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Research paper

Adaptation of aminoacylation identity rules to mammalian mitochondria

Aurélie Fender, Agnès Gaudry, Frank Jühling, Marie Sissler, Catherine Florentz*

Architecture et Réactivité de l'ARN, CNRS, Université de Strasbourg, IBMC, 15 rue René Descartes, F-67084 Strasbourg Cedex, France

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ABSTRACT

Many mammalian mitochondrial aminoacyl-tRNA synthetases are of bacterial-type and share structural domains with homologous bacterial enzymes of the same specificity. Despite this high similarity, synthetases from bacteria are known for their inability to aminoacylate mitochondrial tRNAs, while mitochondrial enzymes do aminoacylate bacterial tRNAs. Here, the reasons for non-aminoacylation by a bacterial enzyme of a mitochondrial tRNA have been explored. A mutagenic analysis performed on *in vitro* transcribed human mitochondrial $tRNA^{Asp}$ variants tested for their ability to become aspartylated by *Escherichia coli* aspartyl-tRNA synthetase, reveals that full conversion cannot be achieved on the basis of the currently established tRNA/synthetase recognition rules. Integration of the full set of aspartylation identity elements and stabilization of the structural tRNA scaffold by restoration of D- and T-loop interactions, enable only a partial gain in aspartylation efficiency. The sequence context and high structural instability of the mitochondrial tRNA are additional features hindering optimal adaptation of the tRNA to the bacterial enzyme. Our data support the hypothesis that non-aminoacylation of mitochondrial tRNAs by bacterial synthetases is linked to the large sequence and structural relaxation of the organelle encoded tRNAs, itself a consequence of the high rate of mitochondrial genome divergence.

1. Introduction

Rules governing interaction of tRNAs and aminoacyl-tRNA synthetases (aaRSs[1]) for efficient and specific aminoacylation have been well established for a large number of aminoacylation systems in the three kingdoms of life (for reviews, see [1–4]). They rely on identity nucleotides, mainly located at both extremities of the tRNA L-shaped 3D structure that contact specific amino acids in the aaRS structure. Identity sets are composed of major and minor elements, with major elements usually conserved during evolution, as is the architecture of tRNA. Accordingly, it became possible to convert the specificity of a given tRNA by simple transplantation of identity sets, and/or by removal of antideterminants (signals hindering recognition by a synthetase of other specificity). Cryptic signals and permissive nucleotides that also contribute to specificity were discovered in this context [5,6]. Cross-species aminoacylation of tRNAs from a given organism by synthetases of the same specificity but from other organisms were reported in great numbers [1]. Exceptions where cross-aminoacylation was not possible were explained by the presence of only partial sets of identity elements in the tRNA or by the existence of alternate sets, and in many cases could be overcome by simple mutagenic approaches *in vitro* [1].

An unsolved cross-aminoacylation barrier concerns mammalian mitochondrial (mt) tRNAs which are not aminoacylated by bacterial synthetases [7,8]. In mammalian mitochondria, aminoacylation systems are of dual origin with the tRNAs coded by a simplified and rapidly evolving mt-genome and aaRSs coded by the slowly evolving nuclear genome and imported into mitochondria. Mammalian mt-aaRSs have various evolutionary origins [9] but many are of bacterial-type ([10,11] and references therein) and do not depart from the already known synthetases. The human mt-aaRSs (*Hsa* mt-aaRS) have no drastic apparent structural peculiarities as compared to their evolutionarily-related bacterial counterparts (except PheRS, which is a monomer instead of an $\alpha_2\beta_2$ tetramer), i.e. they have the same structural organization, possess the expected class-specific signature motifs in their catalytic domains and, accordingly, belong to the expected aaRS-class [12,13]. However, Kumazawa and colleagues [7,8] reported already twenty years ago on aminoacylation of *Escherichia coli* (*Eco*) and *Thermus thermophilus* (*Tth*) tRNAs by bovine mt-aaRSs specific for threonine, arginine, lysine, phenylalanine and serine, but absence of charging of bovine (*Bos taurus* or *Bta*) mt-tRNAs by the corresponding bacterial aaRSs (Table 1). This is intriguing since three of these enzymes (*Bta* mt-

* Corresponding author. Tel.: +33 (0) 3 88 41 70 59; fax: +33 (0) 3 88 60 22 18.
E-mail address: C.Florentz@unistra.fr (C. Florentz).

[1] aaRS stands for aminoacyl-tRNA synthetase with aa for amino acid (for individual aaRS, aa is given in the 3 letter code, e.g. AspRS for aspartyl-tRNA synthetase).

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Table 1
Unilateral aminoacylation of mammalian mitochondrial tRNAs.

aa	tRNA	aaRS			Ref
		Bta mt	*Eco*	*Tth*	
Phe	*Bta* mt	•	NO	NO	[7]
	Eco	YES	•	+	
	Tth	YES	+	•	
Thr	*Bta* mt	•	NO	NO	[8]
	Eco	YES	•	+	
	Tth	YES	+	•	
Arg	*Bta* mt	•	WEAK	NO	[8]
	Eco	YES	•	+	
	Tth	YES	+	•	
Lys	*Bta* mt	•	NO	NO	[8]
	Eco	YES	•	±	
	Tth	YES	+	•	
Ser	*Bta* mt	•	NO	NO	[8]
	Eco	WEAK	•	+	
	Tth	YES	+	•	
		Hsa mt	*Eco*	*Tth*	
Asp	*Hsa* mt	•	NO	NO	[14]
	Eco	YES	•	+	
	Tth	YES	nt	•	

Only aminoacylation capacities for couples involving a single mitochondrial partner, either mitochondrial tRNA or mitochondrial aminoacyl-tRNA synthetase, are emphasized by YES (active), NO (inactive) or WEAK (partially active). Aminoacylation capacities of other couples are indicated as follows: positive control activities of cognate couples (•); aminoacylation activity for systems involving both bacterial tRNA and bacterial synthetase (+/±); non tested (nt). *Bta* mt: *Bos taurus* mitochondrial; *Hsa* mt: *Homo sapiens* mitochondrial; *Eco*: *Escherichia coli*; *Tth*: *Thermus thermophilus*.

ThrRS, -PheRS and -SerRS) are of the bacterial-type [9,11]. A similar barrier was observed for the *Hsa* mt-aspartylation system from which the tRNA is not recognized by a bacterial synthetase, i.e. either *Eco* AspRS or *Tth* AspRS, while the *Hsa* mt-AspRS, which is of prokaryotic-type, recognizes and aminoacylates both *Eco* and *Tth* $tRNA^{Asp}$ ([14]; our unpublished results) (Table 1). These data demonstrate unilateral aminoacylation of mt-tRNAs by mt-aaRSs, and show a strong cross-species barrier for aminoacylation of mt-tRNAs by bacterial synthetases. To sort out the possible molecular reasons for this barrier, the specific case of non-aminoacylation of *Hsa* mt-$tRNA^{Asp}$ by the *Eco* AspRS was investigated as a model system by a mutagenic *in vitro* approach.

As a result, we have observed that the absence of classical aminoacylation signals, namely identity and structural elements, are key elements for non-cross-aminoacylation of the mt-tRNA, but that the intrinsic unusually large thermodynamically-driven structural plasticity of the mt-tRNA also forms an important element in the non-recognition barrier. We propose that non-aminoacylation of mammalian mt-tRNAs by bacterial synthetases is linked to the global divergence of the organelle encoded tRNAs, far beyond loss of identity elements and well-defined structural features, a consequence of the very high mutation rate of mitochondrial genomes.

2. Material and methods

2.1. Materials

Synthetic genes of wild-type *Eco* $tRNA^{Asp}$ [15] and *Hsa* mt-$tRNA^{Asp}$ [10] were cloned previously. T7 RNA polymerase was purified as described [16]. *Tth* AspRS (discriminating version [17]) and the corresponding *in vitro* transcribed $tRNA^{Asp}$ were gifts from H.D. Becker and D. Kern (IBMC, Strasbourg). Oligonucleotides were from Sigma Genosys, restriction enzymes (*BamH*I, *Hind*III, and *Bst*NI) from New England Biolabs, T4 DNA ligase from Qbiogen, and L-[^{3}H] aspartic acid (208 GBq/mmol) from Amersham.

2.2. tRNA gene mutagenesis and in vitro transcription

Hsa mt-$tRNA^{Asp}$ variants displaying individual *Eco* identity elements [18,19] (mt-$tRNA^{Asp}$G73, mt-$tRNA^{Asp}$C38 and mt-$tRNA^{Asp}$G2–C71) or "classical" D- and T-loops as found in $tRNA^{Asp}$ from *Schizosaccharomyces pombe* (mt-$tRNA^{Asp}$DT) have been prepared previously [14]. *Hsa* mt-$tRNA^{Asp}$ variant (G73/DT, C38/G73, idec,[2] idec/DT, idec/U11–A24, and idec/DT/U11–A24) genes were obtained by hybridization of 9 overlapping oligonucleotides, ligation between *BamH*I and *Hind*III sites of plasmid pTFMa [20]. The gene for variant idec/DT/U11–A24/C31–G39 was derived from the idec/DT/U11–A24 gene with the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). All these genes contain a hammerhead ribozyme [21] and tRNA sequences downstream from the T7 polymerase promoter. A *Bst*NI site coincidental with the 3′-end of the tRNA sequences allows synthesis of tRNAs ending with the expected CCA sequence. *Eco* $tRNA^{Asp}$ variant (mtDT and A11–U24) genes were obtained by PCR amplification using two complementary 30 nt-long primers (allowing for introduction of *BamH*I and *Hind*III sites) that contain the T7 polymerase promoter and tRNA sequences. All synthetic genes were transformed into TOP10 cells (Invitrogen). Transcription was as described [21]. tRNAs were purified to single nucleotide resolution on denaturing polyacrylamide gels as described [14]. Quality of tRNAs has been verified on native PAGE which revealed only single populations of conformers.

2.3. E. coli and human mt-AspRS

Eco AspRS was a gift from G. Eriani (IBMC, Strasbourg). *Hsa* mt-AspRS was expressed and purified on a nickel affinity column [10]. Protein concentrations were determined from OD_{280} using the extinction coefficients ($\varepsilon_{(Eco\cdot AspRS)}$ = 42985 M^{-1} cm^{-1} and $\varepsilon_{(Hsa\cdot mt\text{-}AspRS)}$ = 43540 M^{-1} cm^{-1}) and molecular weights ($MW_{(Eco\cdot AspRS)}$ = 65913.4 g mol^{-1} and $MW_{(Hsa\cdot mt\text{-}AspRS)}$ = 69627 g mol^{-1}) calculated with ProtParam from ExPASy tools (expasy.org).

2.4. Aminoacylation of tRNAs

Aminoacylation assays were performed as described [14]. Assays were conducted at 15 °C in 50 mM HEPES-KOH pH 7.5, 25 mM KCl, 12 mM $MgCl_2$, 2.5 mM ATP, 0.2 mg/mL BSA, 1 mM spermine, 32 μM [^{3}H]-aspartic acid (208 GBq/mmol), and adequate amounts of transcripts and aaRS. Assays were performed in 50 μL samples. Transcripts were renatured at 60 °C (for mt-$tRNA^{Asp}$ and variants) or 85 °C (for *Eco* $tRNA^{Asp}$ and variants) for 90 s in water and slow cooling down to room temperature before aminoacylation. Maximal charging levels (plateaus) were determined for 400 nM transcript and optimal concentrations of synthetase (500 nM *Hsa* mt-AspRS or 200 nM *Eco* AspRS). Kinetic parameters k_{cat} and K_M were derived from Lineweaver–Burk plots obtained using a range of tRNA concentration from 200 nM to 8 μM (the very high K_M of 70 μM for one variant is an approximation) and of *Eco* AspRS from 2 to 100 nM. Experiments were performed at least in triplicate on separate preparations of transcripts. Final k_{cat} and K_M values varied up to 40%.

2.5. Thermodynamic stability of tRNAs

Free energies (expressed as kcal/mol) of tRNA structures were calculated with the RNAeval program of the Vienna RNA package (http://rna.tbi.univie.ac.at/cgi-bin/RNAeval.cgi), with fixed secondary

[2] Idec stands for identity elements of *Eco* $tRNA^{Asp}$ for aspartylation by *Eco* AspRS, namely G73, G2–C71, G10, G34, U35, C36, C38.