FEBS Letters 584 (2010) 99–105

FEBS Letters

journal homepage: www.FEBSLetters.org

Changeability of individual domains of an aminoacyl-tRNA in polymerization by the ribosome

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ARTICLE INFO

Article history:
Received 6 October 2009
Revised 31 October 2009
Accepted 2 November 2009
Available online 10 November 2009

Edited by Michael Ibba

Keywords:
$tRNA^{Ala}$
Purified translation
Protein synthesis
Peptide
Unnatural amino acid
Escherichia coli

ABSTRACT

The changeabilities of individual modules of aminoacyl-tRNAs are poorly understood, despite the relevance for evolution, translational accuracy and incorporation of unnatural amino acids (AAs). Here, we dissect the effect of successive changes in four domains of Ala-$tRNA_3^{Ala}$ on translation in a purified system. Incorporating five AAs, not one, was necessary to reveal major effects on yields of peptide products. Omitting tRNA modifications had little affect, but anticodon mutations were very inhibitory. Surprisingly, changing the terminal CCA to CdCA was sometimes inhibitory and non-cognate AAs were sometimes compensatory. Results have implications for translational fidelity and engineering.

1. Introduction

Aminoacyl-tRNAs (AA-tRNAs) contain four modular domains with very precise boundaries: an amino acid (AA)[1], an invariant 3′-terminal CCA, a three-base anticodon and a tRNA body. Domain swaps occurred extensively in evolution [1,2] and are also important for translational fidelity and engineering. Most studies of anticodon swaps have focused on nonsense suppressor tRNAs, which usually function less efficiently in translation than other tRNAs [3]. The misacylated tRNAs characterized best in translation are the natural precursors Asp-$tRNA^{Asn}$ and Glu-$tRNA^{Gln}$, where low affinities for elongation factor Tu (EF-Tu) inhibit delivery to the ribosome, thus preserving translational accuracy [4]. Many unnatural AAs have been incorporated with moderate efficiencies in translation using nonsense suppressor [5] and sense suppressor [6–10] tRNAs.

The limited understanding of the interchangeability of individual domains of AA-tRNAs in translation is mostly due to experimental challenges. One challenge is making single domain changes in a tRNA. For example, an anticodon change frequently also changes the AA charged because anticodons are major positive determinants for AA-tRNA synthetases [11]. Another challenge is interpreting results in in vivo and crude in vitro translation systems: effects may occur at the level of transcription, pre-tRNA processing, tRNA modification, AA charging and/or translation. In order to circumvent these challenges, we combined two technologies: a simplified, purified, *Escherichia coli* translation system lacking AA-tRNA synthetases [12] and chemoenzymatic synthesis of AA-tRNAs [5,13]. The latter technology has the advantage of enabling independent switching of AA and anticodon, but it also generally introduces three additional unnatural changes: a penultimate deoxyribose linkage for ease of chemical synthesis, and the omission of tRNA modifications (Fig. 1) combined with small changes in 5′- and 3′-terminal sequences for ease of tRNA preparation by in vitro transcription. In model cases, these three additional types of changes had little effect on incorporation yields of single AAs [5,14,15]. However, significantly lower product yields were obtained when incorporating three to five unnatural L-AAs in a row using $tRNA^{Asn}$- and $tRNA^{Phe}$-based synthetic adaptors ($tRNA^{AsnB}$ and $tRNA^{PheB}$, [6,16]).

In principle, the effects of individual domain changes on multiple AA incorporations could be determined simply by changing one domain at a time from the wild-type AA-tRNA in a purified system. Unfortunately, this was not practical with $tRNA^{AsnB}$ (for synthetic reasons discussed in Ref. [16]) or with $tRNA^{PheB}$ (because the

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[1] *Abbreviations:* AA or X, amino acid; x-$tRNA_z^y$, x = charged AA- y = AA specificity of either the wild-type isoacceptor or the wild-type isoacceptor upon which the chemoenzymatic sequence is based, z = either the wild-type isoacceptor designation or the anticodon sequence (5′ to 3′) of the chemoenzymatic tRNA sequence; V, cmo^5U; fM, *formyl*methionine; mS, *O-methyl*serine; eU, 2-amino-4-pent*en*oic acid (also know as allylglycine; structure shown in Fig. 1).

0014-5793/$36.00
doi:10.1016/j.febslet.2009.11.006

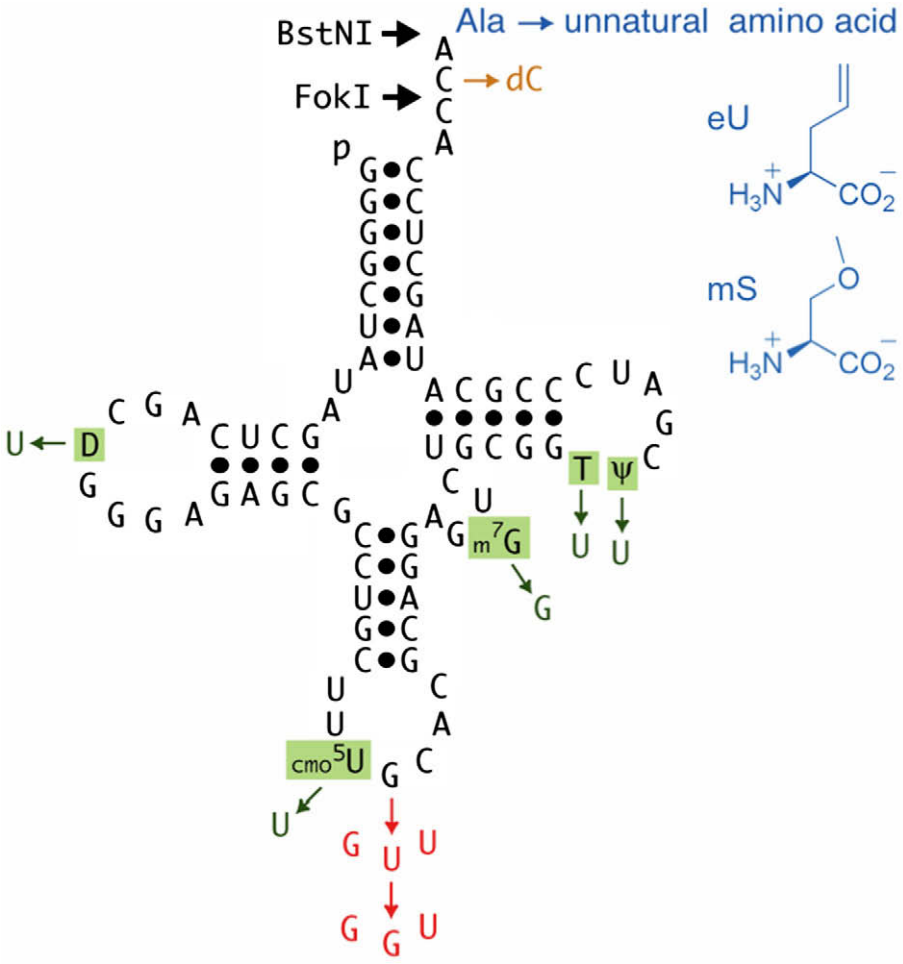

Fig. 1. Wild-type *E. coli* $tRNA_3^{Ala}$ isoacceptor (black [32]) and synthetic $tRNA^{AlaB}$ species which contain from one to four different types of changes (green, red, orange and blue). Run off transcripts of plasmids cut with BstNI or FokI terminate at the positions shown with arrows. Note that $tRNA_2^{Ala}$ is closely related to $tRNA_3^{Ala}$ and that these isoacceptors have identical anticodons that read the same Ala codons (see tRNA database website at http://www.trna.uni-bayreuth.de/).

polyPhe product is insoluble). Here, we overcome these experimental limitations by synthesizing polyAla using a $tRNA^{AlaB}$ adaptor (Fig. 1 [17,18]). This tRNA also has the unusual benefits of having charging determinants independent of the anticodon ([11]; thus allowing charging of anticodon mutants with Ala by AlaRS [17]) and having a 5′-terminal sequence of pGGG that coincides with the optimal T7 RNA polymerase promoter (thus avoiding the need for potentially confounding mutations in 5′- and 3′-terminal sequences [16]).

2. Materials and methods

New materials were prepared by standard methods as described [6,12,16,19]. Purified translations were also performed as described [16], except the final concentration of $tRNA^{Total}$ was adjusted to 160 μM taking into account any $tRNA^{Total}$ added as a component of wild-type $tRNA^{Total}$ charged with Ala, Asn or Thr. Translations also contained 0.5 μM each of initiation factors 1–3 and elongation factors Ts and G, 2.5 μM elongation factor Tu, 0.5 μM purified ribosomes, 1 μM appropriate mRNA, 0.2 μM (limiting) fMet-$tRNA_i^{fMet}$, 0.5 μM C-terminal, ^{3}H-labeled Val-$tRNA^{Val}$, and upstream-encoded, unlabeled elongator AA-tRNAs at the following estimated concentrations: 0.5 μM for single incorporation or 2.5 μM for five straight incorporations. Additional details are given in the Supplementary data.

3. Results

3.1. Effect of incorporating 1, 2 and 5 eU-$tRNA^{AlaB}$ substrates on ribosomal peptide synthesis

Incorporation of unnatural AAs into peptides by the translation apparatus can be inefficient, so we chose a radioactive pure translation assay for sensitivity and quantitation of full-length peptide products. Another advantage of this assay is that it encompasses a number of controls, being specific for products initiated by formylmethionine (fM) and terminated by Val (the only ^{3}H-labeled AA provided), with measured yields being dependent on both added mRNA and test elongator AA-tRNA prepared from pure components. To test the suitability of the $tRNA^{AlaB}$ body for assaying the effects of individual domain changes, we first used a wild-type $tRNA^{AlaB}_{UGC}$ sequence (Fig. 1) for the ribosomal polymerization of 2-amino-4-pent*en*oic acid (eU) (Fig. 1, top right) using mRNAs encoding MTAV, MTA_2V and MTA_5V (Fig. S1A). In comparison with maximal product yields in translations incorporating the wild-type Ala-$tRNA^{Ala}$ substrates (prepared from $tRNA^{Total}$, pure Ala and pure AlaRS), saturation to give the same peptide yields occurred when one or two unnatural eU incorporations were templated (Fig. S1B). However, a significant drop in yield was observed when templating five straight incorporations of eU-$tRNA^{AlaB}_{UGC}$ compared with five of wild-type Ala-$tRNA^{Ala}$, despite using excess unnatural