Studies of Delta N20 Beta 2-Microglobulin at Neutral pH: Thermal Denaturation and Characterization of Fibril Formation

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Candidate for the degree

Bachelor of Sciences

Submitted in partial fulfilment of the requirements for

Departmental Distinction in Chemistry/Biochemistry

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Abstract

This project studied amyloid fibril formation by the delta N20 (ΔN20) variant of the protein beta-2-Microglobulin (β_2m). Kinetic analysis of fibril formation found that the rate of fibril formation although varied for each sample, was complete within a range of eighteen to 63 hours. Thermal denaturation was utilized in study the stability of the ΔN20 β_2m relative to that of the wild type enzyme. After being denatured at 80°C, unlike wild-type, ΔN20 β_2m does not refold upon return to the starting temperature of 10°C. The midpoints of the transition regions were determined to be 55.9° and 45.6°C for wild-type and ΔN20, respectively. This is suggestive of a different structure compared to wild-type β_2m.

Introduction

Proteins fold into unique three dimensional structures, in some occasions the polypeptide chains form alternately folded structures that lead to insoluble aggregates. Such aggregations may form into fibrous structures leading to amyloid disease (1-3). Dialysis related amyloidosis (DRA), one of at least 30 human amyloidogenic diseases, affects long term renal dialysis patients via the deposition of amyloid fibrils in the musculo-skeletal system leading to arthritic symptoms and bone destruction (3-6). Analysis of the fibrils extracted from the carpal tunnel of a DRA patient identified β_2-microglobulin (β_2m) as the primary component of these fibrils and two shorter forms; one missing the first 6 amino acids (ΔN6) and one missing the first 19 amino acids (ΔN19) (4-7). As the soluble-subunit of the type 1 major histocompatibility complex (MHCI), ß$_2$m normally dissociates from MHCI and is degraded and excreted via the kidney. However, when a patient suffers from kidney failure, the inability of β_2m to pass through the dialysis membrane and the subsequent increase in the serum concentration of β_2m leads to development of DRA (8-9).

Beta 2-microglobulin (Figure 1), a small 99 residue protein with a 7-stranded ß-sandwich structure, is a member of the immunoglobulin superfamily of proteins (10). The two ß-sheets are linked by a disulfide bond (11). There have been many studies to initiate the formation of fibrils *in vitro*, in order to begin to understand the cause and the actual process of fibril formation. It was shown that fibrils can be formed *in vitro* by incubating the wild-type protein at low pH with high ionic strength. Under these conditions, the partial unfolding of the protein is favored which may be a precursor to amyloidosis (12-19). Studies with other proteins have shown that equilibrium between a native protein and a partially folded (or highly unfolded) species may be

an initiating event in fibril formation (20). The edge strands in proteins rich in β-sheets are surfaces particularly prone to aggregation (21). Certain amino acid residues, such as proline, function to reduce the probability of fibril formation (22). Eichner and Radford proposed a generic mechanism of amyloid formation by β_2-microglobulin involving a specific *cis* to *trans* proline switch (23).

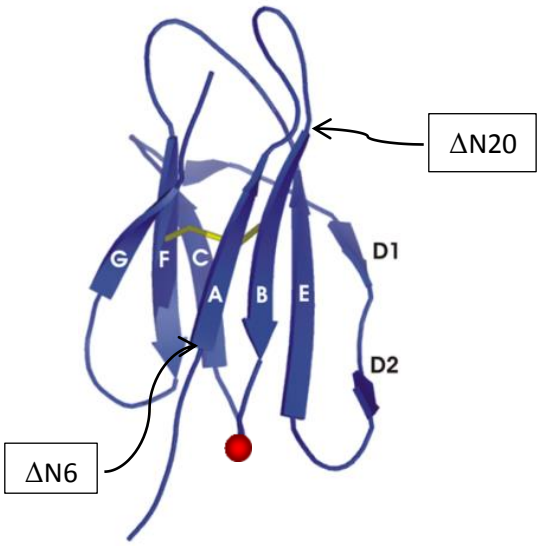

Figure 1. Ribbon structure of beta 2-microglobulin with P32, ΔN6 and Δ20 cleavage sites noted.

In 2005, Dr. Texter began a study in the Radford laboratory in the Astbury Centre, University of Leeds, of a ΔN20 construct of ß$_2$m (Figure 2), a close approximation of the naturally occurring ΔN19 ß$_2$m, to investigate the effect of the loss of the leading A strand. These studies showed the ΔN20 ß$_2$m to be the first near-native structure to form fibrils at physiological pH (24). The structure of ΔN20 ß$_2$m is currently unknown, although attempts to form crystals of ΔN20 ß$_2$m have begun.

WT β2m	ΔN20 β_2m
M(IQRTPKIQV YSRHPAENGK S)NFLNCYVSG FHPSDIEVDL LKNGERIEKV EHSDLSFSKD WSFYLLYYTE FTPTEKDEYA CRVNHVTLSQ PKIVKWDRDM	MNFLNCYVSG FHPSDIEVDL LKNGERIEKV EHSDLSFSKD WSFYLLYYTE FTPTEKDEYA CRVNHVTLSQ PKIVKWDRDM
100 AA; Mass: 11860; est. pI = 6.53; est. charge at pH7.0 = -1.2	80 AA; Mass: 9569 est. pI = 5.15; est. charge at pH 7.0 = -4.4

Figure 2. Comparison of wild-type β_2m and ΔN20 β_2m. The sequence in red is that deleted from ΔN20. The methionine at residue 1 results from expression in a bacterial system.

One method to gain insight into the secondary structure is through the use of far-UV circular dichroism (CD) spectroscopy. In a study done by Eichner, the far-UV CD spectra of wild-type and several variants of ß$_2$m (Figure 3) all showed similar shape with a negative peak at ~220 nm, which indicates an all β-structure. Yet the differences in the spectra, suggest there are some differences in the structures.

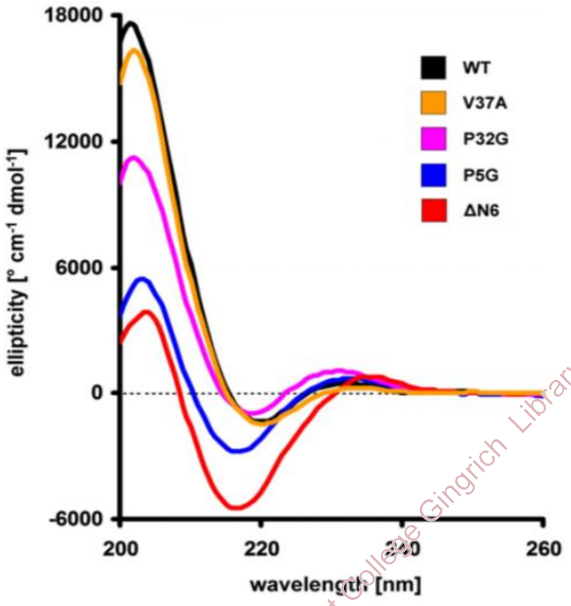

Figure 3. Far-UV CD spectra of wild-type ß$_2$m (black), P5G (blue), P32G (pink), ΔN6 (red) and V37A (orange) in 25 mM sodium phosphate at pH 7.5, 37°C (23).

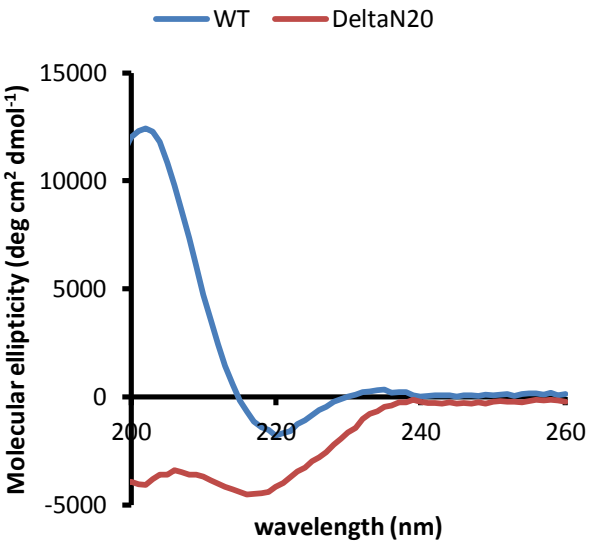

Figure 4. Far-UV CD spectra of wild-type (blue) and ΔN20 (red) ß$_2$m in 25 mM sodium phosphate after overnight incubation at 37°C (25)

However, when this study was performed on ΔN20 by Jennifer Reinhart '13 in the summer of 2011, the characteristic ~220 nm negative peak was not observed for ΔN20 (Figure 4), indicating that this protein is structurally different from wild-type β_2m. There may be a mixture of beta sheet and alpha helix or perhaps a portion of the protein is unstructured.

Eichner compared the stabilities of different ß$_2$m variants to that of wild-type ß$_2$m using equilibrium denaturation by urea followed by fluorescence spectroscopy (Figure 5). Wild-type ß$_2$m was found to be the most stable, with a midpoint at 4.3 M urea. The variant V37A was found to be the least stable, with a midpoint at 2.1 M urea (23). In the summer of 2012, Kaitlyn Pietrusewicz '13 attempted to repeat this experiment with ΔN20 to see where it fits within this group of variants. The results showed an increase in fluorescence with increasing urea concentrations without any apparent shift in the fluorescent peak (26). Fluorescent spectral comparison of the WT and ΔN20 proteins by Dr. Trexler in the presence and absence of 8M urea (24), suggests that this may be due to solvent exposure of both of the tryptophan residues in ΔN20 β_2m.

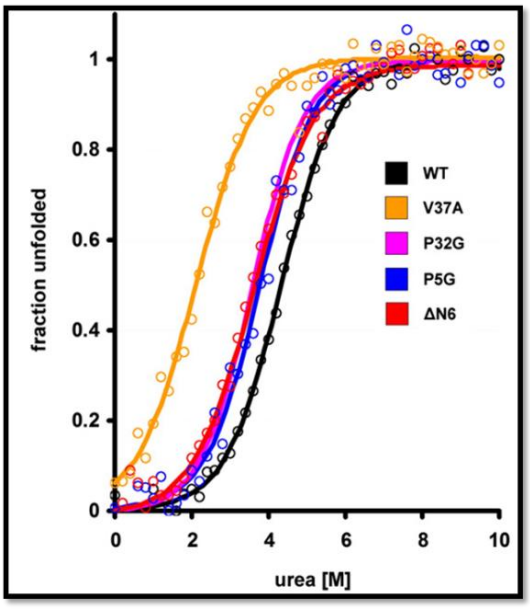

Figure 5: Equilibrium Unfolding by Urea of wild-type β_2m (black), P5G (blue). P32G (pink), ΔN6 (red) and V37A (orange) in 25 mM sodium phosphate buffer, pH 7.5, 37°C (23).

Rieffe Dia '14 carried out a study on equilibrium unfolding of wild-type and ΔN20 β_2m by urea using CD spectroscopy (Figure 6). Dia's value for the midpoint of the transition of the wild type, 4.0 M compares favorably with Eichner's value of 4.3 M. The transition for ΔN20 (2.6 M), shows that that the ΔN20 β_2m is less stable than the wild type protein as well as P5G (3.8 M), P32G (3.6 M) and ΔN6 (3.6 M), yet more stable than V37A with a midpoint of 2.1 M.

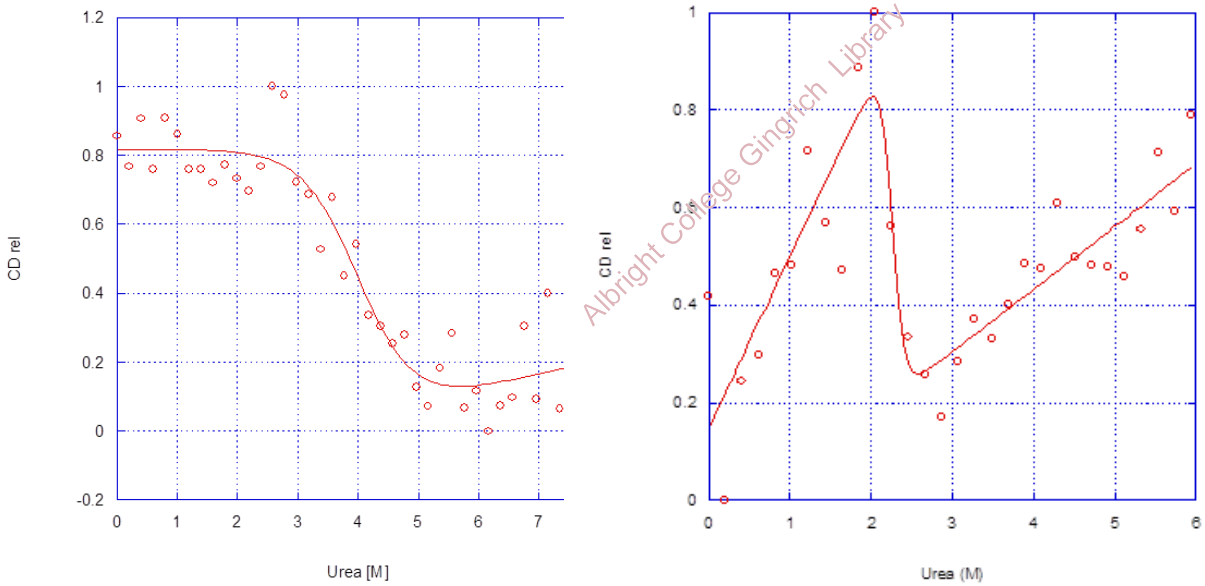

Figure 6: Equilibrium Unfolding by Urea of wild type (left) and ΔN20 (right) β_2m (27)

Analytical size exclusion chromatography (SEC) was used by Eichner (23) to study the size of wild-type β_2m and several variants (Figure 7). This study showed that wild type β_2m protein and the V37A control eluted as a single, sharp peak at a retention volume of 14 mL, indicating the monomeric form. A variant ΔN6 eluted the column a lower volume, 13 mL, suggesting a bigger size. The P5G variant eluted at two retention volumes, 13 mL and 14 mL, which suggests it contains two populations, one behaving like wild type and the other like ΔN6.

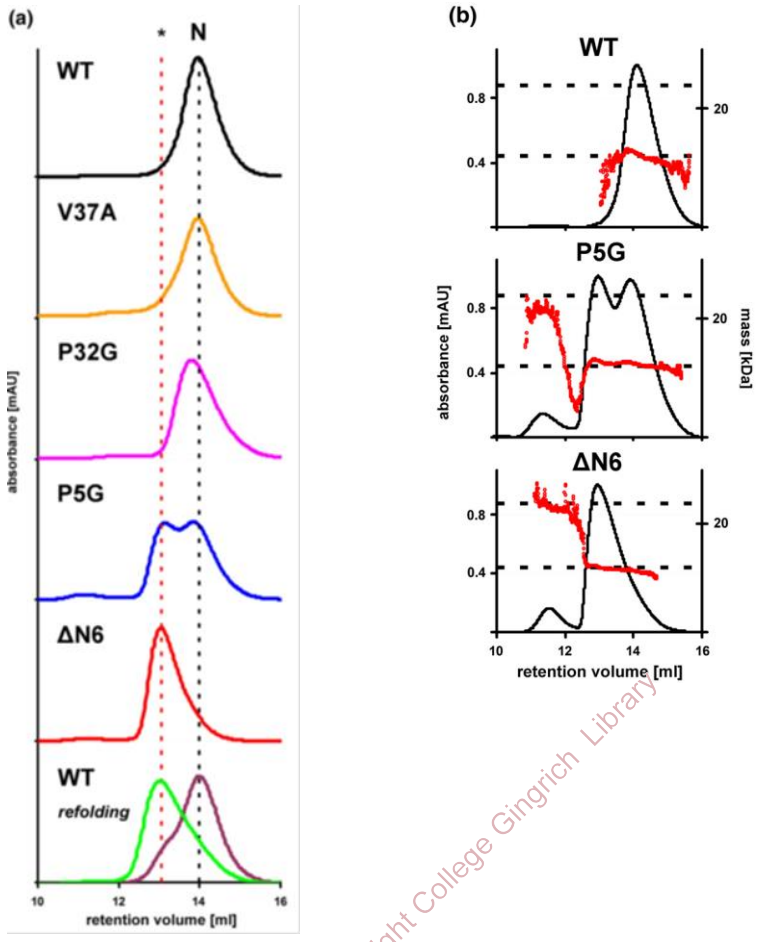

Figure 7. (a) Analytical SEC traces (obtained at 5 °C in 100 mM Tris–HCl, pH 8.0) of 80 μM wild-type β2m (black), V37A (orange), P32G (pink), P5G (blue) and ΔN6 (red) after incubation overnight at pH 7.5 (25 mM sodium phosphate) at 37 °C. (b) Determination of the masses of different β2m assembly intermediates using multi-angle light-scattering (MALS). The figure shows the absorbance at 280 nm in black and the light-scattering trace in red circles. For this analysis 500 μM wild-type β2m, P5G and ΔN6 were incubated for three days at 25 °C before injection onto the column. The horizontal broken lines indicate the masses of monomeric and dimeric β2m (23).

Multi-angle light scattering (MALS) coupled to SEC (Figure 7b) showed that the masses of the corresponding proteins were similar. Thus the species eluting at 13 mL are behaving as if they are a larger size. What this experiment suggests, however, is that the 13 mL species, a non-native monomer, contains a trans P32 conformation. This discovery supports the hypothesis that wild-type β_2m must undergo a *cis* to *trans* proline isomerization prior to forming amyloid fibrils.

Christine Candelora ’13 carried out a similar study of WT and ΔN20 β2m by analytical size exclusion chromatography (SEC) at neutral pH and compared those results to the study done by Eichner. In her experiment, wild-type also eluted at 14 mL, but ΔN20 consistently gave a large peak at 11.3 mL and a smaller peak at 10 mL. Her results suggested that the loss of the first 20 residues, which includes the first beta strand, leads to a less tightly packed protein and results in a conformation of ΔN20 that also behaves like a larger protein (28).

Analytical SEC experiments monitored by MALS carried out at the Astbury Centre in the summer of 2013 found that the peaks observed with ΔN20 β_2m were actually a dimer (11.3 mL) and a hexamer (10 mL). Thus, suggesting that ΔN20 β_2m more readily forms dimeric and oligomeric species than previously demonstrated by wild type β_2m and other variants.

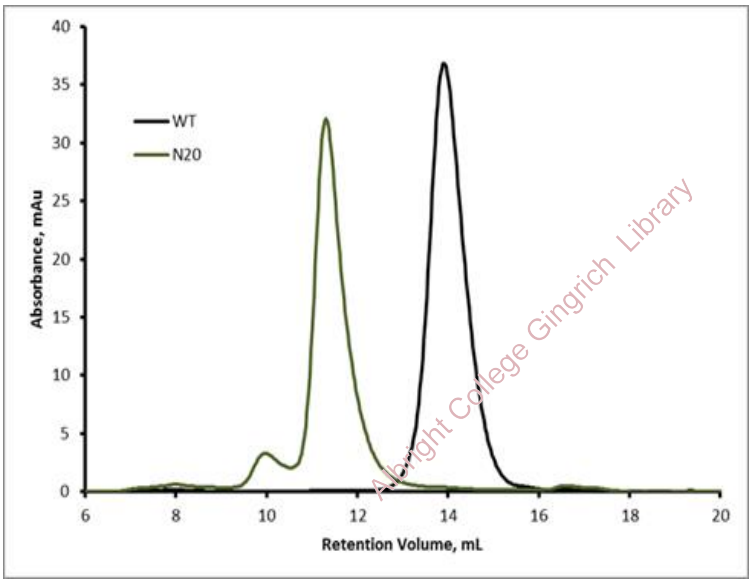

Figure 8. Analytical SEC trace (obtained at 5°C in 100 mM Tris-HCl, pH 8.0) of 80 µM for wild-type (black) and ΔN20 β_2m (green) after incubation overnight at pH 7.5 (25 mM sodium phosphate) at 37°C (28).

Delta N20 was then incubated overnight under the then current conditions used to monitor fibril formation at neutral pH (80 µM in 50 mM MES (2-N-(morpholino)ethanesulfonate buffer, pH 6.5, shaking at 600 rpm, at 37°C. Analytical SEC showed that both peaks had disappeared, suggesting that fibril formation was complete within the 20 h incubation period (24). Dia continued this study to characterize the kinetics of fibril formation using analytical SEC (Figure 9) and native polyacrylamide gel electrophoresis (PAGE). The peaks observed at ~8 mL represent higher order oligomers. As fibril formation proceeds, these higher order oligomers disappear as the hexameric and/or dimeric species appear to go directly to insoluble fibrils. All samples applied to the SEC column were centrifuged prior to loading to remove any insoluble aggregates (fibrils).

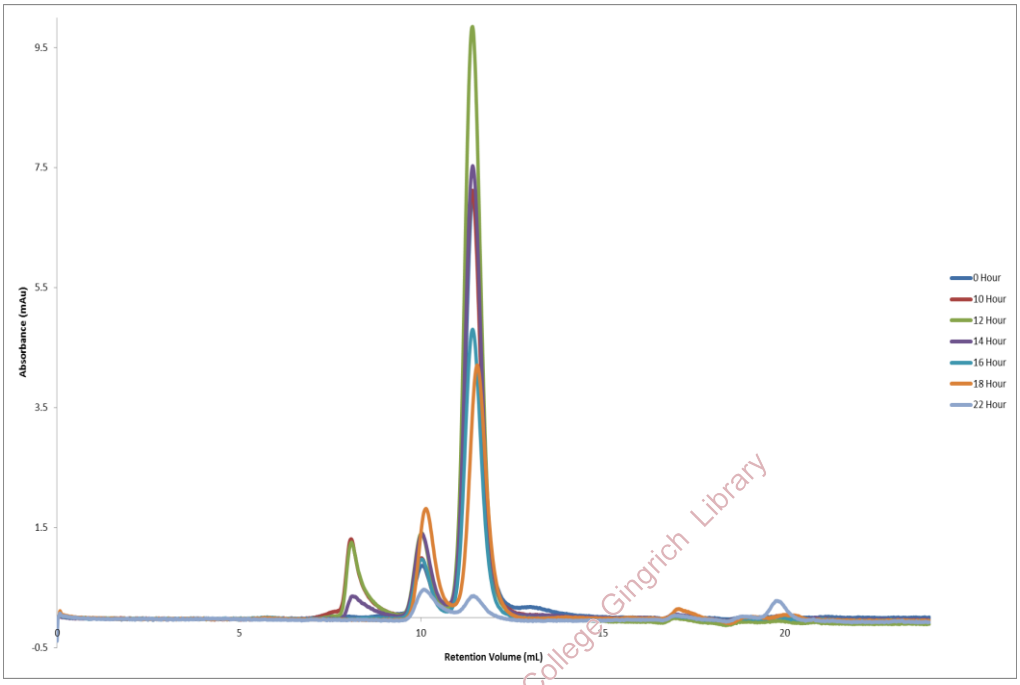

Figure 9. Time course of oligomerization of ΔN20 β_{2}m (50 mM MES buffer, pH 6.5, 600 rpm, 37°C) (27).

Native gels (Figure 10) were used to follow the formation and disappearance of the oligomers as ΔN20 transitions from dimer to fibils. The results are suggestive of hexameric and higher order oligomers, however, since one cannot use standards to accurately determine size with native gels this is not known for sure.

Figure 10. SDS PAGE of time course of oligomerization of ΔN20 β_2m (27)

Eichner carried out an ensemble experiment (Figure 11) to investigate the capability of the variant species in his study to form fibrils under unseeded conditions. Multple samples were prepared in 96- well plates. Fibril formation was measure using a thioflavin-T (ThT) binding assay. Thioflavin-T is a fluorescent dye which binds specifically to amyloid fibrils, while demonstrating no binding to the monomeric species. Samples were analyzed every 48 hours to monitor the rate of fibril formation. The wild type β_2M did not form fibrils over a period of 100 days, while fibril formation by P5G β_2M centered at 27.5 days and by ΔN6 β_2M at 22.5 days. These were the first demonstrations of unseeded fibril formation by any variant other than ΔN20.

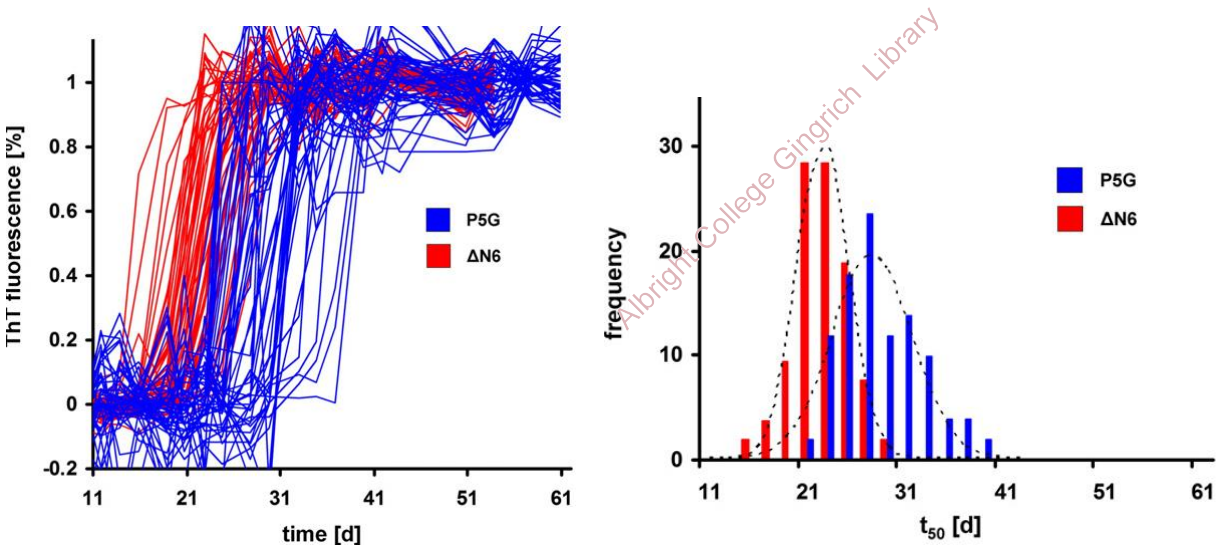

Figure 11. Formation (unseeded) of amyloid like fibrils of different variants in 25 mM sodium phosphate buffer, pH 7.5, 37°C, and 200 rpm. (Left) ThT fluorescence of 50 replicate samples of ΔN6 (red) and P5G

(blue). The fluorescence was normalized to the highest reading after 100 days. (Right) Distribution of t_{50} values for ΔN6 (red) and P5G (blue).

One goal of this project was to further characterize the rate of fibril formation via SEC, SDS (sodium dodecylsulfate) PAGE, electron microscopy and the thioflavin T binding assay. SDS gels allowed us to follow the disappearance of the monomer protein against known standards. A second goal was to use thermal denaturation by CD spectroscopy to compare the stability of wild type and ΔN20 β_2M.

Methods

Preparation of ΔN20 Samples for Kinetics Experiments

Purified protein was stored at -20C. Samples were dissolved in the appropriate buffer, centrifuged at 10,000 x g for 10 minutes at 10°C. The concentration was determined by measuring the A280, using the extinction coefficients 17,400 $M^{-1}cm^{-1}$ and 19,440 $M^{-1}cm^{-1}$ for ΔN20 and wild type, respectively. A final concentration of 80 μM was used in the SEC experiments. Unused solutions were stored at -80°C for later use.

Kinetics Experiments

After the protein samples were prepared, an aliquot was taken immediately for the 0 hour time point. Samples to be used for ThT binding assay or electron microscopy were used immediately. Samples for SEC analysis or SDS PAGE were centrifuged at 10000 x g for 20 minutes at 10°C. The supernatants were immediately loaded on the AKTA or stored at -20°C for SDS PAGE analysis. The remaining sample was placed in the Eppendorf ThermoMixer at 37°C, 600 rpm and aliquots were removed at designated time intervals for analysis.

Analytical SEC

A GE Healthcare AKTA Prime Plus system attached to a Superdex 75™ 10/300 GL column was used for the analytical SEC experiments. The mobile phase was filtered (0.22 μm) and degassed 100 mM Tris-HCl, pH 8.0 buffer using a 0.4 mL/min flow rate at room temperature. All samples were centrifuged for 20 min as noted above prior to injection of a 50 μL sample volume using a 100 μL external loop.

ThT Binding Assay

A HORIBA Jobin-Yvon Fluorolog-3 Model FL3-11 Spectrofluorometer was used for the fluorescence analysis. The instrument was set to the following parameters: temperature (37°C),

slits (3nm), excitation wavelength (440 nm), emission wavelength (480 nm), measurement time (1 minute), measurement rate (one point per second).

A solution of 10 μM ThT in 0.5 M Tris-HCl buffer, pH 8.5, preincubated at 37°C,. was used to determine the blank fluorescence. For each protein reading, 990 μL of the ThT/buffer solution was allowed to equilibrate in the instrument for two minutes. The reading was begun by addition of 10 μL of protein sample. A minimum of three measurements were made for each aliquot.

SDS PAGE gels

Loading buffer was prepared by adding 20 μL of 2-mercaptoethanol to 980 μL of Tricine Sample Buffer (Bio-Rad). Samples were prepared by adding 10 μL of loading buffer to 10 μL of protein sample. The samples were heated at 100°C for 5 minutes and then pulse spun. A 10 μL volume of each sample was run per lane. Empty lanes were loaded with a 1:1 solution of MilliQ water and loading buffer. The gels were run at a constant current of 30 mA until the samples entered the resolving gel, then the current was adjusted to 60 mA. The gels were run until the dye bands ran off of the gel.

The gels were washed 3 times for 5 minutes each in 100 mL milliQ water and then stained for 1 hour with 20 mL of Gel Code Blue Safe Protein Stain. The gels were destained with 100 mL milliQ water for at least 1 hour or overnight. After destaining, a picture of the gel was taken.

Transmission Electron Microscope Grid Preparation

To prepare grids, the protein samples were diluted 1:10 with MilliQ water (2 μL of sample with 18 μL of water). The diluted protein sample (18 μL) was then placed onto Parafilm. A 18 μL sample of 2% uranyl acetate was also placed onto Parafilm. A grid was placed coated (shiny) side down onto the protein sample drop for 30 seconds. The grid was blotted with filter paper (by standing on edge on the paper). The grid was then placed coated side down on the 2% uranyl acetate for 30 seconds, blotted as above, and allowed to air dry before being stored.

Circular Dichroism Spectroscopy

A Jasco Model J-810 spectopolarimeter was used for these experiments. Samples of protein at 10 μM were prepared in 25 mM sodium phosphate buffer, pH 7.5. CD. A buffer blank spectrum was subtracted from each of the protein-containing sample spectra. The instrument was set to the following parameters: Sensitivity (Standard), D.I.T. (8 sec), Band width (1.00 nm),

Start (250), End (190), Data Pitch (0.1 nm), Scanning Speed (100 nm/min), Accumulations 4. A buffer blank spectrum was subtracted from each protein spectrum.

Results

Kinetics of Fibril Formation

In order to further characterize the rate of fibril formation, size exclusion chromatography was used in conjunction with SDS PAGE. The SDS gels allow one to monitor the disappearance of monomer in solution over time, indicating fibril formation. The samples analyzed by SEC are sent through a column that separates on the basis of size. The resultant data is a chromatogram in which large molecules are eluted first since they travel a shorter path through the column than smaller molecules.

Initial experiments showed little change in the relative size of the dimer and hexamer peaks, but a peak at ~8 mL representing the larger higher order oligomers did appear and then disappear as fibrils began to form in the samples. In order to focus more directly on the transition to fibrils, the incubation for the experiment illustrated by Figure 11 was started at 8 pm, so that the 12-24 h samples could be analyzed over the next day.

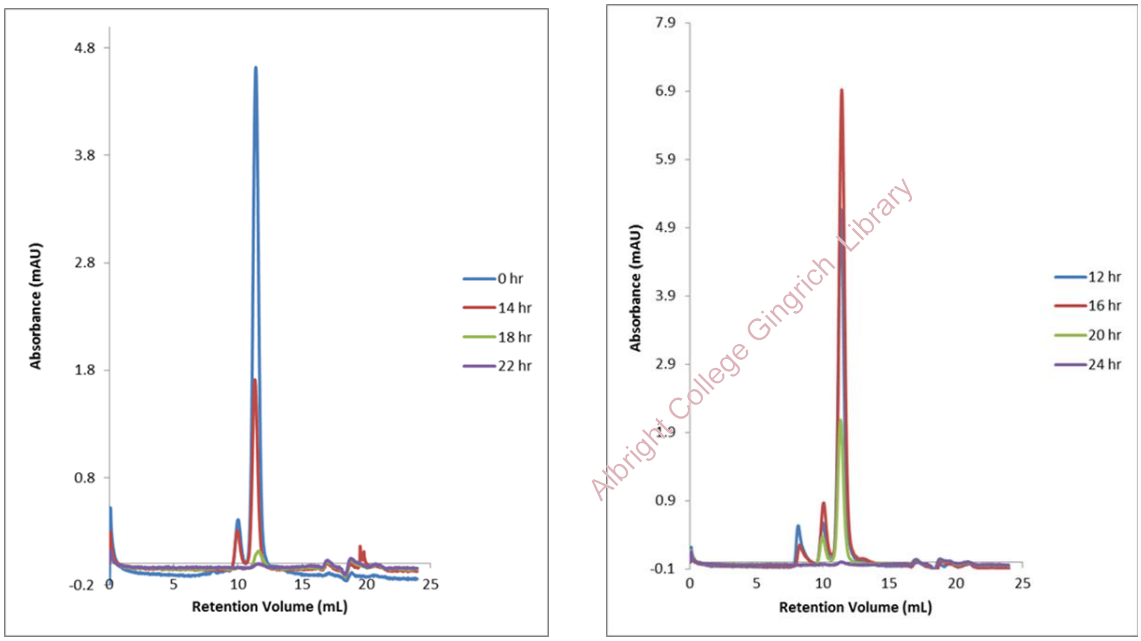

Figure 12. Analytical size exclusion chromatograms of two samples from the same solution in 50 mM MES buffer, pH 6.5, 600 rpm, 37°C. Aliquots were removed every two hours from alternating samples.

The same samples from Figure 11 were analyzed by SDS PAGE. This technique is similar to SEC in that it separates the supernatant on the basis of size. So, again we are looking for the disappearance of fibril building blocks. In Figure 12, the dark bands on the left are for the marker proteins. The bands on the right are samples of ΔN20 β2M which can be compared to the marker protein. Fibril formation appeared to be complete for sample 1 by hours 18-22 and by hour 24 for sample 2.

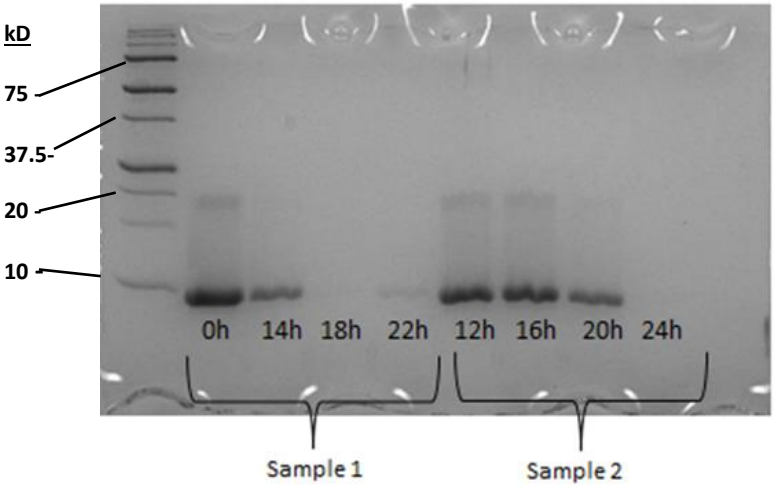

Figure 13. SDS PAGE for the same two samples used in the SEC experiment (Figure 11).

Grids were prepared for visualization by electron microscopy. The image (Figure 13) is from a 36 hour sample of the previously described experiment.

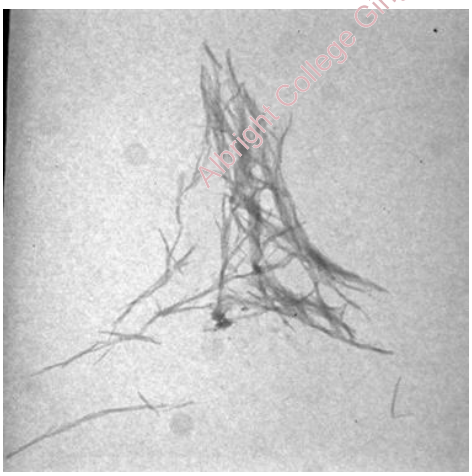

Figure 14. Transmission electron micrograph of sample 2 from Figure 13 after 36 h.

In order to analyze a greater number of aliquots, three samples incubated as described for Figure 12, however, the aliquots were analyzed by SDS PAGE and the ThT binding assay. Grids were also prepared in order to see if it was possible to identify the time at which fibrils were detected by TEM. Figure 15 presents the SDS gels for the three samples over a total time frame of 63 h.

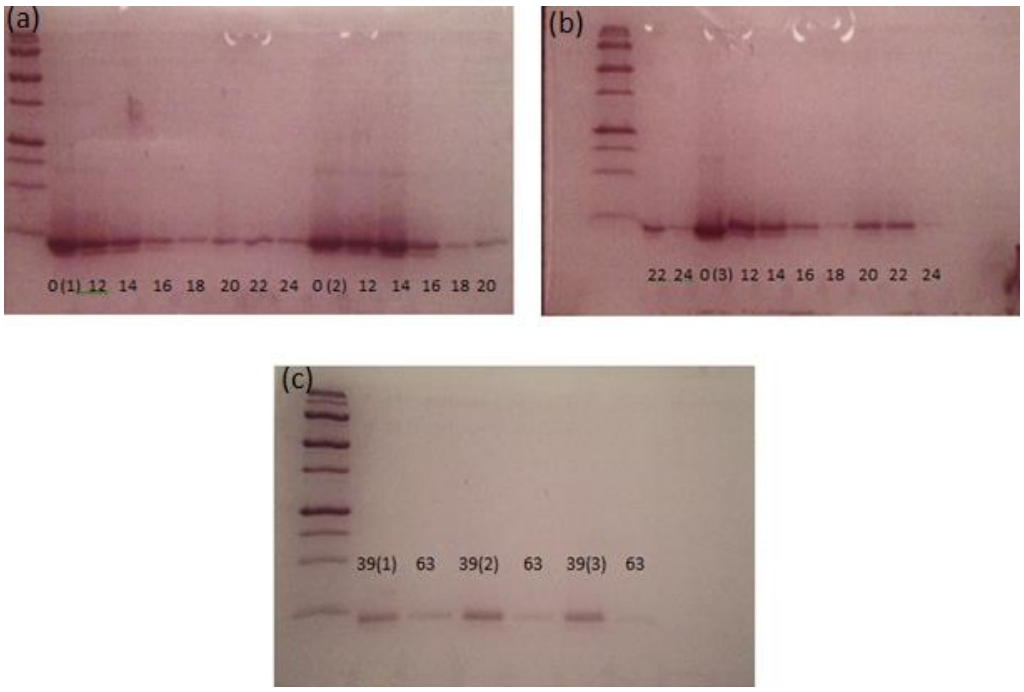

Figure 15. SDS PAGE Gel Data. Three samples 1, 2 , and 3 distributed over gels (a), (b), and (c) were prepared from the same protein and allowed to shake at 37°C, 600 rpm. Aliquots were taken every two hours from each sample. A slight amount of monomer was still noticeable at 63 h incubation.

The same samples from Figure 15 were analyzed for fibril formation by the ThT binding assay as were those from Figure 13. Shown below (Figure 16) is ThT data for both of these experiments. The percent increase in fluorescence over time compared to a ThT buffer blank was plotted after normalizing the samples value measure for the final time point of each data set. Some samples reached maximum fibril formation by 15 hours, others took as much as 24 hours.

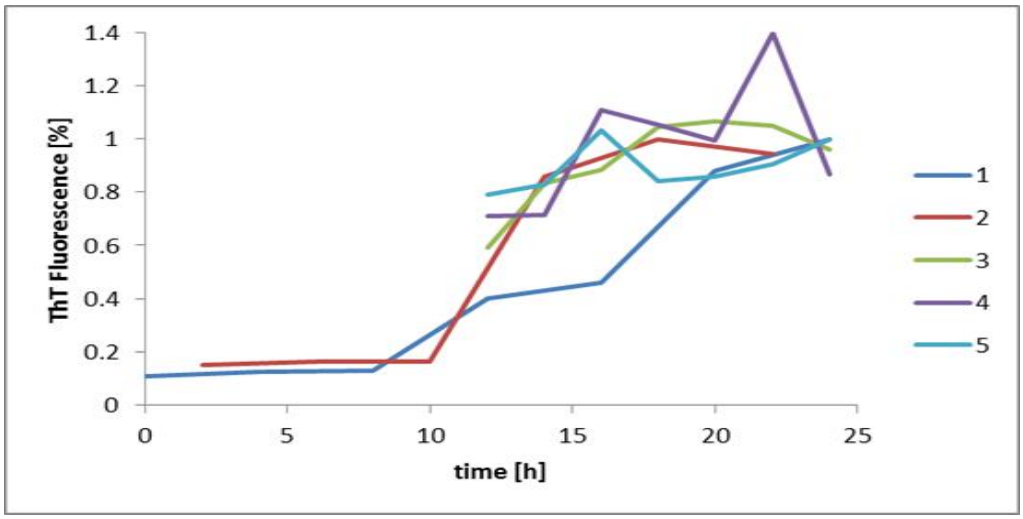

Figure 16. Thioflavin-T binding assay data for experiments corresponding to Figures 11 and 12 (blue and red) and Figure 13 (green, purple and cyan).

Additional grids prepared for both experiments described above were not analyzed due to malfunction of the TEM. It's service is projected for the summer of 2015.

Thermal Denaturation Using Circular Dichroism Spectroscopy

Spectra of wild-type and ΔN20 β_2m were measured using far-UV CD Spectroscopy at 10°C, 80°C, and then brought back to 10°C (Figure 15). This experiment was performed in order to study the refolding capabilities of the protein. Analysis of these spectra allowed determination of the wavelength at which to monitor thermal denaturation over a broader range of temperatures.

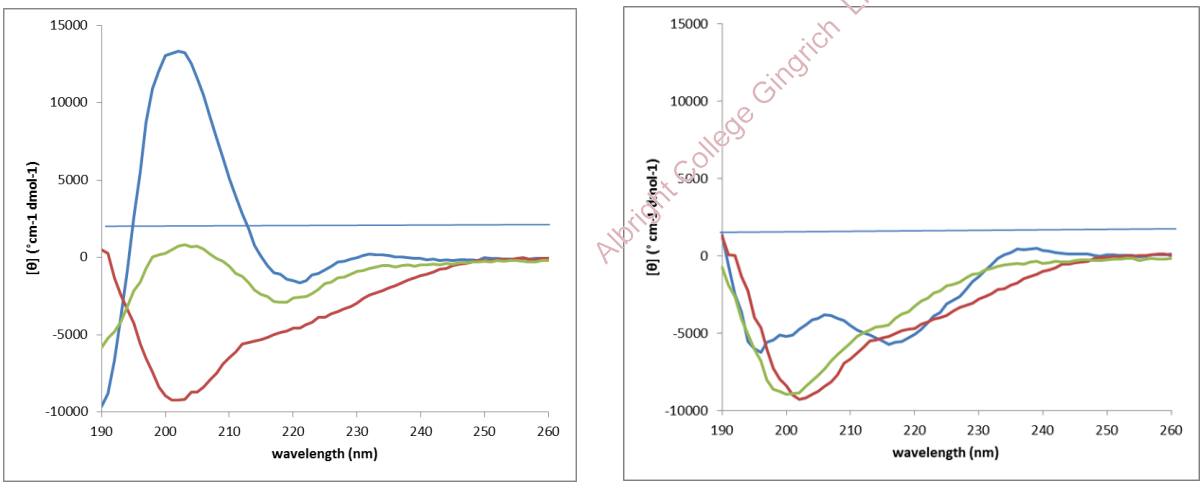

Figure 17. Far-UV CD spectra of (left) and ΔN20 (right) β_2M at 10°Cinitial (blue), 80°C (red), and 10°Cfinal (green). In 25 mM sodium phosphate buffer, pH 7.5.

A thermal denaturation curve was prepared by first recording spectra of the buffer, wild-type, and ΔN20 at 10 degree increments from 10 to 80°C. Each of these experiments was run on a different day. The blank spectrum at each temperature was subtracted from the corresponding spectra for wild-type and ΔN20 β_2m. The values observed at 205 nm were then plotted vs. temperature for both ΔN20 and wild-type (Figure 16). The midpoint of the transition region was determined to be 55.9°C and 44.6 °C for wild type and ΔN20, respectively. The slope of the transition regions were found to be -542.41 $Jmol^{-1}deg^{-1}$ and -713.57 $Jmol^{-1}deg^{-1}$ for wild-type and ΔN20, respectively.

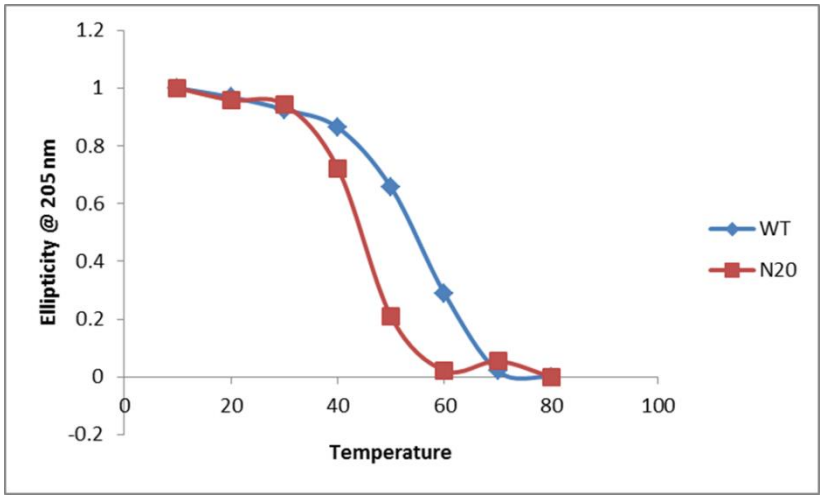

Figure 18. Thermal analysis by far-UV CD spectroscopy of wild-type (blue) and ΔN20 β_2M (red) at 205 nm.

Discussion

Our results from this study suggest that the loss of the wild-type ß$_2$m A strand leads to an increase in the rate of fibril formation and a decrease in the stability of the protein.

Analytical size exclusion chromatography (Figure 12) supports the conclusion that ΔN20 forms fibrils more readily than wild-type. When we interpret this data, we relate a lack of large soluble molecules with an increase in fibril formation. This is because during the centrifuge prior to loading samples on the column, any fibrils formed were sedimented into the pellet. This results in a decrease of fibril building blocks in the supernatant, namely monomers, dimers, and oligomers, over time. Thus, the large molecules are decreasing, because over time, they are incorporated into the fibrils. Fibril formation was complete in the first sample between 18 and

22 hours, since the 22 hour sample is a flat line in the chromatogram. For the second sample, fibril formation was complete within 20 to 24 hours. This study demonstrates that, although these samples were prepared from the same protein, the amount of time that it takes to form fibrils varies. The initiation of fibril formation within an individual sample may be considered to be a nucleation process.

SDS gel analysis further supports the conclusions drawn from the SEC experiments. The key feature of this technique is to note the disappearance of the smaller bands over time, which is the disappearance of monomer. For the first sample (Figure 13), the disappearance of building blocks occurred between 14 and 22 hours. For the second sample (Figure 13), the disappearance occurred between 20 and 24 hours. A greater variation was observed when this experiment was repeated (Figure 15). Slight amounts of monomer were still present at hour 63.

The ThT Binding assay also demonstrated that ΔN20 more readily forms fibrils than wild-type, yet does so over a relatively narrow time frame compared to ΔN6 or P5G (23). The experiments summarized in Figure 16 showed that some samples took as few as 15 hours to complete fibril formation, while others took as much as 24 hours or more.

A comparison of the thermal denaturation of wild-type and ΔN20 wild-type ß$_2$m suggests that the loss of the A strand affects the stability of the protein. Wild-type denatured upon a temperature increase to 80°C and then re-folded upon cooling back to 10°C (Figure 17). This was demonstrated through an initial maximum at ~200 nm which upon an increase in temperature, changed to a minimum. After cooling back to 10°C, the ~200 nm maximum was partially recovered, indicating that the protein refolded. Conversely, ΔN20 denatured in response to the same temperature increase, but did not return to the original structure upon cooling back to 10°C. The initial maximum at ~205 nm was not present upon thermal denaturation. Instead, a minimum at ~200 nm was observed. When the protein was then returned to 10°C, the spectrum looked much like that recorded at 80°C, indicating that the protein had not refolded. This suggests that ΔN20 may be partially disordered in structure. Comparison of the midpoints of the transition regions (Figure 18) of 44.6 °C for ΔN20 versus 55.9°C for wild-type ß$_2$m strongly supports our hypothesis that ΔN20 would be less stable than the full-length wild-type ß$_2$m. The slope of the transition regions were found to be -542.41 $Jmol^{-1}deg^{-1}$ and -713.57 $Jmol^{-1}deg^{-1}$ for wild-type and ΔN20, respectively. The steeper slope of the transition region for ΔN20 compared to that for the wild-type further supports this conclusion.

Future Work

Future work on this project might include an analysis of the thermal denaturation with more points in the transition region. Also, the kinetics experiments may be refined by including more samples and by catching the first appearance of fibrils with time using transmission electron microscopy.

Acknowledgements

I would like to thank my mentor Dr. Frieda Texter for supporting me and being so patient with me throughout this project. I would also like to thank my readers, Dr. Adam Hersperger and Dr. Pamela Artz for providing me with feedback on my thesis.

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