Site Specific Incorporation of Unnatural Amino Acids into Proteins Through Evolution of a Leucyl-tRNA Synthetase

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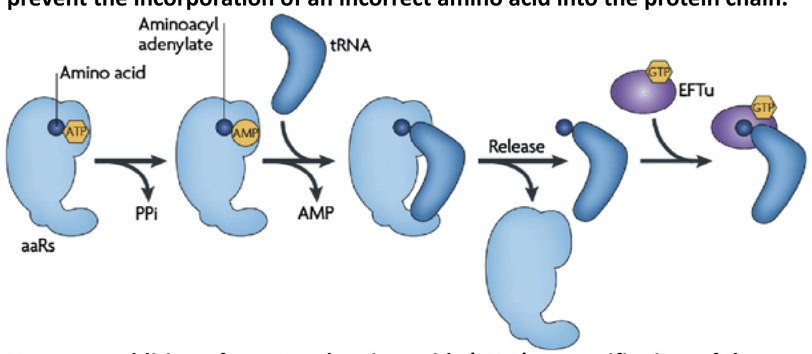
Introduction

Translation is the process of converting messenger RNA to a protein. One of the steps in this process involves the "charging" of transfer RNA (tRNA) by a specific aminoacyl tRNA synthetase (aaRS). The aaRS does this by binding both a specific amino acid and ATP. This leads to the formation of an amino-acyl-adenylate complex releasing pyrophosphate. A hydroxyl group of a tRNA then attacks the aminoacyl-adenylate complex causing the release of AMP and subsequently the release of tRNA which is now attached to its specific amino acid. Charged tRNA proceeds to a ribosome where the amino acid is incorporated into a growing protein chain. aaRSs contain an editing domain that ensures only the correct amino acid is attached to the correct tRNA to prevent the incorporation of an incorrect amino acid into the protein chain.[1]



However, addition of unnatural amino acids (UAA) at specific sites of the protein chain could add novel functions like unsaturation which allows click reactions, fluorescence for tracking and sensing, photo crosslinking, and protein dynamics studies.[1]

Methanobacterium thermoautotrophicum leucyl tRNA synthetase (MLRS) variants were created in which the editing domain was removed to allow for unnatural amino acid binding to the aaRS. In addition, *Halobacterium* sp. NRC-1 leucyl tRNA is used as the tRNA pair to the aaRS. Incorporation occurs through the use of the amber stop codon (TAG). It is used in only 7% of termination in *E. coli* and does not effect growth when the genetic code has been expanded using the TAG codon. MLRS variants had the five amino acids of the binding site randomized, and went through positive and negative selection rounds to test for successful incorporation of Dansyl-Dap. Three clones made it through the selection process and are being tested for successful incorporation of Dansyl-Dap at the 7th position of the Z-domain protein.

Methods

- Dual genetic screening of the MLRS library

T7RNA polymerase with an amber stop codon
CatUpp fusion containing amber stop codon

- Positive selection only allows growth of colonies with functional synthetases that charge tRNA with UAAs
- Negative selection is necessary in the event that natural amino acids are incorporated instead of UAAs. In the presence of 5-fluorouracil, cells incorporating natural amino acids will generate a full length uracil phosphoribosyl transferase (UPRT) which inhibits thymidylate synthase causing cell death.

- Expression of Z-domain TAG mutant

- Electrocompetent cells were transformed with the DNA of the clones that made it through the selection process and plated overnight.
- Cells were then placed in S-DAB (HNC) high oxygen autoinduction media containing Dansyl-Dap (positive) or no Dansyl-Dap (negative) overnight for protein production.

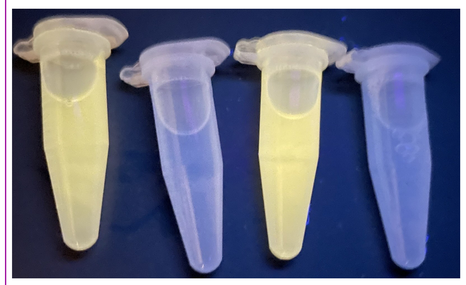
- Z-domain purification and concentration

- Cells were harvested using centrifugation and lysed using sonication.
- The cell lysate was purified using a column containing Ni-NTA resin.
- The protein sample was concentrated and desalted using an Amicon 3K ultrafiltration column.

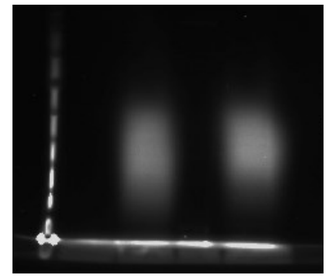
- Visualization with SDS–PAGE

- Both positive and negative protein samples were run on Nu-PAGE gel.
- Samples were then tested for fluorescence under UV light.

Results



Positive solutions showed fluorescence under UV light while negative samples did not.

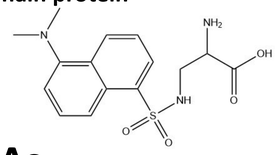


Nu-Page gel in which positive protein solutions showed fluorescence.

Future Directions

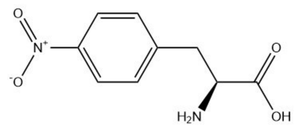
- Further study of Dansyl-Dap incorporation

- Incomplete purification of the Z-domain protein
- Mass spectrometry
- Studies of function



- Addition of other UAAs

- Iodophenylalanine
- 4-Nitro-1-phenylalanine



References

1. Guedez, P. Directed Evolution of Synthetic Riboswitches and a Leucyl tRNA Synthetase. Ph.D. Dissertation, Texas Christian University, Fort Worth, TX, 2022.
2. Shepherd, J.; Ibba, M. Direction of Aminoacylated Transfer RNAs into Antibiotic Synthesis and Peptidoglycan-Mediated Antibiotic Resistance. FEBS Lett. 2013, 587.

Acknowledgments

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