

Biosynthesis of wyosine derivatives in tRNA^{Phe} of Archaea: role of a remarkable bifunctional tRNA^{Phe}:m¹G/imG2 methyltransferase

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ABSTRACT

The presence of tricyclic wyosine derivatives 3'-adjacent to anticodon is a hallmark of tRNA^{Phe} in eukaryotes and archaea. In yeast, formation of wybutosine (yW) results from five enzymes acting in a strict sequential order. In archaea, the intermediate compound imG-14 (4-demethylwyosine) is a target of three different enzymes, leading to the formation of distinct wyosine derivatives (yW-86, imG, and imG2). We focus here on a peculiar methyltransferase (aTrm5a) that catalyzes two distinct reactions: N¹-methylation of guanosine and C⁷-methylation of imG-14, whose function is to allow the production of isowyosine (imG2), an intermediate of the 7-methylwyosine (mimG) biosynthetic pathway. Based on the formation of mesomeric forms of imG-14, a rationale for such dual enzymatic activities is proposed. This bifunctional tRNA:m¹G/imG2 methyltransferase, acting on two chemically distinct guanosine derivatives located at the same position of tRNA^{Phe}, is unique to certain archaea and has no homologs in eukaryotes. This enzyme here referred to as Taw22, probably played an important role in the emergence of the multistep biosynthetic pathway of wyosine derivatives in archaea and eukaryotes.

Keywords: transfer RNA; modification; methyltransferase; archaea; COG2520

INTRODUCTION

Post-transcriptional modifications are critical for the structure and function of tRNA. Those present in the anticodon loop are often chemically complex, and their biosynthesis requires several enzymes (Machnicka et al. 2013). One group of such complex modifications is the fluorescent wyosine derivatives composed of tricyclic imidazo-purine (Fig. 1A) that is found exclusively at position 37, 3'-adjacent to the anticodon of tRNA^{Phe} in eukaryotes and archaea (Urbonavičius et al. 2009). Such wyosine derivatives are important in stabilizing codon-anticodon pairings in the ribosomal decoding site (Konevega et al. 2004) and help to prevent ribosomal frame-shifting (Waas et al. 2007).

In *Saccharomyces cerevisiae*, the enzymatic pathway leading to the biosynthesis of wybutosine (yW) in the cytoplasmic tRNA^{Phe} has been established following comparative genomics analysis, knockout, and complementation studies (Fig. 1B; Noma and Suzuki 2006). It consists of the sequential action of five distinct enzymes, of which only the first reaction occurs in the nucleus (Ohira and Suzuki 2011). In tRNA^{Phe}

of the fungi *Torulopsis utilis*, wyosine (imG) is present in place of wybutosine (Itaya et al. 2002), whereas in several eukaryotes (like *Drosophila melanogaster*, *Bombyx mori*, *Tinca tinca*), a simple N¹-guanosine (m¹G) is found (White and Tener 1973; Mazabraud 1979).

In archaea, the guanosine adjacent to the anticodon of tRNA^{Phe} is also modified to m¹G or wyosine derivatives; however, the diversity of G37-modifications is somewhat different from eukaryotes. In particular, two additional wyosine derivatives have been identified: isowyosine (imG2) and 7-methylwyosine (mimG) (Fig. 1A; McCloskey et al. 2001; Zhou et al. 2004). Moreover, depending on the archaeal species and growth conditions, a mixture of tRNA^{Phe} harboring different wyosine intermediates has often been observed (Table 1 and references therein). Such peculiarities of wyosine metabolism probably result from different nutritional modes and/or harsh physiological conditions in which those archaea are thriving. They can also help to make predictions

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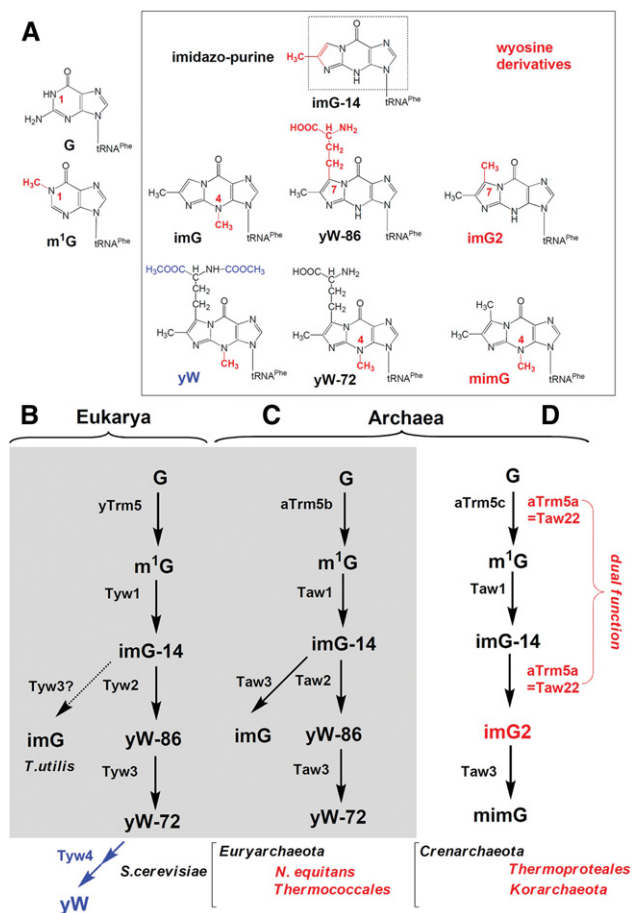


FIGURE 1. Biosynthesis of wyosine derivatives in archaeal tRNA^{Phe}. (A) The chemical structures of the various wyosine derivatives identified in tRNA^{Phe} of archaea and two selected eukaryotes (fungi). (B) The serial steps leading to the final product wybutosine in *S. cerevisiae* and *T. utilis*. (C,D) The sequential biosynthetic steps leading to the various wyosine derivatives found in archaeal tRNA^{Phe}. The steps catalyzed by the bifunctional tRNA^{Phe}:m¹G/imG2 methyltransferase are indicated in red (D). The archaea where such bifunctional aTrm5a (now designated Taw22) may function are indicated in red.

on the evolutionary origin of the wyosine derivatives in tRNA^{Phe} (de Crécy-Lagard et al. 2010).

Biosynthesis of wyosine derivatives in archaea

The first biosynthetic step of all wyosine derivatives is the formation of m¹G³⁷ catalyzed by an S-adenosine-L-methione (AdoMet)-dependent tRNA methyltransferase (Trm5). In yeast, this enzyme, designated yTrm5 (Fig. 1B), is encoded by a single gene (Björk et al. 2001), whereas in archaea, three distinct genes (Fig. 1C,D) code for an yTrm5-homolog (collectively designated below as aTrm5). The next biosynthetic step is the complex radical-mediated formation of 4-demethylwyosine (imG-14), catalyzed by the flavin-binding domain of the *TYW1* gene product in yeast (Young and Bandarian 2013). In all archaea, except for a few halobacter-

iales and *Cenarchaeum symbiosum*, a single homologous gene *taw1* is found (de Crécy-Lagard et al. 2010). However, at variance with the yeast Tyw1, archaeal Taw1 does not have the flavin-binding domain but contains a second Fe-S cluster instead (Perche-Letuvée et al. 2012). The corresponding recombinant Taw1 enzymes of *Pyrococcus horikoshii* (PH1705), *Methanocaldococcus jannaschii* (MJ0257), and *Pyrococcus abyssi* (PAB2039) have been purified; and in the two last cases, their enzymatic activities have been successfully demonstrated in vitro using pyruvate as the key substrate, AdoMet as the cofactor, and dithionite as the source of reducing power (Goto-Ito et al. 2007; Young and Bandarian 2011; Perche-Letuvée et al. 2012).

Genes coding for homologs of yeast Tyw2, the enzymes that catalyze the transfer of 3-amino-3-carboxypropyl (thereafter designated as “acp”) on the C⁷-atom of imG-14, as well as homologs of yeast Tyw3, catalyzing the methylation of the N⁴-atom of the imidazo-purine, are also found in archaea. However, the C⁷-“acp” transferase (Taw2) is present only in Euryarchaeota (Fig. 1C), whereas the N⁴-methyl transferase (Taw3), with the exception of only a few Euryarchaeota, is found mainly in Crenarchaeota (Fig. 1C,D; cf. Fig. 2 in de Crécy-Lagard et al. 2010). The purified recombinant Taw2 of *M. jannaschii* (MJ1557) and of *P. horikoshii* (PH0793) has been produced and their AdoMet-dependent C⁷-“acp” transferase activities have been confirmed in vitro (Umitsu et al. 2009). However, there is currently no experimental report on the archaeal N⁴-methyltransferase. Also, no gene homolog of yeast Tyw4 has been found in archaea. This bifunctional enzyme catalyzes two final steps of wybutosine synthesis in yeast tRNA^{Phe} (Fig. 1B; Suzuki et al. 2009). The distribution of genes coding for Taw2 and Taw3 in different archaea allows prediction of the presence of yW-86, yW-72, and/or imG that generally fit experimentally verified identities of wyosine derivatives in archaeal tRNA (Table 1; cf. Fig. 5 in de Crécy-Lagard et al. 2010). Nevertheless, in the case of Crenarchaeota and a few Euryarchaeota, no obvious candidate gene coding for a methyltransferase capable of adding a methyl group on C⁷-atom of imG-14 to form imG2 derivative has been identified so far (Fig. 1D).

The archaeal Trm5 paradox

Phyletic distribution of *trm5* gene homologs in 62 archaeal genomes allowed identification of three aTrm5 subfamilies: aTrm5a, aTrm5b, and aTrm5c (de Crécy-Lagard et al. 2010). The aTrm5b variant is almost ubiquitous in Euryarchaeota, but in Crenarchaeota it is totally absent. Instead, an aTrm5a is present in all Crenarchaeota, whereas in the Sulfolobales and Desulfurococcales orders an aTrm5c variant is also present. The aTrm5a variant is seldom present in Euryarchaeota, although in the Thermoproteales order of Crenarchaeota, in Korarchaeota phylum, and in *Nanoarchaeum equitans* species, only aTrm5 variant is found (Table 1; cf. Fig. 2 in de Crécy-Lagard et al. 2010).

TABLE 1. Correlation between the presence of genes encoding wyosine biosynthesis enzymes and type of wyosine derivatives in mature archaeal tRNA^{Phe}

Archaea	Enzymes (a)				Wyosine derivatives (b)	Refs
EURYARCHAEOTA Halobacteriales						
<i>Haloferax volcanii</i>	Trm5b	–	–	–	m ¹ G only	(Gupta, 1984; de Crécy-Lagard et al., 2010)
<i>Halobacterium</i> sp. NRC-1	Trm5b	Taw1	Taw2	–	imG-14, yW-86	(de Crécy-Lagard et al., 2010)
Methanosarcinales						
<i>Methanosarcina acetovorans</i>	Trm5a Trm5b	Taw1	Taw2	–	imG-14, yW-86, imG2	(de Crécy-Lagard et al., 2010)
<i>Methanococcoides burtonii</i>	Trm5b	Taw1	Taw2	–	imG-14, imG* (c)	(Noon et al., 2003)
Methanococcales						
<i>Methanococcus maripaludis</i>	Trm5b	Taw1	Taw2	Taw3	imG-14, imG, yW-86 (d)	(McCloskey et al., 2001; de Crécy-Lagard et al., 2010)
<i>Methanococcus vannielii</i>	Trm5b	Taw1	Taw2	Taw3	imG-14, imG, yW-86 (d)	(McCloskey et al., 2001)
<i>Methanocaldococcus jannaschii</i>	Trm5b	Taw1	Taw2	Taw3	imG-14, imG, yW-86 (d)	(McCloskey et al., 2001)
<i>Methanotoris igneus, alias</i> <i>Methanococcus igneus</i> (g)	Trm5b	Taw1	Taw2	Taw3	imG-14, imG, imG* (c)	(McCloskey et al., 2001; Zhou et al., 2004), (Noon et al., 2003)
<i>Methanothermococcus litotrophicus</i>	Trm5b	Taw1	Taw2	Taw3	imG-14, imG (no yW-86)	(McCloskey et al., 2001; Zhou et al., 2004)
Thermococcales						
<i>Pyrococcus furiosus</i>	Trm5a Trm5b	Taw1	Taw2	Taw3x2(e)	imG-14, imG, yW-86, yW-72 imG2, mimG	(de Crécy-Lagard et al., 2010)
<i>Pyrococcus abyssi</i>	Trm5a Trm5b	Taw1	Taw2	Taw3x2(e)	imG, yW-86, yW-72, mimG	(de Crécy-Lagard et al., 2010)
<i>Thermococcus</i> sp. MSB4	Trm5a Trm5b	Taw1	Taw2	Taw3x2(e)	partial data, mimG	(Edmonds et al., 1991)
CRENORARCHAEOTA						
Desulfurococcales						
<i>Pyrodictum occultum</i>	(f)	(f)	(f)	(f)	mimG	(Edmonds et al., 1991) (McCloskey et al., 1987)
<i>Stetteria hydrogenophila</i> (g) <i>Staphylothermus marinus</i> (h)	Trm5a Trm5c	Taw1	–	Taw3	imG, imG2, mimG	(Zhou et al., 2004) (Noon et al., 2003)
<i>Pyrolobus fumarii</i>	Trm5a Trm5c	Taw1	–	Taw3x2(e)	imG-14, imG, imG2, mimG	(McCloskey et al., 2000)
Sulfolobales						
<i>Sulfolobus solfataricus</i>	Trm5a Trm5c	Taw1	–	Taw3	imG-14, imG2, mimG	(Zhou et al., 2004) (McCloskey et al., 1987; Edmonds et al., 1991)
<i>Sulfolobus shibatae</i>	(f)	(f)	(f)	(f)	imG2, mimG	(de Crécy-Lagard et al., 2010)
<i>Acidianus infernus</i>	(f)	(f)	(f)	(f)	mimG	(Edmonds et al., 1991)
Thermoproteales						
<i>Thermoproteus neutrophilus</i>	Trm5a	Taw1	–	Taw3	mimG	(Edmonds et al., 1991) (McCloskey et al., 1987)
<i>Pyrobaculum caldifontis</i>	Trm5a	Taw1	–	Taw3	imG2, mimG	(de Crécy-Lagard et al., 2010)
<i>Pyrobaculum islandicum</i>	Trm5a	Taw1	–	Taw3	mimG	(Edmonds et al., 1991)

The red color enlightens the correlation between the presence of a gene coding for Trm5a and the presence of imG2/mimG in tRNA^{Phe}. There is also a correlation between the presence of Taw2 (blue color) and yW-86 in tRNA^{Phe}.

^aIdentification by homology searches.

^bDetected by HPLC/MS; usually the end products of wyosine biosynthesis accumulate in tRNA^{Phe}, whereas intermediates (such as imG-14) may not be easily detectable.

^cThe chemical structure of compound imG* has not been elucidated; it may be yW-86.

^dCompound with a mass of 422 Da has been identified as yW-86 (Umitsu et al. 2009; de Crécy-Lagard et al. 2010).

^eTwo distinct genes coding for Taw3 have been identified, not discussed in this study (see de Crécy-Lagard et al. 2010).

^fThe complete genome sequence is not yet available.

^gThe name of the archaeon has been changed recently.

^hGenome sequence of *Stetteria hydrogenophila* is unknown but it is known to be closely related to *Staphylothermus marinus*; thus, here the information on the enzymes has been derived from the latter archaeon, whereas tRNA content data come from the former.

Comparison of the amino acid sequences of these three families of methyltransferases demonstrates that no essential differences exist in the AdoMet binding motifs, and only small differences are evident in the NPPY motif known to be important in positioning the target nitrogen atom in several amino-methyltransferases (Bujnicki 2000). In contrast, the N-terminal sequences of these enzyme families differ substantially, a small conserved domain designated D1 is always present in both aTrm5b and aTrm5c, whereas it is absent in a majority of aTrm5a (de Crécy-Lagard et al. 2010).

In the Euryarchaeota *Haloferax volcanii*, the yTrm5 homolog encoded by a *HVO0929* gene is of the aTrm5b type. No genes for other enzymes of the wyosine biosynthetic pathway are found in this archeon (Grosjean et al. 2008). Accordingly, sequencing data of tRNA^{Phe}, as well as of all other G37-containing precursor tRNAs of *H. volcanii*, display an m¹G37 (Gupta 1984). Therefore, HvTrm5b is an N¹-methyltransferase catalyzing the conversion of G37 into m¹G37 in all tRNA species where this modification is present. Likewise, the purified recombinant aTrm5b proteins of the other Euryarchaea *M. jannaschii* (MJ0883) and *Pyrococcus abyssi* (PAB0505) act the same way when tested in vitro (Christian et al. 2006, 2010; de Crécy-Lagard et al. 2010). However, under identical experimental conditions, the paralog PaTrm5a (PAB2272) is more selective for G37-containing tRNA^{Phe} (de Crécy-Lagard et al. 2010).

The crystal structure of MjTrm5b (MJ0883), solved in complex with the substrates tRNA^{Cys} or tRNA^{Leu} and AdoMet, shows an organization that includes three well-identified domains D1, D2, and D3 (Goto-Ito et al. 2008, 2009). The central domain D2 recognizes G37 and the anticodon loop, whereas the C-terminal domain D3, which contains the Rossmann fold of the characteristic class I methyltransferases (Schubert et al. 2003), allows binding of AdoMet and catalyzes the methylation reaction. Enzymatic in vitro tests with the truncated recombinant MJ0883 (aTrm5b) demonstrated that domain D1, attached to the rest of the protein via a flexible link, plays no obvious role in either tRNA binding or methylation of the G37 of the bound tRNA (Goto-Ito et al. 2008). Nevertheless, the crystal structure shows that domain D1 interacts with conserved elements of the T-loop on the opposite side of the anticodon, the elbow of the L-shaped tRNA molecule, thus playing the role of a clamp.

Archaeal Trm5 and Taw2 are two sides of the same coin

All aTrm5 N¹-methyltransferases identified (variants a, b, and c) and paralogous Taw2 C⁷-“acp”-transferases belong to the same cluster of orthologous genes (COG2520/PACE25), attesting to their common evolutionary origin (Matte-Tailliez et al. 2000; Makarova et al. 2007). The crystal structure of purified recombinant Taw2 of *M. jannaschii* (MJ1557) and that of *P. horikoschii* (PH0793), both in complexes with the AdoMet, revealed that as expected, the “acp” group is directed

to the substrate-binding pocket (Umitsu et al. 2009). Unfortunately, the exact positioning of the target imG-14 residue of the bound tRNA^{Phe} is not known. However, based on a plausible tRNA^{Phe}-PfTaw2 docking model, the following scenario has been proposed: (1) The target imG-14 residue of the bound tRNA^{Phe} is probably sandwiched by two conserved hydrophobic residues Met107 and Val201, instead of Tyr177 and Pro267, as is the case with MjTrm5b; (2) a strictly conserved Arg76 residue is hydrogen-bound to O⁹ and N¹ of imG-14, whereas the same conserved Arg in MjTrm5b is bound to O⁶ and N⁷ of guanine; and (3) a Gly199 residue in PfTaw2, corresponding to the Asn265 in MjTrm5b, allows a larger space to accommodate the tricyclic imG-14 base. Thus, juxtaposition and orientation of the target base (G or imG-14) relative to the methyl or the “acp” group of AdoMet within similar catalytic sites appear to be the main clue to understand the catalytic function of Taw2 (C⁷-“acp”-transferase) versus aTrm5b (N¹-methyltransferase). It is noteworthy that the absence of a D1-“clamp” domain in PfTaw2, as well as in Taw2 of all the other archaea, could also be an important signature for the C⁷-“acp”-transferase reaction. These observations support the hypothesis that aTrm5b and Taw2 of Euryarchaeota evolved from a common ancestor.

Bifunctional aTrm5a is very likely the missing link in mimG metabolism

Collectively, the above data favors the hypothesis that in certain archaea, the aTrm5a behaves as an AdoMet-dependent C⁷-imG-14 methyltransferase. In agreement with this hypothesis, the formation of imG2 has been demonstrated by testing in vitro the AdoMet-dependent methylation activity of recombinant PaTrm5a (PAB2272) using imG-14 containing tRNA^{Phe} as a substrate. In contrast, under identical experimental conditions, such methylation of imG-14 has not been detected with recombinant PaTrm5b (PAB0505) (de Crécy-Lagard et al. 2010; J Urbonavičius, unpubl.). Evidently, PaTrm5a, but not PaTrm5b, has a dual activity and catalyzes both N¹-methylation of G37 and C⁷-methylation of imG-14 in the same tRNA^{Phe} (Fig. 1D). As mentioned above, this aTrm5a is omnipresent in Crenarchaeota, which fits with the ubiquitous presence of imG2/mimG in this phylum of archaea (Table 1). Of note, in addition to yW-86 and yW-72, imG2 and mimG are found in the tRNAs of those archaea that possess the full set of corresponding enzymes, including an aTrm5a variant, such as Thermococcales and a few other Euryarchaeota (Table 1; Fig. 1C,D; cf. Fig. 2 in de Crécy-Lagard et al. 2010). A phylogenetic analysis of aTrm5a suggests that this situation arose from multiple independent lateral gene transfers of a gene coding for aTrm5a of Crenarchaeota into Euryarchaeota (de Crécy-Lagard et al. 2010). Although the causes for such diversity remain elusive, the most plausible explanations are adaptation to different stress situations and/or regulatory aspects of the translation process (Chan et al. 2010). The cases of Thermoproteales

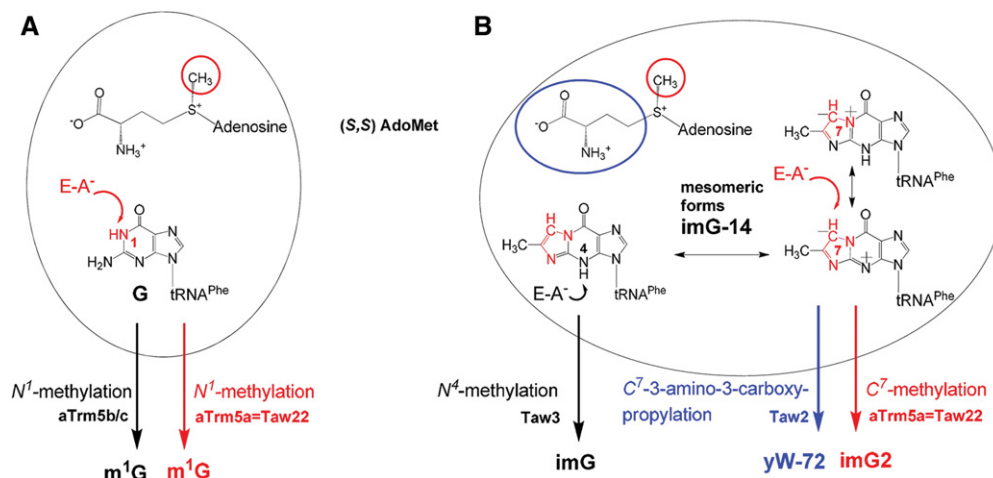


FIGURE 2. Schematic representation of the active sites of selected tRNA methyltransferases acting at guanosine derivatives located on position 37 in precursor tRNA^{Phe}. Depending on the enzyme considered, transfer of a methyl-group (encircled in red) or a 3-amino-3-carboxypropyl group (encircled in blue) on guanine (G) or 4-demethylwyosine (imG-14) may occur, leading to either m¹G, imG, imG2, or yW-72 guanosine derivatives (see formula in Fig. 1). (A) Methylation of the electron-rich N¹-atom of guanine catalyzed by the multi-tRNA specific aTrm5a, b, or c. In the case of *M. jannaschii* Trm5b, a glutamic acid (E185) in the active site (indicated as Enz-A⁻), allows abstraction of the proton at the N¹-atom of guanine and the subsequent addition of the methyl group from the sulfonium ion of AdoMet (Christian et al. 2010). However, in Trm5 of a few other archaea, an aspartic acid (D) is found instead (cf. Supplemental Fig. SS7 in de Crécy-Lagard et al. 2010). (B) Methylation or 3-amino-3-carboxypropylation of electron-rich C⁷-atom in the mesomeric form of imG-14 of tRNA^{Phe} catalyzed by aTrm5a/Taw2 or Taw2, respectively (cf. comments and Fig. 9 in Lin 2011). The amino acid residue in the active site of the enzyme (Enz-A⁻) allowing abstraction of the proton located on the C⁷-atom of imG-14 is probably the same conserved E or D residue as mentioned above in the cases of other aTrm5, including MjTrm5b. As discussed in the text, a certain degree of flexibility of the target base, the AdoMet and/or mobility of the catalytic E or D residue of the active site of the enzyme will probably be required. The S-adenosyl-L-methionine in both A and B is represented in its naturally occurring S,S configuration (Cannon et al. 2002).

order of Crenarchaeota, Korarchaeota, and the single *N. equitans* (with reduced genome of only 490 kb) are special since they encode only three genes of the wyosine biosynthesis pathway (aTrm5a, Taw1, and Taw3) (Fig. 2 in de Crécy-Lagard et al. 2010). Thus, in addition to the C⁷-methylating activity of imG-14 in tRNA^{Phe}, the unique aTrm5a of these archaea should also catalyze the formation of m¹G37 in tRNA isoacceptors other than just tRNA^{Phe}. In the other Crenarchaeota and Euryarchaeota, N¹-methylation of G37 is fulfilled mainly by monofunctional aTrm5b or aTrm5c (Fig. 1C,D). To clearly distinguish between the monofunctional N¹-methyltransferases, such as aTrm5b/aTrm5c acting on several G37-containing tRNAs (including tRNA^{Phe}) and the bifunctional aTrm5a that in addition catalyzes C⁷-methylation of imG-14 in tRNA^{Phe}, we propose to rename all the aTrm5a homologs as Taw22.

CONCLUSION AND FUTURE DIRECTIONS

From an organic chemistry point of view, it may look strange that a Trm5 methyltransferase, probably having emerged to catalyze the methylation of an endocyclic N¹-imino group of a guanine, can also catalyze the methylation of an endocyclic C⁷-atom of a tricyclic imidazopurine. However, from an enzymatic point of view, this might not be a problem (Kozbial and Mushegian 2005). Indeed, once the tRNA^{Phe} substrate is specifically bound to the AdoMet-enzyme com-

plex, the N¹-atom of guanine or the C⁷-atom of the imidazopurine-37 most likely occupies the same position within the cavity of the active site. Because of electron delocalization within the imidazopurine (mesomerism), the C⁷-atom may become sufficiently negatively charged to allow its interaction with the methyl group attached to the positively charged sulfonium ion of AdoMet (Fig. 2B; cf. Fig. 9 in Lin 2011). Proton abstraction from C⁷-atom of imG-14 could be facilitated by an enzyme residue (Asp or Glu) functioning as a general base, as has been demonstrated in the case of N¹-G37 methylation by MjTrm5b (Fig. 2A; Christian et al. 2010, 2013). Spatial orientation of the bound AdoMet, usually in the (S,S) configuration (Cannon et al. 2002), is of course crucial for the precise juxtaposition of the methyl group and the target C⁷-atom of imG-14 or N¹-atom of guanine. Finally, a certain degree of flexibility of the target base within the anticodon loop of tRNA^{Phe} (Sakaguchi et al. 2012), the AdoMet (Gana et al. 2013), or the mobility of certain amino acids of the active site of the enzyme (Christian et al. 2006, 2013) may facilitate the electron donation along the methyl transfer process, probably by an S_N2 type of mechanism. If one accepts that certain Trm5-like enzymes may have a dual activity, it could be anticipated that after the emergence of the radical AdoMet enzyme Taw1, catalyzing the formation of tricyclic imG-14 from m¹G-containing tRNA^{Phe}, the evolving primordial cell might have benefited from the intrinsically relaxed specificity of its N¹-methylating enzyme to also methylate the C⁷-atom of the imG-14 by a similar type of

mechanism, thus allowing to generate imG2 of the wyosine biosynthetic pathway (Fig. 1D). Later, after gene duplication, each duplicate coding for an enzyme acting on two chemically distinct guanosine derivatives located on the same position of tRNA^{Phe} could have evolved to either remain bifunctional (aTrm5a/Taw22) as in some extant archaea, or to become a more base-specific N⁷-G37-methylating enzyme (aTrm5b, c), or a specific C⁷-“acp”-imG-14 transferase (Taw2). Considering the possible archaeal origin of eukaryotes (Gribaldo et al. 2010; Williams et al. 2013), one can even envisage that both yTrm5 and Tyw2 (Fig. 1B) have also emerged from an ancestral aTrm5a/Taw22-type of enzyme. To better understand how these different extant paralogs, which all belong to the same COG2520/PACE25, are functioning, comparative structural and biochemical studies similar to those already reported for a few archaeal aTrm5 and Taw2 enzymes (Christian et al. 2006; Goto-Ito et al. 2008, 2009; Umitsu et al. 2009; Sakaguchi et al. 2012) are urgently needed.

Interesting analogies exist with two other bifunctional modification enzymes acting on RNA. The first is Cfr from *E. coli* that normally mediates the single methylation of the 8-amidine carbon of A2503 of 23S rRNA to form 8-methyladenosine (m⁸A). However, when *E. coli* lacks the methyltransferase RlmN that catalyzes the methylation of the 2-amidine carbon of the same A2503 in 23S rRNA, Cfr can compensate such deficiency and catalyzes the formation of doubly methylated 2,8-dimethyladenosine (m^{2,8}A) (Giessing et al. 2009). Similarly, a single bifunctional methyltransferase NS5 of flaviviruses catalyzes the sequential methylation of the N⁷-atom of the 5'-terminal guanosine and 2'-hydroxyl group of the neighboring adenosine, resulting in the formation of the type 1 cap m⁷GpppAm of viral RNA (Dong et al. 2008a,b). One important common mechanistic aspect of these bifunctional enzymes is that a repositioning of the target nucleotide(s) within a single AdoMet-containing active site of the enzyme is mandatory, and under certain conditions or during the course of evolution, each of the two methylation events have been decoupled and acted independently. Emergence of such bifunctional enzymes, including aTrm5/Taw22, might have been an important step in the evolution of sequential maturation of precursor RNA in particular but also in the development of sequential metabolic pathways in general.

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