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Melting Temperature of MAPK14

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Introduction

Protein kinase domains transfer the γ phosphate of an ATP molecule to a serine, threonine, or tyrosine residue of a protein or peptide substrate[1]. This phosphorylation can activate or deactivate the protein, meaning that kinases are often important regulators of various cell activities. The active sites of kinase domains are highly conserved as they all bind ATP and have similar functions[1]. Kinases are very popular drug targets as they play a significant role in cell signaling pathways. Kinase dysregulation has been shown to play a role in many cancers and other diseases, making them a popular target for drugs[4]. Identifying key differences between different kinase domains could help design highly specific drugs, which would minimize the more severe side effects often seen in other, less specific drugs.

MAPK14, an important mitogen activated protein kinase, is involved in the regulation of the cell cycle in response to environmental stress and proinflammatory cytokines[2]. It is a serine-threonine kinase that has been found to regulate the cell cycle at G0, G1/S, and G2/M transition stages. It can differentially monitor cyclin levels, as well as phosphorylate a number of different tumor suppressor proteins[2]. MAPK14 can also be activated during normal cellular proliferation and differentiation, as it is a key regulator of hematopoiesis and other important processes[3].

Experiments performed in CHEM 406 lab failed to determine the melting temperature of MAPK14, as it never melted during the experiments performed. To understand why this may have occurred, circular dichroism (CD) experiments were performed using the standard phosphate buffer, as well as varying concentrations of GuHCl in conjunction with the phosphate buffer. The His tag was also removed, and circular dichroism experiments were performed again, also with almost no success. It is likely that the protein is melting, as a difference in the CD spectra can be seen as the temperature was increased slowly. However, it is likely that the initial experiments failed because the temperature increased too rapidly and melting of the protein could not be detected.

Methods

The sample of MAPK14 used in the CD experiments was prepped during a previous lab using the following procedure. Kinases were prepped by introducing plasmids expressing the chosen kinase domain into BL21 DE3 RIL *E.coli* cells. The cells were grown overnight and 333mL of this bacterial culture was centrifuged to form a cell pellet. The pellet was then resuspended in lysis buffer containing 50mM Tris HCL pH 8.0, 500mM NaCl, 10% glycerol, and 20mM imidazole. The resuspended pellet was then flash frozen until the laboratory period.

During the laboratory period, the cells were thawed on ice and a few grains of lyophilized DNase I were added to the solution. The cells were then homogenized (1min on, 1 min off, for 2.5min total at 100% intensity). After homogenization, the cell lysate was centrifuged at 4°C for 1 hour at 20,000rpm. To purify the kinase domains, a nickel column was prepared. 1mL of Nickel NTA resin was pipetted into a 15mL conical tube and centrifuged at high speed for 1min. The supernatant was removed, and the resin was rinsed with 2mL of cold dI water. The beads were centrifuged again at high speed for 1min. The supernatant was discarded, and the beads were rinsed 2 more times using 5mL of cold lysis buffer. The centrifuged cell lysate was added

to the beads and put on a nutating mixer for 30min at 4℃. A small gravity flow column with a filter and stopcock were set up and a line at 1mL was marked. After 30min, the cell lysate and bead mixture was added to the column and the flow-through was collected and labeled as "flow-through." The liquid level was never allowed to drop below 1mL as air could poison the column. The column was rinsed with 10mL of cold lysis buffer and the liquid was collected and labeled "wash." Five 1.7mL centrifuge tubes were labeled E1-E5. 1mL of cold elution buffer containing 50mM Tris pH 7.5, 200mM NaCl, 500mM imidazole, and 10% glycerol was added to the column. The flow through was collected in tube E1. Another 1mL of cold elution buffer was added and collected in tube E2. This step was repeated for tubes E3-E5. All fractions were kept on ice to protect protein from degradation. To clean the protein off the beads, 10mL of elution buffer, 5mL of cold lysis buffer, 5mL of cold dI water, and 5mL of 20% ethanol were added to the column. The washed beads were stored in a container for future use. A quick Bradford assay was conducted by labeling 5 glass test tubes E1-E5 and pipetting 40µL of the corresponding elution fraction to each test tube. Bradford reagent was diluted by mixing 90µL of concentrated Bradford with 360µL of water. 160 µL of Bradford reagent was added to each tube. The fractions with the highest protein concentration appeared to be a bright blue color, whereas fractions containing less protein appeared to be a greenish-blue. The highest protein fractions were dialyzed in about 100mL of storage buffer containing 50mM Tris pH 7.5, 300mM NaCl, and 10% glycerol for about 6 hours at 4℃. The dialysis buffer was changed twice.

The first set of circular dichroism experiments were run using the previously prepped MAPK14 samples. A sample of MAPK14 was diluted to about 0.3mg/ml using 10mM phosphate buffer pH 7.5. To dilute, 37µl of 4.1mg/ml MAPK14 was added to a microcentrifuge tube and 463µl of the phosphate buffer was added. A blank was prepared using phosphate buffer and kinase storage buffer (50mM Tris pH 7.5, 300mM NaCl, and 10% glycerol). 37µl of kinase storage buffer and 463µl of phosphate buffer were pipetted into a microcentrifuge tube. Once the sample and the blank were prepared, the CD instrument was set up and the cuvette was rinsed with deionized water and buffer prior to use. Data on melting temperature was obtained by running the sample at different temperatures (20-90℃ at 10 degree intervals) and analyzing the spectra.

The second set of circular dichroism experiments were run using the same MAPK14 samples as before, except at 1M, 2M, and 3M final GuHCl concentration in the sample and blank solutions. To prepare a stock 5M GuHCl solution, 47.7g of GuHCl and 100ml of phosphate buffer were added to a beaker, mixed thoroughly, and adjusted to a pH of 7.5. To prepare the 1M sample, 37µl of MAPK14, 100µl of 5M GuHCl, and 363µl of phosphate buffer were added to a microcentrifuge tube. To prepare the 2M sample, 37µl of MAPK14, 200µl of 5M GuHCl, and 263µl of phosphate buffer were added to a microcentrifuge tube. To prepare the 3M sample, 37µl of MAPK14, 300µl of 5M GuHCl, and 163µl of phosphate buffer were added to a microcentrifuge tube. The blanks for each of the samples were prepared in the same way, except the protein was switched out for the kinase storage buffer.

To remove the His tag from the protein, 37.5µl of MAPK14 and 450µl of dH_2O were added to a microcentrifuge tube. Then, 50µl of 10x TEV reaction buffer (50mM Tris-HCl at pH

8.0, 0.5mM EDTA, and 1mM DTT) and 10µl of TEV protease were added to the tube. This was incubated at 4°C overnight. After incubating, a NEB Express Ni Spin Column[5] was prepped by removing the bottom tab and centrifuging the column at 800 x g for 1 minute to remove the storage buffer. Then, 250µl of the provided lysis buffer[4] (20mM sodium phosphate pH 7.4, 200mM NaCl) was added to the column and centrifuged at 800 x g for 1 minute, making sure to keep the flow through. The column was then placed in a new 2ml microcentrifuge tube and the protein sample was added to the column. We allowed 2 minutes for binding and then centrifuged the column at 800 x g for 1 minute, making sure to keep the flow-through. The column was then washed using 250µl of provided wash buffer[4] (20mM sodium phosphate pH 7.4, 300mM NaCl, and 500mM imidazole) and centrifuged at 800 x g for 1 minute. This was repeated twice, and each wash was collected in a new 2ml tube. To prepare the sample for CD, the MAPK14 sample was dialyzed in 1L of 10mM phosphate buffer overnight at 4°C. The following day, the dialyzed sample of 0.729mg/ml MAPK14 was diluted to 0.3mg/ml by pipetting 206µl of MAPK14 and 294µl of phosphate buffer into a microcentrifuge tube.

To ensure that the His tag was cleaved from the protein, SDS-PAGE was run. The sample of MAPK14 and both the flow-throughs from the wash steps were prepped by pipetting 20µl of the sample and 20µl of SDS sample buffer into corresponding microcentrifuge tubes. The samples were then boiled for 5 minutes on a floating rack. After boiling, the samples were placed on ice and 15µl of the sample mixtures were pipetted into the wells. After loading the samples and the protein marker into the wells, the gel was run for approximately 30 minutes at 200V. After the gel was run, it was removed from the gel cassette and placed in a container filled with deionized water. The gel was rinsed 3 times with deionized water and then placed in a container containing Coomassie blue dye. The gel was microwaved in 30 second intervals until bands could be seen and the gel was blue all the way through. The gel was then placed in a container of deionized water and left to destain for a few days. The deionized water was also changed periodically during the destaining process.

Results

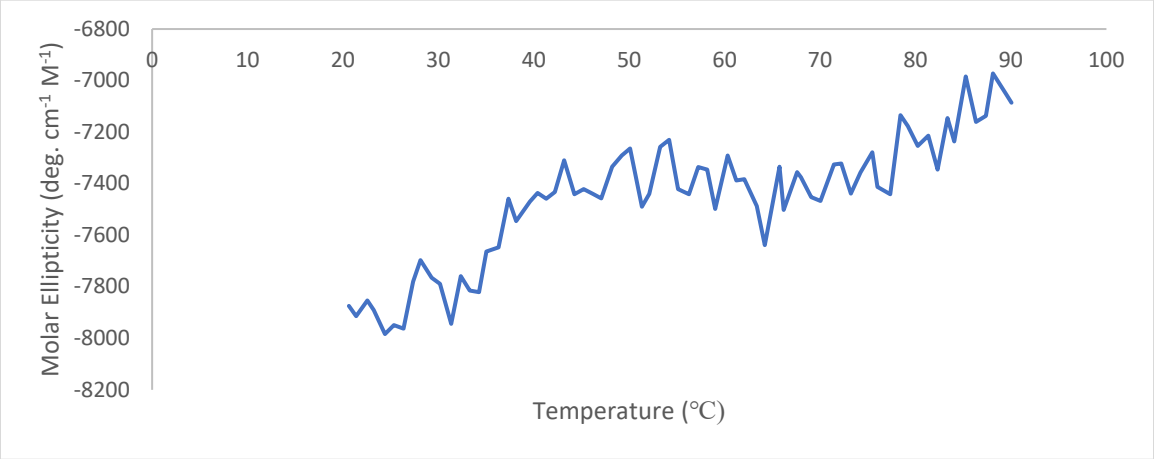

Figure 1. Initial CD thermal denaturation experiment on MAPK14, performed in CHEM 406 lab. This data was inconclusive and did not demonstrate melting. At the end of the experiment, no change was observed in the cuvette – the protein sample remained clear.

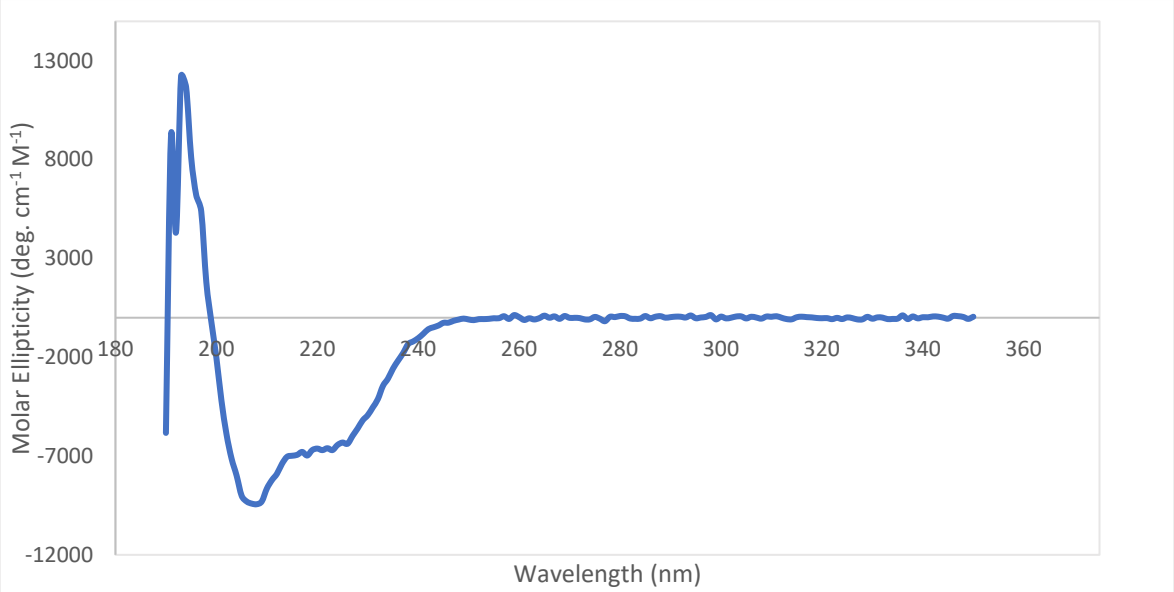

Figure 2. Initial CD spectrum of MAPK performed in CHEM 406 lab. This data was collected at 20°C and showed the relative secondary structure of MAPK14 in its native state.

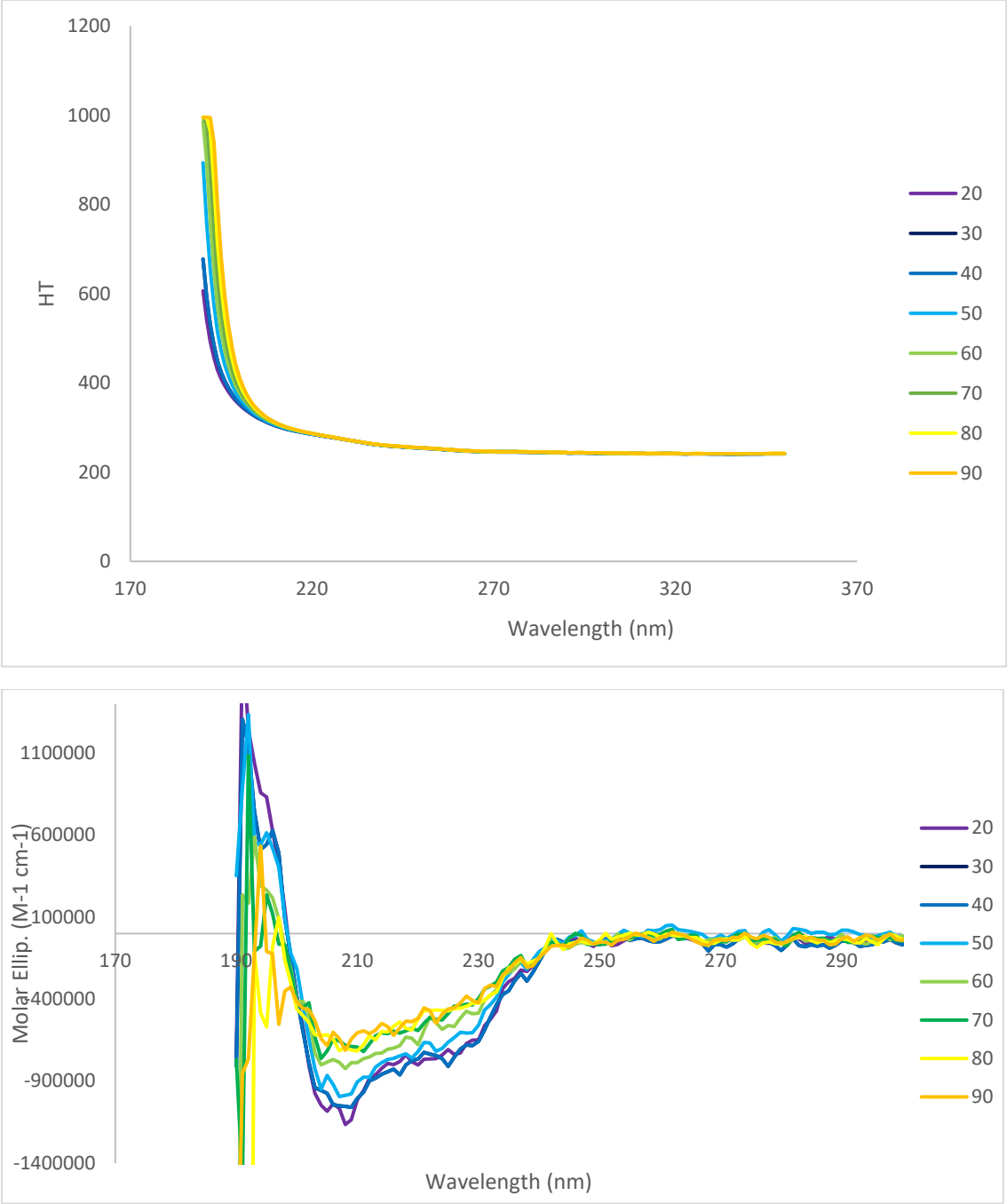

Figure 3. First CD experiment: MAPK14 in phosphate buffer. Thermal denaturation experiment run from 20-90°C. As the temperature was increased, a slight change in the protein's conformation can be seen. This change is most pronounced at about 210 nm. The spectrum at 90 ºC appears characteristic of β sheets, suggesting aggregation – consistent with this, at the end of the experiment a white precipitate was observed in the cuvette. Voltage included as control.

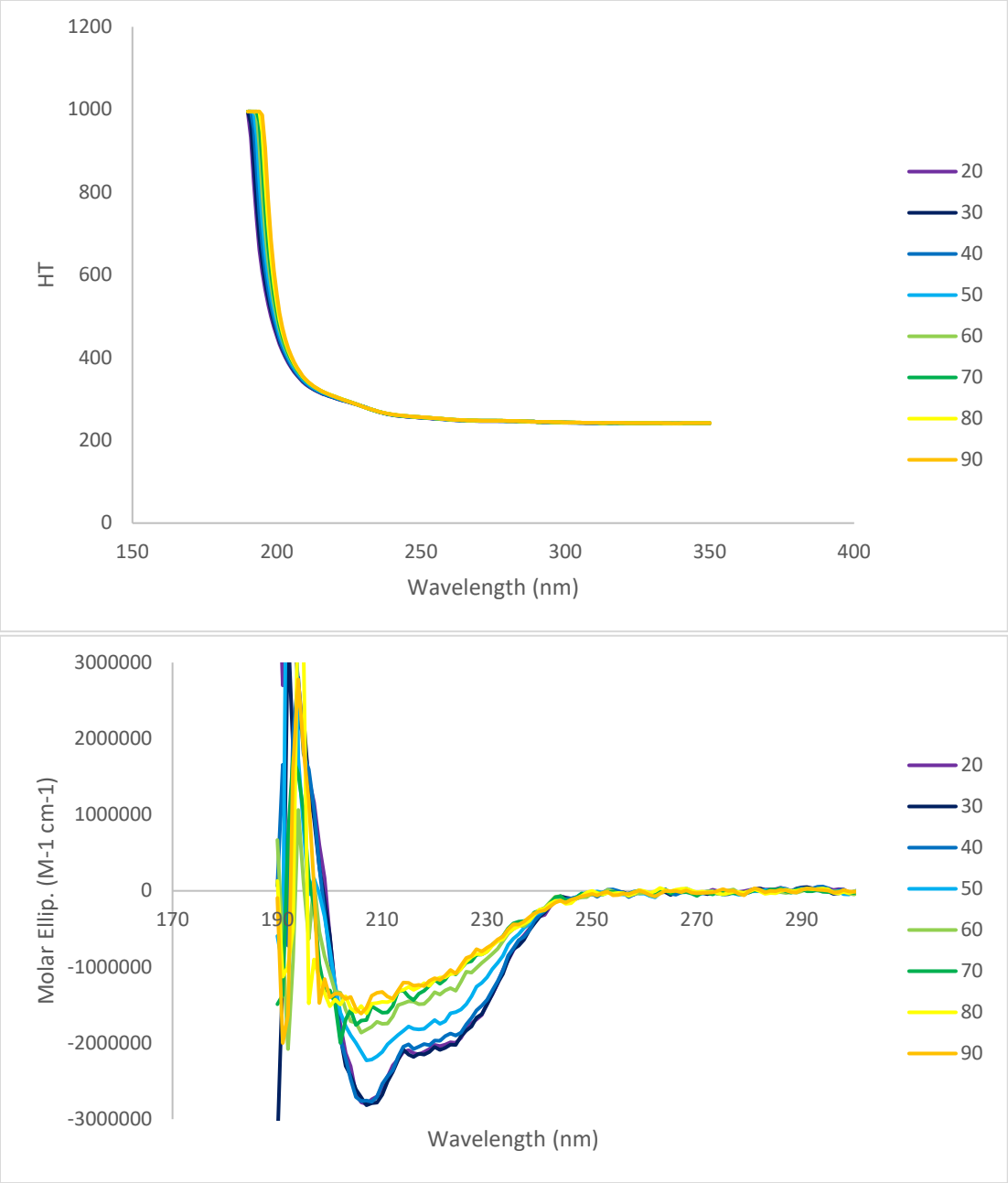

Figure 4. Second CD experiment: MAPK14 in phosphate buffer. Thermal denaturation experiment run from 20-90°C. As the temperature was increased, the signal begins to . Voltage included as control.

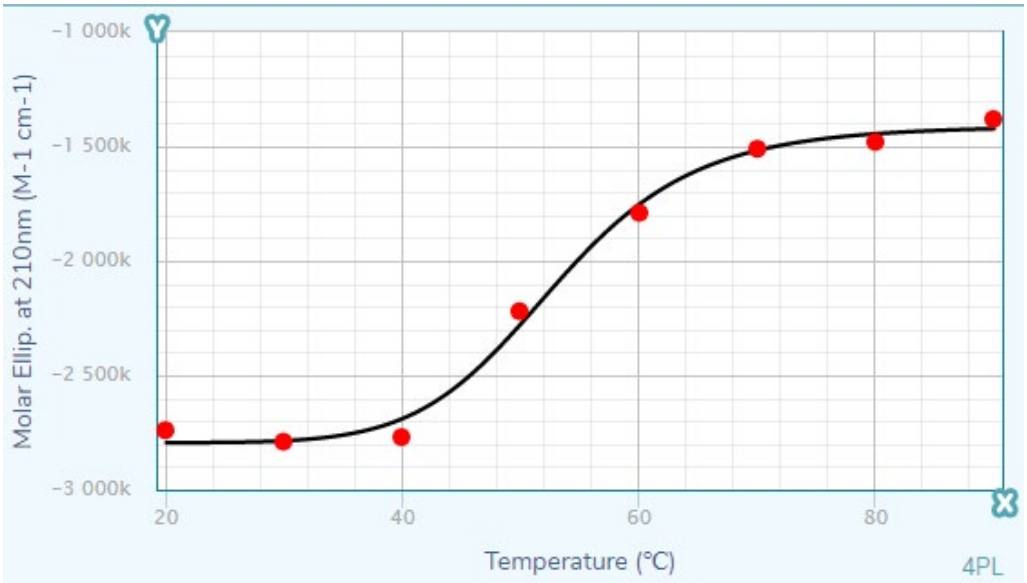

Figure 5. Molar Ellipticity at 210nm vs Temperature. Data was taken from the second set of circular dichroism experiments. Data were fit to a 4PL (symmetric logistic) function and the melting point of the kinase was found to be 52.9°C. The R^2 value was 0.9937 and the equation of the line was $y = -1410217 + (-4204981)/(1 + (x/52.9345)^{8.885241})$.

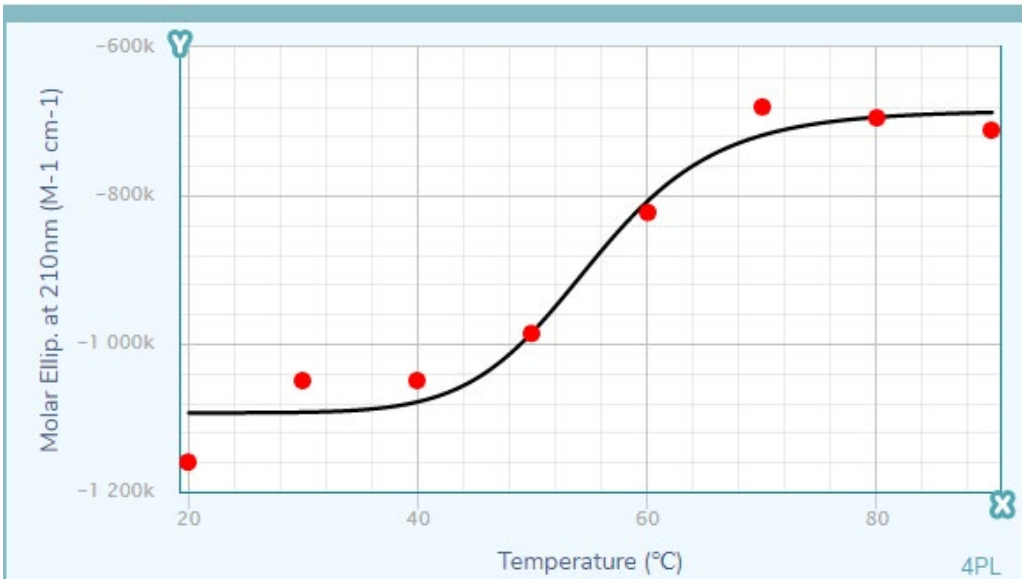

Figure 6. Molar Ellipticity at 210nm vs Temperature. Data was taken from the first set of circular dichroism experiments. Data were fit to a 4PL (symmetric logistic) function and the melting point of the kinase was found to be 55.1°C. The R^2 value was 0.9628 and the equation of the line was $y = -685370.4 + (-408220.6)/(1 + (x/55.17298)^{10.14078})$

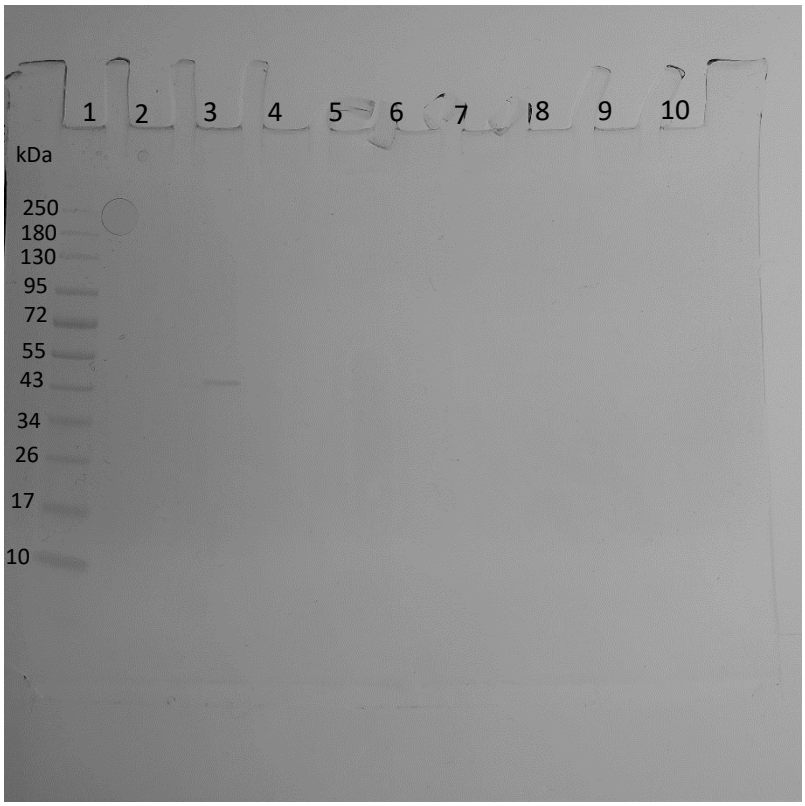

Figure 7. SDS-PAGE MAPK14. Lane 1: Pre-stained protein ladder[7]. Lane 3: His tag cleaved MAPK14 sample. Lane 5: Wash #1 from His tag cleavage. Lane 7: Wash #2 from His tag cleavage. Lane 9: Wash #3 from His tag cleavage. Lanes 2, 4, 6, and 8 all contained SDS-PAGE sample buffer.

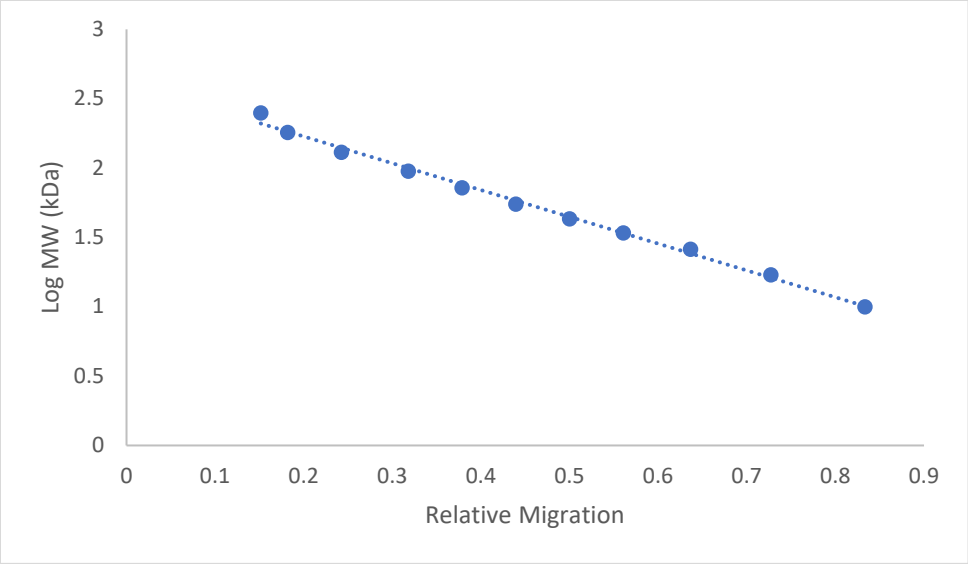

Figure 8. Standard curve of the pre-stained protein ladder[7] used to calculate the molecular weight of the MAPK14 sample. The equation of the line was y= -1.932x + 2.614 and the R^2 value was 0.9945.

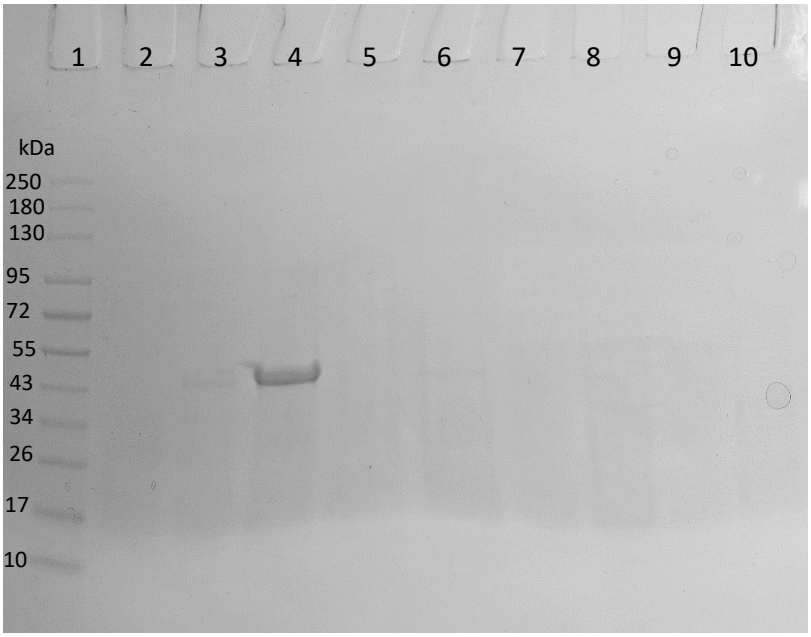

Figure 9. SDS-PAGE MAPK14. Lane 1: Pre-stained protein ladder. Lane 3: His tag cleaved sample. Lane 4: Original MAPK14 sample. Lane 6: Wash #1 from His tag cleavage. Lane 8: Wash #2 from His tag cleavage. Lane 10: Wash #3 from His tag cleavage. Lanes 2, 5, 7, and 9 all contained SDS-PAGE sample buffer.

Initial data collected during previous experiments demonstrated little to no melting (Figure 1). No change in the molar ellipticity was observed, and the protein did not precipitate as white clumps in the cuvette at the end of the experiment. Other kinases tested all melted in accordance with their literature melting values, whereas the sample of MAPK14 showed no evidence of melting during any of the trials. We hypothesized that the kinase was being stabilized in some way, and that was what caused the inconclusive results shown in Figure 1. Figure 2 shows a CD spectrum of MAPK14 at 20°C, characteristic of a well folded protein with a structure containing both α helices and β sheets. In Figures 3 and 4, the lower temperatures (20-40°C) show a very similar structure to the one in Figure 2, whereas higher temperatures (50-90°C) show a very different structure characteristic of β sheet secondary structure, indicating that melting had occurred as the protein's conformation was altered. The predicted melting temperature[6] of MAPK14 is 46.5°C, and the averaged melting point from the first two data sets was 54°C (Figures 5 and 6). This evidence would indicate that the sample of MAPK14 could be denatured, but not by the rapid heating of the sample during the initial failed experiments. When heated slowly, as with the experiments shown in Figures 3 and 4, the kinase demonstrates obvious signs of melting as its conformation was altered when heated above ~50°C. It's very likely that the kinase just exhibits greater kinetic stability than other kinases that were tested during the prior experiments, which is why melting could not be detected when the sample was heated rapidly.

The second set of CD experiments involved adding 1M, 2M, and 3M solutions of GuHCl to the sample of MAPK14 in phosphate buffer. These experiments were not successful as the signal was very noisy, likely due to the GuHCl absorbing too much light in the UV spectrum, and therefore the data could not be analyzed.

The third set of CD experiments involved cleaving the His tag from the sample of MAPK14. The spectra obtained for this experiment were also very noisy and could not be analyzed, although the reason for this is unknown. A gel was also run to ensure that this His tag had been cleaved. The calculated molecular weight of the sample was found to be 52.9 kDa, although by eye the sample band is quite close to the 43 kDa ladder band (Figure 8). The second gel that was run showed two faint bands which may suggest the His tag cleavage reaction did not go to completion. Both the original sample and the His tag cleaved sample running to relatively the same spot on the gel. This experiment should be repeated with higher concentrations of sample run on the gel for better visualization. There also appears to be two bands in the lane with the His tag cleaved protein, however this gel never finished destaining, so it's hard to know what the final result would have been (Figure 9).

Conclusion & Future Directions

The original data on the melting temperature of MAPK14 came back inconclusive as no melting was detected during the initial experiments. To discover why this was happening, several sets of circular dichroism experiments were performed, although with limited success. Circular dichroism is both time intensive and sample intensive, meaning that getting clean data is very difficult. If the above experiments were to be repeated, intrinsic fluorescence may be the best method to use as it's easier to get clean data.

The GuHCl experiments were largely unsuccessful as GuHCl absorbs strongly in the UV spectrum, making it hard, if not impossible, to get a clean spectrum when doing the type of experiments that were conducted above. To run a successful chemical denaturation experiment using CD, either a large number of samples with differing concentrations of denaturant and identical concentrations of protein need to be used or a titration of a protein solution with concentrated denaturant must be used and the experiment is typically run at a specified temperature, or set of temperatures, at a specified wavelength[8]. This data can then be analyzed using the linear extrapolation model[8].

The His tag cleavage experiments were all over the place and had only inconclusive results. The first gel was stained too lightly, which could cause a multitude of errors. It is possible that the second band seen in the second gel in lane 3 was present in the first gel, but the staining was so light that it couldn't be detected. In the second SDS-PAGE, both the original sample of MAPK14 and the His tag cleaved MAPK14 had the same relative migration, which is odd, and could mean that the results obtained from the first gel are inaccurate. Because of the mystery second band in the second SDS-PAGE, the fact that both the original and His tag cleaved samples of MAPK14 had the same relative migration, and because the CD spectra were noisy, the experiment should be repeated.

One final experiment that could be run is a fluorescence based thermal denaturation. To perform this type of experiment, SYPRO orange is added to a protein solution (≥0.216mg/ml) and pipetted into a PCR plate[9]. A thermofluor melt is then performed using a thermocycler and the Tm can be determined by plotting fluorescence as a function of temperature[9]. In Albanese et al, the melting point of MAPK14 was determined to be 57°C using this SYPRO orange fluorescence-based method and the melting peak was very broad. This is similar to the ~55 °C melting temperature measured here.

Citations

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