

Unravelling the Structure of SecA: Analysis of WT- vs. Y134S-SecA



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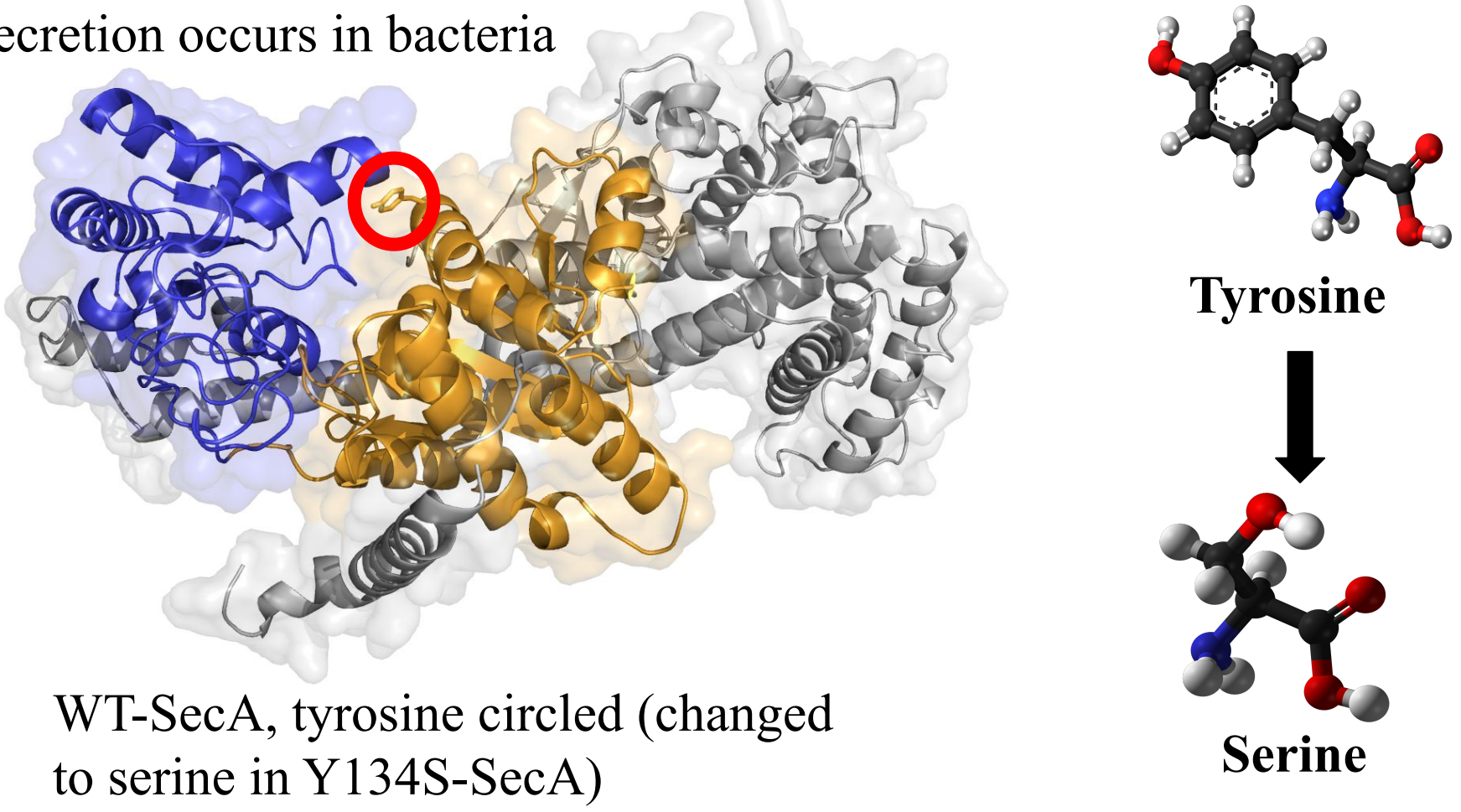


Abstract

- SecA and the integral membrane proteins SecYEG control the secretion of most proteins across the plasma membrane in bacteria
- Understanding the structure/function of SecA mutants provides insight into the functions of domains in SecA, and consequently SecA as a whole
- The *WT-secA*- and *Y134S-secA*- gene was expressed in BL21 *E. coli* cells, purified via Immobilized-Metal Affinity Chromatography, concentrated with a desalting column, and analyzed via tryptophan fluorescence (thermal unfolding from 310 nm to 400 nm)
- WT-SecA and Y134S-SecA were shown to have two distinct temperatures at which they converted from a folded to a fully unfolded state
- This difference indicates that tyrosine (amino acid 134 in WT-SecA) plays a significant role in determining the stability of SecA- particularly in the two domains surrounding this amino acid
- Results provide a greater understanding of SecA's involvement in the secretion of preproteins; humans homologs of the SecA complex (Sec61 α), can be compared against SecA to characterize protein secretion in humans

Background Research

- SecA is a motor protein (102 kDa) that plays a pivotal role as the main protein transport across the plasma membrane of *E. coli* cells, working in conjunction with the SecYEG translocon complex
- A functional copy of SecA is essential for the survival of *E. coli* bacteria
- Y134S-SecA is a crucial mutant because it can secrete preproteins with a modified signalling peptide (attached to protein exports from the ribosome) more than WT-SecA
- Understanding how this mutation affects the stability of the protein would begin the process of characterizing the differences between WT and Y134S-SecA, and contribute to the general understanding of how protein secretion occurs in bacteria



Investigative Question

How does the conversion of WT-SecA to the Y134S-SecA mutant in *E. coli* affect the tertiary structure and stability of SecA?

Hypothesis

The amino acid in position 134 is tyrosine in WT-SecA and serine in Y134S-SecA. Since this mutation is located exactly between two structural domains of SecA (see Figure above), it might affect the overall stability of the protein. Indeed, changing the tyrosine, a hydrophobic residue, to serine, a polar amino acid, will probably change the possible interactions between the residues located at the interface of the two domains. To compare the stability of WT- versus Y134S-SecA, the temperatures at which they unfolded were compared using tryptophan fluorescence.

Materials and Procedures

1. Culture Preparation and Protein Expression

1. Prepare a flask with 20 mL of autoclaved LB medium and 20 μ L of each antibiotic (ampicillin and chloramphenicol); add 2 μ L of *E. coli* (BL21 (DE3) containing the plasmid (T5) with either the *WT-secA*- or *Y134S-secA*- gene; place in the shaker overnight at 37°C.
2. Start a culture by adding 8 mL of the overnight culture to 400 mL of autoclaved LB medium (use previous antibiotics); transfer to shaker at 37°C.
3. Monitor the absorbance of the culture at 600 nm using a spectrophotometer every 20 minutes until the OD reaches a value of 0.6-0.8; induce protein expression by adding 0.5 mM of IPTG (400 μ L from 0.5 M stock solution / 400 mL of LB medium) and transfer the culture to a shaker at 30°C.
4. Sample the culture every 30 minutes to monitor the protein expression level (250 μ L for 2.5 OD). (See Figure 1)
5. After 2 hours, centrifuge the culture for 15 minutes (4000 rpm at 4°C), discard the supernatant; freeze pellets at -20°C until needed.
6. Add bleach to supernatant overnight to kill all remaining bacteria, discard accordingly in biohazard trash can.



Left to right: shaker spectrophotometer, centrifuge (temperature controlled)

2. Solubilization and Bacterial Lysis

1. Solubilize the pellets in 48 mL of Buffer A (shake, vortex).
2. To lyse the cells, pour solubilized mixture into a French Pressure Cell, collect lysed material and transfer material to the sonication machine (output- 4, duty cycle- 50, time- 30 s, pulsed) in increments of 5-6 mL of solution per cycle.
3. Transfer the solution into 24 2-mL test tubes, centrifuge for 10 minutes (13,300 rpm and 4°C), keep the supernatant (and save the pellets for later).

3. Purification by Immobilized-Metal Affinity Chromatography

1. Equilibrate the column with 3 column volumes (CV) of water followed by 3 CV of Buffer A (0.8 ml/min).
2. Load the supernatant on the column (0.4 mL/min) (keep 20 μ L to load on a gel later, and collect the flow-through (FT).
3. Wash the column with 3x1 CV of buffer A (collect flow-through from washes (labeled W1, W2, and W3).
4. Elute the protein with 2 CV of buffer B and collect 2-mL fractions.
5. Run a SDS-PAGE to monitor the protein elution. Prepare a sample (20 μ L of protein + 5 μ L of loading buffer) for insoluble fraction (I, pellet), supernatant (S), washes (W1-3) and eluted protein (imidazole). (See Figure 2)

4. Desalting

1. Pool the fractions containing the protein of interest into centrifugal filter unit (CFU) with a cutoff membrane of 50 kDa.
2. Centrifuge CFU (full speed, 5°C) for cycles of 10 minutes until the volume is 2.5 mL or less. Between each centrifugation cycle, ensure that the protein does not precipitate (milky or flocky solution).
3. Equilibrate a PD-10 desalting column with 25 mL of Buffer C.
4. Load 2.5 mL of the concentrated protein (from the CFU) onto the PD-10 column.
5. Once the protein sample is fully incorporated into the resin, load 3.5 mL of Buffer C and collect the flow-through in a tube.

5. Determining Protein Concentration

1. Prepare standards as shown below:

	0	0.5	1	2	3	4
BSA (μ L)	0	5	10	2	3	4
Water (μ L)	10	5	0	8	7	6
BSA Stock 1 (1 mg/mL)			BSA Stock 10 (10 mg/mL)			

2. Prepare a tube with 5 μ L of the purified protein with 5 μ L of water.
3. Add 1 mL of Bradford's reagent to all tubes; shake, sit for 5 minutes.
4. Measure absorbance at 595 nm using spectrophotometer for all tubes. (See Figure 3)

6. Fluorescence Microscopy

1. Prepare the sample (200 μ L): 0.5 mg/mL of WT or Y134S-SecA in buffer C and transfer to quartz cuvette.
2. Record tryptophan fluorescence (emission wavelength of 280 nm, and record fluorescence between 310 and 400 nm) at temperatures ranging from 20 to 60°C. (See Figure 4)



Left to right: French Pressure Cell, Centrifugal Filter Unit, PD-10 Desalting column, fluorescence microscope (overview)

Results

SDS-PAGE Analysis of SecA Expression after IPTG Induction

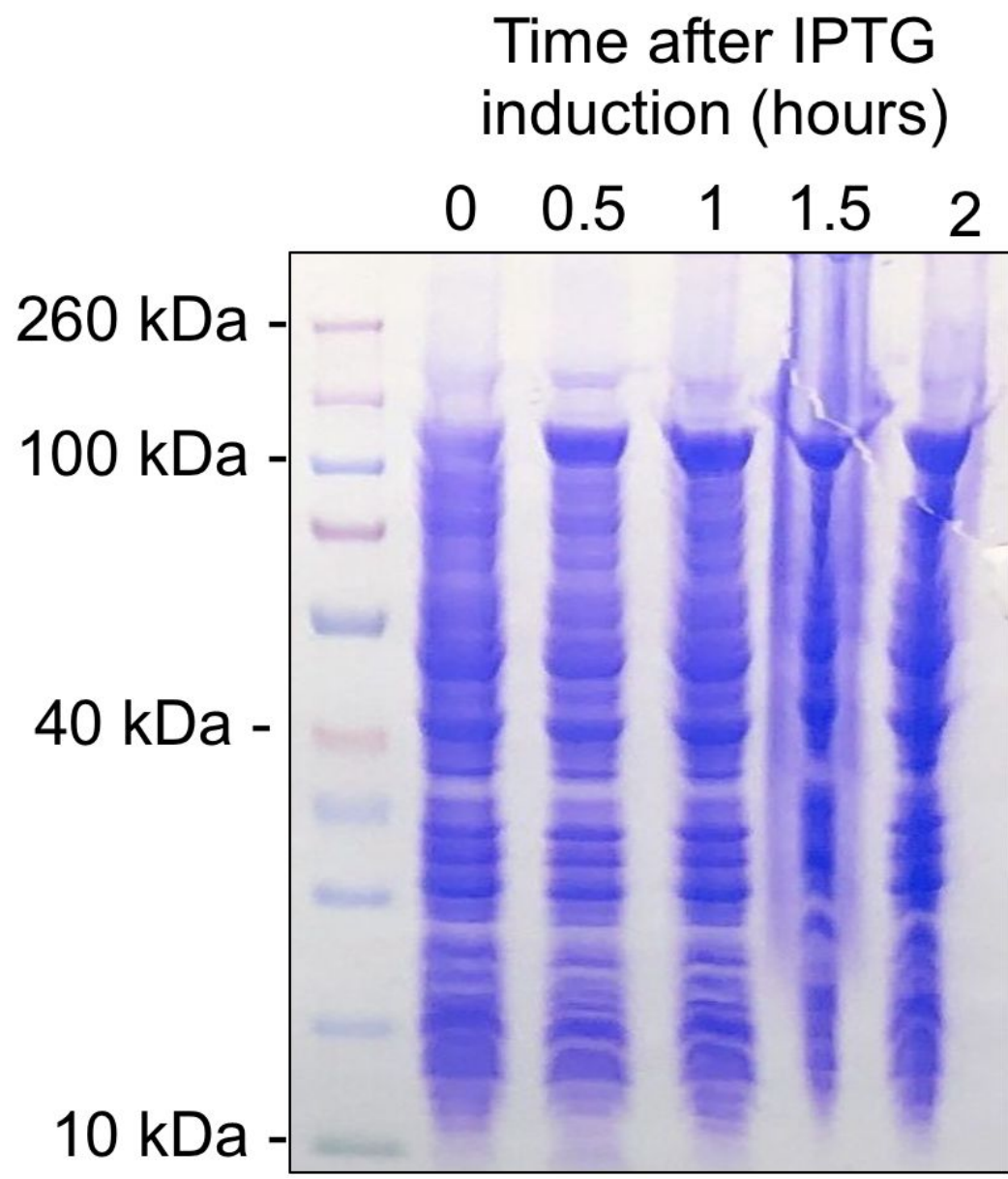


Figure 1. Protein profile of log-phase cultures (WT-SecA, OD 0.8) after Isopropyl β -D-1-thiogalactopyranoside (0.5 mM) induction for two hours at 30°C. Columns (left to right): ladder, WT-SecA (0-2 hours after IPTG induction)

SDS-PAGE after IMAC Purification of WT and Y134S-SecA

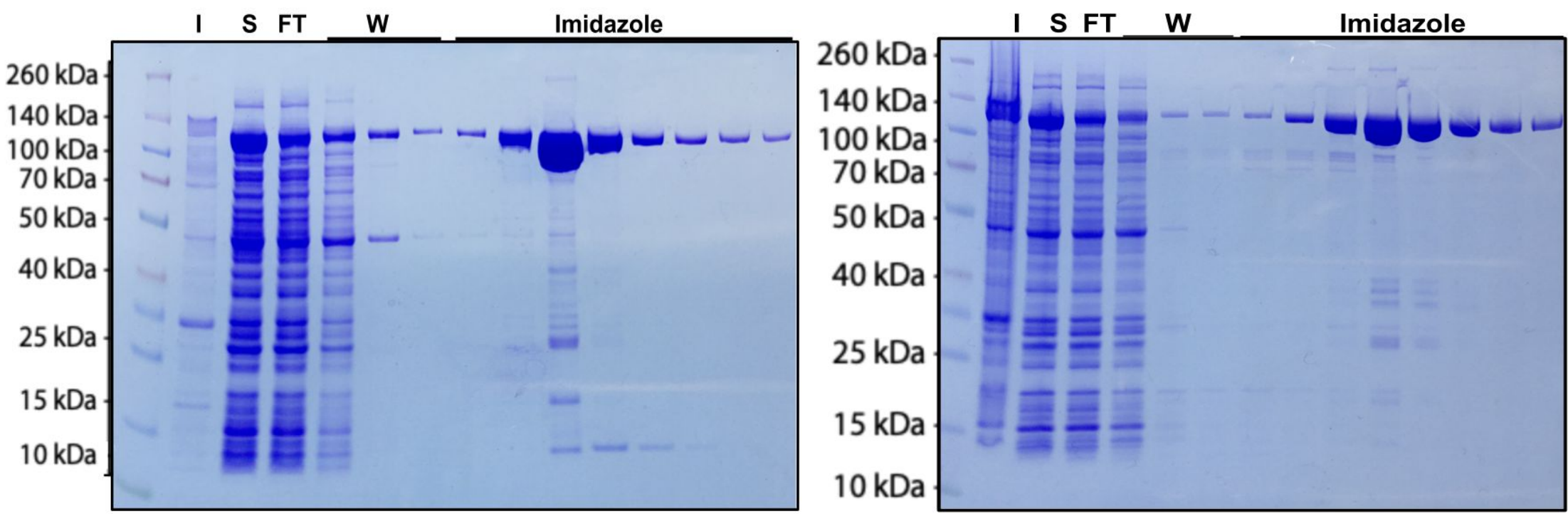


Figure 2a. WT-SecA protein profile after IMAC.

Figure 2b. Y134S-SecA protein profile after IMAC.

Figure 2. Columns (left to right): ladder, (I) insoluble fraction (pellet after centrifugation), (S) soluble fraction (supernatant after centrifugation), (FT) flow-through (fraction that does not bind to the column), (W) washing (1, 2, 3), imidazole (fraction containing protein)

Results (cont.)

Concentration of Y134S-SecA After Purification and Desalting

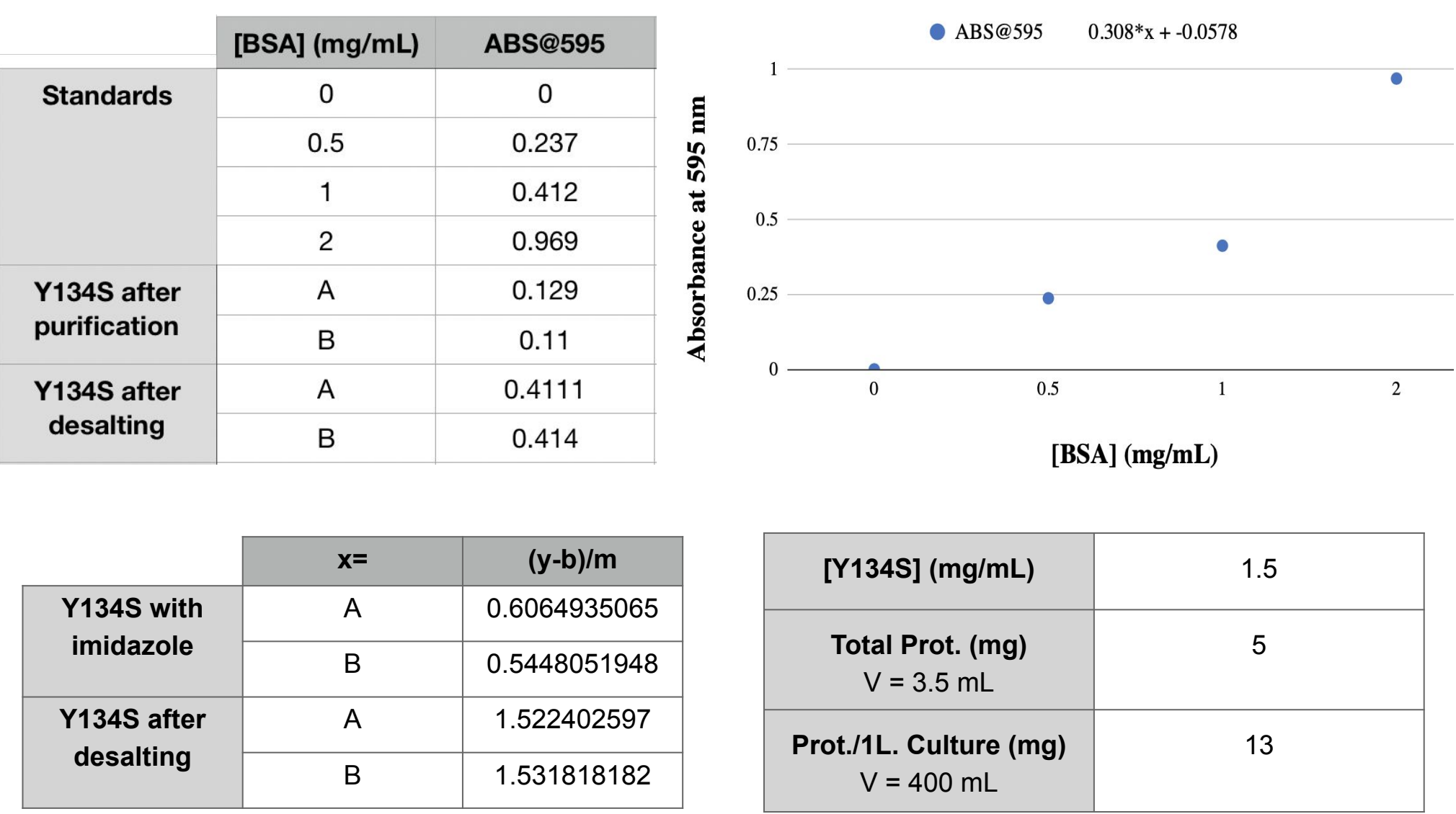


Figure 3. Top: Absorbance of BSA standards and Y134S-SecA after purification and desalting at 595 nm. Bottom: Left- Calculation of Y134S-SecA concentration. Right- Yield of protein production.

Thermal Unfolding of WT and Y134S-SecA (20°C to 60°C) at 340 nm

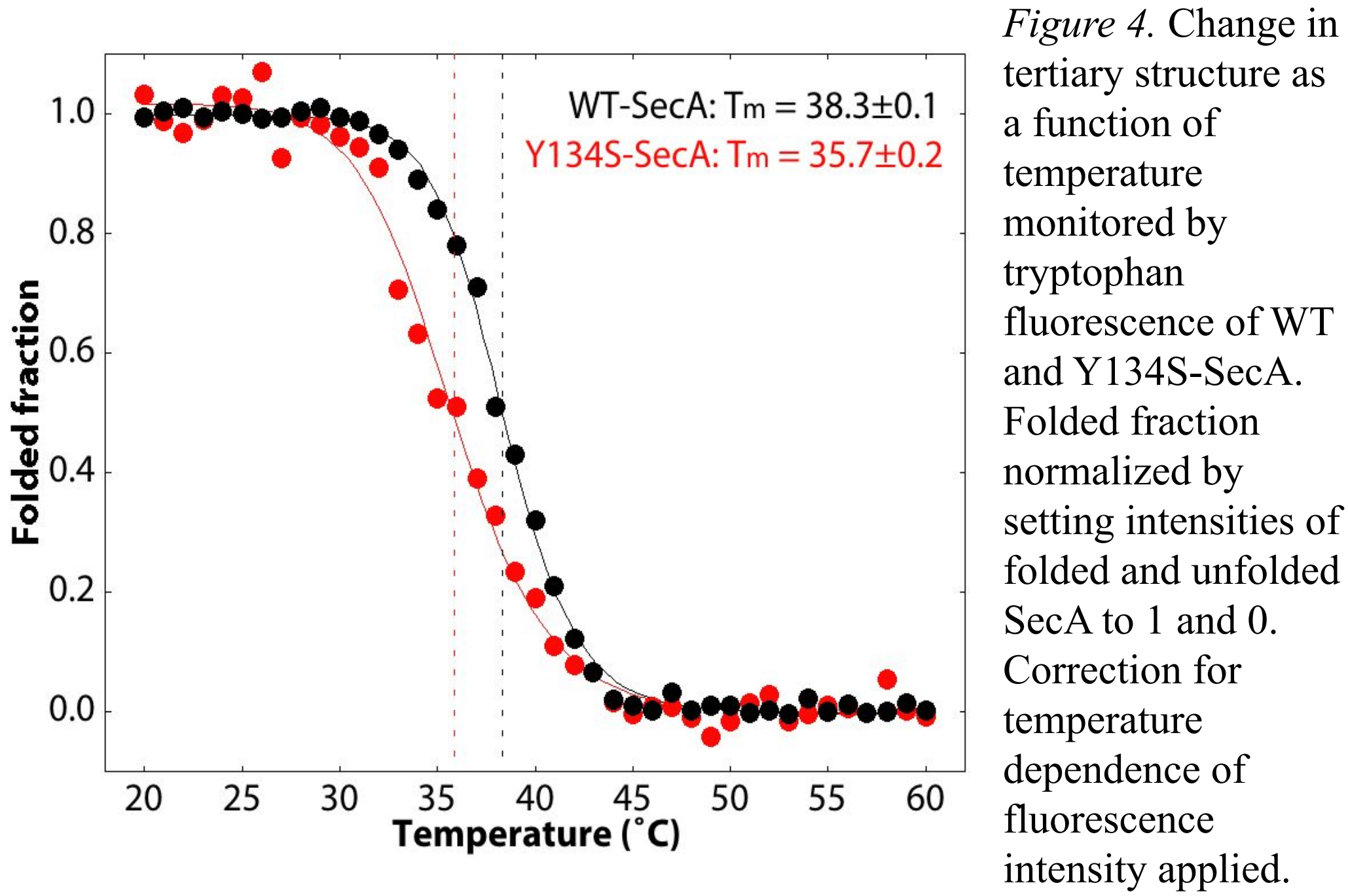


Figure 4. Change in tertiary structure as a function of temperature monitored by tryptophan fluorescence of WT and Y134S-SecA. Folded fraction normalized by setting intensities of folded and unfolded SecA to 1 and 0. Correction for temperature dependence of fluorescence intensity applied.

Conclusion

- Mutation of tyrosine to serine at position 134 (Y134S-SecA) resulted in a shift in the unfolding threshold of about 2.6°C (See Figure 4)
- Distinction between Y134S-SecA and WT-SecA is still apparent after the correction for temperature dependence of fluorescence intensity is applied-difference is significant after external variables are removed from the data
- Results indicate that overall structure of Y134S-SecA is less stable than WT-SecA; tyrosine residues in WT-SecA appear to play a role in protein stability
- Amino acid 134 lies between two domains of SecA that are involved in ATP hydrolysis; possible link between mutation and cell metabolism
- Future research includes investigating effects of intracellular salts (i.e. K⁺, Cl⁻) on Y134S-SecA (stability as compared to WT-Sec), and ATPase activity of WT and Y134S-SecA

Applications

- Identifying the significance of the 134 amino acid in SecA paves the way for more research into its role in facilitating the secretion of preproteins
- Researchers can better understand protein secretion/transport across the plasma membrane in bacteria and humans (via homologs of the SecA complex) if the function of SecA is more thoroughly researched
- Relationship between increased signalling peptide recognition (increased protein export) by Y134S-SecA and the 134 amino acid can be identified