Determining the Structure of Thrombin Using Hydrogen Deuterium Exchange Mass Spectroscopy

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ABSTRACT

Blood clotting is a vital role in the human body, and one of the most important proteins involved in this pathway is thrombin. When activated, thrombin is able to initiate coagulation and promote clot formation. However, when it forms a complex with thrombomodulin, its function changes drastically to an anticoagulant, halting the coagulation cascade & preventing further clotting. Understanding how TM is able to alter thrombin's function provides insights into the mechanisms of clotting, as well as insights that may guide the design of better anticoagulant drugs.

One method that can be used to reveal the subtle changes in thrombin upon binding to TM is hydrogen deuterium exchange mass spectroscopy (HDXMS). Bovine thrombin from cow's blood and TM456m from Pichia pastoris was purified, and preliminary HDXMS data was collected for thrombin & the complex. This data gives insight into the three dimensional solution structure of thrombin and how it changes upon binding to TM456m. Through HDXMS experiments, we can unlock the mysteries of TM and use it to help countless people around the world

Background Information

The coagulation cascade is started when there is a cut or a breaking of the blood vessel and the trauma activates tissue factor. This factor activates a chain of serine which in the end activate prothrombin, the precursor to thrombin. Thrombin is essential to blood clotting. It promotes coagulation by promoting the activation of upstream coagulation factors, and most importantly, converting soluble fibrinogen into insoluble fibrin. This insoluble fibrin is the primary component of the clot. However, when thrombin is over-activated, issues occur like atherosclerotic plaque, apoptosis, and neural diseases. The body balances thrombin's procoagulant activity with a membrane protein called thrombomodulin. Thrombomodulin (or TM) is located on the surface of endothelial cells in blood vessels, and it forms a complex with thrombin. When this complex is formed, thrombin's function is changed from converting fibrinogen into fibrin toward cleaving Protein C which starts the anticoagulant pathway. Protein C stops the clotting mechanism by inhibiting the activation of upstream coagulation cascade serine proteases.

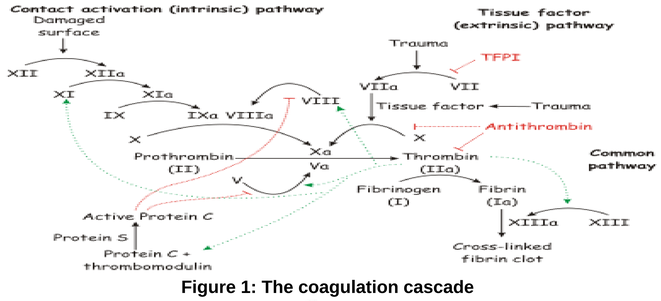


Figure 1: The coagulation cascade

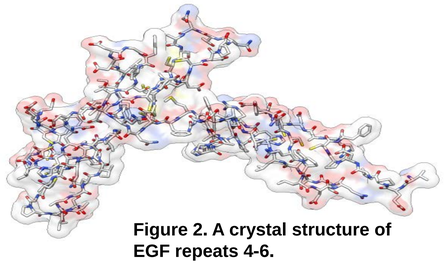


Figure 2. A crystal structure of EGF repeats 4-6.

For this experiment, we used TM456m which is the smallest active fragment of thrombomodulin that alters thrombin's activity. We purified this protein using *Pichia pastoris*, which has been genetically engineered to express this segment of the full TM protein. The full-length wild type TM contains six epidermal growth factor-like (EGF) domains, but only the last three EGFs have full thrombin-altering activity. The sixth domain enhances the binding affinity of thrombin. The fifth domain binds to thrombin, but also inhibits fibrinolysis. The fourth domain does not directly bind to thrombin, but is absolutely necessary for the thrombin-altering activity of TM because it is essential in Protein C activation.

Methods

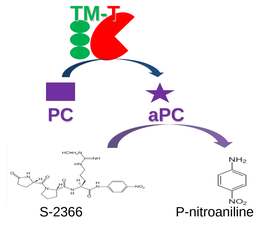
Bovine Thrombin Preparation

- Purification by $(NH_4)_2SO_4$ cuts
- Centrifuge and extract bTh pellet, resuspend in TBS
- Remove $(NH_4)_2SO_4$ over G-25 size exclusion column
- Activate w/ E_c arin venom
- Stop reaction by removing Ca^{2+} (G-25 column)
- Final Purification w/ MonoS column

Thrombomodulin Preparation

- Growth and expression of TM456m in *Pichia*
- Purification with QAE column, PC assay
- Purification with HLQ column, PC assay
- Purification with HPLC, PC assay
- Lyophilization
- Final Purification with S75 size exclusion

PC Assay



1. Add 20 µL of BSA/Ca2+ to each well
2. Add 100 µL of 1x TBS to the negative control, 90 µL to the others
3. Add 15 µL of Th solution to each well
4. Add 10 µL of TM samples to each well, wait 10 minutes
5. Set timer for 22 minutes, add 20 µL PC to first well, start timer, and finish adding
6. Set timer for 12 minutes, after 22 minutes is over, add 40 µL HAT to the first well, start timer, and finish adding
7. Add 20 µL of 100mM Tris to each well and then add 15 µL of S2366 to each well
8. Measure absorbance at 405nm over time.

Figure 3: We measure concentration of TM by seeing how much activated protein C is available through P-nitroaniline, which is converted by protein C and shows its activity and in turn how much TM is present

Hydrogen Deuterium Exchange Mass Spectroscopy

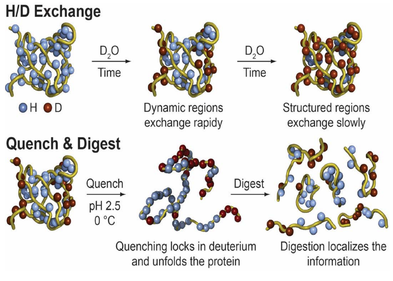
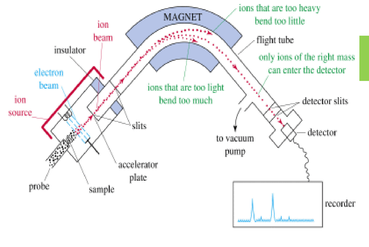


Figure 4: This shows the process behind HDX and how to prepare the protein for mass spectroscopy.

When looking at protein backbones, the hydrogens are mainly the isotope protium(1 proton). However we can place these proteins in a deuterium solution, which is another isotope of hydrogen with an added neutron, and they will exchange with the hydrogens in the protein backbone. This can tell us many things about proteins by looking at where the exchanges occurred, how much exchange there was, and the time taken for the exchanges. For example, if a peptide fragment is on the surface of the protein, there will be a lot of deuterium exchange whereas a peptide embedded inside the core of the protein, will have little to no exchange. This can be a useful tool ,especially when we can take it at different time points to look at folding. This technique can be used to determine protein structure, physical properties and biochemical functions.

Mass spectroscopy provides us a method to analyze and see the results of the hydrogen deuterium exchange. It does this by taking the digested fragments and separating them by charge as well as mass. This way we can analyze each fragment and see changes to its mass after being in the deuterated solution . We know that deuterium has been exchanged if we can see a shift because deuterium has more mass compared to protium, telling us where exchanges occurred. This data provides many clues into understanding proteins as listed in the section above. Instead of having to look into complex proteins, mass spectrometry gives us easily analyzable and comparable data to use on a mass scale.



Mass Spectroscopy Results

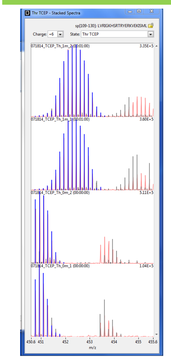


Figure 6 : Mass spec data for peptide sequence LVRIGKHSRTRYERKVE KISML taken at timepoints 0 and 1

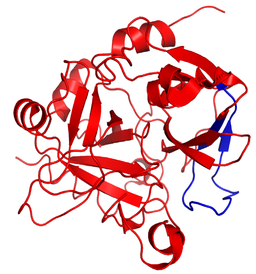


Figure 7: Bovine thrombin with the peptide segment LVRIGKHSRTRYERKVEKIS ML highlighted in blue.

For this particular fragment, the data tells us that there was a large shift, which means there was extensive deuterium exchange in this part of the backbone. This tells us it is on the surface and exposed to the deuterated solution. By looking at the peptide's placement in bovine thrombin, we can confirm the data by seeing its location on the surface.

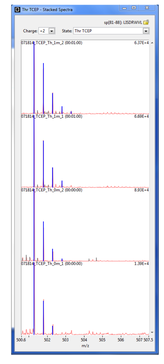


Figure 8 : Mass spec data for peptide segment LISDRWVL taken at time points 0 and 1 minutes.

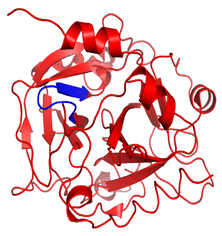
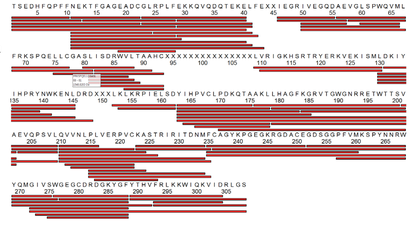


Figure 9: Bovine thrombin with peptide segment LISDRWVL highlighted in blue

In contrast with the last peptide, this peptide has little to no shift after one minute. This can tell us that this fragment is enclosed and embedded in the protein, not allowing it to exchange with the solution. This is shown true by Figure 9 where we see that this peptide is in the inner part of the protein.



These are just two examples and the data has given us over a 100 peptides from different parts of the protein to analyze and draw conclusions about thrombin and its structure as well as comparison to other proteins and complexes to understand how they function.

Figure 10: This is a coverage map of all the peptides that were analyzed by the mass spectroscopy machine. Many overlap because there were many different thrombin proteins that were broken apart and run.

Further Research

- Comparing data with that of TM-Thrombin complex to see changes to the active site and which parts of thrombin that TM covers and inhibits by seeing where exchanges occur compared to thrombin by itself
- Taking different time points to see how the complex induces different conformational changes in thrombin as well as how long the complex is able to stay together.
- Further tests on human thrombin as it has subtle differences from bovinethrombin.