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Rewiring Protein Synthesis: From Natural to Synthetic Amino Acids

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Abstract

Background—The protein synthesis machinery uses 22 natural amino acids as building blocks that faithfully decode the genetic information. Such fidelity is controlled at multiple steps and can be compromised in nature and in the laboratory to rewire protein synthesis with natural and synthetic amino acids.

Scope of review—This review summarizes the major quality control mechanisms during protein synthesis, including aminoacyl-tRNA synthetases, elongation factors, and the ribosome. We will discuss evolution and engineering of such components that allow incorporation of natural and synthetic amino acids at positions that deviate from the standard genetic code.

Major conclusions—The protein synthesis machinery is highly selective, yet not fixed, for the correct amino acids that match the mRNA codons. Ambiguous translation of a codon with multiple amino acids or complete reassignment of a codon with a synthetic amino acid diversifies the proteome.

General significance—Expanding the genetic code with synthetic amino acids through rewiring protein synthesis has broad applications in synthetic biology and chemical biology. Biochemical, structural, and genetic studies of the translational quality control mechanisms are not only crucial to understand the physiological role of translational fidelity and evolution of the genetic code, but also enable us to better design biological parts to expand the proteomes of synthetic organisms.

1. Introduction

The genetic information flows accurately from DNA to messenger RNA (mRNA) and to protein. The standard genetic code features 20 natural amino acids encoding 61 triplet sense codons, with the remaining three codons (TAA, TAG, and TGA) signaling translation termination [1]. In some organisms, certain TGA and TAG codons are recoded by

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selenocysteine (Sec) and pyrrolysine (Pyl), known as the 21st and 22nd natural amino acids, respectively [1–3]. Except for Sec, each of the 22 amino acids used as protein building blocks is ligated to the correct transfer RNA (tRNA) by a specialized aminoacyl-tRNA synthetase (aaRS) [4] (Figure 1). The resulting aminoacyl-tRNA (aa-tRNA) is delivered to the ribosome by an elongation factor (EF-Tu in bacteria and EF1A in archaea and eukaryotes), and correct matching of tRNA anticodon and mRNA codon leads to peptide elongation. To ensure translational fidelity, multiple quality control mechanisms are utilized by aaRSs, elongation factors, and the ribosome [5–8]. However, such quality control is frequently escaped by many natural amino acids and analogs [9]. For example, L-canavanine is a structural analog of L-arginine produced by higher plants, and causes toxicity to insects through co-translational incorporation into the insect proteome [10]. In the last couple of decades, many laboratories have engineered the protein synthesis machinery to insert a wide variety of synthetic amino acids into proteins [11–14], which have demonstrated broad applications ranging from structural determination, fluorescent labeling, purification of nascent proteins, studies of protein modifications, and engineering of synthetic organisms [14–18]. In this review, we provide an update on the mechanisms of amino acid selection by the protein synthesis machinery, and the implications in cell physiology and synthetic biology.

2. Selection of amino acids by aminoacyl-tRNA synthetases

2.1. Selection and editing of natural amino acids by aaRSs and trans-editing factors

Picking the correct amino acid by the aaRS poses a significant challenge due to the structural similarity between some amino acids [19]. For example, valine (Val) and isoleucine (Ile) differ by a small methyl group in the side chain. The initial selection at the active site of isoleucine-tRNA synthetase (IleRS) only provides 180-fold discrimination between Val and Ile [20], which does not satisfy the average amino acid misincorporation rate of approximately 10^{-4} [21]. Additional proofreading is provided by an appended editing domain in IleRS, which hydrolyzes the misacylated Val-tRNA^{Ile} in a post-transfer editing reaction [20]. Editing domains are also present in valyl- (ValRS) [22, 23], leucyl- (LeuRS) [24], prolyl- (ProRS) [25], threonyl- (ThrRS) [26], alanyl- (AlaRS) [27], and phenylalanyl- (PheRS) [28] tRNA synthetases. The editing site specifically hydrolyzes the misacylated aa-tRNA but excludes the correct aa-tRNA, therefore preventing waste of resources. For example, the editing site of PheRS hydrolyzes tyrosyl- and meta-tyrosyl-tRNA^{Phe}, but not Phe-tRNA^{Phe} [28–30]. A conserved glutamate residue in the editing site specifically recognizes the hydroxyl group of tyrosine and meta-tyrosine for substrate selection [30–32] (Figure 2). Besides *cis*-editing domains within aaRSs, post-transfer editing is also achieved by free-standing *trans*-editing factors [33, 34]. To date, *trans*-editing factors that are homologous to the editing domains of ThrRS, AlaRS and ProRS have been identified [33–35]. Unlike *cis*-editing domains that recognize specific aa-tRNAs, *trans*-editing factors have recently been shown to exhibit broader substrate recognition patterns [36, 37]. For example, a homolog of the ProRS editing domain (ProXp-y) hydrolyzes Ser-tRNA^{Thr} and Thr-tRNA^{Val} produced by ThrRS and ValRS, respectively [36], indicating a generic role of *trans*-editing factors in the improvement of aminoacylation fidelity.

Editing mechanisms of aaRSs are mostly conserved throughout evolution. However, mitochondria and certain organisms with reduced genomes have lost some editing functions. In yeast mitochondria, both PheRS [38] and ThrRS [39, 40] have lost the entire *cis*-editing domains. Mitochondrial PheRS fidelity is achieved by a more selective active site [38], and mitochondrial ThrRS utilizes an alternate proofreading mechanism, namely pre-transfer editing [41], to reduce aminoacylation errors [40]. Interestingly, PheRS and ThrRS in certain mycoplasma species are also editing-defective [42, 43]. Mycoplasma PheRS and ThrRS with defunct editing sites are intrinsically error-prone and not able to support growth in *Escherichia coli* and *Saccharomyces cerevisiae* that have lost their native cytosolic counterparts [43, 44]. It is still unclear why these mycoplasma species have lost the editing function during evolution. One possibility is that reduced translation fidelity is not only tolerated, but may also be advantageous during the life cycle of mycoplasma.

2.2. Stress-induced misacylation

Aminoacylation fidelity is a prerequisite for accurate proteins synthesis. Mutations in the aaRS or tRNA could increase errors during aminoacylation [6]. More recent studies revealed that certain stress conditions also increase specific aminoacylation errors via distinct mechanisms [29, 45–48]. In mammalian cells, viral infection and oxidative stress substantially increase attachment of methionine to noncognate tRNAs [45]. Such global misacylation is induced by phosphorylation of methionyl-tRNA synthetase (MetRS), and may provide transient protection against oxidative stress thanks to reversible oxidation of Met in the proteome [49]. Oxidative stress also leads to misacylation by two other aaRSs – ThrRS and PheRS. The ThrRS editing site contains a conserved cysteine (C182 in *Escherichia coli* ThrRS) that is essential for the editing activity [26, 50]. We have shown that C182 is sensitive to oxidative stress and can be modified to a sulfenic acid, which can be reverted to cysteine when the oxidative stress is removed [46, 51]. Oxidation of C182 is facilitated by two conserved histidines in the editing site [51], and leads to an editing deficiency and misacylation of serine (Ser) to tRNA^{Thr}. C182 thus serves as a redox switch to control ThrRS editing. For PheRS, oxidized Phe is attached to tRNA^{Phe} when the editing activity is compromised [29].

Microorganisms frequently encounter environmental changes. In the hyperthermophilic archaeon *Aeropyrum pernix*, low temperature enhances misacylation of tRNA^{Leu} with Met by MetRS [47], although the underlying mechanism remains unclear. In *E. coli*, growth under anaerobic conditions suppresses a succinyl-lysine modification of MetRS that is required for high fidelity during aminoacylation, resulting in increased Met misacylation [48]. These recent advances suggest that aminoacylation fidelity is remarkably flexible under stress conditions and likely serve as novel regulatory mechanisms (see discussion below in **Physiological impact of rewiring protein synthesis**).

2.3. Plasticity of wild-type aaRSs towards synthetic amino acids

Quality control mechanisms in aaRSs have evolved to discriminate against amino acids and analogs that are present in the natural environments. Synthetic amino acids that are structurally similar to the cognate amino acids can be effective substrates for aaRSs. For example, the wild-type PheRS has been shown to activate several Phe analogs [52].

Screening of a synthetic amino acid library reveals that wild-type *E. coli* tryptophanyl- (TrpRS) and tyrosyl- (TyrRS) tRNA synthetases uses a wide range of synthetic amino acids as substrates [53]. An exciting application that takes advantage of the substrate plasticity of aaRSs is bioorthogonal noncanonical amino acid tagging (BONCAT) [54]. BONCAT uses endogenous MetRS to incorporate a Met analog azidohomoalanine (AHA) into Met positions, and further click chemistry allows fluorescent tagging or purification of the nascent proteome. This residue-specific approach has been broadly used to visualize and quantitate newly synthesized proteins, to probe the dynamics of protein modifications, and to produce new materials [14].

2.4. Engineering of aaRS to expand the genetic code with novel synthetic amino acids

As discussed above, some wild-type aaRSs are capable of activating certain synthetic amino acids. However, such synthetic amino acids need to compete with the cognate amino acid for the aaRS and are often inserted into the entire proteome with low homogeneity. In contrast to residue-specific incorporation of synthetic amino acids, a site-specific approach uses an engineered aaRS/tRNA pair to insert the synthetic amino acid only at particular locations of the target protein, typically mutated to the UAG codon [55]. The most commonly used aaRSs for such purposes are TyrRS from *Methanocaldococcus jannaschii*, and pyrrolysyl-tRNA synthetase (PylRS) from *Methanosarcina* species. These systems have been reviewed extensively elsewhere [12, 55, 56]. Recently, this site-specific approach has provided important insights into the dynamics of post-translational modifications, such as lysine acetylation and serine phosphorylation [16, 57]. Another intriguing application of site-specific incorporation of synthetic amino acids is biocontainment. Two recent studies have engineered genetically modified organisms that depend on the addition of a synthetic amino acid for growth, thanks to an engineered aaRS suppressing an in-frame stop codon of an essential gene with the synthetic amino acid [17, 18].

3. Recognition of aminoacyl-tRNAs by elongation factor

Elongation factors deliver a variety of aa-tRNA substrates to the ribosome. However, not all amino acids are recognized with equal efficiency [58]. The amino acid binding pocket of EF-Tu is relatively small and negatively charged [59] (Figure 3); therefore it binds negatively-charged and bulky amino acids with low affinity [7, 58, 60]. For natural proteinogenic amino acids with negative charges, such as glutamate (Glu) and aspartate (Asp), weak binding of the amino acid by EF-Tu is compensated by strong binding of the tRNA moiety [7, 61]. To co-translationally incorporate negatively-charged phosphoserine (Sep) into the proteins, EF-Tu has been engineered to improve recognition of Sep [62]. Similar EF-Tu engineering has been conducted to effectively insert selenocysteine into proteins in a SECIS-independent manner [63]. Not surprisingly, these laboratory-evolved EF-Tu variants contain mutations of negatively-charged or neutral residues to positively-charged arginines in the amino acid binding pocket [62–64]. In addition to EF-Tu engineering, tRNA optimization has also been successfully performed to enhance ribosomal incorporation of long-chain amino acids [65]. In this study, Foster and colleagues engineered a synthetic tRNA^{AlaB}, which is derived from a natural tRNA^{Ala} and has high affinity to EF-Tu. Using tRNA^{AlaB} as the tRNA body to carry long-chain amino acids substantially

increases EF-Tu affinity and the incorporation speed of synthetic amino acids into peptides [65].

4. Discrimination of amino acids by the ribosome

4.1. Recognition of natural proteinogenic amino acids by the ribosome

The ribosome uses 22 natural amino acids to synthesize proteins. When the crystal structure of the large ribosomal subunit was solved, it was found that the amino acid side chain of the aa-tRNA directly contacts the rRNA residues in the ribosomal aa-tRNA binding (A) site [66], suggesting that different amino acids may be recognized by the ribosome with distinct efficiencies. Later *in vitro* kinetic studies showed that amino acid side chains of aa-tRNAs do contribute to ribosome binding [61, 67]. Fast kinetic analyses of dipeptide formation revealed that the N-alkylamino acid proline (Pro) is a poor substrate for peptide elongation on the purified ribosome [68]. *In vivo*, a specialized elongation factor (EF-P in bacteria and eIF5A in eukaryotes) promotes efficient translation of Pro codons [69–71]. Enhancement of Pro translation by EF-P or eIF5A requires diverse post-translational modifications, including β -lysylation in *E. coli* and *Salmonella* [72, 73], rhamnosylation in *Pseudomonas aeruginosa* [74], lysine modification with 5-aminopentanol group in *Bacillus* [75], and hypusine in yeast [71].

4.2. Ribosomal translation of D- and β -amino acids

Ribosomes use *L*- α -amino acids as building blocks for peptides. *D*- and β -amino acids (Figure 4) attached to the tRNA have been shown to be poor substrates for the wild-type ribosome [76]. Hecht and colleagues tested ribosome variants with mutations in the peptidyl-transferase center, and found several ribosome mutants with substantially increased efficiency to incorporate *D*-amino acids into a reporter protein *in vitro* [77]. A recent study by Englander *et al.* provided mechanistic insights into discrimination of *D*-amino acids by the wild-type ribosome [78]. It was shown that *D*-aa-tRNAs are accepted by the ribosomal A site and used as substrates for peptide formation. However, ribosomes with *D*-amino acid in the peptidyl-tRNA (P) site are partitioned into translating and arrested subpopulations, therefore impeding further peptide elongation [78].

Similar to *D*-amino acids, β -amino acids have also been inserted into peptides by mutant ribosomes *in vitro* [79, 80]. A recent study shows that some β -amino acids can even be used by the wild-type ribosome as substrates [81]. Ribosomal incorporation of β -amino acids into proteins has also been achieved *in vivo* [82]. Using mutant ribosomes and endogenous aaRSs and EF-Tu, Czekster *et al.* successfully inserted β -Phe analogs into reporter proteins in *E. coli* [82]. Including *D*- and β -amino acids in the protein alphabet will significantly expand the toolbox for de novo protein design [83]. Synthetic peptides containing *D*- and β -amino acids can be used as new biomaterials with novel properties, such as resistance to degradation by proteases. *D*-amino acids are key components of the bacterial cell wall and many antibiotics. Site-specific insertion of *D*-amino acids into peptides could also have potential applications in the development of novel antimicrobial strategies.

5. Physiological impact of rewiring protein synthesis

5.1. Fitness costs of rewiring protein synthesis

Rewiring protein synthesis with natural or synthetic amino acids leads to production of novel proteomes comprised of proteins with noncanonical sequences. Many proteins with amino acid replacements fail to fold correctly and even form aggregates [84–86], causing both gain of toxicity and loss of function [29, 87, 88]. Reduced translational fidelity causes various cell defects, ranging from growth defects and cell death in bacteria [89–91], mitochondrial dysfunction in yeast [92], shortened life span in flies [93], and neurodegeneration and cardioproteinopathy in mammals [85, 94]. Editing defects in aaRS further increase DNA mutations in bacteria [95] and activates DNA damage response in zebrafish [96]. Recent work also suggests that a PheRS editing defect suppresses stringent response in *E. coli* [97], and a ribosomal error-prone mutation suppresses bacterial motility [98].

In addition to reduced translational fidelity, genome-wide codon reassignment also leads to reduced fitness. In *Methanosarcina acetivorans*, deleting the tRNA^{Pyl} gene reduces the genetic code from 21 to 20 amino acids, which causes downregulation of amino acid metabolism and growth defects in methanol [99]. This work suggests a selective advantage of an expanded genetic code in nature. In another study, evolved *E. coli* cells that completely replaces Trp with an analog (Tpa) in the proteome appear longer and thinner, and need to accumulate beneficial mutations to support robust growth [100].

5.2. Benefits of reduced translational fidelity

Despite the fitness cost of translational errors, reduced translational fidelity paradoxically provides benefits under certain stress conditions [101, 102]. In *Candida albicans*, ambiguous translation of CUG codons with Ser and Leu increases phenotypic diversity [103]. In the budding yeast, depletion of a release factor that forms prion increases resistance of cells to various stresses [104]. Further, methionine misacylation has been suggested to increase cell resistance to oxidative stress [45] and antibiotics [48], and produce alternate protein variants that better adapt to low temperatures [47]. We have also shown that reduced translation fidelity caused by a ribosomal mutation or canavanine improves bacterial tolerance to oxidative stress by activating the general stress response [105]. Reduced translational fidelity may also directly modify drug targets to increase resistance. For example, in *Mycobacteria*, mistranslation of glutamine (Gln) and asparagine (Asn) codons with Glu and Asp in the RpoB protein creates variant proteins that resist rifampicin [106, 107].

6. Conclusions and perspectives

The flexibility of the genetic code has been demonstrated in numerous cases from natural evolution and adaptation to laboratory engineering. Increased knowledge of the mechanisms and physiological impact of rewired protein synthesis has reshaped our understanding of evolution, microbial pathogenesis, and synthetic biology. In future studies, novel sensitive tools need to be developed to detect alternate translation events in microorganisms and higher eukaryotes under native environments. To further understand evolution and the physiological role of translational fidelity and facilitate design of synthetic organisms with

altered proteomes, genetically-modified model organisms and systems are warranted. In addition, the role of translational errors on protein misfolding and human diseases needs to be clarified.

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Highlights

- The protein synthesis machinery is selective for natural amino acid substrates
- Translational fidelity can be compromised under stress conditions
- Engineering of aaRSs, EF-Tu, and the ribosome expands the genetic code

Figure 1. Amino acid selection during protein synthesis

Each aminoacyl-tRNA synthetase (e.g., GlnRS, PDB ID: 1EUQ) selectively pairs the cognate amino acid with corresponding tRNA. EF-Tu (PDB ID: 1OB2) then delivers the aa-tRNA to the ribosome, where codon-anticodon match triggers transfer of the amino acid to the growing peptide in the peptidyl-transferase center.

Figure 2. Substrate selection of PheRS editing site

The crystal structure of *Thermus thermophilus* PheRS (PDB ID: 2AMC) reveals that the hydroxyl group of Tyr is recognized by the carboxyl group of E334.

Figure 3. Structure of the amino acid binding pocket of EF-Tu (PDB ID: 1OB2)

Two conserved acidic residues prevent effective binding of negatively-charged amino acids, such as Asp, Glu, phosphoramino acids, and selenocysteine.

Figure 4. Structures of amino acids with different backbones

D- and β -amino acids are discriminated against by the wild-type ribosome, but can be co-translationally inserted into peptides by certain mutant ribosomes.