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Review

Misacylation of tRNA in prokaryotes: a re-evaluation

A. A. Stortchevoi

Yale University School of Medicine, Yale University LH-420, 310 Cedar Street, New Haven, Connecticut 06510 (USA), Fax: +1 203 785-6899, e-mail: alexei.stortchevoi@yale.edu

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Abstract. Misacylation of tRNA by a non-cognate amino acid is a natural phenomenon and occurs with a frequency of approximately 1 in 10,000 due to occasional mistakes in aminoacyl transfer RNA (tRNA) synthesis. In a number of prokaryotic organisms, misacylation of selenocysteinyl tRNA, glutaminyl tRNA and aspartyl tRNAs has particular physiological meaning. Recently, misacylation has emerged as a powerful tool for studying specific interactions between aa-tRNAs and associated protein factors. The present review provides an overview of the application of misacylated tRNA in research.

Keywords. Misacylation, aminoacylation, non-cognate trna/amino acid, translation, prokaryotes, suppression.

Introduction

Translation is the process of decoding nucleic acid sequence (messenger RNA, mRNA) into a corresponding amino acid (aa) sequence. Major translation elements include ribosomes, transfer RNAs (tRNAs), aminoacyl tRNA synthetases (aaRSs) and protein factors that take part in initiation (initiation factors, IFs), elongation (elongation factors, EFs) and termination (release factors, RFs) of translation. Characteristic features of translation in prokaryotes include (i) transcription and translation occurring simultaneously, directly influencing one another and (ii) formylmethionine used in initiation of protein synthesis where eukaryotes utilize methionine for this purpose. Initiation of protein synthesis requires a specialized tRNA recognizable by initiation factors and not by elongation factors. Since interactions of a tRNA molecule with protein factors determine its pathway *in vivo*, it is important that recognition be highly specific. On the other hand, elongation factor-Tu (EFTu), which carries aminoacyl-tRNAs (aa-tRNAs) to ribosomes seems relatively non-specific because it is able to engage 20 or more aa-tRNA species with relatively equal affinity. The exceptions are initiator tRNA and selenocysteinyl tRNA, which bind their own factors (initiation factor 2 (IF2) – for initiator tRNA, and selenocysteinyl tRNA binding factor (SELB) – for selenocysteinyl tRNA), and are not bound to EFTu. The characteristic properties of initiator tRNA have been investigated intensively, and a detailed map of specific nucleotide determinants responsible for $tRNA^{fMet}$ specificity to initiation factors, to ribosomal P-site, as well as to MetRS, EFTu and peptidyl tRNA hydrolase (PTH) is now available. Recently, the focus of attention has moved from solely tRNA specificity to shared specificities of tRNA and its esterified amino acid to protein factors. Misacylation of tRNA provides a powerful tool for studying the thermodynamic synergy of tRNA and amino acid moieties in interactions with translation factors. In the present review examples of tRNA misacylation by non-cognate amino acids and their effects on aa-tRNA specificity will be highlighted.

tRNA as the central translation unit of protein synthesis

There are about 30 different tRNA molecules in the prokaryotic cell [1], including canonical elongator tRNAs species and special types of tRNA, such as initiator

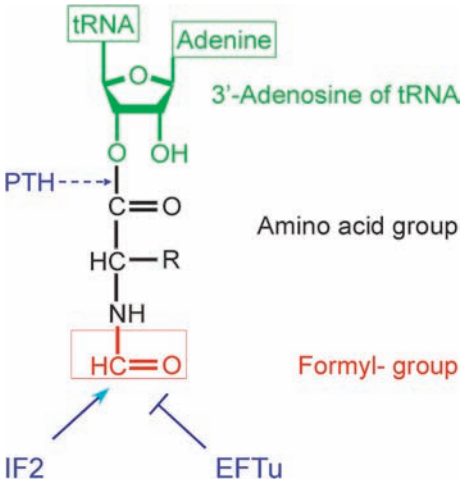

Figure 1. Formyl-aminoacyl-tRNA. tRNA – in green, amino acid – in black, formyl group – in red. Formyl group inhibits aa-tRNA binding to EFTu but serves as positive determinant for IF2 binding.

tRNAs [2] and selenocysteinyl tRNA [3, 4]. The apparent discrepancy between the number of tRNAs and the 61 amino acid codons is explained by the wobble theory: the same tRNA molecule can pair with a triplet of nucleotides that differs by one nucleotide from its cognate codon [5, 6]. In translation the term 'cognate codon' designates a trinucleotide sequence complementary to a tRNA's anticodon (e.g. an AUG codon is cognate for a CAU anticodon). Consequently, a cognate amino acid is a substrate for esterification of a given tRNA by its cognate aminoacyl tRNA synthetase [7]. For instance, methionine is cognate to both $tRNA^{Met}$ and $tRNA^{fMet}$ since both have CAU anticodon complementary to AUG. Likewise, histidine acts as the cognate amino acid to $tRNA^{His}$ with the GUG anticodon complementary to CAC and so on according to the universal genetic code [8, 9] (Table 1). Some amino acids have more than one cognate tRNA species, called isoacceptor tRNAs. Isoacceptor tRNA species usually exist for translation of high-redundancy codons such as leucine and serine (Table 1), where even the third base wobbling is not sufficient for pairing with the anticodon of a single tRNA [10].

tRNA is the central unit in the process of translating the nucleic acid code into an amino acid sequence. Overall fidelity of protein synthesis depends, on the one hand, on correct tRNA anticodon matching to an mRNA sequence (accomplished by the ribosome), and on the other hand, on correct aminoacylation of tRNA by its cognate amino acid (accomplished by aaRSs). Mistakes in either tRNA anticodon matching to mRNA sequence and/or in tRNA aminoacylation can lead to biosynthesis of mutated and misfolded proteins with potentially grave consequences to metabolism. Nevertheless, in some special cases misacylation of tRNA occurs naturally in prokaryotes. This will be discussed in the following section.

Table 1. Genetic code and amino acid codon redundancies

Amino acid	Codon 1	Codon 2	Codon 3	Codon 4	Codon 5	Codon 6
Methionine	AUG					
Tryptophan	UGG					
Cysteine	UGU	UGC				
Phenylalanine	UUU	UUC				
Histidine	CAU	CAC				
Lysine	AAA	AAG				
Glutamic acid	GAA	GAG				
Glutamine	CAA	CAG				
Aspartic acid	GAU	GAC				
Asparagine	AAU	AAC				
Tyrosine	UAU	UAC				
Isoleucine	AUU	AUC	AUA			
Proline	CCU	CCC	CCA	CCG		
Threonine	ACU	ACC	ACA	ACG		
Alanine	GCU	GCC	GCA	GCG		
Glycine	GGU	GGC	GGA	GGG		
Valine	GUU	GUC	GUA	GUG		
Leucine	CUU	CUC	CUA	CUG	UUA	UUG
Arginine	CGU	CGC	CGA	CGG	AGA	AGG
Serine	UCU	UCC	UCA	UCG	AGU	AGC
Selenocysteine	UGA	STOP	OCHRE			
Pyrrolysine	UAG	STOP	AMBER			
	UAA	STOP	OPAL			

Naturally occurring misacylation of tRNA in prokaryotes

Correct aminoacylation of tRNA with its cognate amino acid is achieved through highly discriminative synthesis by endogenous aaRSs [7]. These enzymes are highly specific to their substrate tRNA and amino acid molecules, and usually have two sterically separated catalytic centers, both having proofreading activities, reviewed in [11]. For example, IleRS is able to exclude amino acids larger than isoleucine at its first catalytic center (molecular sieve #1), but is unable to effectively discriminate valine, which differs by only a single methyl group from the cognate substrate isoleucine.

If Val becomes erroneously activated by IleRS during the first stage of synthesis, Valyl-AMP, it is translocated to the second proofreading center of the enzyme for alternative fitting (molecular sieve #2) where IleRS is able to hydrolyze either the enzyme-bound aminoacyl-adenylate (pre-transfer editing) or the enzyme-bound aa-tRNA (post-transfer editing). The tRNA-independent pre-transfer editing mechanism at molecular sieve #2 involves cyclization of homocysteine to yield thiolactone, found in a number of aaRSs, including IleRS, MetRS and LeuRS [12, 13]. The bond between tRNA and non-cognate amino acid created in the previous step is hydrolyzed, and both the tRNA and the amino acid are recycled. Such a 'double control' mechanism ensures that the error rate of aaRSs will in general not exceed 1 in 10,000 [7], which in combination with downstream checkpoints by other factors (discussed later) provides one of the two major conditions of translation fidelity. However, there are known examples when tRNA is misacylated *in vivo* by aaRSs without consequences to protein synthesis. These include misacylations of $tRNA^{Asn}$ and $tRNA^{Gln}$ with Asp and Glu, selenocysteinyl tRNA misacylation with Ser, formation of O-phosphoserine-$tRNA^{Cys}$ in archea, charging of a suppressor tRNA ($tRNA^{Pyl}$) in archea with pyrrolysine, and misacylation of $tRNA^{pro}$ with cysteine in *Methanococcus jannaschii*.

Alternative aminoacylation of $tRNA^{Asn}$ and $tRNA^{Gln}$

'Relaxed specificity' of AspRS and GluRS enables synthetases to recognize two tRNA species instead of one (Asp-Asn or Glu-Gln) and allows misacylation of $tRNA^{Asn}$ by Asp and $tRNA^{Gln}$ by Glu [14–16]. The potential basis of such non-discrimination between Asp-Asn and Glu-Gln is the lack of recognition of the last nucleotide in the tRNA anticodon by some AspRSs and GluRSs: the GUC anticodon in $tRNA^{Asp}$ differs by only one nucleotide from the GUU anticodon in $tRNA^{Asn}$ [17], and the UUG anticodon in $tRNA_1^{Gln}$ differs by only a single nucleotide from the UUC anticodon in $tRNA^{Glu}$ [18]. However, misacylated Asp-$tRNA^{Asn}$ and Glu-$tRNA^{Gln}$ are excluded from translation because they are not recognized as substrate by EFTu and subsequently are not carried to the ribosome [14, 19].

Misacylations of $tRNA^{Gln}$ with gtutamic acid (Glu-$tRNA^{Gln}$) or $tRNA^{Asn}$ with aspartate (Asp-$tRNA^{Asn}$) have particular significance in organisms lacking GlnRS or AsnRS, or both [20, 21]. In such organisms Gln-$tRNA^{Gln}$ and/or Asn-$tRNA^{Asn}$ synthesis proceeds in two stages: (i) Misacylation of $tRNA^{Gln}$ or $tRNA^{Asn}$ correspondingly with Glu- or Asp- and (ii) transamidation of esterified Glu- or Asp- moieties, turning them into Gln- or Asn-, which is carried out in a tRNA-dependent manner by special amidotransferases, glutamate amidotransferase (Glu-AdT) or aspartate amidotransferase (Asp-AdT), respectively [22, 23] (Fig. 2a). In some instances, one amidotransferase has dual specificity and transamidates both Glu-$tRNA^{Gln}$ to Gln-$tRNA^{Gln}$ and Asp-$tRNA^{Asn}$ to Asn-$tRNA^{Asn}$ (Glu/Asp-AdT) [24].

Misacylation of $tRNA^{Sec}$ with serine

Selenocysteinyl tRNA ($tRNA^{Sec}$) has been best characterized in *Escherichia coli* (reviewed in [3, 4]), although the genes encoding it have been found in many organisms [25, 26]. It is not recognized by EFTu due to antideterminants present [27] but is carried to the ribosome by its own selenocysteinyl tRNA binding factor, SELB. $tRNA^{Sec}$ has

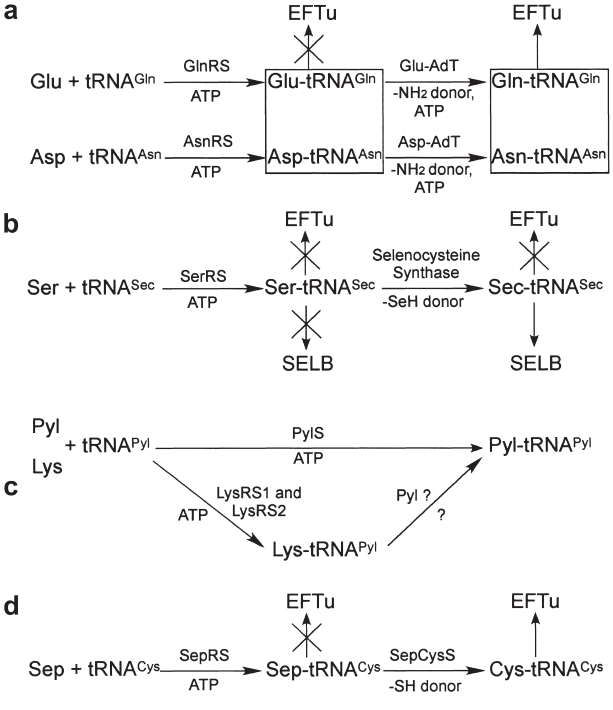

Figure 2. Examples of natural misacylations of aa-tRNAs. (*a*) Alternative synthesis of Gln- and Asn-tRNAs by transamidation pathway. (*b*) Misacylation of selenosysteinyl tRNA with serine. (*c*) Misacylation of suppressor $tRNA^{Pyl}$ with lysine. (*d*) Alternative synthesis of Cys-$tRNA^{Cys}$.

the UCA anticodon, complementary to UGA (Ochre) stop codon, and translation mediated by $tRNA^{Sec}$ 'suppresses' the UGA stop codon in certain contexts of the mRNA sequence environment [28] by adding selenocysteine to the growing peptide chain instead of termination of translation at that point. Besides being a natural ochre suppressor, selenocysteinyl tRNA is unique in the fact that a synthetase for direct aminoacylation of $tRNA^{Sec}$ does not exist in nature. Alternative synthesis goes in two stages (Fig. 2b). First, $tRNA^{Sec}$ is misacylated with serine by SerRS. At this stage neither EFTu nor SELB can recognize Ser-$tRNA^{Sec}$ as a substrate. Subsequently, transformation of esterified serine into selenocysteine is catalyzed by a special factor, selenocysteinyl synthase, in a tRNA-dependent manner. The transformation turns Sec-$tRNA^{Sec}$ into a substrate for SELB, and from this point translation of UGA into Sec can proceed.

As in the case of transamidation of Glu-$tRNA^{Gln}$ to Gln-$tRNA^{Gln}$ and Asp-$tRNA^{Asn}$ to Asn-$tRNA^{Asn}$, misacylation of $tRNA^{Sec}$ can be considered a temporary measure, and non-cognate Ser-$tRNA^{Sec}$ can be corrected into cognate Sec-$tRNA^{Sec}$ without Ser-$tRNA^{Sec}$ reaching the ribosomal complex. In both scenarios, special modifying activities present in the cells compensate for the absence of cognate aaRSs, and misacylation of the tRNAs during the initial step does not result in mistranslation during protein synthesis.

Misacylation of amber-suppressor $tRNA^{Pyl}$ with lysine in methanogenic archea

Another example of naturally occurring stop-codon suppressor tRNA (in addition to $tRNA^{Sec}$) is pyrrolysine tRNA ($tRNA^{Pyl}$ amber). The gene encoding this tRNA (pylT), as well as mRNA sequences with internal UAG codons for pyrrolysine insertion, have been discovered in methanogenic archea [29–31]. An initial study reported misacylation of $tRNA^{Pyl}$ with lysine by LysRS class I and LysRS class II complex *in vitro*, leading to speculation about tRNA-dependent synthesis of pyrrolysine by a specialized protein factor PylS in a way similar to the conversion of serine into selenocysteine in the case of $tRNA^{Sec}$ [32].

More recent studies, however, revealed that PylS is able to directly aminoacylate $tRNA^{Pyl}$ with pyrrolysine with high specificity [33, 34], providing the first example from nature of direct aminoacylation of non-canonical tRNA-suppressor (Fig. 2c).

Future research will show whether misacylation of $tRNA^{Pyl}$ with lysine takes place *in vivo* as well as *in vitro*.

The formation of O-phosphoserine-$tRNA^{Cys}$ in archea

Many archea organisms either lack the genes encoding CysRS or can dispense with them [35–37]. Alternative synthesis of Cys-$tRNA^{Cys}$ involves misacylation of $tRNA^{Cys}$ with O-phosphoserine (Sep) by a non-canonical member of the aaRS family, O-phosphoserine tRNA synthetase (SepRS) during the first stage of the synthesis, and conversion of the Sep-$tRNA^{Cys}$ to Cys-$tRNA^{Cys}$ by a specialized factor, Sep-tRNA:Cys synthase (SepCysS), during the second stage [38] (Fig. 2d).

The examples of O-phosphoserine-$tRNA^{Cys}$, Asp-$tRNA^{Asn}$, Glu-$tRNA^{Gln}$ and selenocysteine-$tRNA^{Sec}$ synthesis as well as Met-$tRNA^{fMet}$ formylation by MTF represent pre-translational modifications of amino acids in aa-tRNAs.

Misacylation of $tRNA^{Pro}$ in *M. jannaschii*

An additional case of natural misacylation has been reported for ProRS in *M. jannaschii*, which misacylates $tRNA^{Pro}$ to Cys-$tRNA^{Pro}$ [39, 40]. This curious example of dual amino acid specificity (Pro and Cys) remains poorly investigated. It is unknown whether Cys-$tRNA^{Pro}$ is a surrogate which can undergo further modification (such as in the case of Ser-$tRNA^{Sec}$ and Glu-$tRNA^{Gln}$), or whether it has a particular function of its own. Further studies are thus needed.

A single nucleotide modification prevents misacylation of the $tRNA_2^{Ile}$ with methionine

E. coli $tRNA_2^{Ile}$, specific for the codon AUA, has a modified nucleoside lysidine (L – a modified cytidine – at the wobble position (L34) of its anticodon sequence (Fig. 3). Unmodified LAU anticodon appears indistinguishable from CAU, the anticodon of methionyl tRNA. $tRNA_2^{Ile}$(LAU) is specific to isoleucine, whereas $tRNA_2^{Ile}$(CAU) is specific to methionine. The only feature which prevents misacylation of $tRNA_2^{Ile}$ with methionine is the modification of C34 to L34 [41].

Molecular basis of cognate aminoacyl tRNA synthesis

While all cellular tRNA molecules are similar, they bear minor differences in nucleotide composition, base modifications and tertiary structure. It has been shown that aaRSs can exploit such differences for specific substrate tRNA recognition (reviewed in [42]). Identity of cognate tRNA is determined by a few key elements in tRNA structure rather than the molecule as a whole [43, 44]. Depending on the tRNA species, one or more 'key' elements in its structure interact directly with cognate aaRSs. The recognition elements form a so-called identity set of a given tRNA species. Identity switch experiments have provided convincing demonstration of the role of identity sets. When the key elements from one tRNA (donor) were transplanted into another tRNA (acceptor), it caused the 'acceptor' tRNA to assume the do-

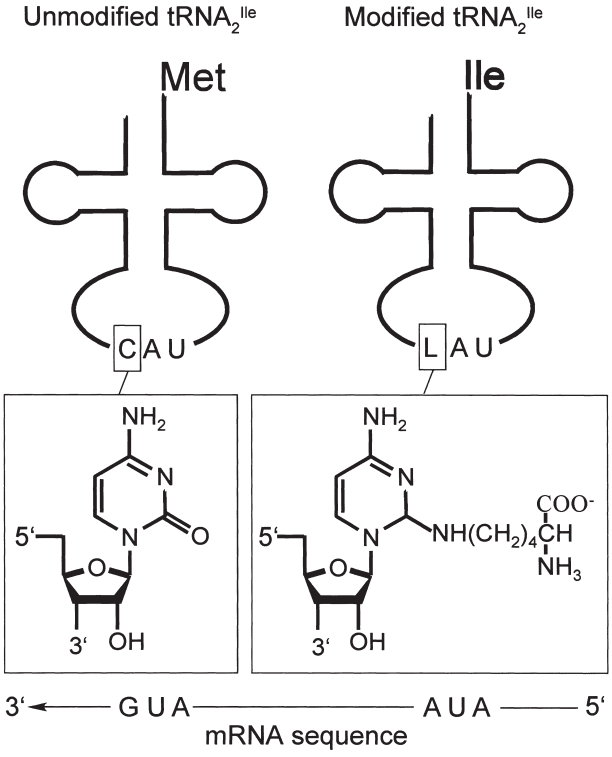

Figure 3. C34 to L34 modification in anticodon of $tRNA_2^{Ile}$.

nor tRNA type, such that it was no longer recognized by its own cognate aaRS, but instead was aminoacylated by the donor type aaRS with a donor type amino acid *in vivo* and *in vitro* (reviewed in [44]).

Although identity sets are individual for each tRNA, the elements that comprise them are often found within the same locations in tRNA structure, which is determined by the way a physical contact is formed between tRNA and aaRS. The trinucleotide anticodon sequence (N34-N36) for example is unique for any given tRNA species and most frequently is included in the identity set (as mentioned in relation to the 'relaxed specificities' of GluRS and AspRS). The anticodon sequence is not involved in the identity set for aaRS recognition only in cases where aaRS must recognize too large a number of potential isoacceptor tRNAs (i.e. $tRNA^{Ser}$ which has six different anticodons plus UCA, the anticodon of $tRNA^{Sec}$) [45, 46]. Among the other frequently used identity nucleotides are the discriminator base (N73) and those located in the acceptor stem of tRNA (N1-7, N66-72), the tRNA variable loop (reviewed in [47]), the D stem [48, 49] and the phosphate backbone [45, 50]. Modifications of nucleotides throughout the tRNA structure present yet another type of identity element serving cognate aaRS-tRNA recognition (reviewed in [51]). Identity elements have different relative 'strengths' and different relative influence on the overall cooperative effect on tRNA specificity [52].

One important benefit of knowing the identity elements in tRNA required for aminoacylation is the ability to generate misacylated tRNA molecules or tRNA mimics for research purposes. Although misacylation of tRNA *in vitro* is possible using chemical methods, enzymatic synthesis tremendously enhances the process and allows *in vivo* application of misacylated tRNA as well.

Methods and limitations of misacylation will be discussed followed by review of its practical applications.

Misacylation of tRNA in research: methods and limitations

Acid/urea Northern blot analysis

Direct measurement of tRNA aminoacylation/formylation levels *in vivo* represented an important step in tRNA research [53]. The method is now generally known as acid/urea gel Northern blot. At the core of the technique lies the observation that the chemical bond between amino acid and tRNA remains stable at low pH and low temperatures. Under optimized conditions, the free tRNA form can be resolved from aa-tRNA and faa-tRNA in denaturing polyacrylamide gels and all three forms can be detected with the help of a radioactive probe complementary to a specific tRNA sequence (usually the anticodon loop)

Acid/urea Northern blot is routinely used for *in vivo* analysis of tRNA, but it is also a very efficient tool for visualizing *in vitro* aminoacylation/formylation of tRNA, which is particularly important in studies where misacylation is done *in vitro*. It allows an accurate estimate of misacylation by a non-cognate amino acid of choice, and also shows the extent of formylation of non-cognate aa-tRNAs when necessary.

Acceptable limits of tRNA mutagenesis

Mutant tRNAs or tRNA mimics can be generated either by *in vitro* transcription (see for instance [54, 55]) or *in vivo* expression [56–58] of mutated tRNA genes.

Because tRNA has a complex tertiary structure [59, 60], some mutations and modifications will inevitably lead to misfolding [61]. Misfolded tRNA cannot be used in research, so general guidelines have to be followed to avoid the unwanted effects of mutations. As a rule, any single nucleotide change on one side of tRNA stem must be accompanied by the complementary nucleotide change on the other side of the stem to preserve base pairing. Even seemingly non-perturbing nucleotide changes, such as those in the anticodon and the loops, or complementary changes in tRNA stems may also have general destabilizing effects because of numerous intrinsic links in tRNA molecule that can be missing as a result of a single nucleotide change [62]. It is not always acceptable to sub-

stitute a purine by a pyrimidine, because the natural pattern of stacking of the bases may be compromised. Even correctly transcribed tRNA molecules can sometimes assume unnatural configurations due to poorly optimized conditions of folding, storage and handling [63] and because posttranscriptional modifications are not present [64]. There are many other more confined limitations dictated by the design of a particular study.

In conclusion, not every tRNA can be misacylated with every amino acid of choice because of structural limitations that prohibit certain nucleotide changes, and not all structurally sound mutant tRNAs can be suitable substrates for a particular study.

Misacylation of initiator tRNA

Methionine is the amino acid for initiation of protein synthesis in all living systems. This is because initiator tRNA is universally charged with methionine in all organisms studied up to date [65, 66]. In prokaryotes and cellular organelles (including, among others, mitochondria and chloroplasts), a formyl group is attached to the -NH- residue of methionine after the latter has been esterified to the -OH 3′-end of $tRNA^{fMet}$ (Fig. 1) (reviewed in [2]). Among the known functions of the formyl group of fMet-$tRNA^{fMet}$ are recognition by IF2 and abolition of binding to EFTu [67].

Why methionine is the amino acid for initiation of protein synthesis remains unknown. While *in vitro* misacylation of wild-type initiator $tRNA^{fMet}$ is possible, *in vivo* it is always charged with methionine. Interestingly, when an anticodon mutant of $tRNA^{fMet}$ was expressed and misacylated in *E. coli*, it was not for testing of an alternative amino acid as initiator of translation in place of methionine, but for initiation from a nonsense codon [68]. Selenocysteinyl tRNA, as mentioned, provides an example of a natural stop-codon (UGA) suppressor during the elongation step. RajBhandary and co-workers changed the anticodon sequence of initiator $tRNA^{fMet}$ of *E. coli* from CAU to CUA, matching the UAG (amber) stop-codon to test the possibility of initiation from the amber stop-codon. Their study proved that such initiation was possible *in vitro* and *in vivo*. But they also revealed that fGln-$tRNA^{fMet}$ rather than fMet-$tRNA^{fMet}$ acted as the initiator from amber codon [68], because the CAU to CUA anticodon change turned $tRNA^{fMet}$ into a substrate for GlnRS, which aminoacylated it with glutamine *in vivo*, as reported earlier for *in vitro* aminoacylation [69]. Interestingly, the same tRNA with modifications in the acceptor stem that allowed it to bind to the ribosomal aminoacyl (A) site was later used for suppression of termination at the UAG codon in *E. coli* and was also misacylated with glutamine [53, 70], but the same mutant tRNA could also be misacylated with tyrosine in yeast [71]. Initiator tRNA was a popular substrate for misacylation. It has been modified to accept tryptophane [72], valine and phenylalanine [73], and so on described in the other sections of this review.

The evidence that initiation could happen with fGln- instead of fMet- in *E. coli* focused interest on the role of the amino acid part of aa-tRNA in recognition by initiation factors. Since Gln-$tRNA^{fMet}$ was formylated by MTF *in vivo* and fGln-$tRNA^{fMet}$ was recognized by IF2 and the P-site of the ribosome, does this mean that the initiation function is determined solely by $tRNA^{fMet}$ itself and any other amino acid, if attached to $tRNA^{fMet}$, can initiate protein synthesis just as well? Why does methionine ubiquitously fulfill the function of initiator? Do initiation factors discriminate between amino acids attached to $tRNA^{fMet}$, and if so, do they prefer methionine to other amino acids? Studies involving misacylation of $tRNA^{fMet}$ have provided answers: it appears that methionine has its unique position as initiator not only due to $tRNA^{fMet}$ but also due to amino acid sensitivity of associated protein factors.

Misacylated $tRNA^{fMet}$ and peptidyl-tRNA hydrolase

PTH [74] plays an important role in recycling of aa-tRNAs that for some reason are not utilized in translation. PTH acts on the ester bond between the tRNA and the amino acid (Fig. 1) and causes its hydrolysis. In the absence of such hydrolysis, accumulation of non-recycled peptidyl-tRNAs would be toxic to the cell [75, 76].

fMet-$tRNA^{fMet}$ is the only N-blocked aa-tRNA resistant to PTH hydrolysis *in vivo* [77]. If PTH hydrolyzed fMet-$tRNA^{fMet}$, depletion of the initiation substrate would stall the entire protein synthesis.

Would PTH hydrolyze any other amino acid (not methionine) attached to $tRNA^{fMet}$ and N-formylated?

An interesting study conducted by Varshney and co-workers has shown that unlike fMet-$tRNA^{fMet}$, fGln-$tRNA^{fMet}$ is hydrolyzed by PTH [78]. Although only Gln has been tested thus far, the result provides evidence that PTH is able to discriminate between amino acids attached to $tRNA^{fMet}$. These findings open up the possibility that methionine may have a unique protective role in preserving the initiation substrate.

Misacylated $tRNA^{fMet}$ and methionyl-tRNA formyltransferase

MTF is expressed in eukaryotic organelles and in all prokaryotes except archea, organisms which use unformylated Met-$tRNA^{fMet}$ for initiation of protein synthesis [2]. Elongator aa-tRNAs are not formylated by MTF because they do not have positive determinants for MTF

recognition present in the initiator tRNA structure [79]. The formyl group of fMet-tRNA^{fMet} provides a positive determinant for IF2 recognition [80, 81] and negatively regulates EFTu binding [82, 83].

Hence, formylation fulfills the important functions of (i) improving the efficiency of the selection of tRNA^{fMet} by IF2 and (ii) sequestration of fMet-tRNA^{fMet} exclusively for initiation and not for elongation [67, 83–85].

The most striking demonstration of the importance of formylation in initiation of protein synthesis came from Blanquet and co-workers, who discovered the MTF gene in *E. coli* and produced bacterial strains carrying a disrupted copy of this gene: *E. coli* growth was seen to be severely impaired [86]. A somewhat less dramatic but similar effect was observed in *Pseudomonas aeruginosa* [87] and a knockout of the mitochondrial MTF gene in *Saccharomyces cerevisiae* also resulted in negative growth effects [88]. Interestingly, expression of a recombinant MTF in yeast cytoplasm led to formylation of yeast initiator Met-tRNA^{Met} but severely impaired the growth of this eukaryotic host [89].

A number of *E. coli* mutants, lacking formyl group donor tetrahydrofolate and bacterial species grown in the presence of inhibitors of folate metabolism were reported to survive using Met-tRNA^{fMet} in place of fMet-tRNA^{fMet} [90–94]. Taken together, these findings suggest that formylation of Met-tRNA^{fMet} may be dispensable under certain growth conditions and genetic contexts, and the degree of the dispensability varies among organisms.

The MTF gene of *E. coli* has been identified and cloned [86], and affinity-purified recombinant MTF has been used in assays measuring binding constants of misacylated tRNA^{fMet} mutants *in vitro*. In a recent study involving misacylated tRNA^{fMet} and MTF [95], the anticodon sequence mutants of tRNA^{fMet} (AUG, UAG, CAG, GUC, AUC and UUC) were aminoacylated by different aaRSs *in vitro* and *in vivo*. The order of relative affinity to MTF was Met > Gln > Phe > Ile ~Val > Lys. MTF does not interact with the anticodon of tRNA^{fMet} [96], and therefore the differences in formylation were exclusively due to the difference in amino acids. The finding is consistent with earlier reports that MTF is amino acid-sensitive. The relative differences in Vmax/Km values were Met (1), Gln (1/3), Phe (1/6) and Val (1/130) [73, 97].

It is interesting to note that MTF, being an initiation factor of translation, shows better affinity for methionine than for the other amino acids tested thus far.

Misacylated tRNA^{fMet} and initiation factor 2

IF2 plays a role in assembling the initiation complex, which includes (in addition to IF2) the ribosomal 30S subunit, fMet-tRNA^{fMet}, mRNA and two other initiation factors denominated IF1 and IF3. Although the exact order of events whereby fMet-tRNA^{fMet} binds the ribosome is not known, one suggestion is that the 30S ribosome-IF2 complex selects fMet-tRNA^{fMet} from the tRNA pool [80, 98]. At the same time a large body of evidence favors the hypothesis that IF2 carries fMet-tRNA^{fMet} to the P-site of the 30S ribosomal unit just as EFTu carries a charged elongator tRNA to the A-site of elongating ribosomes [79, 99, 100].

Formylation of initiator tRNA provides a very important determinant for IF2 recognition [82, 101]. Unformylated Met-tRNA^{fMet} and free formylmethionine or short fMet-oligonucleotides are very poor substrates for IF2 [81], but IF2 forms binary complexes with tRNA structures covalently attached to N-blocked methionine or other amino acids (faa-tRNA). *In vitro* binding assays show equal efficiency of acetyl group and formyl group as determinants for IF2 recognition [81, 102]. Even acylated elongator aa-tRNA species were reasonable substrates, although there were interspecies differences in binding affinities such that tyrosine, valine and phenylalanine > lysine, alanine and leucine tRNAs [81]. It is important to note, however, that IF2-faa-tRNA complexes are not particularly stable *in vitro*, with K_Ds on the order of 2–10 μM [100] and are easily dissociated by Mg^{2+} ions [103].

In earlier studies, only cognate aa-tRNA pairs were tested for their affinities to IF2, whereas the question of amino acid specificity could only be approached by using a misacylated tRNA. On the other hand, it was important to develop an assay system sensitive enough to detect marginal differences in K_D values in the micromolar range in the absence of Mg^{2+} ions.

In an elegant study that employed surface plasmon resonance and a recombinant IF2 affixed to the surface, RajBhandary and co-workers were able to measure the interactions between IF2 and a number of tRNA^{fMet} mutants misacylated in accordance with nucleotide changes in the anticodon [95, 104]. Binding affinities of initiator tRNA anticodon mutants for IF2 were related (depending on the amino acid attached) in the order fMet > fVal > fIle > fPhe > fGln.

IF2 as well as MTF is sensitive to the amino acid moiety of aa-tRNA. In addition, methionine is the preferred amino acid for forming the IF2-faa-tRNA^{fMet} complex, although the amino acid is a weaker determinant than the formyl group and the tRNA^{fMet} (reviewed in [79]).

Misacylated tRNA^{fMet} and EFTu

EFTu forms a ternary complex with GTP and any elongator aa-tRNA. EFTu binds to initiator tRNA^{fMet} with much less affinity than to methionine-accepting elongator tRNA^{Met} because of antideterminants present in the tRNA^{fMet} [70, 105]. Interactions of EFTu with unformylated Met-tRNA^{fMet} are detectable *in vitro* [70, 106]; how-

ever, since formylated fMet-tRNA^{fMet} is not a substrate for EFTu, there is practically no ternary complex formation of fMet-tRNA^{fMet}-EFTu•GTP *in vivo*, despite EFTu being the most abundant cytoplasmic protein (5–10% of total protein), exceeding in concentration all other elongation factors and the ribosomes by a factor of almost 10 [107].

In vitro studies have shown a 73-fold difference in EFTu binding k_{off} between Met-tRNA^{fMet} and Gln-tRNA^{fMet} [70], providing a striking example of how charging with a different amino acid can switch tRNA type from initiator to elongator. The fact that glutamine is much preferred by EFTu over methionine is yet another clue as to why methionine is the leading candidate amino acid for initiation: MTF, which is present in very small amounts in *E. coli* [108], can compete for Met-tRNA^{fMet} with the much more abundant EFTu. In this regard it is interesting to examine the preference of EFTu to the other amino acids attached to tRNA^{fMet}.

Misacylated elongator tRNAs and EFTu

Misacylated tRNA was an important tool in analysis of EFTu specificity.

Until recently, EFTu was considered a relatively non-specific protein factor. After all, it recognizes over 20 different aa-tRNAs, binding them with very similar K_D values, varying only 12-fold among all non-initiator aa-tRNAs [109, 110].

Known exclusions from EFTu binding of tRNA, initiator tRNA^{fMet} and selenocysteinyl tRNA^{Sec}, both have antideterminants in their structures which eliminate EFTu affinity to the tRNA body [27, 70, 105]. By analogy, a 'generic' tRNA feature was thought to assure a uniform binding to EFTu of all elongator tRNA species. The role of amino acids attached to tRNA was also posited because non-aminoacylated tRNA is a very poor substrate for EFTu [111]. However, thermodynamically the contribution of different amino acids to formation of the EFTu•GTP/aa-tRNA ternary complex was thought to be equal.

In recent years, previously existing views of EFTu specificity have changed. Several factors have contributed to that change.

First, it was seen that in prokaryotic organisms (where, as discussed earlier, misacylation of tRNA^{Gln} and/or tRNA^{Asn} occurs naturally) misacylated Glu-tRNA^{Gln} and Asp-tRNA^{Asn} are excluded from elongation because they are not bound to EFTu-GTP and as a result are not carried to the ribosome [14], [19].

Second, there are examples of mutant tRNAs which can be aminoacylated with more than one amino acid. Su^{+7} is a mutant tryptophan tRNA from *E. coli* whose anticodon is changed to CUA [112]. Su^{+7} can be efficiently aminoacylated by both TrpRS and GlnRS, *in vivo* and *in vitro* [113]. Gln-tRNA^{Su+7} binds to *E. coli* EFTu•GTP three times more strongly than Trp-tRNA^{Su+7}, and glutamine gets incorporated into the peptide chain at the CUA codon during *in vivo* protein synthesis nine times as often as tryptophan [114].

As discussed in a previous section, initiator amber suppressor tRNA from *E. coli* aminoacylated with glutamine binds EFTu with much higher affinity than Met-tRNA^{fMet} [70].

Uhlenbeck and co-workers have systematically studied the role of amino acids in EFTu•GTP binding by aa-tRNAs. Their landmark study led to formulation of the theory of thermodynamic compensation [115], which postulates that EFTu can recognize tRNAs and amino acids independently and, furthermore, that the seemingly uniform binding of different aa-tRNAs to EFTu•GTP results from the combined free energies of the amino acid and tRNA moieties which complement each other. Uniform binding is therefore not to be attributed to 'poor' specificity of EFTu.

In one approach taken by Uhlenbeck and co-workers, four different tRNA molecules, tRNA^{Ala}, tRNA^{Val}, tRNA^{Gln} from *E. coli* and tRNA^{Phe} from yeast, were each aminoacylated with Ala, Val, Gln or Phe [115]. Mutations in tRNA molecules where needed to facilitate misacylations by non-cognate aaRS were limited to the regions known not to interact with EFTu of *Thermus aquaticus* [82, 116]. The affinities of the resulting 4 cognate aa-tRNA pairs and 12 misacylated aa-tRNA pairs for EFTu•GTP from *T. aquaticus* were measured and compared *in vitro* by a ribonuclease protection assay. As expected, the K_D values of the four cognate aa-tRNAs were within a close (10×) range of affinities [109], but 5000-fold range differences were reported for misacylated aa-tRNAs. According to K_D calculation, the strongest binding amino acid proved to be glutamine, followed by Phe, Val and Ala. The affinities of the subsequent tRNAs to EFTu followed the reverse order: tRNA^{Ala} > tRNA^{Val} ≅ tRNA^{Phe} > tRNA^{Gln} [115]

In another approach, 21 different tRNAs, including each one of 19 isoacceptor species (except tRNA^{His}) plus tRNA^{fMet} and tRNA^{Sec}, were aminoacylated with either Val or Phe [106]. Specificity of ValRS from *E. coli* and PheRS from yeast is heavily reliant on the anticodon sequences of their cognate tRNAs [117, 118], and that reliance was exploited in preparation of misacylated substrates. The anticodon sequences of all tRNA molecules chosen for the experiment were mutated to the valine anticodon GAC. Since PheRS can recognize the GAC anticodon almost as successfully as its cognate GAA anticodon [119], there was no need to create another set of mutant tRNAs with the GAA (Phe) anticodon. The mutations did not affect EFTu binding due to the fact that EFTu only binds to the acceptor stem and T-arm of tRNA [82, 116]. All valylated and phenylalanylated tRNAs were subjected to the *in vitro* EFTu•GTP binding assay,

and the binding constants were compared among the different species of tRNA. Comparison has allowed recognition of an expanded range of -ΔG (kcal/mol) that different tRNAs contribute to EFTu•GTP binding, independent of the nature of the amino acid (Val or Phe) with which they have been acylated showing, in order: $tRNA^{Glu} > tRNA^{Asp} > tRNA^{Gly} > tRNA^{Thr} \geq tRNA^{Ala} > tRNA^{Cys} \geq tRNA^{Leu} > tRNA^{Met} \geq tRNA^{Pro} > tRNA^{Phe} \geq tRNA^{Lys} \geq tRNA^{Arg} \geq tRNA^{Ser} > tRNA^{Asn} > tRNA^{Val} > tRNA^{Ile} > tRNA^{Trp} > tRNA^{fMet} > tRNA^{Gln} > tRNA^{Tyr} >> tRNA^{Sec}$. The differences between -ΔG varied from ~–11.7 kcal/mol ($tRNA^{Glu}$) to –7.5 kcal/mol ($tRNA^{Sec}$), i.e. a range of 4.2 kcal/mol. The difference between the strongest ($tRNA^{Glu}$) and the weakest ($tRNA^{Tyr}$) elongator tRNA species was ~3.6 kcal/mol, which is comparable to the difference between aminoacylated and deacylated $tRNA^{Phe}$ (~4.3 kcal/mol)! [111]. A similar difference is expected between the amino acids Glu and Tyr, but in the reverse order, with Tyr being the 'tightest' amino acid, and Glu the 'weakest'.

Misacylated tRNA and ribosome

Is the ribosome selective against misacylated tRNAs?

On the one hand, there are multiple examples of incorporation of amino acids attached to non-cognate tRNAs into the peptide chain during protein synthesis, and those examples downplay the role of ribosome in recognition. The earliest example, wherein Cys-$tRNA^{Cys}$ was reduced to Ala-$tRNA^{Cys}$ and Ala successfully incorporated into globin instead of Cys, was reported in 1962 [120]. Mutant $tRNA^{fMet}$ misacylated with Gln has been used to suppress an internal UAG codon in the *β*-galactosidase gene [70], while Su7+ showed an even better rate of non-cognate Gln incorporation than cognate Trp incorporation *in vivo* [113, 114].

On the other hand, the role of ribosome in recognition of misacylated aa-tRNAs has not yet been studied systematically, and the number of random examples of 'ribosomal indifference' is limited to several amino acids and tRNAs. Furthermore, experiments were often designed to 'pressure' the incorporation of non-cognate amino acid by overexpressing misacylated aa-tRNAs *in vivo*, or by utilizing a reporter system which works only with misacylated aa-tRNA.

Meanwhile, the possibility of ribosomal refusal of misacylated aa-tRNAs pairs whose binding to EFTu•GTP is exceedingly strong (i.e. Gln-$tRNA^{Trp}$ and so on) is quite real, as discussed [106]. The rate with which aa-tRNA enters the ribosomal A-site after GTP hydrolysis and dissociation of EFTu•GDP can be a critical factor [121]. And if aa-tRNAs were bound too tightly to EF-Tu•GDP, the peptide bond formation might occur too slowly, possibly leading to overall rejection of such aa-tRNA from the process.

Misacylation of tRNA: the prospects

Misacylated tRNA has already become an invaluable tool in determining the specificities of many components of the translational machinery, and it will likely remain in use in the foreseeable future. It has helped our understanding of why methionine occupies a unique place in initiation, and it has dramatically changed our view of the specificity of EFTu. Among the upcoming potential goals are ribosomal recognition of mischarged aa-tRNAs and introduction of artificial amino acids. It will be of great interest to see whether established relations between canonical aa-tRNAs and associated factors can lead to artificial components.

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