THESIS

CHARACTERIZATION OF THE DISSOCIATION EQUILIBRIA OF THE HISTONE H3H4 TETRAMER USING SEDIMENTATION VELOCITY ANALYTICAL ULTRACENTRIFUGATION

Submitted by

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ABSTRACT

CHARACTERIZATION OF THE DISSOCIATION EQUILIBRIA OF THE HISTONE H3H4 TETRAMER USING SEDIMENTATION VELOCITY ANALYTICAL ULTRACENTRIFUGATION

By performing sedimentation velocity analytical ultracentrifugation experiments under various ionic and pH conditions the k_d can be measured. This system was found to be pH dependent with a change of Svedberg (S) value distribution in between pH 5.2 and pH 5.5. The H3H4 system was found to be an interacting one due to the change in S value distribution with increasing the histone protein concentration. We found that the S value distribution is highly dependent on the ionic conditions of the solution with 2M NaCl solution showing higher S values then 5mM KPO_4 solution at the same concentration and pH. Oddly enough adding HEPES to a KPO_4 buffer will destabilize the H3H4 species present with 5mM KPO_4 having a higher S value distribution than 5mM KPO_4 10mM HEPES at the same concentration and pH. I was unable to model these systems to a H3H4 dimer to tetramer equilibrium model which leads me to believe the conditions I was using did not stabilize the tetramer save for the 2M NaCl.

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Chapter 1 – Introduction

Chapter 1.1 – The Nucleosome

The DNA in Eukaryotic cells wraps around proteins called histones in a complex called the nucleosome. The nucleosome consists of about 147 base pairs of DNA being wrapped around a octameric complex of histones. The central 80 base pairs of DNA is wrapped around the $(H3H4)_2$ tetramer with H2AH2B dimers flanking on either side (Luger 1997). The nucleosome is a target for post translational modifications on the histones which will control the accessibility of the chromatin to transcription factors. The nucleosome also acts as a barrier for replication and transcription by disallowing polymerases from proceeding past it.

Chapter 1.1.1 Disassembly and Assembly

To overcome the nucleosome barrier during replication the nucleosome is disassembled by protein factors. The disassembly of the nucleosome is known to occur ahead of the DNA replicase complex as shown by studies that have shown there are no nucleosomes 250bp behind the replication fork (De Bernardin, W 1986) or 300 bp ahead of the replication fork (Gasser 1996). Similar protein factors will disassemble or slide the nucleosome down the DNA to allow for RNA transcription to occur. As such the nucleosome must have a dynamic structure that can be readily broken down and rebuilt.

The in vitro kinetic folding of the unfolded H3 and H4 monomers associate in a twostep reaction, first undergoing a burst phase reaction into a dimer intermediate. This is followed by a first order folding reaction into the correctly folded H3H4 dimer (Banks and

Gloss 2004). These histone chaperones can bind H3 and H4 histone proteins only once they have been dimerized. In vivo assembly occurs as an ATP dependent process which involves a host of histone chaperone proteins as seen by biochemical fractionization experiments that isolate the proteins involved to than assemble chromatin in vitro (Stillman 1986).

When the H3H4 dimer is formed they can than associate with histone chaperones such as Anti-silencing function 1 (Asf1), chromatin assembly factor 1 (CAF-1) and histone regulatory homolog A (HIRA) (Das C. 2010). After this association with a chaperone the H3H4 dimers can then be brought to the nucleosome binding site on the DNA where they tetramerize (Liu, W. H 2016). The details of this process are still being investigated as there may be many more proteins involved helping regulate and assist this process.

Chapter 1.2 Structure of the Octamer

The histone octamer is made of two copies of each histone H2A, H2B, H3 and H4. Each histone protein has a conserved domain known as the histone fold which consists of an α helix-turn-α helix-turn-α helix. This histone fold helps the proteins dimerize in a handshake motif and is a core component of the final octamer structure (Arents, G 1995). These eight proteins form a tripartite complex with the association of a $(H3H4)_2$ tetramer with two H2AH2B dimers (Arents, G 1991). Isothermal heat burst microcalorimetry experiments have shown the H3H4 tetramer to first associate with one H2AH2B dimer to form a hexamer and then subsequently associate with another H2AH2B dimer to form an octamer (Benedict, R. C 1984).

Chapter 1.2.1 Stability of the H2AH2B System

Calorimetric and spectroscopic experiments of H2A and H2B have shown that at a pH below 4 the proteins are unfolded. The H2AH2B dimer is stable at pH 6.5-8.5 with no evidence of H2A or H2B monomer. This is evident as there is only one melting temperature for structures at this pH range indicating that the H2A and H2B monomer will not even exist in a folded state outside of dimerization. Ionic strength and concentration effected the enthalpy values gathered from the melting experiments, with increased melting temperature correlating with increased ionic strength and protein concentration (Karantza, V 1995).

Chapter 1.2.2 Stability of the H3H4 System

Thermal denaturation experiments a low ionic strength have shown that at pH 2.5 – 5.0 the H3H4 system behaves as a H3H4 dimer with only a single cooperative melting temperature for a single structure. Below a pH of 2.5 no secondary or tertiary structures were observed by thermal denaturation. This infers that like the H2AH2B system the individual monomers do not exist as intermediates and only as dimers or as part of a greater complex. The $(H3H4)_2$ tetramer and H3H4 dimer structures were shown to be stable at low ionic strength solutions at a pH range between 3.0 - 9.5. Since tetramer thermal denaturation is irreversible it was not quantified to the same degree as the H3H4 dimer. (Karantza, V. 1996).

The ionic effects on the H3H4 system have been explored through cross linking experiments where the H3H4 was examined using circular dichroism and native PAGE. Under both high and low salt conditions the tetramer was present suggesting high

tetramer stability under most ionic conditions. There was a change in secondary structure in higher 2M Chloride conditions then that was not observed in phosphate and sulphate. In low sodium chloride ionic conditions both dimer and tetramer rare detected as well as species thought to be aggregates which increase in population size as the salt concentration is raises. In phosphate and sulfate aggregation products were observed as well as dimer and tetramer in the cross-linking experiments and AUC equilibrium experiments (Baxevanis, A. D. 1991). These results inform that the type of ion in solution can lead to different structures in theH3H4 system.

To further delve into the thermodynamic properties of the H3H4 tetramer I will sedimentation velocity analytical ultracentrifugation (SV-AUC) to attempt to model the dissociation equilibria.

Chapter 2 – Equilibria of the H3H4 System

Chapter 2.1 pH Effects on the H3H4 System

Decreasing the pH of the system prior to running SV-AUC experiments will help highlight which amino acids are critical in the association of the H3H4 dimers to tetramers. At a pH of 4 the there is an SV-AUC Svedberg (S) value distribution in between 1-2 s. Increasing the pH to a value of 7 showed a shift of the distribution to 2 - 3 S (Figure 1). This shift happens in between a pH of 5.2 and a pH of 5.5. Once the shift in S value distribution happens no further shift will happen with increasing the pH beyond 5.5 (Figure 2).

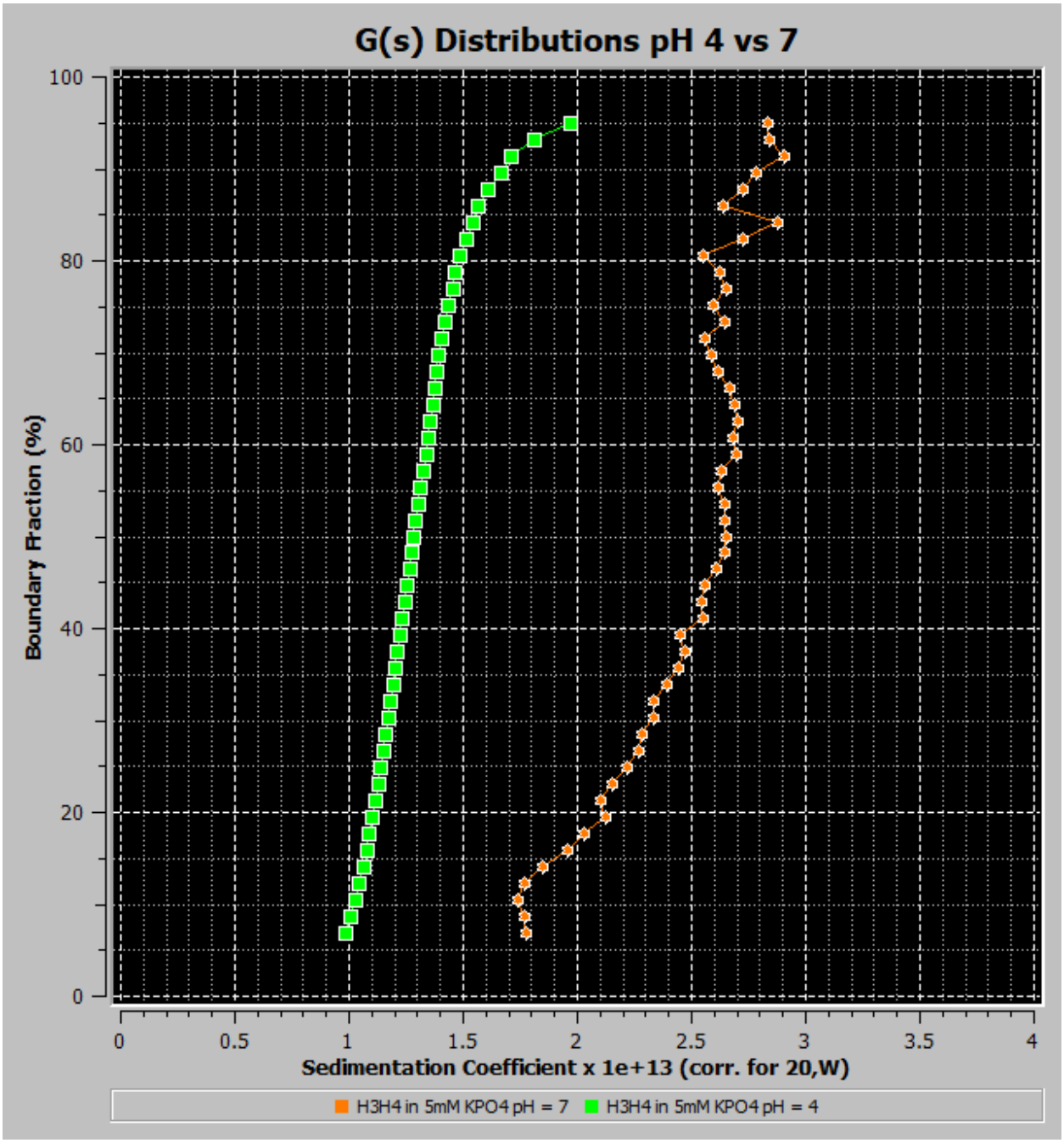

Figure 1: pH Change of S value distribution between pH 4 and 7. Green Squares: pH = 4. Orange Circles: pH = 7.

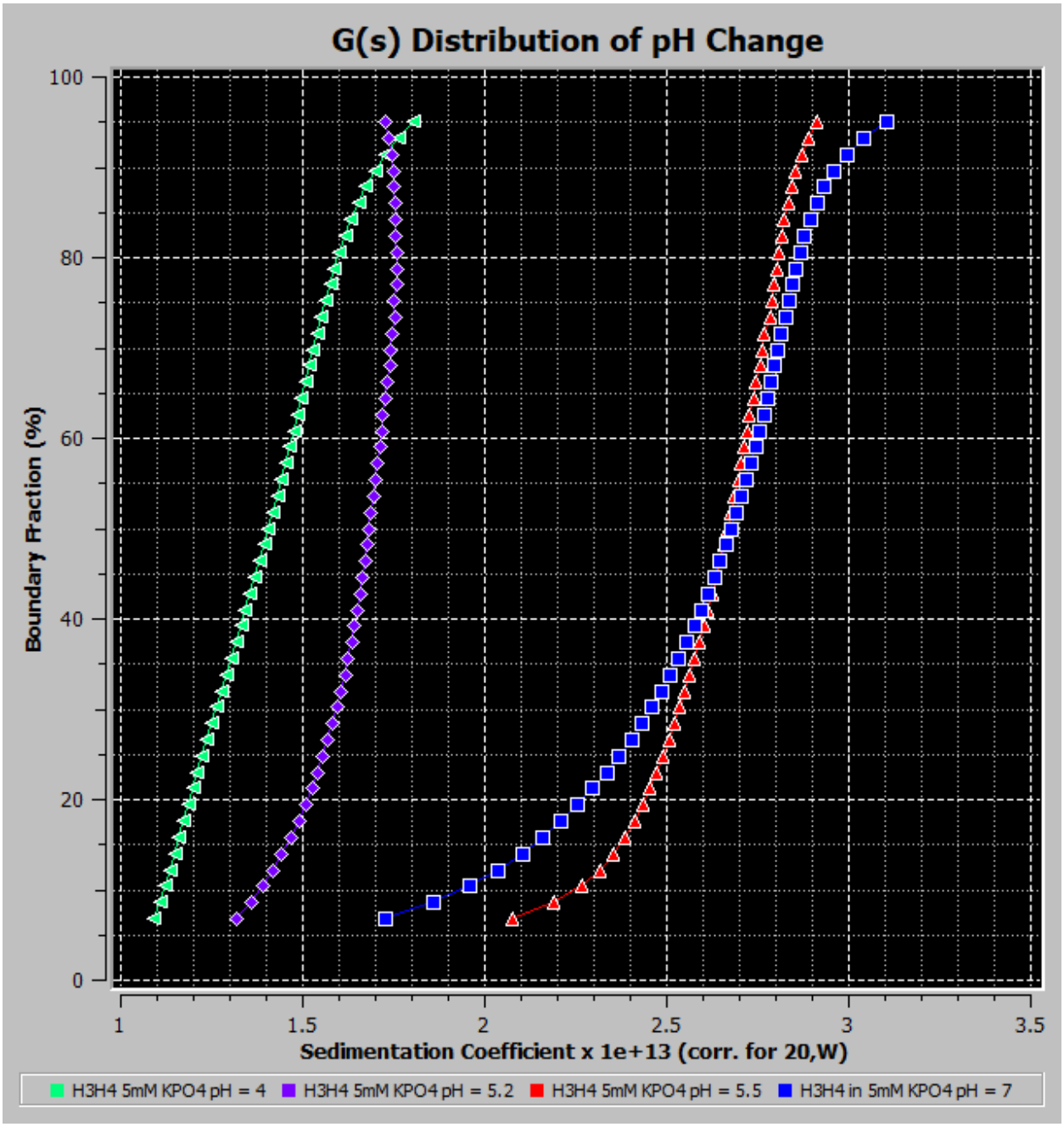

Figure 2: Svedberg Value Distribution for pH Changes. Green Triangles: pH = 4. Purple Circles: pH = 5.2. Red Triangles: pH = 5.5. Blue Squares: pH = 7.

Chapter 2.2 Concentration Effects on the H3H4 System

Running SV-AUC on the H3H4 system at various concentrations shows that the S value distribution is concentration dependent. An interacting system will change the S distribution with a change of concentration while the shape of the distribution is

indicative of the reaction rate. The shape of the H3H4 system distribution looks like the right half of a parabola that is a smooth change in S value between the proposed dimer S value and the proposed tetramer S value. This smooth change shows the dimer to tetramer equilibrium reaction happens rapidly.

As concentration shifts from 0.19 mg/ml to 2.6 mg/ml in 5mM KPO_4 10mM HEPES pH 6.8 the S distributions increase as more of the tetramer species is present (Figure 3). This is indicative of a mass action effect as the greater the concentration of H3H4 dimers there are in solution increases the chance that these dimers will find each other and tetramerize.

Each SV-AUC experiment with increased concentration does shift the S value distribution to the right. However increasing concentration from 0.06 mg/ml to 0.19 mg/ml or 0.48 mg/ml to 0.7 mg/ml shows very little change in the S value distribution (Figure 4). And increasing the concentration past 0.7 does not seem to affect the S value although due to optics restrictions runs past 2.6 mg/ml were not performed. Lack of seeing a concentration effect on octamer formation makes me suspect the ionic conditions of the experiments may be preventing their formation.

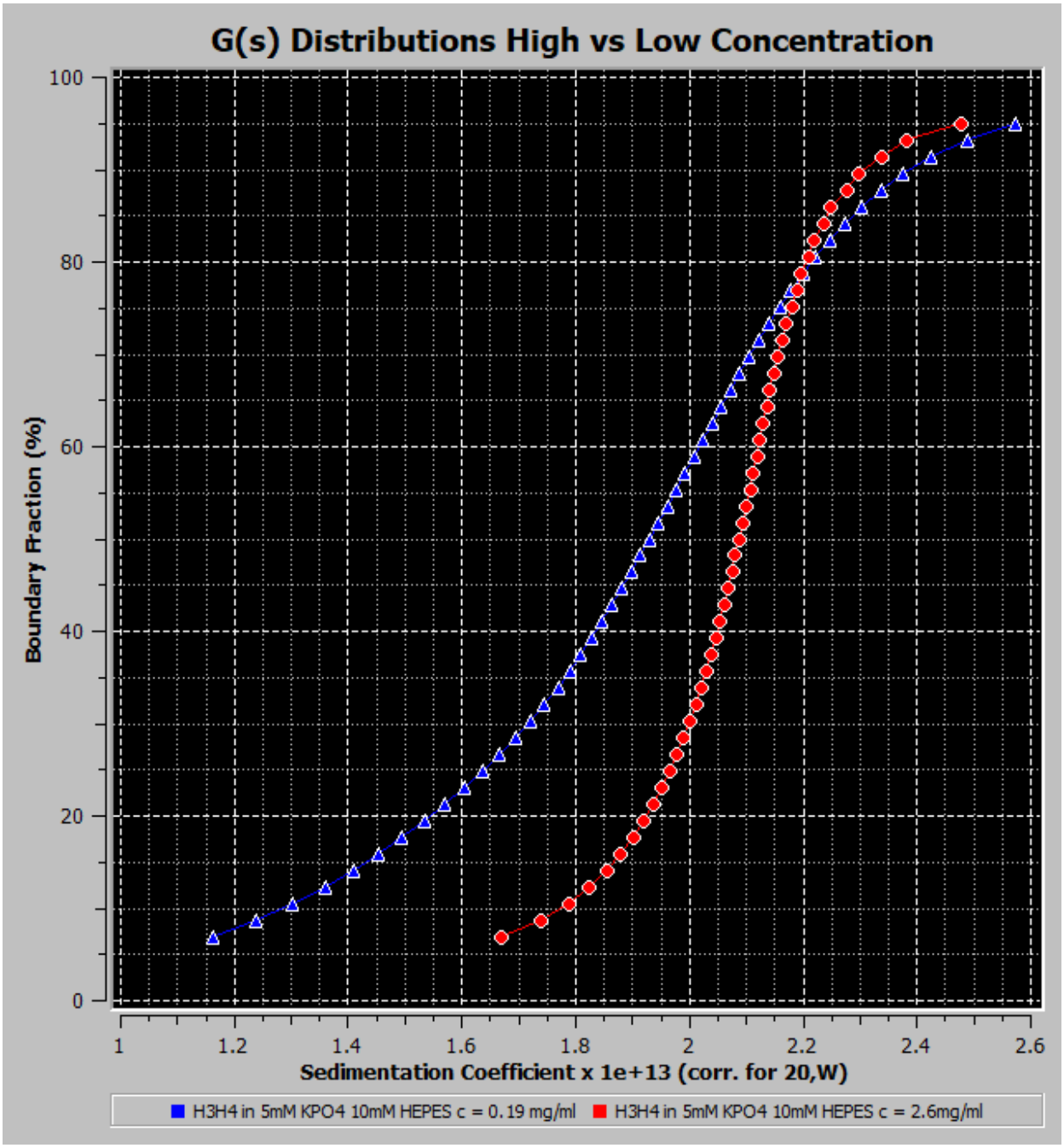

Figure 3: Shift of S value distributions due to concentration change in 5mM KPO_4 10mM HEPES pH = 6.8. Blue Triangles: Concentration = 0.19 mg/ml. Red Circles: Concentration = 2.6 mg/ml.

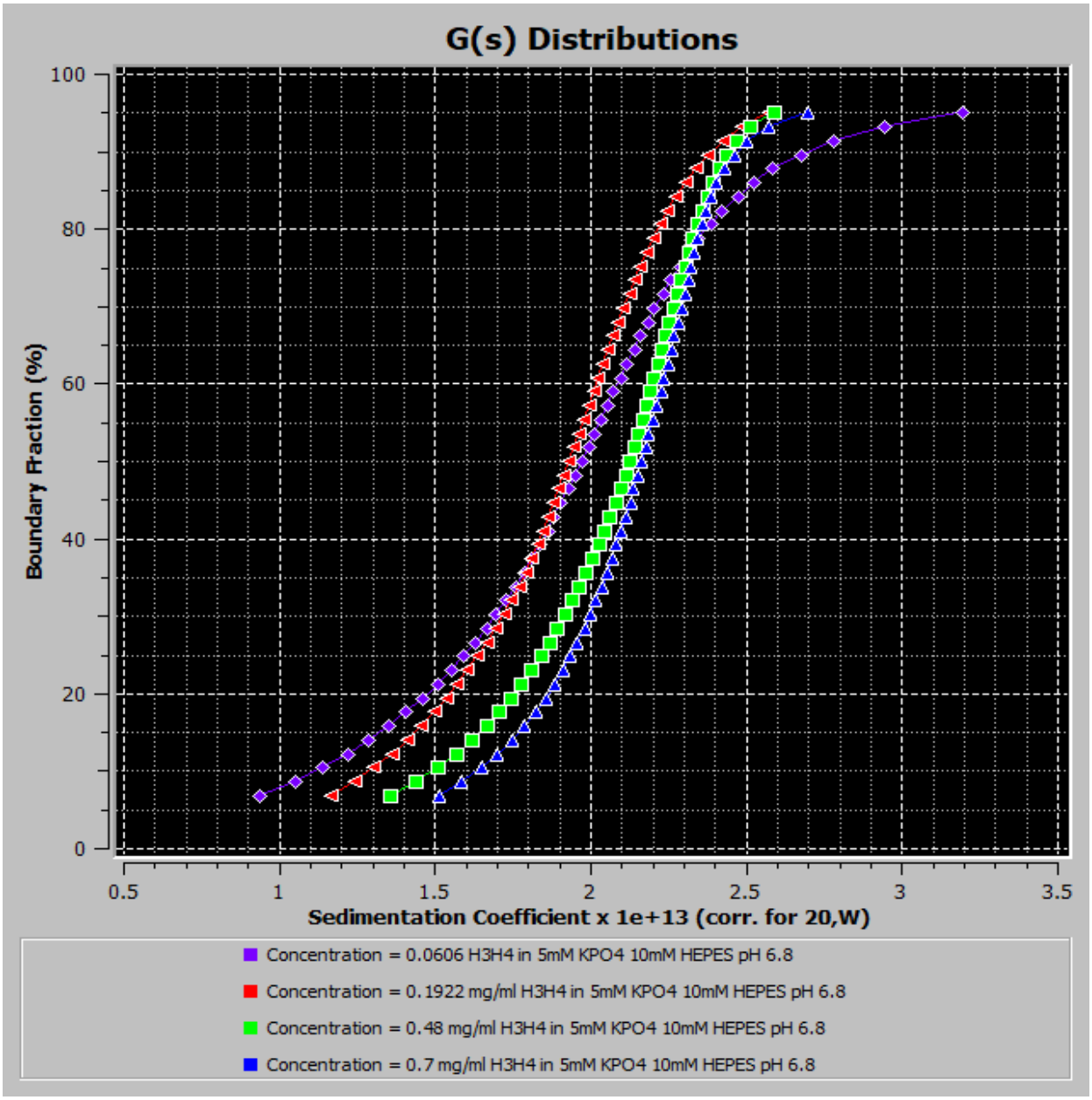

Figure 4: Shift of S value distributions due to concentration change in 5mM KPO_4 10mM HEPES pH = 6.8. Purple Diamonds: 0.0606 mg/ml. Red Triangles: 0.1922 mg/ml. Green Squares: 0.48 mg/ml. Blue Triangles: 0.7 mg/ml.

Chapter 2.3 Ionic Effects on the H3H4 System

Runs performed at 2M NaCl generated much higher apparent S values than experiments run with 5mM KPO_4 10mM HEPES. In these experiments' concentration and pH was held constant at 0.7mg/ml and 6.8 respectively for each run. The S value

distribution of the 2M NaCl H3H4 system was about 2.5 – 4.5 while the S value midpoint was about 3.5 s. At an equivalent pH and concentration in 5mM KPO_4 10mM HEPES the S value distribution was 1.5 -2.5 with a midpoint at about 2.2 S (Figure 5). This suggests that the high salt concentration of NaCl provides a stabilizing effect on the H3H4 system.

The 2M sodium chloride experiment had an increased stabilizing effect when compared against the potassium phosphate. However just adding a high amount of salt may not be universally stabilizing. With the concentration and pH held constant at 0.2 mg/ml and 6.8 the effects of HEPES was tested. In only 5mM KPO_4 the S value distribution was 1.7 – 3 with a midpoint of 2.4 S. In 5mM KPO_4 10mM HEPES the S value distribution was 1.2 – 2.5 with a midpoint of 1.9 S (Figure 6).

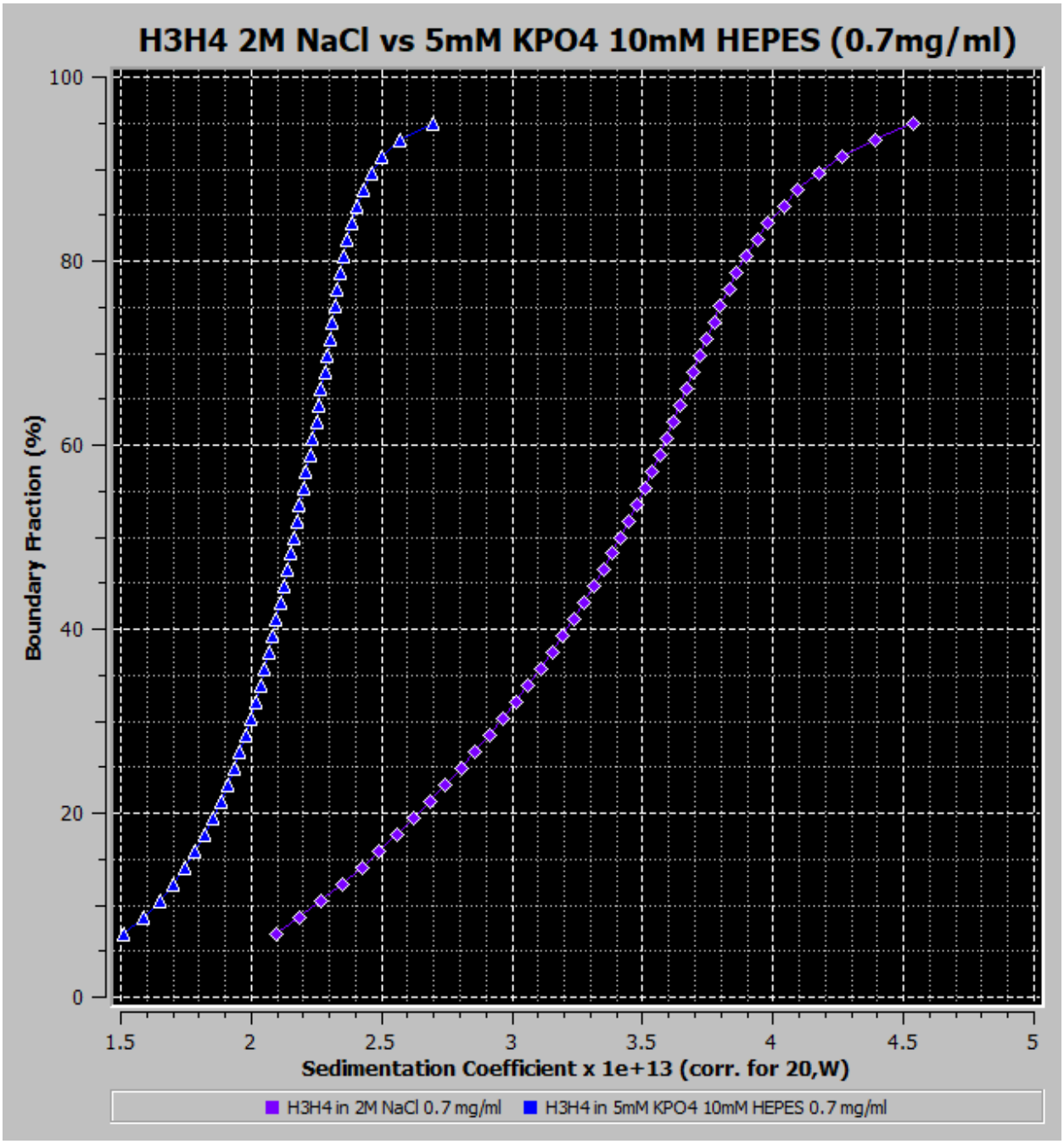

Figure 5: Ionic Effects on H3H4 System at 0.7 mg/ml protein concentration and pH = 6.8. Blue Triangles: 5mM KPO_4 10mM HEPES. Purple Squares: 2M NaCl 10mM Tris 1 mM EDTA.

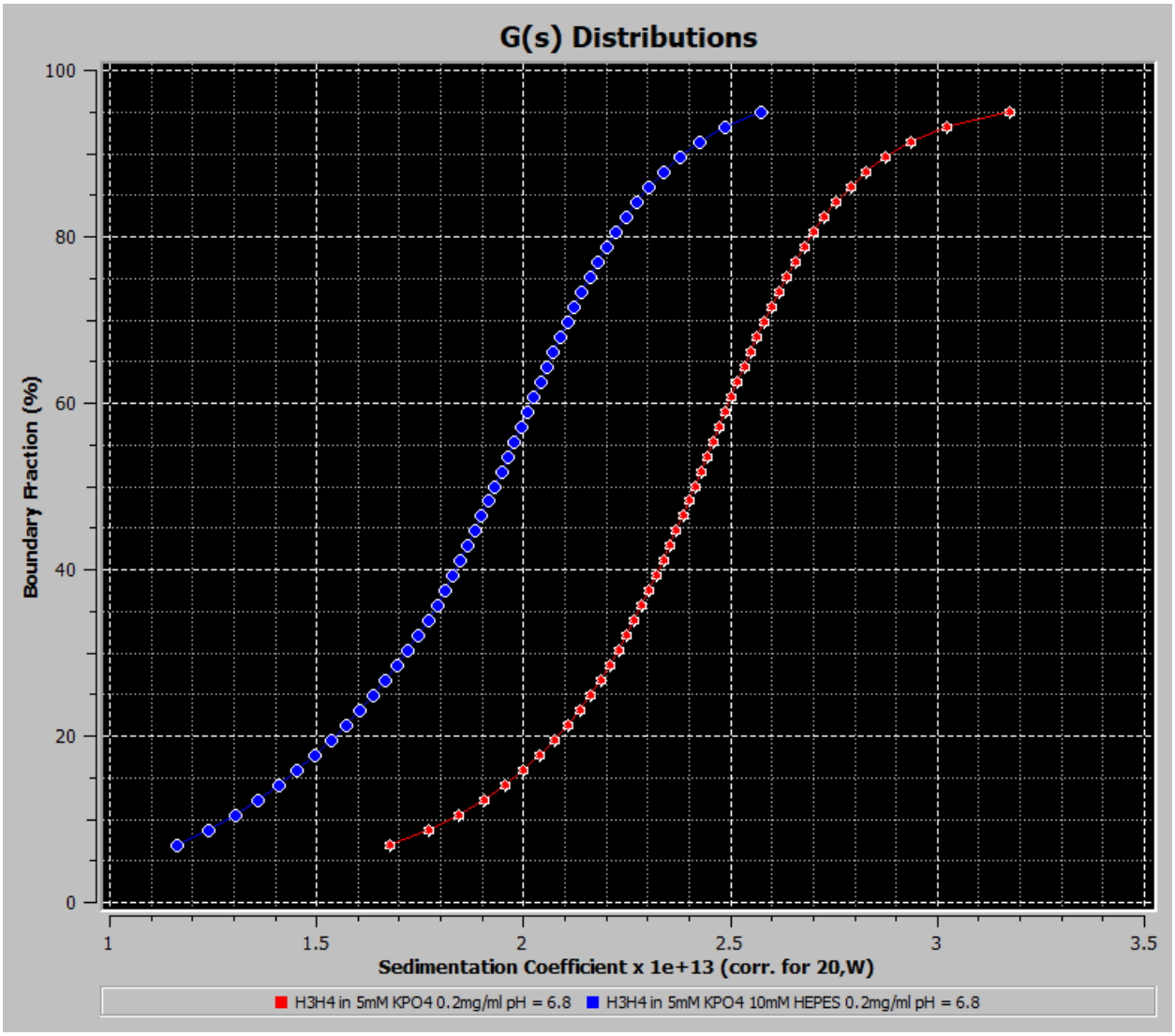

Figure 6: Ionic Effects on H3H4 System at 0.2 mg/ml protein concentration and pH = 6.8. Blue Circles: 5mM KPO_4 10mM HEPES. Red Circles: 5mM KPO_4.

Chapter 2.4 Genetic Algorithm to Calculate k_d

To generate a k_d value from the SV-AUC experiments a model was built using the reaction that H3H4 dimer oligomerizes to tetramer. While H3H4 octamer can be formed at higher concentrations I did not initially model to this as my concentration conditions suggested there would be little to no octamer present from past unpublished. I did however include a variable solute called aggregate where I floated a wide range of molecular weight values to account for any nonspecific aggregation.

Guessing k_d values I should have been able to continuously refit the data changing the values until the genetic algorithm showed a normal distribution of fits around my guessed value indicating a reasonably good fit of the value. The root mean squared deviations from the model were nonrandom when I ran my data through the genetic algorithm (Figure 7). This indicates the model is fundamentally wrong and at my conditions I am seeing something other then a dimer to tetramer reaction.

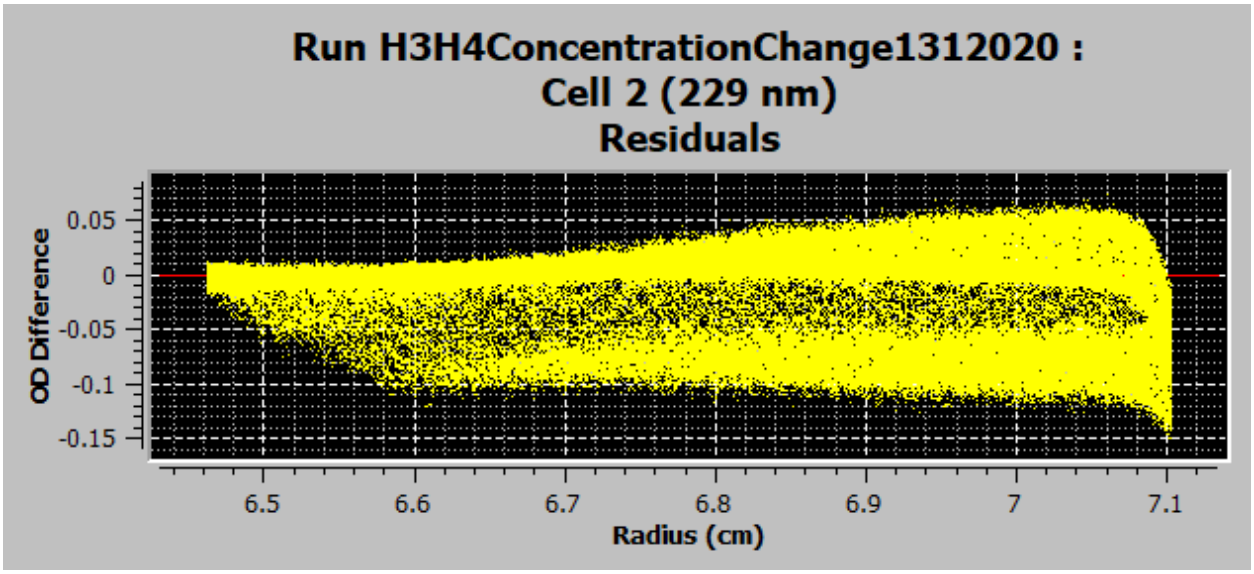

Figure 7: Root Mean Squared Deviations of the curve fitting from the H3H4 dimer to tetramer model.

Chapter 3 – Discussion

The H3H4 tetramer is a fundamental component of chromatin that helps regulate its structure as well as provides a barrier for transcription and replication (Tse, C. 1998). A quantitative value of the k_d between H3H4 dimers is crucial for a comparative look at the competitive interactions between histone chaperones and the H3H4 dimers with themselves. While the S distribution of SV-AUC experiments in the H3H4 system led me to believe I was looking at the reaction of H3H4 dimers into $(H3H4)_2$ tetramers the model ultimately proved to be ineffective at calculating k_d values.

An interacting system can be distinguished from a non-interacting system by running increasing concentrations of protein and performing an SV-AUC experiment. In an interacting system there will be a change in the S value that correlates to a change in the concentration and its shape will give information on the speed of the reaction taking place (Demeler, B. 1997).

The shape of the curve for almost every SV-AUC experiment under a variety of conditions was that of the left half of a parabola. There were some additional points almost making the shape sigmoidal, but this could be explained by the presence of higher S value structures such as a H3H4 octamer or some aggregate species being present during the experiment.

Based on the shape of the distribution I suspect that in each sample there is a population of both monomer, dimer, tetramer and octamer with their respective percent

compositions dictated by the conditions of each experiment. I expect in all conditions however there is always a fraction of each species present.

This gradual left half of a parabola shape indicates a fast reaction taking place. If this were not the case and the interaction was slow there would be multiple discrete S values distributions that would be straight up and down at each respective S value of each oligomer. However, the gradual distribution of S values leads me to believe my system was at rapid equilibrium between dimers and tetramers.

As the concentration increases the curve begins to straighten out at a S value single value rather than be distributed throughout many values. This indicates that as I increase concentration more H3H4 dimers will come into contact and associate due to the law of mass action. The more crowded the environment becomes in an interacting system the more probability that the system is shifted towards a more oligomeric state. From the results of the concentration experiments it looks as if I have a mixed sample of dimers and tetramers with the ratio favoring the tetramer the concentrated the sample becomes.

The pH effect is extremely pronounced while at a pH 4 the 1.1 – 1.7 S species is dominate. At a pH 5.5 and above the 1.7 – 2.7 S population is the main species present which would fit a dimer to tetramer transition at this pH. The protonation state of amino acid side chains as well as their amino and carboxylic acid termini is dictated by the isoelectric point (PI) of each amino acid in relation to the pH of the solution. The only amino acid with a PI around 5.5 is histidine. Examining the crystal structure of the tetramer there is a histidine at position 113 on the dyad axis where the two H3 proteins

of each H3H4 dimer are in proximity. This could inform on a key amino acid residue in the H3H4 dimer interface that could be tested with a histidine mutation at position 113.

The interface that exists at the interactions between H3H4 dimers is heavily affected by the ions in solution. This effect is to some degree concentration dependent as a low KPO_4 solution had a lower S distribution then a higher NaCl solution. However, the effect could also be the specific cations and anions involved rather than their concentration. This is further supported by the evidence that the addition of HEPES to the solution destabilized the structure present by lowing the S value distribution.

The hypothesis was that the dimer and tetramer would be the most prevalent species in low ionic conditions and at most pHs and concentrations. The model built reflected that hypothesis in that it used 2 analytes, the dimer and the tetramer to try and fit a guessed k_d value. Each analyte had a predefined molecular weight, viscosity, partial specific volume and extinction coefficient as calculated by UltraScanIII from their amino acid sequence. The root mean square deviations to this model in the genetic algorithm was nonrandom which would imply the model being incorrect. One possible explanation for the model not fitting is in such low salt concentrations is that the histone proteins had begun to aggregate causing many different sized structures. Another explanation would be that rather than a dimer to tetramer model I am measuring a monomer to dimer.

Further evidence supporting an alternate explanation of my original hypothesis is when I measured the H3H4 system in 2M NaCl and obtained a 3.8 midpoint S value that this might have been the value of a tetramer and not a H3H4 octamer as I originally assumed. 2M NaCl has been shown to stabilize the octamer during histone purification

so I can be reasonably sure that the tetramer would have been present under these conditions (Hake 2007 et. al, Chandra et al 1979).

Chapter 4 Future Directions

I am going to further test the oligomerization states over the summer by first performing a native polyacrylamide gel on H3H4 which have been cross linked under a variety of ionic conditions. Ideally, I would see at which conditions the octamer, tetramer, dimer and monomers are present. I suspect there is a minimum concentration of salt I will see these species and I can then redo the SV-AUC experiments at these conditions. I can then re-model the system from the new data and hopefully achieve random residuals indicating that the hypothesis was correct.

Once verified I would like to continue the experiment with the H113A H3 mutant. This mutant will test an amino acid residue experimentally proven to destabilize the interface of the tetramer. By measuring the k_d in this mutant it could be compared to the wild type to illustrate the residues contribution to the association. While discerning the k_d was not possible from the current data gathered I believe it is still possible to calculate this way if the conditions of the experiment was different.

Chapter 5 Methods

Chapter 5.1 Histone Preparation

Histone H3 and H4 were obtained in lyophilized samples and resuspended in 6M Guanidinium unfolding buffer for 1 hour. They were than combined in equimolar amounts and the final concentration was brought to 1 mg/ml with excess unfolding buffer. The sample was dialyzed against 2M NaCl 1mM EDTA 10mM Tris refolding buffer for 4 hours 3 times. The samples were the concentrated to a volume of 1 ml in a centrifugal concentrator for loading into a fast protein liquid chromatographer (FPLC) AKTA start. The H3H4 octamers, tetramers and dimers were separated from aggregates using FPLC. The fractions with the H3H4 in it was concentrated to 1 ml.

Chapter 5.2 Analytical Ultra Centrifugation

Sedimentation Velocity Experiments were run on a Beckman XL-I Analytical ultracentrifuge. Runs were performed at AN-60 TI 516 4 cell rotor. Each run was at 20C at 40,000 RPM for 600 scans at either 230 or 280 nanometer wavelengths.

Chapter 5.3 Data Analysis

Data analysis was done in UltraScanIII. Each run was processed for time invariant and radially invariant noise. The data was then analyzed by the van Holde-Weischets module to subtract for the effects of diffusion on the system. The genetic algorithm module was then used to curve fit the k_d and k_{off} values.

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