Investigating Catalytically Important Residues in *Escherichia coli* Prolyl-tRNA Synthetase through Site-directed Mutagenesis.

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ABSTRACT

Prolyl-tRNA synthetase (ProRS) is a modular enzyme, which is responsible for catalyzing the covalent attachment of the amino acid proline to its cognate tRNA. This reaction is essential for protein biosynthesis in all living organisms. It has been established that the chemical properties of amino acid residues in the protein structure influence its catalytic function. In the present study, we have employed site-directed mutagenesis to probe the role of four residues in *E. coli* ProRS (*Ec* ProRS) on the overall catalytic function. Specifically, we examined the impact of changes in amino acid properties on the interactions between the enzyme (*Ec* ProRS) and the substrates, proline and ATP. The results of our study can be used to experimentally validate the findings of a quantum mechanical/molecular mechanical simulation of the prolyl adenylate formation, and has potential applications towards the design of a drug that selectively inhibits *Ec* ProRS. The preliminary results of our study are presented herein.

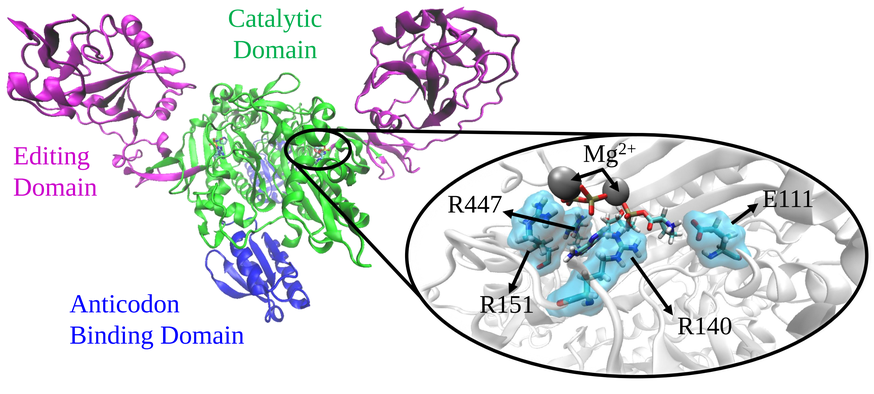
BACKGROUND

- **Aminoacyl-tRNA Synthetases (AARSs)**
  - Multi-domain, allosterically-regulated enzymes [*1*].
  - Catalyze the covalent attachment of amino acids to their cognate tRNAs, an important step in protein synthesis.

$$AA + ATP + AARS \rightleftharpoons AARS{\bullet}AA\text{-}AMP + PPi \quad (1)$$

$$AARS{\bullet}AA\text{-}AMP + tRNA \rightarrow AARS + AA\text{-}tRNA + AMP \quad (2)$$

- **Prolyl-tRNA Synthetases (ProRSs)**
  - Catalyze the covalent attachment of proline to $tRNA^{Pro}$.
  - Bacterial ProRS occasionally misactivates alanine and cysteine.
  - *E. coli* ProRS possesses an editing mechanism and a separate domain to hydrolyze $Ala\text{-}tRNA^{Pro}$ [*2*].



- **Catalytically Important Residues**
  - A Quantum Mechanical/Molecular Mechanical (QM/MM) computational study revealed that residues E111, R140, R151, and R447 are important for catalysis in *Ef* ProRS.
    - Elll and R140 bring proline and ATP into close proximity within the active site, and help anchor the prolyl-adenylate.
    - R151 and R447 interact with the gamma-phosphate of ATP, and assist in the release of PP_i from the active site pocket.
  - All four residues are located in the catalytic domain and have positive charges.

OBJECTIVE

- To experimentally validate the findings of the QM/MM computational study using site-directed mutagenesis of E111, R140, and R447 in *Escherichia coli* prolyl-tRNA synthetase.
- Neutralize the charges on the wild-type residues by mutating to alanine.

METHODS [AND] RESULTS

E111A_C 5' GGCCCAACTCAT**GCA**GAAGTTATCAC 3'
R140A_C 5'CCAGACCAAGTT**CGC**CGACGAAGTGCG 3'
R450A_C 5'CGGGGTAAC**GCG**TGTGGTAGCTGCGG 3'

Primer Design

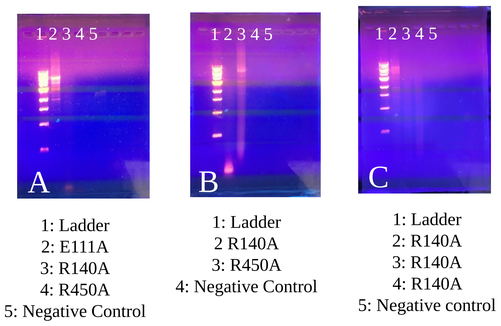
- Single point mutations
- WT *Ec* ProRS plasmid used as template

E111A: Glutamic acid(GAA)→ Alanine
R140A: Arginine(GCC)→Alanine
R450A: Arginine(GCG)→ Alanine

PCR

- Polymerase Chain Reaction [*3*]
  - Amplification of mutant DNA strands
  - Thermocycler Settings (30x)
    - 95° C Denaturing 30 seconds
    - 55°(60°)C Annealing 45 seconds
    - 75°C Elongation 6 minutes

Gel Electrophoresis



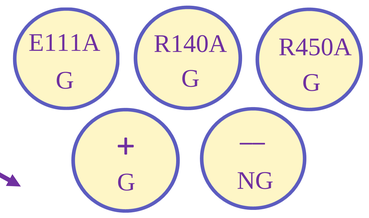
A: 1: Ladder 2: E111A 3: R140A 4: R450A 5: Negative Control
B: 1: Ladder 2 R140A 3: R450A 4: Negative Control
C: 1: Ladder 2: R140A 3: R140A 4: R140A 5: Negative control

DPN1 Digestion

- DPN1 enzyme degrades DNA template strand
  - Template strand has methylated adenines recognized by DPN1
  - Mutant strand remains

Transformation with XL1 Blue Cells

- Amplified purified DNA
- Successful transformation screened for using agar plates with 0.1 mg/mL ampicillin



G= Growth
NG= No growth

DNA Quantification and Sequencing

DNA Purification

1. P1 lyses cells and contains RNase A, which will degrade RNA
2. P2 precipitates protein out of solution
3. N3 neutralizes acidic solution pH
4. Centrifuge and transfer supernatant to QIA miniprep spin column
5. PE contains ethanol and will rinse away residual buffer solutions
6. DNA eluted from electrostatic QIA column with sterile H_2O [*4*]

- UV-Vis Spectroscopy to measure absorption of purified DNA
- DNA concentration calculated using Beer's Law [*5*]
  - $A=\varepsilon cl$
- Sent to UW-Madison for Sanger sequencing [*6*]
- Sequence results show successful mutagenesis

Mutant	Conc. (µg/mL)
E111A	149.6
R140A	481.7
R450A	260.7

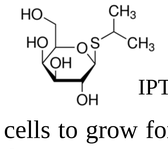
Transformation with SG13009 Cells

- Used to express a large quantity of protein
- Successful transformation screened for using agar plates with 0.1 mg/mL ampicillin and 0.025 mg/mL kanamycin

FUTURE DIRECTIONS

Overexpression

- *E coli.* SG13009 expression vector in luria bertami containing 0.01mg/mL AMP and 0.0025 mg/mL KAN.
- Induce with 1 mM Isopropyl β-D-1-thiogalactopyranoside.

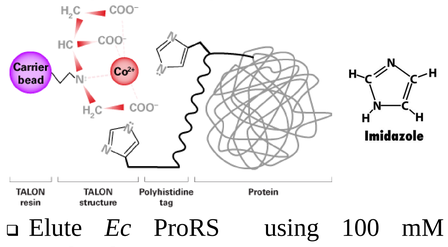


IPTG

- Allow cells to grow for 4 hours post-induction.
- Harvest and collect cell pellets from liquid culture.

Protein Purification

- Lyse *E. coli* cells and remove debris.
- Isolate *Ec* ProRS using His-tag and Co^{2+} affinity chromatography column



- Elute *Ec* ProRS using 100 mM imidazole.
- Isolate *Ec* ProRS from imidazole using differential centrifugation filters.

Michaelis-Menten Kinetics

$$E + S \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

- Determine K_M, k_{cat}, and V_{max} of enzymes using Michaelis-Menten equation [*7*].
- Compare kinetic parameters of mutants EllA, R140A, and R450A to wild-type *Ec* ProRS.

$$V_0 = \frac{V_{max}[S]}{(K_M + [S])}$$

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