Comparing two different preparations of the W215A/E217A Mutant Thrombin by Hydrogen-Deuterium Exchange

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Abstract

Thrombin is a serine protease found in the bloodstream that cleaves fibrinogen into fibrin, assembling networks large enough to create blood clots. Activating protein C, thrombin can also promote anticoagulation, only with thrombomodulin present. However, thrombin is not always activated. There are many types of thrombin, each with different levels of activity; alpha-Thrombin is the fully active form. Thrombin is activated when there is tissue damage, which triggers molecular signaling that start the protease cascade. Errors in thrombin activation can cause heart attacks and strokes. To understand more about the behavior of thrombin, we mutated two side chains, W215A and E217A, to create WE-Thrombin. We ran the Hydrogen-Deuterium Exchange Mass Spectrometer (HDXMS) to compare two differently prepared WE-Thrombin. We discovered that there was a decrease in amide exchange in residues 33-39(54-61) and residues 161-170(202-211). There was a slight increase in residues 66-80(97-112). Altogether, the results show how different preparations of WE-Thrombin affect the catalytic activity and the regions that are solvent accessible.

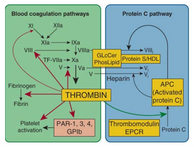

Figure 1. Alpha-Thrombin can cleave both procoagulative and anticoagulative substrates.

Methods

Growing and Purifying W215A/E217A Mutant Thrombin

We ran a transformation of 100 microliter of BL21 (DE3) pLysS cells with 2 microliter miniprep containing the human Thrombin gene with W215A and E217A mutations following with 30 minutes on ice. We plated 100 microliter onto each CAM/AMP plate and placed them in the incubator overnight at 37°C.

We placed one colony from the plate in the M9ZB media that we made. We let the solution vortex, aliquoted 10mL of M9ZB media into four autoclaved test tubes, and shook the starter cultures overnight at 37°C.

Using two autoclaved 4L growth flasks, we made two 1L M9 solutions and Ampicillin with the overnight starters. The flasks were in the shaker at 37°C until an OD600 of 0.6-0.8 was reached.

To induce the protein production, we added 5mL of diluted IPTG in MQ water to each growth flask in the incubator. The flasks shook for another four hours.

We spun down the cells at 5000 rpm for 15 minutes using 1L centrifuge bottles. Then, we poured out the supernatant and scraped the pellets into two separate 50 mL tubes to store in the -80°C freezer.

We thawed a 1L cell pellet and performed sonication and centrifugation to break open cells. After the solubilization, we loaded the G25 column with the supernatant, collected fractions, and added Glutathione to five pooled fractions. After adjusting the pH and three hours of incubation at room temperature, the solution was transferred to dialysis tubing and set in the cold room to stir overnight.

After making the refolding buffer, we transferred the protein that was in dialysis beforehand to a tube, gradually dripping the protein into the buffer solution. We used a magnetic stirrer to let the protein and buffer mix overnight.

Running the HLS column, we purified the protein separating correctly folded and misfolded. We collected ten fractions and only used the two fractions corresponding to the latest eluting peak to mix in with the activating solution, ecarin, and Wild Type (WT) Thrombin that was in a 1:50 ratio with WE Thrombin in milligrams.

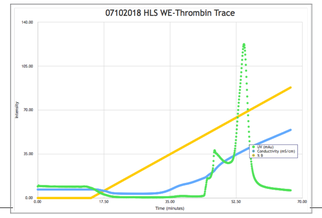

Figure 2. The trace from the HLS column of WE-Thrombin

Running the Experiment by HDXMS

3mL of the filtered activated sample was injected into the MonoS column to purify. We took two pooled fractions to make two 500 microliter aliquots and froze at -80°C.

Following the thawing of one frozen WE-Thrombin, we filtered the sample and concentrated it to 10 micromolar. Then, we ran the ID runs on the HDXMS.

Next, we thawed the second WE-Thrombin from the freezer, filtered it, and concentrated it to 5 micromolar. We ran the WE-Thrombin on the HDXMS overnight to measure how each region of the protein exchanges with deuterated buffer.

How the HDXMS Works

The HDXMS explores the changes in protein conformation and dynamics. It also measures the mass difference to identify if the protein is solvent accessible. After deuterium oxide is injected, proteins may take a deuterium atom from the solution to form a bond that is more stable. Then, the protein passes through two columns in the machine. The first column is the pepsin column. Because pepsin is a protease, its job is to cleave protein. Through the pepsin column, thrombin would be split into smaller sections called peptides. The second column is the Carbon-18 (C-18) column, whose purpose is to slow the peptides from flowing too quickly to the mass spectrometer, preventing the machine from being overwhelmed. The longer it takes for a peptide to elute off the column, the more hydrophobic it is. The HDXMS data graphs from **Figure 4** show the deuterium uptake graphs for peptides of thrombin.

Figure 3. The HDXMS machine we used for the experiment is shown on the left.

HDXMS Results

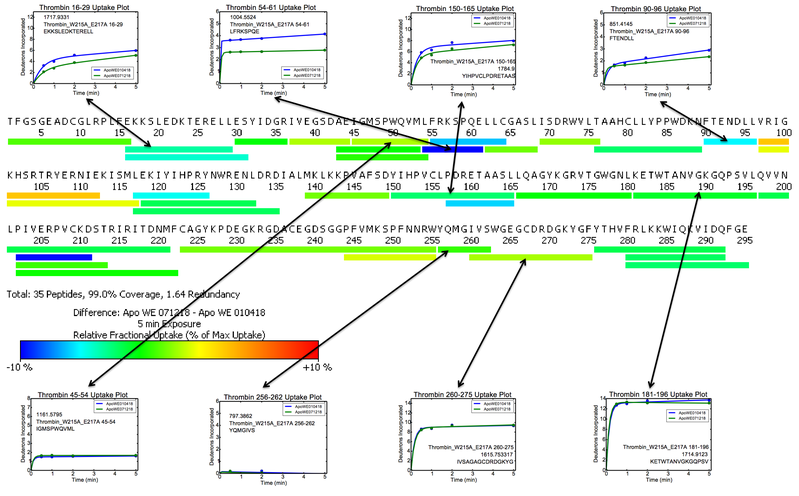

Figure 4. Coverage Map of the Difference Between HDXMS Data from January and July
The graphs show the deuterium uptake in certain residues. From 295 amino acids, I chose these eight peptides because it shows the difference and similarities between both data. The thrombin uptake plots on the top row all show a decrease in amide exchange from the data from July compared to the data from January. On the other hand, the thrombin uptake plots on the bottom row show little difference between the two data sets.
Note: The W215A and E217A mutants are in the uptake plot of residues 260-275 in the coverage map.

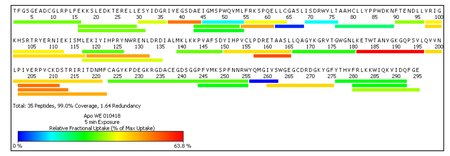

Figure 5. Coverage map of HDXMS results from January 4, 2018

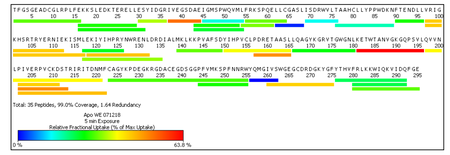

Figure 6. Coverage map of HDXMS results from July 12, 2018

Analysis and Conclusions

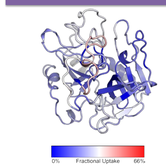

Figure 7. The percentage uptake for the WE thrombin from July is represented from the ratio of deuterium uptake and the maximum uptake for each peptide. This corresponds to Figure 5.

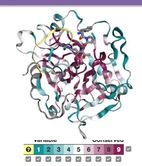

Figure 8. We analyzed the sequence of thrombin to see which amino acids in the sequence are conserved in evolution. The core of the protein is most conserved among most blood coagulative proteases while the residues on the surface are variable or are neither conserved nor variable.

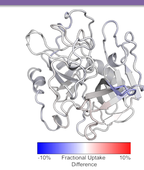

Figure 9. The difference of the fractional uptake values of the new minus the old Thrombin data. Blue shows lesser uptake of new compared to old. Red shows more uptake of new compared to old. Little difference corresponds to a white color. This matches with the map in Figure 4.

As seen in **Figure 9**, the only noticeable difference between the new and old data is in residues 54-63, which is in light blue, meaning that the new data has a slight decrease in deuterium exchange. Similarly in **Figure 7**, residues 54-63 have a fairly low deuterium exchange compared to the maximum exchange in the July HDXMS run. This region in **Figure 8** is variable compared to most blood coagulative proteases. Interestingly, this region is also where thrombomodulin binds to thrombin. It would be a useful comparison if we also did the HDXMS experiment with thrombomodulin bound to WE-Thrombin.
The two preparations were different on how the ecarin and WT-Thrombin samples were mixed in the activation solution. The WE-Thrombin mutant is overall less stable and low in catalytic activity. WT-Thrombin is injected into the sample to help WE-Thrombin with the activation. In January, my mentor, Riley, prepared the activated sample inserting ecarin and WT-thrombin separately to the activation solution. For our preparation in July, we mixed the ecarin and thrombin beforehand, and then added the mixed solution to the activated solution. Both WT-Thrombin and ecarin cleave a certain region of thrombin, and if one cleaved before the other, there could have been less activated thrombin in one protein sample. Therefore, the January preparation may have produced less activated protein than in July due to the different preparation. Referring to **Figure 9**, we suspect that the blue region, where the thrombomodulin binding site is, is correlated to how the activated protein samples were prepared.

References

Peacock, Riley B.; Davis, Jessie R.; Markwick, Phineus R.L.; Komives, Elizabeth A. (2018) Dynamic Consequences of Mutation of Tryptophan 215 in Thrombin, Biochemistry 57, 2694-1703

Acknowledgements

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