Engineering bacterial translational machinery for incorporation of D-amino acids

Po-Yi Huang[1] (pyhuang@hms.harvard.edu), Narasimhan Kamesh[1], and **George M. Church**[1]

[1]Harvard Medical School, Genetics Department, Massachusetts, Boston

http://arep.med.harvard.edu (Church lab)

Project Goals: Life is an anti-entropic phenomenon with two mutually reinforcing characteristics—homochirality and stereospecific catalysis—that have resulted in the exclusive presence of L-amino acids in proteins in the living world. Molecules from the mirror kingdom, such as proteins with D-amino acids (D-AAs), will be more resistant to existing biodegradation systems, and could provide new tools for biotechnology, classes of pharmaceuticals, and pathways for producing recalcitrant carbon. By gradually rewiring existing bio-machineries we aim to build a bridge to the space of mirror-imaged biomolecules. Here we investigated translational mechanisms relevant to protein synthesis with D-AAs, including tRNA amino acylation, EF-Tu binding of amino acyl-tRNAs, and ribosome catalysis of peptidyl transfer. Our data demonstrate that affinity between EF-Tu and amino acyl-tRNAs is critical to D-AA incorporation, and suggest that ribosome stalling on D-AAs is due failure of a substrate-assisted mechanism.

D-amino acid (D-AA) containing peptides (DAACP) are widely present in bioactivities within and secretions from microbes, fungi and amphibians[1]. In nature, these molecules are made through non-ribosomal pathways, such as non-ribosomal peptide synthesis or post-translational modifications such as epimerization. D-AA-containing proteins and peptides, mimicking the concept of DAACP, have been shown to have prolonged half lives in serum and resistance toward proteases without immunogenicity, desirable properties for pharmaceuticals. However, current protein expression methods cannot be applied to explore this immense space of potential compounds due to evolved barriers in translational machinery against incorporating D-AAs into proteins.

We used a purified *E. coli* protein synthesis system[2] as a model system in which to study how to best engineer translation machinery to tolerate D-AAs without interference of D-AA oxidase and D-aminoacyl-tRNA deacylase. While exceptions exist, it is believed that mechanisms have evolved to block the incorporation of D-amino acid at almost every step of protein synthesis.[3] There are three major steps at which D-AAs are discriminated from L-AAs in core translation machinery: aminoacylation of tRNAs by aminoacyl-tRNA synthetases (aaRSes), formation of ternary complexes with EF-Tu-GTP and their delivery to the ribosome, and the ribosome's own catalysis of peptide bond formation.

We began by surveying the amino acylation specificity of all 20 aaRSes toward D-AA, and then compared this against both chemical and ribozyme-catalyzed acylation methods. Next, we tested the overall effect of EF-Tu's binding to D- or L-AA-tRNAs, based on different tRNA backbones

and anticodons.[4] Our data suggested that tRNA backbones with high affinity to EF-Tu could improve the delivery of D-AA-tRNA to ribosome and hence D-AA incorporation.

It has long been argued if *E. coli* ribosome can catalyze peptide bond formation with D-AAs. Recent studies indicate that D-AA containing peptides can be produced, but with greatly reduced kinetics.[5–8] Our data agree with reports of single incorporations of D-AA in elongating peptide chains but we have not observed measurable consecutive D-AA incorporations. However, in our initial ribosome engineering experiments, we have found a few mutants in which D-AAs can be incorporated with increased efficiency relative to L-AAs, although with lower overall peptide synthesis activity. This leads us to hypothesize that L-AA incorporation involves a substrate-assisted mechanism that is not available with D-AA[9].

References

1. Ollivaux, C., Soyez, D. & Toullec, J.-Y. Biogenesis of d-amino acid containing peptides/proteins: where, when and how? *J. Pept. Sci.* **20,** 595–612 (2014).
2. Shimizu, Y. *et al.* Cell-free translation reconstituted with purified components. *Nat. Biotechnol.* **19,** 751–5 (2001).
3. Ahmad, S. *et al.* Mechanism of chiral proofreading during translation of the genetic code. *Elife* **2,** e01519 (2013).
4. Dale, T., Sanderson, L. E. & Uhlenbeck, O. C. The Affinity of Elongation Factor Tu for an Aminoacyl-tRNA Is Modulated by the Esterified Amino Acid†. *Biochemistry* **43,** 6159–6166 (2004).
5. Dedkova, L. M., Fahmi, N. E., Golovine, S. Y. & Hecht, S. M. Enhanced d-Amino Acid Incorporation into Protein by Modified Ribosomes. *J. Am. Chem. Soc.* **125,** 6616–6617 (2003).
6. Fujino, T., Goto, Y., Suga, H. & Murakami, H. Reevaluation of the d-Amino Acid Compatibility with the Elongation Event in Translation. *J. Am. Chem. Soc.* **135,** 1830–1837 (2013).
7. Englander, M. T. *et al.* The ribosome can discriminate the chirality of amino acids within its peptidyl-transferase center. *Proc. Natl. Acad. Sci.* **112 ,** 6038–6043 (2015).
8. Achenbach, J. *et al.* Outwitting EF-Tu and the ribosome: translation with d-amino acids. *Nucleic Acids Res.* **43 ,** 5687–5698 (2015).
9. Wallin, G. & Åqvist, J. The transition state for peptide bond formation reveals the ribosome as a water trap. *Proc. Natl. Acad. Sci.* **107,** 1888–1893 (2010).

This project has been funded by DOE grant DE-FG02-02ER63445, the Harvard Origin of Life Initiative, and EMD Millipore Inc.