Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Chemical Biology

Ribosomal incorporation of unnatural amino acids: lessons and improvements from fast kinetics studies

Jinfan Wang[1] and Anthony C Forster[2]

Technologies for genetically programming ribosomal incorporation of unnatural amino acids are expanding and have created many exciting applications. However, these applications are generally limited by low efficiencies of the unnatural incorporations. Here we review our current mechanistic understanding of these limitations delineated from *in vitro* fast kinetics. Rate limitation occurs by different mechanisms, depending on the classes of the unnatural amino acids and the tRNA adaptors. This new understanding has led to several ways of improving the incorporation efficiencies, as well as challenges of dogma on rate-limiting steps in protein synthesis in natural cells.

Addresses
[1] Department of Structural Biology, Stanford University School of Medicine, Stanford, CA 94305, United States
[2] Department of Cell and Molecular Biology, Uppsala University, Uppsala 75124, Sweden

Corresponding author: Forster, Anthony C (a.forster@icm.uu.se)

Current Opinion in Chemical Biology 2018, **46**:180–187

This review comes from a themed issue on **Synthetic biology**

Edited by **Michael Ledbetter** and **Floyd E. Romesberg**

https://doi.org/10.1016/j.cbpa.2018.07.009

Introduction

The protein synthesis apparatus accommodates more chemical variation in its natural monomer substrates, amino acids (AAs), than other cellular polymer synthesis machineries. This innate plasticity has been harnessed to expand the genetic code by site-specifically incorporating unnatural AAs into biopolymers *in vitro* and *in vivo*. These designer moieties have been used to probe translation mechanism, post-translational modification, protein catalysis and protein structure and have been applied for peptidomimetic drug discovery via mRNA display [1–9].

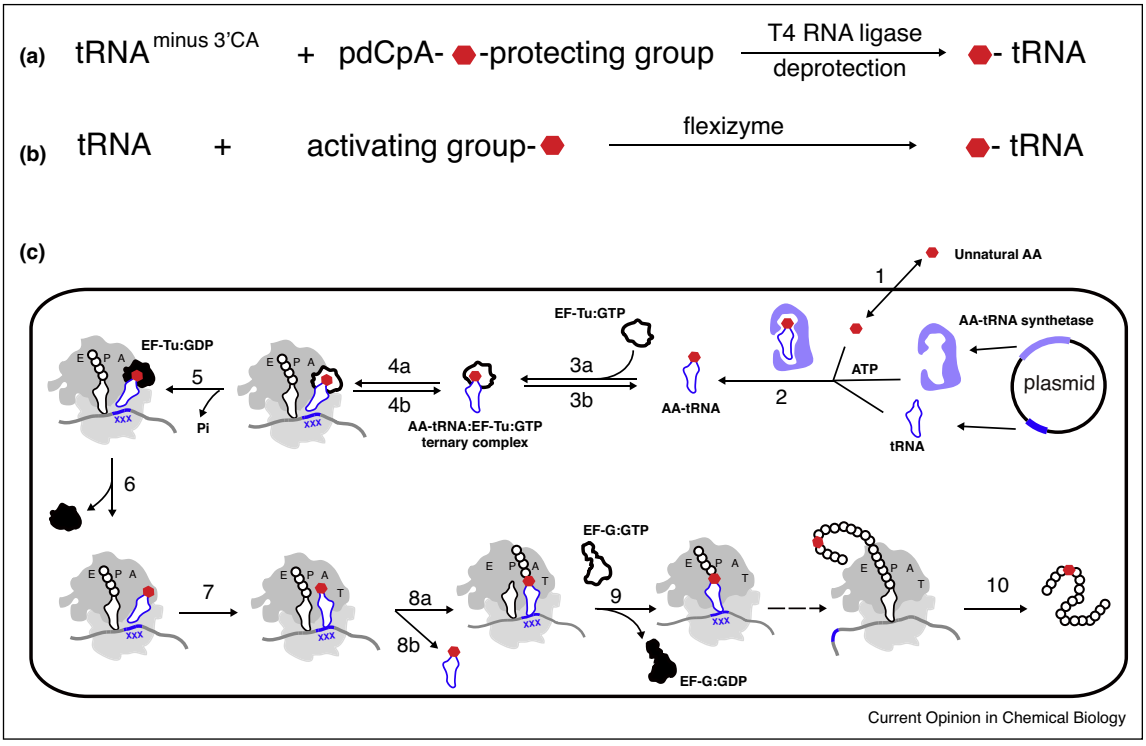
By far the most useful unnatural moieties incorporated to date have been non-proteinogenic L-α-AAs because they most closely resemble the natural AAs and incorporate most efficiently. But even *N*-alkyl-AAs, D-AAs, α-hydroxy acids, β-AAs and dipeptides have been incorporated to various extents. Although a low incorporation efficiency can be problematic for both yield and specificity, it is often acceptable when only a single unnatural incorporation step is required and a small yield is sufficient. But when multiple unnatural AA incorporations are needed per polymeric chain, as in peptidomimetic drug selection, low individual efficiencies multiply (or worse), limiting the library size of full-length peptidomimetics. Therefore, it is important to understand the limiting determinants of unnatural incorporations as this should help guide improvements.

Early understanding of determinants that limit unnatural AA incorporation efficiencies was gained empirically. This was mostly achieved by synthesizing many AA-tRNA analogs and incorporating them at the UAG stop codon in an *Escherichia coli* crude *in vitro* translation system [10]. Major limitations were incorporation yield due to competition with translation release factors (RFs), and expansion of unnatural AA incorporation to any other stop or sense codon due to competition with natural AA-tRNAs. These limitations became even greater (and more difficult to probe) upon transitioning to *in vivo* systems due to the difficulty of engineering unnatural AA:unnatural tRNA:unnatural AA-tRNA synthetase triples that were orthogonal to all the natural triples yet still functioned efficiently enough [11,12•]. RF1 knock-out strains and UAG knock-out strains have been engineered to improve efficiencies of UAG reprogramming *in vivo* [13–15]. But the biggest gains in yield, specificity and understanding of unnatural AA incorporation were enabled by moving to a purified translation system [16,17] which liberated all 64 codons for reassignment and facilitated multiple unnatural incorporations per product [18–20]. However, incorporation efficiencies were still limited in purified systems for reasons not understood [17,18], despite exciting breakthroughs in 3D imaging of the ribosome. Prolonging the incubation time did not improve the final yields, indicating the reactions were limited by processes occurring in the early time course and that methods with higher temporal resolution were needed to investigate the mechanisms. We thus turned to pre-steady-state fast translation kinetics with unnatural AA-tRNAs [21]. Although we covered this topic earlier as a subset of a review [22••], results have expanded considerably since then, so it is now time for this topic to be the main subject of a review.

Translation kinetics methods

For translation kinetics, the unnatural AA is first pre-charged onto a tRNA by one of several methods (Figure 1;

Figure 1



tRNA charging methods and translation reaction scheme. To be co-translationally incorporated, one natural or unnatural AA needs to go through many steps. For translation *in vitro*, the AA (red hexagon) is activated *in vitro* by either chemical synthesis **(a)** and **(b)** [58–60] or AA-tRNA synthetase-catalyzed adenylation by ATP *in situ* (not shown) before it is linked to the tRNA enzymatically (step 2 in **(c)**). To improve consecutive incorporations of unnatural AAs [54], AAs should be acylated to pCpA [61], not pdCpA (see supplementary material and Table 1). **(c)**. For translation *in vivo*, the steps are: first, passage across the cell membrane (can be bypassed by synthesis *de novo* by the cell); second, AA is adenylated by ATP, then linked to its cognate tRNA via an ester bond, both steps being catalyzed by the cognate AA-tRNA synthetase; (3a) the AA-tRNA forms a ternary complex with a translation elongation factor (EF-Tu, or SelB for the $tRNA^{Sec}$ case, in bacteria) and GTP; (3b) the ternary complex dissociates; (4a) the ternary complex binds to the ribosomal A/T site; (4b) the ternary complex is rejected by the ribosome without GTP hydrolysis on EF-Tu; (5) when the A-site codon is cognate, GTP hydrolysis on EF-Tu is triggered; (6) EF-Tu:GDP is released from the ribosome; (7) the AA-tRNA is accommodated to the A/A site; (8a) the peptidyl transfer reaction occurs; (8b) the AA-tRNA is rejected by the ribosome; (9) the newly formed peptidyl-tRNA is translocated to the P site by a translation factor (EF-G:GTP in bacteria); and (10) steps 1–9 are repeated until the A site displays a stop codon that is recognized by one of the release factors (rather than a suppressor AA-tRNA), which catalyzes the release of the newly synthesized protein from the tRNA.

see supplementary material). Most of the steps (*i.e.* steps 3–9 of Figure 1c) that occur in cellular translation are then monitored kinetically in a purified peptide-synthesis system (Figure 2a). We applied fast kinetics to ribosomal incorporation of various kinds of unnatural AAs (Figure 2b) and now have sufficient data to draw conclusions about all the main classes of unnatural AAs and tRNAs. Unexpectedly different rate-limiting/competing steps were discovered for the different unnatural classes and all proved instructive for improving incorporation efficiencies.

N-alkyl AAs: rate-limiting peptidyl transfer

N-alkyl AAs are invaluable substrates for peptidomimetic drug discovery because the *N*-alkylation of the backbone gives the peptidomimetic better pharmacological properties, *i.e.* higher protease resistance and cell membrane permeability, and potentially even oral availability. Among the 22 natural AAs, Pro is the only *N*-alkyl AA; its five-membered ring structure distinguishes it from other *N*-alkyl AAs (Figure 2b, yellow). Early attempts at incorporation of *N*-methyl AAs within proteins and peptides were successful, albeit sometimes with lower yields than might have been expected based on chemical similarity to Pro [23,24,25]. Surprisingly, when the alkylation was a larger *N*-butyl group, incorporation was blocked altogether [26]. The mechanism by which the translation machinery discriminated different *N*-alkyl AAs was unclear, but reasonable culprits based on literature at the time were delivery by EF-Tu to the ribosome (given that EF-Tu:AA-tRNA co-crystals showed recognition of the amino group [27]) or accommodation (believed to be the rate-limiting step in translation elongation [28]). However, based on our data, we proposed that *N*-alkyl AA discrimination occurred at the peptidyl transfer step [26] (see below).