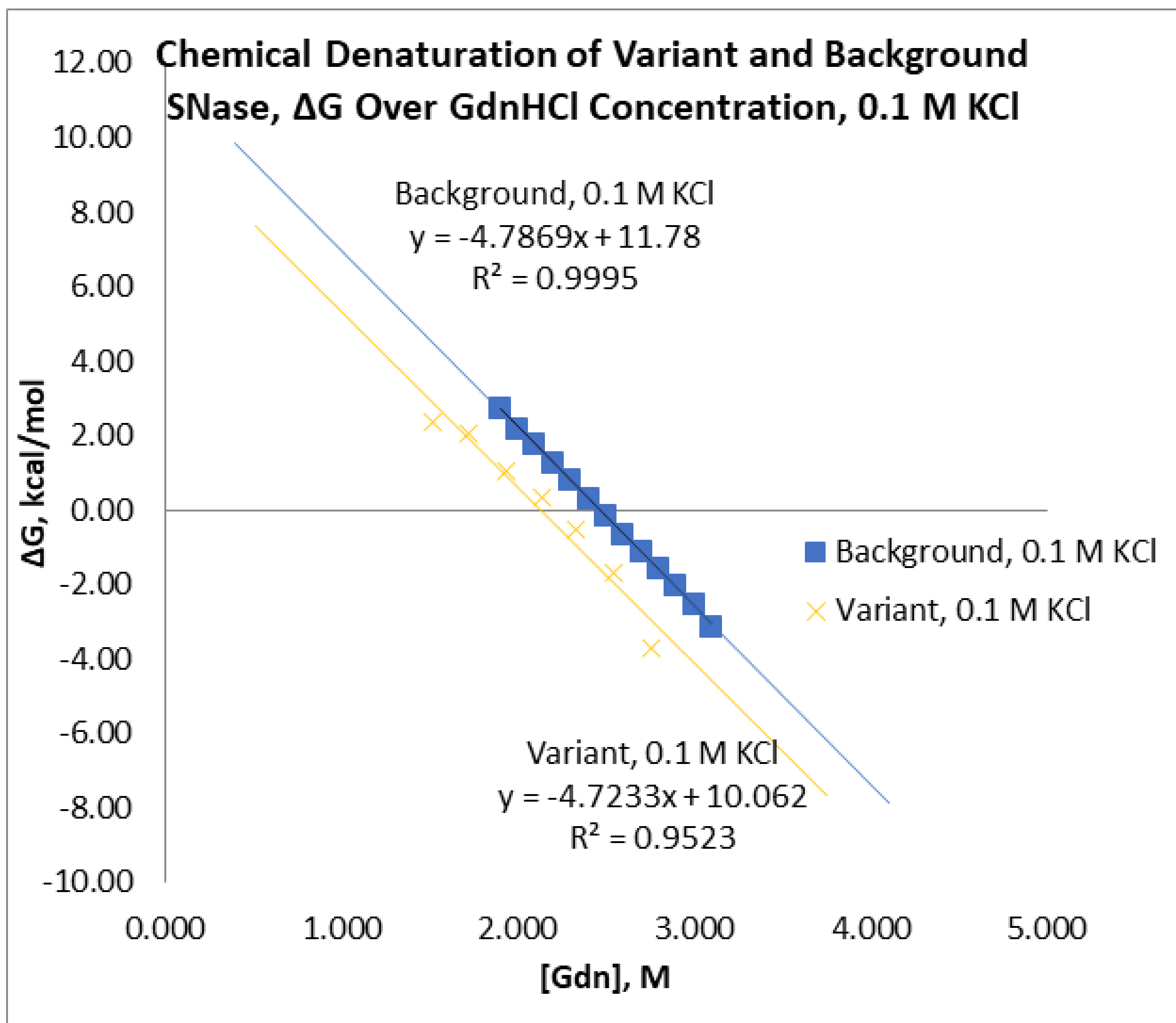
1C, Left: Variant SNase Spectra

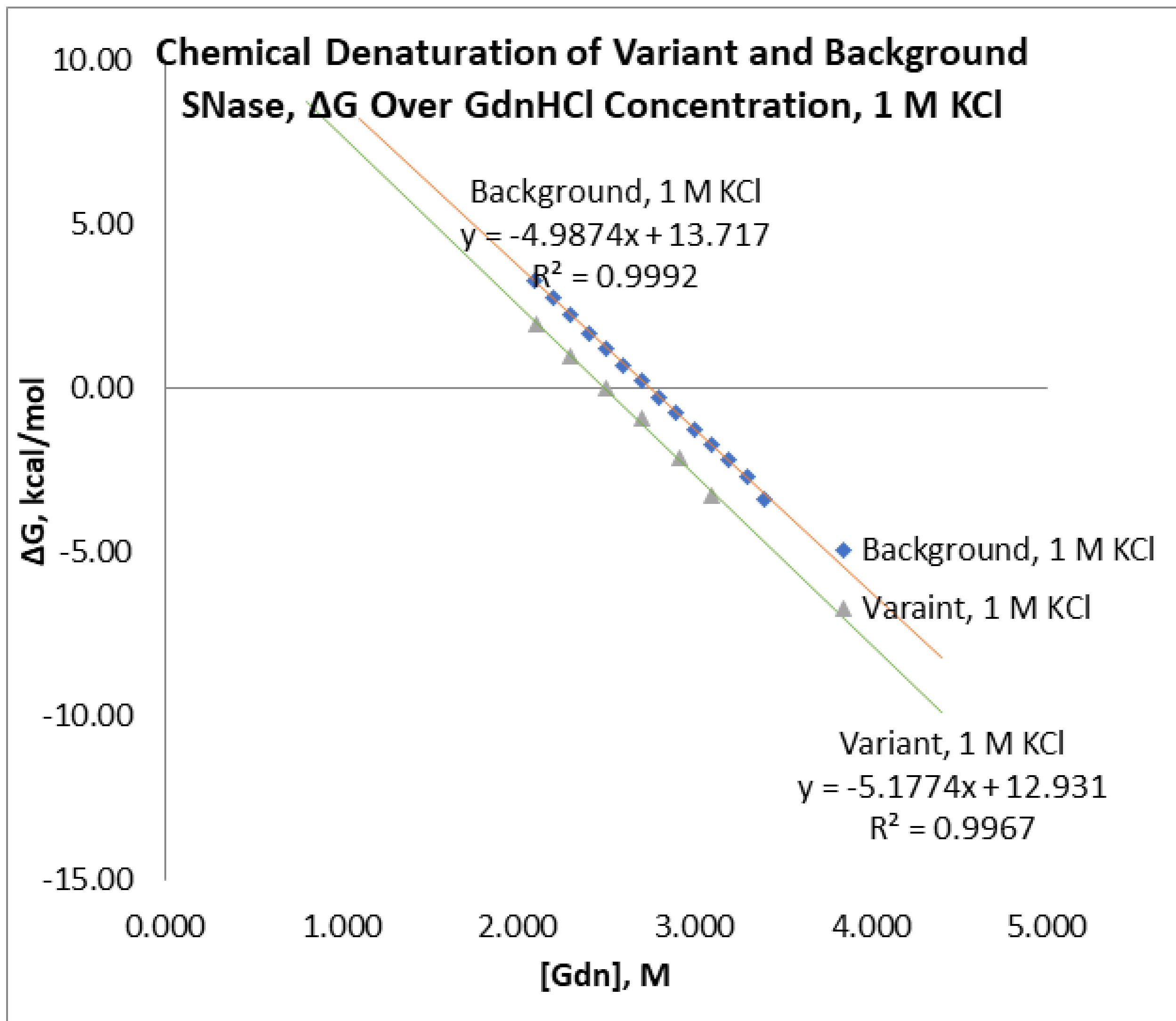
1D, Right: Background SNase Spectra

Figure 2:






Chemical Denaturation of Variant and Background SNase, ΔG Over GdnHCl Concentration, 0.1 M KCl
Background, 0.1 M KCl
y = -4.7869x + 11.78
R² = 0.9995
Variant, 0.1 M KCl
y = -4.7233x + 10.062
R² = 0.9523
Background, 0.1 M KCl
Variant, 0.1 M KCl
ΔG, kcal/mol
[Gdn], M
12.00
10.00
8.00
6.00
4.00
2.00
0.00
-2.00
-4.00
-6.00
-8.00
-10.00
0.000
1.000
2.000
3.000
4.000
5.000



Top to bottom: 2A) Acid titration of variant and background SNase with pH_{mid} values annotated. 2B) Temperature denaturation of variant and background SNase at two salt concentrations with T_m values annotated. 2C) ΔG values plot for variant and background SNase from chemical denaturation at 0.1 M KCl. 2D) ΔG values plot for variant and background SNase from chemical denaturation at 1 M KCl.

Table 1:

Ca++ at pH 9

Total Minutes	Time	Measured Diameter (mm)				
		Variant	Water	Background	Folded/ Inactive	Unfolded
0	7:38	0	0	0	0	0
42	8:20	4	0	3	0	2
92	9:10	4.5	0	4	0	2.5
138	9:56	8	0	8.5	0	4.5

EDTA at pH 9:

Total Minutes	Time	Measured Diameter (mm)				
		Variant	Water	Background	Folded/ Inactive	Unfolded
0	7:38	0	0	0	0	0
44	8:22	0	0	0	0	0
91	9:09	0	0	0	0	0
138	9:56	0	0	0	0	0

Mg++ at pH 9

Total Minutes	Time	Measured Diameter (mm)				
		Variant	Water	Background	Folded/ Inactive	Unfolded
0	7:38	0	0	0	0	0
45	8:23	3	0	3	3	2
91	9:09	3	0	3	4	2
138	9:56	3	0	4	4	2

Ca++ at pH 7

Total Minutes	Time	Measured Diameter (mm)				
		Variant	Water	Background	Folded/ Inactive	Unfolded
0	7:38	0	0	0	0	0
47	8:25	5	0	5	0	2
90	9:08	6	0	6	0	2
138	9:56	6.5	0	7.5	0	3.5