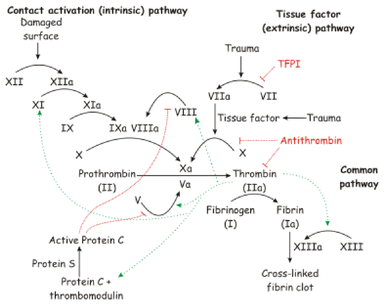
Refolding the Smallest Active Fragment of Human Thrombomodulin Expressed in *E. coli*

Cooper L. Wedge, Lindsey D. Handley, and Elizabeth A. Komives

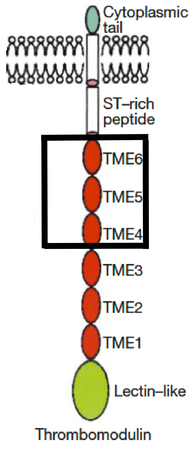
Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0378

Coagulation Cascade

Hemostasis is the halt of blood flow through a damaged blood vessel. Trauma that causes hemostasis to occur through the exposure of tissue factor to factor VIIa which activates factor X, activating prothrombin. When hemostasis occurs, thrombin cleaves fibrinogen into fibrin which promotes clot formation (the coagulation cascade). When thrombin binds thrombomodulin, on the surface of cells, thrombin's activity changes from procoagulant to anticoagulant, resulting in the activation of protein C. Protein C is an inhibitor of the coagulation cascade, thus stopping the formation of clots.

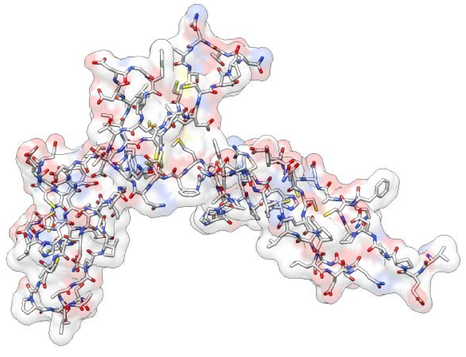


Thrombomodulin



Thrombomodulin is a protein with 557 amino acids that is present on the endothelium of all cells lining blood vessels. It binds to thrombin, a protease that, unbound, promotes coagulation by cleaving fibrinogen to make clots. Thrombomodulin has five domains; the tail has an unknown function, the transmembrane domain anchors the protein, the EGF like repeats change the function of thrombin, and the lectin domain is an anti-inflammatory molecule.

TM456t



We are working with the smallest active fragment of thrombomodulin that changes thrombin's activity, TM456t, which we are purifying from the inclusion bodies of *E. coli*. The primary difference between *E. coli* and mammalian cells in regard to thrombomodulin is that *E. coli* can not glycosylate proteins, and normally thrombomodulin is glycosylated in mammals at asparagines N364 and N391. The purpose of our research is to refold thrombomodulin from inclusion bodies in order to find an easier, more simple way to make thrombomodulin for future research. In order to assist in refolding, we have made three different mutants (N364D, N391D, and N364D-N391D) which change N364 and N391 to aspartates.

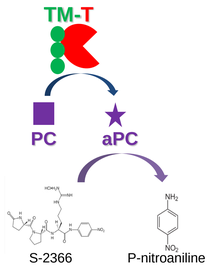
Methods

Large Scale Refolding

- Growth and expression of TM456t in *E. coli*
- Inclusion body prep
- Resolubilized inclusion bodies in 8M urea
- Load resolubilized protein onto Ni column
- Refold protein on column three days in refolding buffer
- Elute with imidazole

Small Scale Refolding Screen

- Growth and expression of TM456t in *E. coli*
- Inclusion body prep
- Resolubilized inclusion bodies in 8M urea
- Load resolubilized protein onto Ni resin in epp tubes
- Replace resolubilizing buffer with various refolding buffers
- Refold protein in epp tube for three days
- Elute with imidizole



PC Assay

1. Add 20 µL of BSA/Ca^{2+} 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. Start the run in the plate reader

Results for N364D TM456t Screen

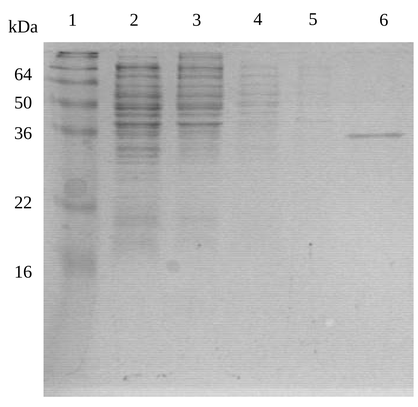


Figure 1. 15% reducing SDS-PAGE gel of our N364D TM456t prep.
Lane 1 is the molecular weight ladder.
Lane 2 is the pre-induction sample of our 1L *E. coli* growth.
Lane 3 is the post-induction sample of our 1L *E. coli* growth.
Lane 4 is the supernatant after the first sonication from the inclusion body prep. No TM should be present because TM should be in the inclusion bodies in the pellet.
Lane 5 is the supernatant after the second sonication from the inclusion body prep.
Lane 6 is the TM eluted with 250mM imidazole from the Ni resin after three days of refolding. The Ni resin was washed with 25mM imidazole before the step to remove un-specifically binding and specifically binding proteins.

CHAPS (%w/v)

Glycerol(%v/v)	0.05%	0.5%	2%	5%	10%	20%
0%	1.49	1.38	1.32	1.30	1.11	1.32
2%	1.34	1.33	1.37	1.22	1.08	1.11
10%	1.01	0.990	1.00	0.962	0.918	0.841
20%	0.839	0.693	0.809	0.650	0.779	

Best refolding condition ⟵ ⟶ Worst refolding condition

Figure 2. Relative activities of N364D TM456t refolded at each of the given conditions. Activity of the protein was divided by the activity of the positive control in each PC assay. Each of the refolding buffer conditions also contained 50mM Tris pH 8.2, 200mM NaCl, 10mM CaCl, 0.1mM ox glutathione, and 1mM red glutathione.

Results for N364D/N391D TM456t Large Scale Refolding

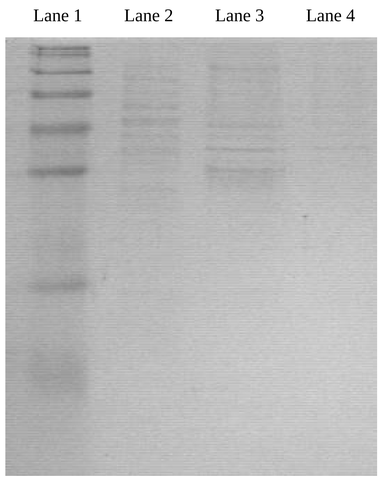


Figure 3. 15% reducing SDS-PAGE gel of our N364D/N391D TM456t prep.
Lane 1 is the molecular weight ladder.
Lane 2 is the supernatant after the first sonication from the inclusion body prep. No TM should be present because TM should be in the inclusion bodies in the pellet.
Lane 3 is the lysis before centrifugation after the second sonication.
Lane 4 is the supernatant after the second sonication from the inclusion body prep.

Column Number	1	2	3	4	5	6	7	8	9	10
Fraction Number	-	+	4	6	8	10	12	14	16	18
Activity	16.86	147.3	99.69	27.01	17.13	20.33	9.46	7.14	6.26	4.58

Figure 4. Results of the PC Assay of 2mL fractions eluted from the Ni column of the N364D/N391D large scale refolding prep. Refolded protein was eluted with 1x TBS 250mM imidazole after washing the column with 1x TBS 25mM imidazole.

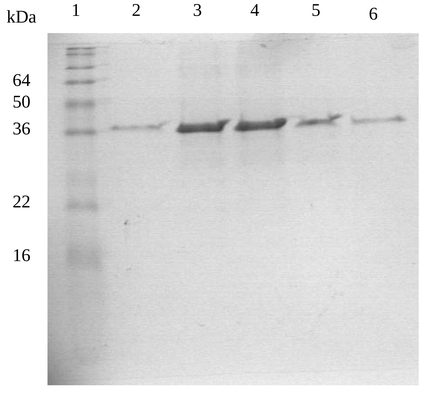


Figure 5. 15% reducing SDS-PAGE gel of our N364D/N391D TM456t Ni fractions.
Lane 1 is the molecular weight ladder.
Lanes 2-6 correspond to Ni fractions 4-8 eluted from our large scale N364D/N391D on-column refolding prep.

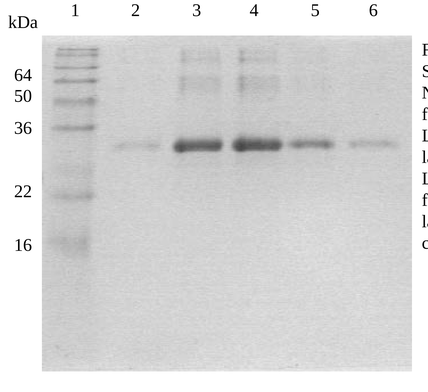


Figure 6. 15% non-reducing SDS-PAGE gel of our N364D/N391D TM456t Ni fractions.
Lane 1 is the molecular weight ladder.
Lanes 2-6 correspond to Ni fractions 4-8 eluted from our large scale N364D/N391D on-column refolding prep.

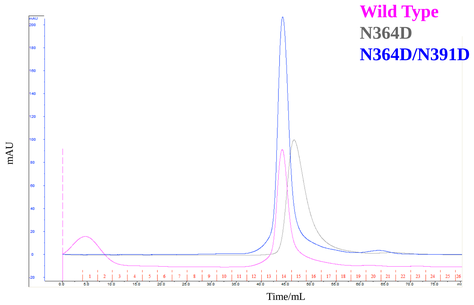


Figure 7. Ni fractions 4-11 of the N364D/N391D large scale on column prep were concentrated and loaded onto an S75 size exclusion column. The S75 trace was overlaid onto the traces of previous wild type and N364D TM456t S75 purifications.

Results for N391D TM456t Large Scale Refolding

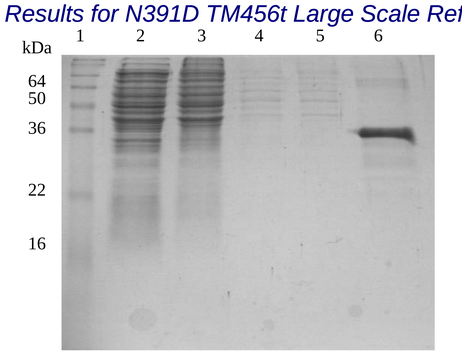


Figure 8. 15% reducing SDS-PAGE gel of our N391D TM456t prep. Lane 1 is the molecular weight ladder. Lane 2 is the pre-induction sample of our 1L *E. coli* growth. Lane 3 is the post-induction sample of our 1L *E. coli* growth. Lane 4 is the supernatant after the first sonication from the inclusion body prep. No TM should be present because TM should be in the inclusion bodies in the pellet. Lane 5 is the supernatant after the second sonication from the inclusion body prep. Lane 6 is Ni fraction 6 from N364D/N391D prep.

Column Number	1	2	3	4	5	6	7	8	9	10
Fraction Number	-	+	4	6	8	10	12	14	16	18
Activity	11.8	125	11.4	16.1	8.05	3.48	2.00	1.21	0.97	0.78

Figure 9. Results of the PC Assay of 2mL fractions eluted from the Ni column of the N391D large scale refolding prep. Refolded protein was eluted with 1x TBS 250mM imidazole after washing the column with 1x TBS 25mM imidazole.

Conclusions

- Low glycerol, low CHAPS refolding buffers better promote the refolding of N364D TM456t.
- N364D/N391D oligomerizes to a similar extent as the wild type TM456t. Therefore, the N364D mutant will likely be used in further refolding studies.

Acknowledgments

The UCSD Research Scholars program for letting me conduct research over the summer and all of my lab mates in the Komives lab for all of the support. Special thanks to Nick Treuheit for his schematic of the PC assay.

References

1. Wikipedia: http://en.wikipedia.org/wiki/File:Coagulation_full.svg
2. Fuentes-Prior, P. et al. "Structural basis for the anticoagulant activity of the thrombin-thrombomodulin complex" *Letters to Nature* **2000**, *404*, page 518-525.