Quality Control and Infiltration of Translation by Amino Acids Outside of the Genetic Code

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Abstract

Translation of the genome into functional proteins is critical for cellular life. Accurate protein synthesis relies on proper decoding of mRNAs by the ribosome using aminoacyl-tRNAs. During aminoacyl-tRNA synthesis, stringent substrate discrimination and rigorous product proofreading ensure tRNAs are paired with the correct amino acid, as defined by the rules of the genetic code. What has remained far less clear is the extent to which amino acids that are not part of the genetic code might also threaten translational accuracy. Here, we review the broad range of nonproteinogenic, or nonprotein, amino acids that can naturally accumulate under different conditions, the ability of the translation quality control machinery to deal with such substrates, and their potential impact on the integrity of the genetic code and cellular viability.

INTRODUCTION

Transfer RNA (tRNA): alanine tRNA or $tRNA^{Ala}$ denotes tRNA specific for alanine

Aminoacyl-tRNA synthetases (aaRSs): denoted by their three-letter amino acid designation, e.g., AlaRS for alanyl-tRNA synthetase

NPA: nonproteinogenic amino acid

The genetic code defines the rules by which information stored in the nucleic acid sequences of genes is translated into the corresponding amino acid sequences in proteins. How accurately the genetic code is translated depends mainly on two steps in protein synthesis: precise decoding of mRNAs and accurate synthesis of aminoacyl-tRNAs (aa–transfer RNAs). aa-tRNAs are made by aminoacyl-tRNA synthetases (aaRSs), which match specific amino acids with the corresponding tRNAs as defined by the genetic code. As described in more detail below, tRNA aminoacylation is the subject of extensive quality control (QC), and a reduction in the accuracy of this step in protein synthesis can have catastrophic effects on the cell. Such effects are well illustrated in a landmark study that linked QC by alanyl-tRNA synthetase (AlaRS) to protein-folding defects and neurodegeneration in mice (52). In a related study in mammalian cells, inactivation of the valyl-tRNA synthetase (ValRS) QC mechanism that discriminates valine (Val) from threonine (Thr) disrupted cell morphology and led to membrane blebbing, activation of caspase-3, and apoptosis (67). A notable finding of the latter study was that the phenotypes observed upon disruption of QC were exacerbated dramatically by the introduction of α-aminobutyrate into the growth media. α-Aminobutyrate is a naturally occurring metabolite that can potentially act as a substrate for protein synthesis despite the fact that it is not encoded within the genetic code; i.e., it is a nonproteinogenic, or nonprotein, amino acid (NPA; see sidebar, Genetically Encoded Nonproteinogenic and Nonnatural Amino Acids). One of the broader implications of this study is that mistranslation of Val codons with the NPA α-aminobutyrate is more disruptive to the synthesis of functional proteins than misincorporation of the genetic code amino acid Thr. Although other examples of the harmfulness of NPAs are well documented (e.g., 8), their use in translation can also be beneficial, for example, to maintaining growth during amino acid starvation (79).

Although NPAs can dramatically reduce the accuracy of protein synthesis, the majority of studies to date on QC and mistranslation have focused on errors within the confines of the genetic code, i.e., the substitution of one canonical amino acid for another canonical amino acid. The ability of the QC machinery to recognize and proofread amino acids outside the genetic code, the natural occurrence of different NPAs, and how translation with nonproteinogenic substrates ultimately impacts cell growth have received comparatively little attention and are the focus of this review (**Figure 1**).

GENETICALLY ENCODED NONPROTEINOGENIC AND NONNATURAL AMINO ACIDS

The genetic code contains 22 amino acids: the 20 canonical amino acids found in all organisms plus selenocysteine and pyrrolysine, which are encoded in only some genomes. In addition to these genetically encoded protein amino acids (GPAs), protein synthesis is able to use a vast range of other natural and synthetic substrates. Nonproteinogenic, or nonprotein, amino acids (NPAs) is a term used to refer to naturally occurring amino acids that are not part of the genetic code but nevertheless can act as substrates for protein synthesis. Examples of NPAs include D–amino acids and other precursors and products of GPA metabolism. The other category of amino acids that can be used as substrates for translation is the synthetic nonnatural amino acids (NNAs). Although translation can naturally utilize a modest range of NNAs, genetic engineering has allowed synthetic expansion of the genetic code to accommodate a wide range of potentially useful NNAs (reviewed in 69).

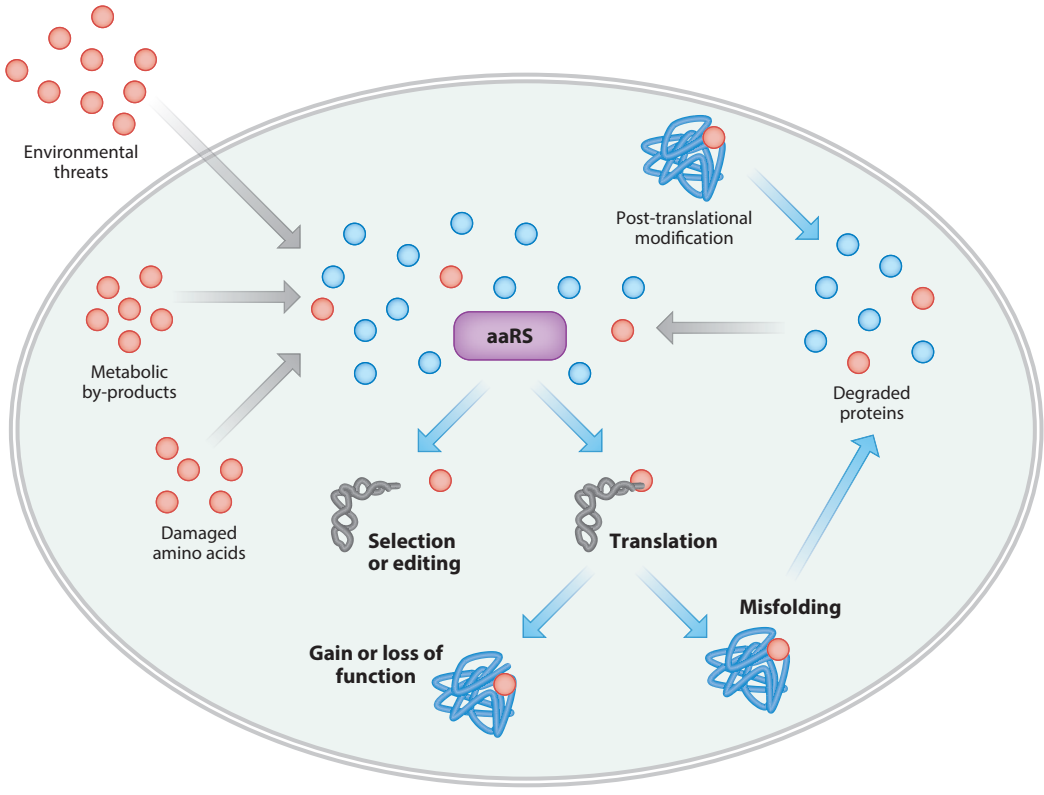


Figure 1

The central role of aminoacyl-tRNA synthetase (aaRS) enzymes in determining the fate of nonproteinogenic amino acids (NPAs; *red circles*) in the cell. Sources of NPA in the cellular pool of translation substrates are shown as gray arrows, and the outcomes of aaRS recognition of these compounds are shown as blue arrows. If an NPA is recognized by an aaRS, then it is either edited or used in protein synthesis. NPAs that are capable of making their way into the proteome can have various effects on protein functions in the cell, many of which are still unknown. NPAs in the proteome can also be recycled back into the amino acid pool following protein degradation. Genetically encoded cognate amino acids are shown as blue circles.

AMINO ACID QUALITY CONTROL

The potential utilization of NPAs during translation depends in part on their ability to be recognized by aaRSs. The aaRS family comprises a total of 23 enzymes, various combinations of which are found in different organisms (14). Aminoacylation of tRNA by aaRSs is a two-step reaction, consisting of ATP-dependent amino acid activation followed by ligation of amino acid to the 3′ end of tRNA, forming an aminoacyl ester bond (40). During amino acid activation, approximately half of the aaRSs display a level of specificity of 3,000:1 or greater for cognate versus noncognate proteinogenic amino acids, which is similar to overall error rates typically observed during protein synthesis. For the other aaRSs, the existence of closely related near-cognate substrates precludes a high level of cognate amino acid discrimination, and for QC these enzymes depend on an additional proofreading function called editing (109) (**Figure 1**). Editing is categorized as pre- or post-transfer, depending on whether products of the first or second step of the aminoacylation reaction are hydrolyzed, respectively (37, 57, 111).

AaRS editing contributes to QC as part of a double-sieve mechanism (30, 31). The first sieve is the active site of the aaRS, which determines the specificity for the cognate substrate. The second

sieve is an editing or proofreading activity to clear either activated near-cognate amino acids or mischarged tRNAs. For example, isoleucyl-tRNA synthetase (IleRS) activates Ile only ~200-fold more efficiently than the noncognate amino acid Val, which differs by a single methyl group (92). To maintain translation fidelity, IleRS employs a form of proofreading that results in hydrolysis of misactivated Val-AMP (pre-transfer editing) and misacylated Val-tRNA^{Ile} (post-transfer editing), preventing mischarged tRNA from being released for translation (5, 23, 27). Similarly, the editing activities of the enzyme phenylalanyl-tRNA synthetase (PheRS) prevent the delivery of Tyr (tyrosine)-tRNA^{Phe} to the ribosome and protect against the mistranslation of Phe codons as Tyr (58). Editing activities have also been described for ValRS, leucine-tRNA synthetase (LeuRS), methionine-tRNA synthetase (MetRS), ThrRS, AlaRS, proline-tRNA synthetase (ProRS), lysine-tRNA synthetase (LysRS), and serine-tRNA synthetase (SerRS) in a broad range of organisms, consistent with the widespread use of aaRS editing to maintain translational fidelity (109 and references therein). Finally, if mischarged tRNAs are released before editing has occurred, in some instances they are subsequently edited in *trans* either by aaRSs or by freestanding *trans*-editing factors. This resampling of aa-tRNA synthesis errors in *trans* provides an additional QC step before protein synthesis.

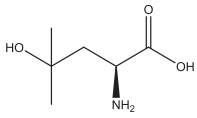
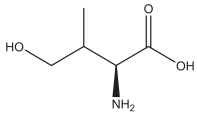
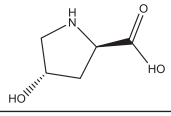
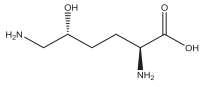
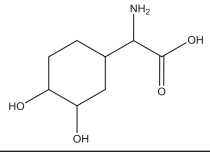
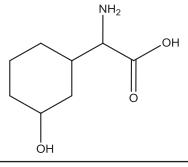
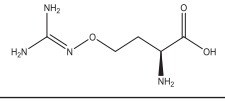
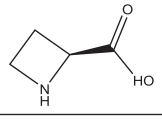
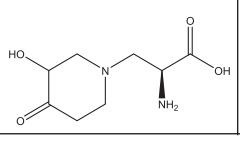
GPA: genetically encoded protein amino acid

Although the ability of aaRSs to discriminate and proofread noncognate, genetically encoded protein amino acids (GPAs) is well documented, the importance of their role in preventing infiltration of the genetic code by NPAs is considerably less well understood. Several aaRSs have been demonstrated to efficiently proofread both GPAs and NPAs; for example, LeuRS edits norvaline and Ile (36), and PheRS edits *meta*-Tyr (*m*-Tyr) and *para*-Tyr (*p*-Tyr) (49) (see **Table 1**). The ability of aaRS QC to target substrates outside the genetic code with adequate substrate specificity to prevent protein synthesis errors suggests that NPAs pose a threat to accurate translation in the cell (22). This idea is supported by previous studies showing that NPAs present major challenges for QC when supplied exogenously to cells, and under some conditions this is even more problematic than near-cognate GPA replacements (e.g., 35, 67). The known naturally occurring NPAs include D-enantiomers, which are used for cell wall biosynthesis but are excluded from protein synthesis by broad specificity D–amino acid deacylase *trans*-editing factors, and a range of naturally occurring metabolites (**Table 1**; see below for details). A typical NPA metabolite is *m*-Tyr, a product of Phe oxidation that is partially excluded from translation by the PheRS editing pathway in some organisms but not others (49). In *Escherichia coli*, the editing of *m*-Tyr-tRNA^{Phe} produced after cells are exposed to reactive oxygen species (ROS) constitutes a major component of the cell's oxidative stress response, underscoring the potential threat to cellular viability posed by NPAs (15). These findings illustrate that in order to fully appreciate the potential impact of NPAs on cell growth, it is necessary to examine conditions under which they are expected to accumulate to significant levels and how this might affect protein synthesis and cellular physiology.

MODIFIED AMINO ACID POOLS

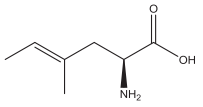
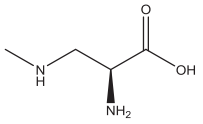
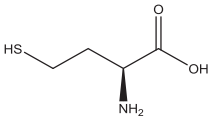
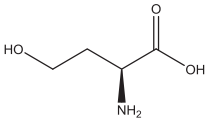
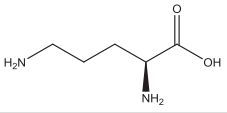
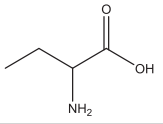
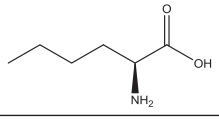
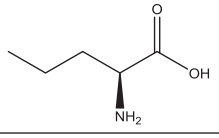
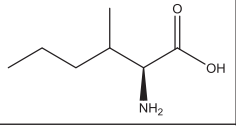
The accumulation of NPAs in the cell can occur in a number of ways. One is via the degradation of proteins containing residues that have undergone post-translational modification. Post-translational modifications involve various chemical changes, such as acylation, methylation, phosphorylation, and oxidation. Degradation of modified proteins introduces NPAs into the pool of free amino acids in the cell, potentially making them available as substrates for aaRS enzymes and, subsequently, use in protein synthesis. It is not entirely clear how drastically free NPA pools are altered by these modified amino acids and whether they serve as substrates for translation or affect the need for aaRS proofreading. For example, phosphorylated amino acids are not substrates for protein synthesis, and substantial manipulation of the translation machinery is needed

Table 1 Naturally occurring nonproteinogenic amino acids that are substrates for aminoacyl-tRNA synthetases (aaRSs)

Source	Nonprotein amino acid	Structure	aaRS	Edited by aaRSs	Cotranslationally inserted into proteins	References
Post-translational oxidation	Leucine hydroxides (e.g., γ-hydroxyleucine)		LeuRS	Yes	Yes	(28, 33, 83)
Post-translational oxidation	Valine hydroxides (e.g., 4-hydroxyvaline)		NA	NA	Yes	(33, 83)
Post-translational oxidation	*Trans*-4-hydroxyproline		ProRS	Yes	Yes	(13)
Post-translational oxidation	Hydroxylysine (5-hydroxy-L-lysine)		LysRSII	Yes	NA	(2)
Oxidation of free amino acids; post-translational oxidation	3,4-dihydroxyphenylalanine (L-dopa)		PheRS	Yes	Yes	(64, 83, 105)
Plant product; post-translational oxidation	*Meta*-tyrosine		PheRS TyrRS	Yes	Yes	(15, 16, 35, 49)
Plant product	Canavanine		ArgRS	No	Yes	(86)
Plant product	Azetidine-2-carboxylic acid		ProRS	No	Yes	(78, 94, 101)
Plant product	Mimosine		PheRS	Yes	NA	(34)

(*Continued*)

Table 1 (*Continued*)

Source	Nonprotein amino acid	Structure	aaRS	Edited by aaRSs	Cotranslationally inserted into proteins	References
Plant product	2-Amino-4-methylhex-4-enoic acid (AMHA)		PheRS	Yes	NA	(3)
Plant product	β-N-methylamino-L-alanine (BMAA)		SerRS	NA	Yes	(24)
Biosynthesis by-products	Homocysteine		Several	Yes	NA	(45, 46)
Biosynthesis by-products	Homoserine		LysRSII	Yes	NA	(42)
Biosynthesis by-products	Ornithine		LysRSII	Yes	NA	(2, 43)
Biosynthesis by-products	α-Aminobutyrate		ValRS, LysRSII	Yes	Yes	(2, 66)
Biosynthesis by-products	Norleucine		MetRS	Yes	Yes	(11, 60)
Biosynthesis by-products	Norvaline		LeuRS, MetRS	Yes	Yes	(4, 48)
Biosynthesis by-products	β-Methylnorleucine		IleRS	NA	Yes	(65, 100)

Abbreviations: ArgRS, arginine-tRNA synthetase; IleRS, isoleucyl-tRNA synthetase; LeuRS, leucine-tRNA synthetase; LysRS, lysine-tRNA synthetase; MetRS, methionine-tRNA synthetase; NA, not available; PheRS, phenylalanyl-tRNA synthetase; ProRS, proline-tRNA synthetase; SerRS, serine-tRNA synthetase; TyrRS, tyrosine-tRNA synthetase; ValRS, valine-tRNA synthetase.

to accommodate them (74). The other potential sources of NPAs in the cellular metabolite pool are chemical modification of free GPAs and enzymatic synthesis, for example, of amino acid biosynthesis intermediates. Examples of the different routes of NPA accumulation that have been demonstrated to generate substrates used in protein synthesis are discussed in more detail below.

Oxidation of Amino Acids

Carefully controlled oxidation-reduction reactions within proteins have critical physiological roles in the cell, such as regulating the function of enzymes containing active cysteine (Cys) residues. However, unregulated oxidation resulting from the presence of elevated levels of ROS can both damage proteins and transform GPAs into NPAs. Protein modification and oxidative damage are well characterized in eukaryotic systems and have been found to be strongly correlated with aging and disease (99). More recently, oxidation of free amino acids prior to translation has also been shown to be a source of damaged residues in proteins (35). In both cases, the generated oxidized amino acids, whether they contribute directly or indirectly to the cellular NPA pool, can have a significant impact on the accuracy of translation and the overall functionality of the proteome.

In addition to the sulfur-containing amino acids Met and Cys, there are several other amino acids prone to oxidation, in particular the aromatic amino acids (98). Phenylalanine can undergo hydroxyl radical attack, which places a hydroxyl group at either the *ortho* or *meta* position of the carbon ring, generating *o*-Tyr and *m*-Tyr, respectively. *m*-Tyr is activated efficiently by the catalytic site of PheRS, and in *E. coli*, post-transfer editing by PheRS is required for growth in the presence of this NPA (15). In the absence of PheRS editing, *m*-Tyr is readily misincorporated at Phe codons, which limits, and can even inhibit, cell growth at elevated NPA levels. The primary role of *E. coli* PheRS proofreading, at least under laboratory conditions, seems to be to protect the cell from *m*-Tyr misincorporation, as similar experiments with the noncognate GPA *p*-Tyr do not have any discernible effects on growth. In yeast, by contrast, PheRS editing directly prevents *p*-Tyr incorporation but provides little protection against *m*-Tyr. The variability in the roles of the same pathway in different organisms, protecting the proteome from NPA incorporation in *E. coli* and GPA misincorporation in yeast, illustrates how the QC machinery can adapt to the demands posed by different growth environments and cellular physiologies.

Hydroxyl radicals can also generate other amino acids in the cell; for example, attack on Tyr results in addition of a hydroxyl group at the *meta* position producing the NPA L-3,4-dihydroxyphenylalanine (L-DOPA). L-DOPA is a precursor for dopamine and is used as a treatment for Parkinson's disease. The potential effects of L-DOPA misincorporation into proteins as a secondary outcome of therapeutic use of this NPA are of great interest. In vitro experiments show that L-DOPA is a substrate for PheRS and also that it can be incorporated into mammalian proteomes via TyrRS, an aaRS that lacks editing mechanisms to correct aminoacylation errors (64, 70, 83). *E. coli* and *Bacillus subtilis* TyrRS have also been shown to use L-DOPA as a substrate (16). The location within proteins at which L-DOPA is misincorporated determines the impact of this NPA on the cell, as only the replacement of Tyr residues that are not exposed to solvent leads to protein unfolding and aggregation (25, 26).

Aliphatic amino acids can also be targets for oxidation, leading to formation of noncognate, nonprotein substrates for aaRSs and the translation machinery. Oxidized GPAs, such as Leu hydroxide and Val hydroxide, have been shown to be incorporated into nascent proteins in mammalian cells (33, 83). In addition to oxidation of the host cell amino acid pool, these nonprotein amino acids are also produced post-translationally in plants, providing a dietary source of NPAs (82). Proline hydroxides (e.g., *trans*-3-hydroxyproline and *trans*-4-hydroxyproline) are also found in plants, where they are used for cell wall biosynthesis and may constitute as much as 16% of the residues

in potato lectin (1). In animals, post-translational hydroxylation of Pro residues contributes to the role of collagen in cell stability and regulates the activity of the hypoxia-inducible factor, which is central to oxygen homeostasis (93). *E. coli* ProRS is able to edit the Pro analog *trans*-4-hydroxyproline, but the QC machinery can be overwhelmed at high intracellular concentrations of this NPA, leading to efficient incorporation into recombinant proteins (9, 13).

De Novo Synthesis of Nonprotein Amino Acids

In addition to the accumulation of damaged amino acids in the cell, there are numerous examples of GPA mimics that are produced in large quantities by plants and microbes as part of what appear to be defense mechanisms, with toxic effects on protein synthesis in neighboring species. Such NPAs, in particular, plant-produced amino acids, have been studied mainly for their toxic effects on humans through consumption or in some cases their neurological benefits, as with theanine found in green tea (reviewed in 81). The targets of extracellular NPAs are often biosynthetic pathways, where they mimic intermediates, resulting in direct disruption of these critical processes. However, in other cases these NPAs also have high similarity to GPAs and therefore are recognized by aaRSs and used as substrates for translation.

Nonprotein Amino Acid–Based Defense Mechanisms in Plants and Microbes

Two NPAs produced in plants, L-canavanine and azetidine-2-carboxylic acid, have been well characterized for their ability to be used for protein synthesis by some organisms but not others. L-Canavanine, a mimic of arginine (Arg), is produced by a subfamily of Leguminosae and was shown to be incorporated into the proteins of insect larvae in place of Arg (32, 85). Ingestion of canavanine by humans becomes toxic at high levels and is being investigated in combination with Arg starvation for its potential anticancer properties (107). Differences exist in the ability of ArgRS from various organisms to use canavanine as a substrate and correlate with the presence of this amino acid in the organisms' environments, indicating the selection pressures such NPAs place on translation and the development of QC mechanisms, in this case increased enzyme specificity for the GPA Arg. The jack bean plant, which produces high levels of canavanine, and some species of insects such as the brucid beetle, have highly selective ArgRSs that do not efficiently use canavanine as a substrate and so provide a mechanism of resistance to the NPA in these organisms (41, 62, 87). Azetidine-2-carboxylate is produced in large amounts by several plant species, including sugar beets (*Beta vulgaris*) (78). This NPA is effectively misincorporated into proteins at Pro codons by several plant species as well as animals, where it has been shown to lead to neurodegeneration and autoimmune disorders (89, 90). Similar to L-canavanine and ArgRS, the ProRSs of azetidine-2-carboxylate-producing plant species are able to discriminate effectively against the NPA. Azetidine-2-carboxylate is toxic to *E. coli*, where it has been shown to elude post-transfer editing by ProRS (9).

Several plant NPAs chemically similar to Phe have been investigated for their ability to target PheRSs of other organisms. *Aesculus californica* seeds produce AMHA (2-amino-4-methylhex-4-enoic acid), which is a poor substrate for the host PheRS but is efficiently activated by other plant PheRSs. Whether AMHA is excluded during transfer to $tRNA^{Phe}$ or is edited by the proofreading activity of PheRS is unknown (3). Mimosine (β-3-hydroxy-4-pyridone) is an aromatic amino acid analog found in high amounts in the genus *Mimosa* and in *Leucaena leucocephala*, and is toxic to animals, *E. coli*, and mung bean seedlings (96). This NPA is known to have cytotoxic effects outside of protein synthesis, and it is interesting to note that mimosine is activated by mung bean PheRS as well as PheRSs from mimosine-producing plants (34, 96). However, transfer of mimosine to $tRNA^{Phe}$ has not been observed, and the NPA is not incorporated into proteins (96).

These observations suggest that PheRS pre-transfer and/or post-transfer QC mechanisms protect the proteomes of producer and target plants from mimosine incorporation, and that differences in mimosine sensitivity are unrelated to the potential impact of the NPA on protein synthesis. *m*-Tyr, a product of phenyalanine oxidation discussed above, is produced at high concentrations by fescue grasses and is toxic to other competing plant species (8). L-DOPA, another hydroxylation product, is made by the macuna plants, and is incorporated into mammalian cells in place of Tyr, indicating its use as an NPA by TyrRS of nonproducing species (83, 84). BMAA (β-*N*-methylamino-L-alanine) is an NPA that mimics Ser and is produced by cyanobacteria (genus *Nostoc*), which include free-living organisms and root symbionts of the cycad palms native to Guam (20). BMAA is found in contaminated seafood and drinking water, and can accumulate in seeds of *Cycas circinalis* as well as in the animals that feed on such plants. This NPA is associated with neurological diseases of the South Pacific that resemble amyotrophic lateral sclerosis (ALS), Parkinson's, and dementia (6). BMAA is found in proteins in the place of Ser and upon accumulation results in protein misfolding and aggregation, which is particularly problematic in nerve cells (24, 108).

The vast number of NPAs produced by plants, many of which target protein synthesis, presents the question of what evolutionary roles these toxins play. NPAs present an effective mechanism to limit unwanted competitors in a particular niche as a form of defense against competing plants, insect herbivores, or microbial pathogens that live in the surrounding rhizosphere. However, the selective pressure cytotoxic NPAs exert on aaRS specificity and proofreading in the producer suggests that to remain effective against targeted species, the development of widespread resistance is limited. More broadly, as with other antibiotics (21), the fact that sensitive organisms have persisted in niches where NPAs are synthesized also suggest other as yet unknown, perhaps positive, roles beyond interfering with protein synthesis.

PRIMARY METABOLITES AND NONPROTEIN AMINO ACIDS

The complexity and diversity of cellular metabolism lead to various by-products and intermediates, many of which are, or resemble, amino acids. Owing to the high number of NPAs and other metabolites in the cell, the translational machinery is constantly faced with an immense pool of compounds to select from, in addition to the coded amino acids normally found in proteins. In most cases, a low concentration of these intermediates and high aaRS enzyme specificity prevent any threat to protein synthesis. However there are several examples where metabolites that fall outside of the coded set of amino acids used in translation have been shown to function as efficient aaRS substrates and in some cases have been seen to be incorporated into the protein. Different editing mechanisms are used to limit the incorporation of these metabolites in protein biosynthesis, including aaRS-dependent pre-transfer editing of homocysteine (Hcy) and post-transfer editing of norvaline, and editing by an independent editing domain in the case of D–amino acids. Metabolic imbalances caused by mutations or changes in the organisms' environment can lead to significant upshifts in the levels of these metabolites, which in the case of Hcy is associated with significant toxicity. However, many questions remain regarding the full range of conditions that can lead to accumulation of these metabolites and the complete list of cellular mechanisms to prevent these NPAs from entering protein synthesis and eliciting cellular toxicity.

Pre-Transfer Editing of Metabolites: Homocysteine, Homoserine, and Ornithine

Several biosynthetic intermediates (Hcy, homoserine, and ornithine) are activated by aaRSs and then subsequently hydrolyzed in a tRNA-independent, pre-transfer editing reaction. In each case,

a cyclized lactone or lactam is formed, which in the case of Hcy-thiolactone is a highly toxic molecule that requires an additional enzyme for further breakdown. How frequently the levels of homoserine or ornithine exceed the levels of the cognate amino acid for the relevant aaRSs (LysRS, ValRS, and IleRS in the case of homoserine and LysRS in the case of ornithine) is unknown, but for Hcy, the end product of Hcy editing, Hcy-thiolactone, can accumulate to significant levels in humans (46).

Hcy is the precursor of Met and differs from Met by one methyl group (19, 77). As a consequence, Hcy is readily activated by MetRS and, to a lesser extent, by IleRS, LeuRS, ValRS, and LysRS (46). Hcy-AMP is subsequently hydrolyzed in the aaRS active site through an intramolecular reaction between the activated carboxyl group and the thiolate to yield Hcy-thiolactone and AMP (46). Hcy-thiolactone is itself a toxic molecule that reacts with the amino side chain of lysines in proteins, resulting in protein N-homocysteinylation (77). In the cases in which N-homocysteinylation has been studied, it typically has resulted in protein inactivation and commonly caused the protein to aggregate (44, 73). Thus, it is not surprising that at least some organisms have a lactonase (PON1 in humans) that can degrade Hcy-thiolactone (45). In fact, PON1 lactonase activity was inversely correlated with serum protein N-homocysteinylation in humans (76).

As part of the biosynthetic pathway for Met, from bacteria to mammals, and also for cysteine in mammals, it would seem unlikely a priori that Hcy would accumulate to sufficient levels to compete with the cognate amino acids of the aaRSs. However, several genetic disorders and dietary deficiencies lead to increased levels of Hcy and Hcy-thiolactone in humans, a condition called hyperhomocysteinemia, which is a risk factor for a number of diseases, including cardiovascular disease (45, 47). The conversion of Hcy to Met is a folate- and vitamin B_{12}–dependent reaction. Human mutations in the gene for methylenetetrahydrofolate reductase, the enzyme that generates the folate substrate, or dietary deficiency in either folate or vitamin B_{12} lead to hyperhomocysteinemia (103). Similarly, the conversion of Hcy to cysteine is a vitamin B_6–dependent reaction, and genetic defects in cystathionine beta-synthetase, the first step in the cystenine biosynthesis pathway, and dietary deficiencies in vitamin B_6 lead to hyperhomocysteinemia, or high Cys in the blood (53).

Although defects in Hcy processing are well known in humans, it is less readily apparent whether other organisms encounter elevated levels of Hcy. For the bacterial species that can synthesize their own vitamins, Hcy should not readily accumulate. However, most bacterial species utilize a LuxS pathway to degrade *S*-adenosylhomocysteine (SAH), a by-product of reactions involving *S*-adenosylmethionine (SAM). LuxS catalyzes the second step in the pathway that generates Hcy and 4,5-dihydroxy-2,3-pentanedione (DPD) (75). DPD spontaneously cyclizes to form the AI-2 quorum-sensing molecule, which regulates processes such as biofilm formation and pathogenesis (29, 91). When bacteria produce high levels of AI-2, are the levels of Hcy high enough that MetRS and other aaRS are able to transform Hcy to Hcy-thiolactone? Although mammals and certain vertebrates and nematodes have homologs of the human PON enzymes that can degrade Hcy-thiolactone (7), it is unclear whether bacteria have a mechanism to degrade to do the same. Bacterial lactonases have been identified that degrade acyl-homoserine lactones, a class of quorum-sensing molecules of bacteria (54). Mammalian PON enzymes have also been found to degrade bacterial acyl-homoserine lactones, indicating the broad specificity of this class of enzymes (71). Bacterial genes recently identified as having >30% identity to mammalian PON1 demonstrate the phylogenetic relationship between the mammalian PON enzymes and the bacterial lactonases that degrade acyl-homoserine lactones (7). This finding raises the possibility that bacterial PONs and other lactonases may have a second function in degrading toxic Hcy-thiolactone, in addition to a quorum-quenching role.

Post-Transfer Editing of Metabolites from Branched-Chain Amino Acid Biosynthesis

By-products of the branched-chain amino acid pathways that have been shown to be substrates for aaRSs include norleucine, norvaline, and β-methylnorleucine. These have all been shown to be used in protein synthesis, particularly in the case of over-produced recombinant proteins in *E. coli*. Norleucine has been shown to be incorporated in place of Met in many different proteins in *E. coli* (60). Norvaline is incorporated at Leu codons in human hemoglobin expressed in *E. coli* (4), and β-methylnorleucine is misincorporated in overexpressed hirudin (65). These NPAs are charged onto tRNAs by aaRSs, including norvaline by LeuRS, norleucine by MetRS, and β-methylnorleucine by IleRS, but are then removed by a post-transfer editing reaction that hydrolyzes the aminoacylated tRNA to release the free amino acid and tRNA (102). Therefore, misincorporation must occur at concentrations at which these NPAs saturate the editing mechanism. Norvaline and norleucine have been shown to accumulate in cells when there is an imbalance in branched-chain amino acid biosynthesis, particularly when there is a downshift in oxygen concentration (10, 97). However, much remains to be learned about the environmental growth conditions that affect the accumulation of these NPAs.

A Free-Standing Editing Domain and D–Amino Acids

Another class of NPAs is D–amino acids, which are by-products of the synthesis of the corresponding L–amino acids. D–amino acids are highly toxic if incorporated into cellular proteins; however, many cell types do not exclude D–amino acids but rather use them for specific cellular functions and even actively produce D–amino acids (112). Bacteria specifically produce D–amino acids for incorporation into peptidoglycan and into nonribosomally synthesized antimicrobial peptides (17). Interestingly, several bacterial species have recently been shown to secrete micromolar concentrations of D–amino acids during entry into the stationary phase (for reviews, see 17, 39). In *Vibrio cholerae* and *B. subtilis*, for example, these stationary-phase secreted D–amino acids are not the canonical D-Ala and D-Glu found in peptidoglycan but instead are D-Leu, D-Met, and D-Val for *V. cholerae* (51) and D-Leu, D-Met, and D-Tyr for *B. subtilis* (50). Although the full range of function for these secreted D–amino acids is unknown, they can be incorporated into peptidoglycan, resulting in a change in structure and function. In *V. cholerae*, for instance, incorporation of D-Met and D-Leu into peptidoglycan increased osmotic stress resistance 20-fold (51).

For *B. subtilis* and possibly other organisms, the increased production of D–amino acids during the stationary phase poses a problem, as it can compromise protein synthesis. Addition of D–amino acids [D-Leu, D-Tyr, and D-Trp (tryptophan)] to *B. subtilis* cells resulted not only in incorporation of these amino acids into peptidoglycan but also in inhibited growth (50, 51, 55). This growth inhibition appears to be due to the incorporation of these D–amino acids into proteins. The addition to cells of the D–amino acid along with the specific L-enantiomer prevented growth inhibition and did not affect incorporation of the D–amino acid into peptidoglycan (55). Interestingly, the *B. subtilis* strain used in these studies has a loss-of-function mutation in the *dtd* gene that encodes D-tyrosyl-tRNA deacylase, an editing enzyme with broad specificity for D-aminoacylated tRNAs. Replacing the mutant *dtd* allele with a wild-type allele of the gene resulted in a strain that was now resistant to D–amino acids with regard to growth inhibition (55). Although it has not been formally shown that these D–amino acids are incorporated into proteins, the data clearly support the idea that these D–amino acids can be charged onto tRNAs.

D-tyrosyl-tRNA deacylases are widespread and represent the major mechanism cells use to edit tRNAs that are mischarged with D–amino acids (112). However, for particular D–amino

acids that are not effectively selected against by the tRNA synthetases, the D-tyrosyl-tRNA deacylase may not be sufficient to resist the surge of D–amino acids produced by bacteria on entry into stationary phase. TyrRS is able to aminoacylate $tRNA^{Tyr}$ with either D- or L-Tyr (16, 95). In *B. subtilis*, there are two TyrRS encoding genes, *tyrS* and *tyrZ*. TyrS is the major TyrRS used by the cell during vegetative growth (38), and like TyrRS in other organisms, TyrS is able to use D-Tyr as a substrate (16; R. Williams-Wagner, M. Raina, M. Ibba, and T. Henkin, personal communication). TyrZ, by contrast, exhibits a slower rate of aminoacylation than TyrS but is 20-fold more selective for L-Tyr than for D-Tyr (R. Williams-Wagner, M. Raina, M. Ibba, and T. Henkin, personal communication). Although the function of TyrZ in *B. subtilis* is not known, its ability to select against D-Tyr strongly suggests that this aaRS may be important during stationary phase, when the processes of biofilm formation and sporulation are induced and levels of D-Tyr produced by the cell dramatically increase. The observation that *B. subtilis* encodes a TyrRS that is able to effectively discriminate D-Tyr presents the question of why TyrZ is not the major TyrRS of the cell. Is the slower aminoacylation rate of TyrZ insufficient to support a fast growth rate?

CONCLUSIONS AND FUTURE OBJECTIVES

In general, cells go to great lengths to minimize errors during protein synthesis, employing extensive QC mechanisms to ensure that the genetic code is translated with high fidelity (113). The role of aminoacylation QC in limiting mistranslation of the genetic code and the diverse outcomes, both positive and negative, of coding errors have been studied extensively and are the subject of several recent reviews (72, 80). Although the general assumption is that there are no specific hot spots for mistranslation, there are proteins (called intrinsically disordered proteins) that are more prone to aggregation, such as α-synuclein and tau proteins (106). However, despite the widespread use of QC and proofreading, it is not always safe to assume that aminoacylation errors lead to protein unfolding and aggregation and limited growth and viability. *E. coli* can tolerate up to 10% mistranslation of some codons (88), microbes such as *Mycoplasma mobile* have lost aaRS QC mechanisms leading to widespread mistranslation (56, 110), and the yeast *Candida albicans* uses misaminoacylation to generate morphological diversity and increase competiveness (63). Perhaps one of the most striking examples is the mischarging of up to 1% of all tRNAs, with Met in eukaryotic cells exposed to viruses, Toll-like receptor ligands, or chemically induced oxidative stress (68). The common feature of all these examples is that the reported errors involve the replacement during aminoacylation of one GPA by another that is often closely related in structure. Consequently, the resulting errors in mRNA decoding are confined to amino acids within the genetic code and frequently lead to conservative substitutions. This is not the case with NPAs, which by definition are outside the genetic code and whose use during protein synthesis is often considerably more detrimental than the equivalent GPA substitutions. For example, substitutions with norleucine increase sensitivity to oxidative stress, and mistranslation with α-aminobutyrate or *m*-Tyr is considerably more problematic for the cell than are the corresponding GPA errors (15, 61, 67). As a better understanding of the challenges posed to translation by NPAs emerges, it is likely that a broader appreciation will develop for their role in both healthy and stressed cells.

The observation that NPAs sometimes pose a greater threat than misincorporated GPAs to the synthesis of a functional proteome is consistent with the evolution of aaRS QC to recognize and deal with such threats. For some aaRSs, such as TyrZ and PheRS, the NPA targeted by QC is known, and in other instances divergent evolution suggests as yet undefined challenges to protein synthesis. Yeast, for example, encodes two GlyRS (glycine-tRNA synthetase) enzymes, the second of which (GlyRS2) is dispensable for growth under normal conditions but is expressed under certain

stress conditions (heat, oxidation, pH) in which NPAs might accumulate (18, 104). Similarly, *E. coli* encodes a second LysRS, LysU, which is synthesized as part of the heat shock response and exhibits differences in amino acid specificity compared with the corresponding housekeeping enzyme LysS (12). In another case, ThrRS editing activity against the GPA Ser is altered under oxidative stress conditions via modification of a critical Cys residue. These examples suggest that more NPAs that impact protein synthesis remain to be discovered. Recent developments in analytical approaches have considerably facilitated the ability to characterize and quantify cellular metabolite pools and are likely to dramatically change our future appreciation of the changing balance of GPAs and NPAs under different growth conditions, as well as our understanding of how this influences protein synthesis.

SUMMARY POINTS

1. Studies of translation QC have typically focused primarily on errors within the genetic code.
2. AaRS QC pathways can proofread nonproteinogenic amino acids outside of the genetic code.
3. Proofreading and editing of nonproteinogenic amino acids are essential for normal cell growth.
4. Nonproteinogenic amino acids can accumulate to potentially toxic levels in cells.
5. Growth under stress conditions can significantly increase nonproteinogenic amino acid synthesis.
6. QC of nonprotein amino acids is more important than proofreading of genetic code amino acids under some growth conditions.
7. Continuing advances in metabolomics are starting to reveal the full range of NPAs that accumulate in different cells under varied growth conditions.

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