Aminoacyl-tRNA Quality Control Provides a Speedy Solution to Discriminate Right from Wrong

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Translational fidelity must be maintained with a high degree of accuracy for optimal cell homeostasis. While the cell is able to tolerate low levels of erroneous protein synthesis, with basal levels of translational errors occurring 10^{-4} amino acids [1], gross accumulation of mistranslated proteins leads to a strong decrease in viability [2–4]. Given the dynamic and fluctuating nature of the cytoplasm with respect to nutritional availability, the cell must be equipped to accommodate rapid fluctuations in substrate availability that might otherwise compromise translational fidelity. Translational errors can occur through two mechanisms: errors in decoding within the A site of the ribosome or aberrant pairing of amino acids with their cognate tRNAs [5]. To counteract potential errors in tRNA aminoacylation, the cell has evolved proofreading mechanisms within the enzymes responsible for pairing free amino acids with their cognate tRNAs, the aminoacyl-tRNA synthetases (aaRSs).

To perform their role, aaRSs catalyze a two-step process. First, aaRS must discriminate non-cognate and cognate amino acids from a complex pool of substrates. Upon successful cognate amino acid recognition and in complex with ATP, an aminoacyl adenylate is formed releasing inorganic pyrophosphate. After amino acid activation, the aaRS transfers the amino acid to its cognate tRNA where it is available for protein synthesis [6].

Half of the aaRS in *Escherichia coli* are able to efficiently select cognate over non-cognate amino acids solely on ground-state discrimination within the enzyme's synthetic site [7]. In other words, non-cognate amino acids are unable to be activated due to steric occlusion of the synthetic site and therefore are no longer a threat to translational fidelity. However, due to the chemicophysical similarities of certain amino acids, some aaRSs must rely on additional proofreading mechanisms to prevent tRNA misaminoacylation. Pre-transfer editing is employed by some aaRSs to hydrolyze or selectively release misaminoacyl adenylates. In addition, some enzymes utilize a post-transfer editing mechanism that will transfer the non-cognate amino acid on to their tRNA where it can then by hydrolyzed by a distinct editing domain [6].

One of the most studied aminoacyl-tRNA proofreading systems is that of the class I aaRS, leucyl-tRNA synthetase (LeuRS), which utilizes a post-transfer editing mechanism through its conserved CP1 domain (connective polypeptide 1) [8,9]. Recent studies have challenged the idea that the main target for LeuRS editing was against misaminoacylation of isoleucine [10]. It appears that one of the primary roles of LeuRS editing may actually be to also prevent the non-proteinogenic amino acid, norvaline (Nva) from being incorporated into the proteome. What remained unclear was how Nva is targeted for proofreading over cognate Leu. In this issue of the *Journal of Molecular Biology*, Dulic *et al.* now sought to further characterize the biochemical processes that not only lead to hydrolyses of the non-cognate Nva-tRNA^{Leu} but also successfully prevent hydrolysis of cognate Leu-tRNA^{Leu}, which would otherwise be lethal to the cell. Through the use of substrate analogs, structural data, and molecular dynamic simulations (MDSs), the authors were able to dissect the discrete mechanistic interactions between LeuRS and its various potential substrates. Their results challenge our understanding of LeuRS editing and further substantiate the role of the terminal adenosine of tRNA for post-transfer hydrolysis for LeuRS.

As ground-state discrimination has been the prevailing model for cognate omission of aaRS editing sites [7], Dulic *et al*. used non-hydrolyzable analogs that mimic the 3′ end of the aminoacylated tRNA to determine dissociation constants for these substrates. Using isothermal calorimetry, the authors measured the binding affinities for leucine and norvaline analogs with full-length LeuRS, the CP1 editing domain, and LeuRS variants that have been previously characterized to alter editing function. The authors observed that, 2′-(L-leucyl)amino-2′-deoxyadenosine (Leu2AA) has only a 10-fold weaker affinity for the CP1 editing site compared to its non-cognate 2′(L-norvayl)amino-2′deoxyadensoine (Nva2AA) counterpart. This observation suggests that ground-state discrimination is not sufficient to efficiently discern cognate from non-cognate amino acids during tRNA translocation.

To determine if substrate discrimination is due to the chemical rate of hydrolysis rather than steric occlusion of the editing domain, the authors used single-turnover kinetics and measured deacylation rates of Leu-tRNA^{Leu} and Nva-tRNA^{Leu}. In contrast to the modest difference in binding affinities for the cognate or non-cognate substrate, the authors observed a ~10^4-fold increase in the rate of Nva-tRNA^{Leu} deacylation compared to Leu-tRNA^{Leu}. These results highlight the novel finding in this report that leucine is not sequestered from cognate hydrolysis due to structural preclusion of the editing site but rather substrate coordination that leads to differences in reaction kinetics.

This model was further supported through the utilization of a previously characterized variant of LeuRS T252A that has been shown to hydrolyze cognate Leu-tRNA^{Leu} [11]. The T252A variant had no effect on binding affinities for Leu2AA relative to the wild-type enzyme, while the binding affinity for Nva2AA was decreased in this variant nearly 40-fold. This result suggests that threonine 252 does not alter binding of cognate leucine, rather it contributes to the productive binding of non-cognate norvaline. While T252A does not affect the binding affinity of leucine to the editing domain, single-turnover kinetics revealed a 400-fold increase in deacylation of Leu-tRNA^{Leu} relative to the wild-type enzyme.

With these kinetic data in hand, the authors employed the use of structural and MDSs to identify the physical coordination of LeuRS residues with cognate and non-cognate substrates in the editing site. The authors determined the structure of the T252A variant to 2.6-Å resolution and the T252A variants with Leu2AA or Nva2AA in the editing site at 3.1-Å resolution. MDSs revealed that leucine is able to enter the editing site of wild-type LeuRS but primarily adopts a conformation in which the N-Cα-Cβ-Cγ torsion angle is $-170^\circ \pm 20^\circ$. In contrast, when MDS is performed using the T252A variant, the primary N-Cα-Cβ-Cγ torsion angle was $-70^\circ \pm 20^\circ$. Importantly, the latter conformation permitted the productive orientation of a catalytic water molecule in the editing site with the terminal 3′-OH of the tRNA. This suggests that leucine will predominantly be oriented in a non-productive orientation in the editing site and that threonine 252 is critical for maintaining this coordination. Interestingly, MDSs with the norvaline analog and the wild-type CP1 domain showed that norvaline can adopt the -70° and -170° N-Cα-Cβ-Cγ torsion angle, but even in the -170° conformation, it is still oriented in productive conformation with the catalytic water molecule leading to hydrolysis.

The 3′-OH of the terminal adenosine of tRNA^{Leu} has previously been implicated in the deacylation reaction, and the author's simulations have substantiated this interaction [12]. To experimentally confirm the role of the 3′-OH for LeuRS hydrolysis, the authors removed the terminal adenosine and replaced it with 3-deoxyadenosine generating 3′dtRNA^{Leu}. Using single-turnover kinetics, the authors found a significant decrease in deacylation rates of cognate and non-cognate aminoacyl-tRNA. When preforming the kinetic experiments with the 3′ dtRNA substrate and the D345A variant, deacylation rates were even further impeded indicating that both the 3′-OH and aspartate 345 play critical and independent roles in maintaining editing function in LeuRS.

The study by Dulic *et al*. provides new and surprising insights into the mechanism of LeuRS proofreading in *E. coli*. This work has challenged the dogma of ground-state discrimination by class I synthetases within the editing domain. In addition, this work provides conclusive evidence for the role of the terminal 3′-OH of the tRNA for efficient hydrolysis in LeuRS. More broadly, this work highlights the utility of structural analogs for dissecting discreet mechanistic processes. The authors note that when working with the full-length aminoacyl-tRNA substrates, binding results using microscale thermophoresis were indistinguishable for Leu-tRNA^{Leu}, Nva-tRNA^{Leu}, or uncharged tRNA^{Leu}. This observation is inconsistent with the modest, but relevant observed differences when using the Leu2AA and Nva2AA analogs. The authors attribute this discrepancy to the contributions of the tRNA body sequence that mask subtle differences with amino acid substrate binding.

In addition to their utility for similar biochemical studies, application of these analogs has the potential to influence more applied drug discovery-based research. AaRSs and their proofreading sites have become a promising target for antimicrobial therapies due to their essential role in the cell [13]. The ability to target discreet interactions leads to the possibility of establishing therapy cocktails that may have been ignored when investigating gross biochemical function.

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