New archaeal methyltransferases forming 1-methyladenosine or 1-methyladenosine and 1-methylguanosine at position 9 of tRNA

Morgane Kempenaers¹, Martine Roovers², Yamina Oudjama², Karolina L. Tkaczuk³,⁴, Janusz M. Bujnicki³,⁵ and Louis Droogmans¹,*

¹Laboratoire de Microbiologie, Université Libre de Bruxelles (ULB), ²Institut de Recherches Microbiologiques Jean-Marie Wiame, Avenue E. Gryson 1, B-1070 Bruxelles, Belgium, ³International Institute of Molecular and Cell Biology in Warsaw, Trojdena 4 St., PL-02-109 Warsaw, ⁴Institute of Technical Biochemistry, Technical University of Lodz, B. Stefanowskiego 4/10, PL-90-924 Lodz and ⁵Institute of Molecular Biology and Biotechnology, Faculty of Biology, Adam Mickiewicz University, PL-61-614 Poznan, Poland

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ABSTRACT

Two archaeal tRNA methyltransferases belonging to the SPOUT superfamily and displaying unexpected activities are identified. These enzymes are orthologous to the yeast Trm10p methyltransferase, which catalyses the formation of 1-methylguanosine at position 9 of tRNA. In contrast, the Trm10p orthologue from the crenarchaeon Sulfolobus acidocaldarius forms 1-methyladenosine at the same position. Even more surprisingly, the Trm10p orthologue from the euryarchaeon Thermococcus kodakaraensis methylates the N¹-atom of either adenosine or guanosine at position 9 in different tRNAs. This is to our knowledge the first example of a tRNA methyltransferase with a broadened nucleoside recognition capability. The evolution of tRNA methyltransferases methylating the N¹ atom of a purine residue is discussed.

INTRODUCTION

Cellular RNAs possess numerous chemically modified nucleosides, but the largest number and the greatest variety are found in transfer RNA (tRNA). These modifications are introduced by many different enzymes during the complex process of RNA maturation. The functions of these modified nucleosides are not well known, but it seems that modifications in the anticodon region play a direct role in increasing translational efficiency and fidelity, while modifications outside the anticodon region are typically involved in the maintenance of the structural integrity of tRNA. Among naturally occurring nucleoside modifications, base and ribose methylations are by far the most frequently encountered (1,2). These methylations are catalysed by tRNA methyltransferases (MTases) that use S-adenosyl-L-methionine (AdoMet) as the methyl donor, with a single exception of a recently identified enzyme, which uses a folate as the methyl donor (3).

AdoMet-dependent MTases belong to at least seven evolutionarily and structurally unrelated classes/superfamilies (4–6). Most of the known RNA MTases belong to class I. They possess a fold similar to the Rossmann fold, and are therefore called Rossmann fold MTases (RFM). The structure comprises a seven-stranded b sheet with a central topological switch-point and a characteristic reversed b hairpin at the carboxyl end of the sheet (6†7†5†4†1†2†3†). This b sheet is flanked by α helices to form an αβα sandwich (4). RFM enzymes are typically monomeric although di-tri- or tetrameric structures have been reported. Class IV MTases, also named the SPOUT class MTases, a nomenclature coming from the first two members of this class, SpoU and TrmD, act also on RNA, but are structurally different from the class I MTases (7). They possess a five-stranded b sheet core (5†3†4†1†2†) flanked by seven α helices. The most characteristic feature of these enzymes is the presence of a deep topological knot in the C-terminal part of the sheet. This knot is responsible for AdoMet binding. All known SPOUT MTases are dimers, with the catalytic site formed at the interface of two monomers (8).

There are only a few archaeal tRNA specific MTases functionally characterised to date. Those MTases can be...
very different in terms of structure, of target or in their mechanism. Some act as base MTases, while others are ribose MTases. Among the MTases acting on ribose, there exist two kinds of methylation pathways. In the first one, called ‘all-protein enzyme’, the MTase recognizes on its own the target nucleoside and catalyses the methyl transfer on the ribose. The second mechanism involves an MTase taking part of a ribonucleoprotein (RNP) complex, in which the recognition of the target nucleoside is carried out by a small guide RNA. In different organisms, the same modification can be accomplished by the two different mechanisms. This is the case of the CmS6 ribose methylation of tRNA in Archaea. In Pyrococccus abyssi, this modification is catalysed by a RNP complex, where the small RNA sR35 targets the C56 ribose methylation of tRNA in Archaea. In addition, P. abyssi contains a SPOUT MTase homologous to aTrm56 from Pyrococcus abyssi, which catalyses non-guided methylation of C56 (9,10).

The six archaeal RFM MTases characterised to date act on the base of nucleosides. Trm-G10 from P. abyssi methylates or dimethylates guanosine 10 from tRNA to form m1G10 or m2G10 (11,12). The same modification can be found at position 26 of tRNA and is catalysed by Trm-G26 (also known as Trm1) (13,14). The MTase encoded by the open reading frame (ORF) PAB1947 from P. abyssi acts on several cytosines in tRNA to form mC, with a preference for C49. The specificity of this MTase for C49 is increased by an archaease (15). Another important tRNA MTase identified in Archaea is Trm5, which methylates G37 in tRNA (16). Guanosine in position 37 is commonly methylated to form m1G37 in tRNA from organisms belonging to the three domains of life, and this modification prevents frame-shifting by assuring correct codon–anticodon pairing (17). The tRNA MTase TrmU54 catalyses the methylation of atom C1 in uridine to form ribothymidine in tRNA from P. abyssi (18). This modification is invariably found at position 54 in the TΨC loop of tRNAs of most organisms. And finally, the MTase TrmI from P. abyssi catalyses the methylation of position A1 of adenosine to form m1A. This enzyme displays region specificity, methylating A58 and A57 from TΨC loop of tRNA, m1A57 being the obligatory intermediate in the biosynthesis of 1-methylinosine (m1I) (19).

In this article, we show that the S. acidocaldarius Saci_1677p enzyme indeed acts at position 9 of tRNA, catalysing mA formation. Furthermore, in Euryarchaeota, the homologous protein from Thermococcus kodakaraensis also acts at position 9 of tRNA, but catalyses both mA and mG formation. To our knowledge, this is the first MTase found to methylate the two purine bases at the same position.

**MATERIALS AND METHODS**

**Strains, media, growth conditions and general procedures**

Pwo DNA polymerase, T4 DNA ligase, T7 RNA polymerase, and T4 polynucleotide kinase were purchased from Roche. Ribonuclease A was from Fermentas. Genomic DNA from S. acidocaldarius was a gift from H. Grosjean (CNRS, France) and T.J. Santangelo (Ohio State University, USA). Genomic DNA from S. acidocaldarius was a gift from D. Charlier (VUB, Belgium). The S. cerevisiae Trm10-GST clone plasmid (pYCG_YOL93w) and S. cerevisiae Y16243 strain (BY4742; MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; yol093w::kanMX4) were purchased from Euroscarf.

**Cloning of the S. kodakaraensis TK0422 ORF, S. acidocaldarius Saci_1677 ORF and of S. cerevisiae TRM10 ORF**

The TK0422 ORF was amplified from S. kodakaraensis genomic DNA using Pwo polymerase (Roche) and the primers TKF (5’-CTAGCATATGAAAGACCTCGAGCATG-3’) and TKR (5’-CTAGCTGGAGTCAAAGGTTTTTTCGCG-3’) containing the NdeI and XhoI restriction sites, respectively. After cloning the PCR product in pCR-Blunt vector (Zero Blunt, Invitrogen), the NdeI/XhoI fragment was extracted and cloned in pET-28b expression vector (Novagen), generating the pTK1 plasmid, allowing expression of an N-terminal His-tagged T. kodakaraensis protein in Escherichia coli. The Saci_1677 ORF was amplified from S. acidocaldarius genomic DNA using Pwo polymerase (Roche) and the primers SAF (5’-CTACTCTGGAGGCTCTGAAAGTTTTTTCGCG-3’) and SAR (5’-CTAGCTGGAGTCAAAGGTTTTTTCGCG-3’) containing the NdeI and XhoI restriction sites, respectively. After cloning the PCR product in pET-28b expression vector (Novagen), generating the pTK1 plasmid, allowing expression of an N-terminal His-tagged S. acidocaldarius protein in E. coli. The TRM10 ORF was amplified from the Trm10-GST clone using Pwo polymerase (Roche) and the primers SCF (5’-CTACTCTGGAGGCTCTGAAAGTTTTTTCGCG-3’) and SCR (5’-CTACTCTGGAGGCTCTGAAAGTTTTTTCGCG-3’) containing the NdeI and XhoI restriction sites, respectively. After cloning the PCR product in pET-28b expression vector, generating the pSC1 plasmid, allowing expression of an N-terminal His-tagged S. cerevisiae protein in E. coli. The sequences of all clones were checked.
Expression and purification of the recombinant T. kodakaraensis TK0422p, S. acidocaldarius Saci_1677p and S. cerevisiae Trm10p

The His-tagged TK0422p, Saci_1677p and Trm10p recombinant proteins were expressed in E. coli strain Rosetta (DE3) (Novagen) carrying extra copies of tRNA genes ([argU, argW, ileX, glyT, leuW, proL, metT, thrT, tyrU and thrU] specific for rare E. coli codons, to aid this expression. Freshly transformed cells were grown to an OD660 of 0.5–0.6 at 37°C in 11 l of Luria broth with kanamycin (30 μg/ml). Isopropyl-β-d-thiogalactopyranoside (IPTG) (Roche Diagnostics) was then added to a final concentration of 1 mM to induce recombinant protein expression. Cells were harvested after 3 h incubation at 37°C and resuspended in 100 ml of buffer A (Tris–HCl 50 mM pH 8, KCl 500 mM) complemented with protease inhibitors (Complete, EDTA-free protease inhibitor; Roche Diagnostics) prior to cell disruption by sonication. The lysate was cleared by centrifugation (20 000g for 30 min), and was applied to a Chelating-Sepharose fast flow column (GE Healthcare) charged with Ni2+ and equilibrated with buffer A. The column was washed with the same buffer, and the adsorbed material was eluted with a linear gradient (210 ml, from 0 to 1.0 M) of imidazole in buffer A. The fractions containing TK0422p, Saci_1677p and Trm10p were separately pooled. The purified proteins were then submitted to a gel filtration chromatography (Superdex G200; GE Healthcare), leading to almost completely pure TK0422p, Saci_1677p and Trm10p.

T7 in vitro transcription of tRNA genes

The general procedure for generating in vitro transcripts of tRNA genes is based on the method described previously (22). The sequence of the DNA product obtained after amplification of S. acidocaldarius genomic DNA with oligonucleotides MK1 (5′-TCTGGATAATACGCTACTATAGGGCGGTAGGGAAGCTGGTATCC-3′) and MK2 (5′-TCTGGCTCGACATGGTGTCGGGCG GCCTGGAATTGGAACAGGAGGACTCGGTTA-3′) together with the sequence of this region of the genome in the database revealed differences with that of the tRNAsequence (20). The amplification product for the tRNAMetsequence (20) was thus PCR amplified using primers and oligonucleotides MK1 (5′-TCTGGATAATACGCTACTATAGGGCGGTAGGGAAGCTGGTATCC-3′) and MK2 (5′-GATTGGAAGCCTGGATTTGAACCCGGGTTCAAA-3′) as primers and oligonucleotides MK11 (5′-GACGCCGGCCCATCATACGGGACTCTAGGCTTTGACCCGGTTCAAA-3′) and MK10 (5′-TCTGGATAATACGCTACTATAGGGCGGTAGGGAAGCTGGTATCC-3′) as template DNA. The DNA sequences encoding tRNAAla and tRNAMet were cloned in the SmaI site of the pUC18 vector, giving plasmids pUC18-tRNAAla and pUC18-tRNAMet. The DNA sequences encoding tRNAAla and tRNAMet were cloned in the EcoRI site of pUC18, giving plasmids pUC18-trNRAla and pUC18-trNMet. The sequences of all the clones were checked. These plasmids allow T7 transcription of S. acidocaldarius tRNAAla and tRNAMet (A9G) and T. kodakaraensis tRNAAla and tRNAMet, respectively. Radioactive [32P] in vitro transcripts were obtained using PstI (for S. acidocaldarius tRNA) or MvaI-digested plasmids (for T. kodakaraensis tRNA). [23S]ATP and [32P]GTP were purchased from Perkin Elmer. Radioactive transcripts were purified by 10% polyacrylamide gel electrophoresis.

tRNA MTase assays

The two types of tRNA MTase assays used in this work were described in (23). The first method consisted of measuring the amount of [14C] transferred to total yeast tRNA (Y16243 strain), or total E. coli tRNA using [methyl-14C]AdoMet as the methyl donor. The reaction mixture (300 μl) consisted of 50 mM Tris–HCl pH 8, 10 mM MgCl2, 100 μM total tRNA, and variable amount of TK0422p (5, 2.5, 1, 0.5 g). The effect of pH on MTase reaction was measured using 50 μg E. coli tRNA as substrate and 5 μg enzyme. The buffers were: acetate buffer pH 4.8, MES buffer pH 5.5, phosphate buffer pH 7, HEPES buffer pH 7, CHES buffer pH 9 and CAPS buffer pH 9.75. All buffers concentrations were 50 mM. The second type of tRNA MTase assay involved in vitro transcribed, [32P]-labelled tRNA as substrates. Modified nucleotides were analysed by 2D thin layer chromatography (2D–TLC) on cellulose plates (Merek). First dimension was with solvent A (isobutyric acid/concentrated NH4OH/water; 66/1/33; v/v/v); second dimension was with solvent B [0.1 M sodium phosphate pH 6.8/solid

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(NH₄)₂SO₄/n-propanol; 100/60/2; v/w/v]). The nucleotides were identified using a reference map (24).

Analysis of the presence of m¹A or m¹G in E. coli tRNA in vivo

Prior to tRNA extraction, the E. coli strain overexpressing TK0422p and the control strain (no expression of TK0422p) were incubated for 2 h at 50°C. Then, total tRNA was extracted. An amount of 200 μg of totally hydrolysed tRNA was injected on a Supelco Discovery C18 (250 × 4.6) mm HPLC column equilibrated with ammonium acetate 0.25 M, pH 6.5. The column was eluted with a linear gradient of acetonitrile/water (40/60; v/v) at a flow rate of 1.2 ml/min. The nucleosides were detected by measuring UV absorbance at 254 nm. The standard HPLC curve was obtained by injecting 20 μl of 1 mM canonical nucleosides together with m¹A and m¹G modified nucleosides.

Localization of m¹A and m¹G in S. acidocaldarius tRNA

The transcript of tRNA⁰Met or tRNA⁰Met(A9G) from S. acidocaldarius was used as substrate for the MTases TK0422p or Saci_1677p. An amount of 2 μg of tRNA was incubated in presence of 4 μg of enzyme and 0.3 mM AdoMet (Sigma) for 1 h at 50°C. As a control, transcripts were incubated in the MTase assay conditions, but without enzyme. Then the reaction was stopped by phenol extraction, transcripts were ethanol precipitated and the pellet was resuspended in 30 μl of RNase A buffer (Tris–Cl 10 mM pH 7.5, 15 mM NaCl). The resuspended solution was heated at 85°C for 5 min and then slowly cooled down to 37°C. Then 10 μg of RNase A was added and the digestion was carried out for 1 h at 70°C. Half of the RNase A digestion was 5'-end radiolabelled with 50 μCi of [γ³²P]ATP and 20 units of T4 polynucleotide kinase. The radiolabelled fragments were then separated by 20 or 30% polyacrylamide gel electrophoresis and revealed by autoradiography. The 8-nt long fragments (A9 or G9 at position 9) were identified using a reference map (24). Interestingly, the 8-nt long fragments (A9 or G9 at position 9) were identified using a reference map (24). By iterative Psi-Blast analysis, the authors identified proteins from S. solfataricus and P. furiosus which are distantly related to S. cerevisiae Trm10p. We used these two archaeal sequences as query for a Psi-Blast analysis of archaeal proteomes, and identified the proteins Saci_1677p from S. acidocaldarius (phylum Crenarchaeota) and TK0422p from T. kodakaraensis (phylum Euryarchaeota). These two enzymes are orthologues of S. cerevisiae Trm10p.

RESULTS

Sulfolobus acidocaldarius Saci_1677p and T. kodakaraensis TK0422p are distantly related to Trm10p from S. cerevisiae

The tRNA⁰Met from S. acidocaldarius is the only tRNA from an hyperthermophilic archaeon sequenced to date (20). It bears an unknown modification at position 9. In yeast, the enzyme Trm10p catalyses m¹G formation at this position (21). By iterative Psi-Blast analysis, the authors identified proteins from S. solfataricus and P. furiosus which are distantly related to S. cerevisiae Trm10p. We used these two archaeal sequences as query for a Psi-Blast analysis of archaeal proteomes, and identified the proteins Saci_1677p from S. acidocaldarius (phylum Crenarchaeota) and TK0422p from T. kodakaraensis (phylum Euryarchaeota). These two enzymes are orthologues of S. cerevisiae Trm10p.

The proteins Saci_1677p from S. acidocaldarius and TK0422p from T. kodakaraensis show different tRNA MTase activities in vitro

Since Saci_1677p and TK0422p are distantly related to S. cerevisiae Trm10p, the activities of these enzymes were tested in vitro using unfractionated (bulk) tRNA from the S. cerevisiae Y16243 strain (in which the TRM10 gene is inactivated) as substrate. The purified recombinant proteins (see ‘Materials and Methods’ section) were incubated at 30°C for the yeast enzyme, and at 50°C for the archaeal enzymes for an hour with [methyl-¹⁴C]AdoMet and tRNA. The choice of a reaction temperature of 50°C was based on a compromise between hyperthermophilic enzyme activity and mesophilic tRNA stability. After incubation, the tRNA was recovered by phenol extraction and ethanol precipitation and further completely hydrolysed into 5'-phosphate nucleosides by nuclease P1. The resulting hydrolysates were then analysed by 2D–TLC followed by autoradiography. The results presented in Figure 1 confirm that S. cerevisiae Trm10p catalyses formation of one single radioactive compound with migration characteristics identical to 1-methylguanosine (m¹G) 5'-phosphate according to reference maps (24). Interestingly, the S. acidocaldarius enzyme catalyses the formation of one single radioactive compound, but it corresponds to 1-methyladenosine (m¹A) 5'-phosphate. Surprisingly, the T. kodakaraensis TK0422p enzyme catalyses formation of two radioactive compounds, m¹A and m¹G in tRNA. The enzyme TK0422p forms approximately the same amount of m¹A and m¹G when the tRNA of the yeast strain Y16243 is used as substrate. Given that occurrence of A9 and G9 in this tRNA population is almost equal (~50% each) this result indicates that the enzyme TK0422p does not show any preference for one of these two nucleosides.

To further confirm the activities of the archaeal enzymes, the MTase activity assay was carried out as described above on unfractionated E. coli tRNA. The rationale behind using bulk E. coli tRNA arises from the fact that position 9 of E. coli tRNA is never modified. The
results shown in Figure 2 confirm those obtained using tRNA from the yeast Y16243 (trm10::kan) strain. Indeed, the Saci_1677p from S. acidocaldarius catalyses the formation of m^1A, while the TK0422p from T. kodakaraensis catalyses the formation of two radioactive compounds, m^1A and m^1G. The ratio m^1A/m^1G is higher than what we observed using tRNA from the yeast Y16243 strain. This is consistent with the fact that there are about two times more tRNAs with A9 than with G9 in E. coli. Again, this confirms the absence of preference of TK0422p for one of these two nucleosides. To our knowledge this is the first description of a tRNA MTase with a broadened substrate recognition capability.

Saci_1677p catalyses m^1A formation, while TK0422p catalyses m^1A and m^1G formation on in vitro transcripts of archaeal tRNAs

The activity of Saci_1677p from S. acidocaldarius was confirmed under identical experimental conditions as above, but using [\(\alpha\)^32P]ATP-labelled tRNA substrates obtained after in vitro transcription by T7 RNA polymerase of synthetic S. acidocaldarius tRNA^Met^9 gene or tRNA^A9G gene. After incubation, the formation of N^1-methyladenosine was analysed as above, and the ^32P phosphate is only present in AMP (5'P) and AMP (5'P) derivatives. Figure 3 demonstrates unambiguously the presence of m^1A in the WT tRNA^Met^ from S. acidocaldarius, showing that the enzyme is indeed a tRNA m^1A MTase. Interestingly, no m^1A was formed in a mutant of the tRNA^Met^ where position 9 is occupied by a guanosine (tRNA^A9G), suggesting that S. acidocaldarius Saci_1677p acts at position 9 of tRNA, as its yeast orthologue does. Quantification of the relative amount of ^32P in the different radioactive spots on TLC plates revealed that about 1 mole of m^1A was formed per mole of tRNA after 1 h incubation at 50°C.

Similarly, the activity of T. kodakaraensis TK0422p was also confirmed using in vitro [\(\alpha\)^32P]ATP- and [\(\alpha\)^32P]GTP-labelled transcripts of T. kodakaraensis tRNA^Ala^ and tRNA^Asp^ genes. The tRNA^Ala^ contains an adenosine at position 9, while tRNA^Asp^ contains a guanosine at this position. Figure 4 shows that TK0422p catalyses formation of m^1A in the [\(\alpha\)^32P]ATP-tRNA^Ala^ (A in position 9), but not in the [\(\alpha\)^32P]ATP-tRNA^Asp^ that possesses G in position 9. Similarly, there was only formation of m^1G in the GTP-labelled tRNA^Asp^ (G9), but
not in the tRNA$^{A_{16}}$ (A9). Quantification of the relative amount of $^{32}$P in the different radioactive spots on TLC plates revealed that about 1 mol of m$^1$A or 1 mol of m$^1$G was formed per mole of tRNA after 1 h incubation at 50°C. Taken together, these results confirm that TK0422p is capable of methylating the two purine bases, and they suggest that the T. kodakaraensis enzyme acts also at position 9 of tRNA.

The archaeal enzymes act at position 9 of tRNA

The MTase Trm10p from S. cerevisiae modifies guanosine at position 9 of tRNA (21). The results described above suggest that the archaeal enzymes also target this position. To precisely identify the localisation of nucleosides methylated by the archaeal enzymes, modified and unmodified S. acidocaldarius tRNA$^{Met}$ or S. acidocaldarius tRNA$^{Met}$A9G were digested by RNase A and the products of this reaction were $^{32}$P-labelled at the 5’ extremity (see ‘Materials and Methods’ section). The resulting 8-nt long fragments bearing A9 or G9, and 5-nt long fragments (controls) were hydrolysed by nuclease P1 and the 5’-phosphate mononucleosides were separated by 2D–TLC. The nature of the nucleotide present at the 5’-end of each fragment was revealed by autoradiography. Figure 5 shows that m$^1$A or m$^1$G is only present at the 5’-end of the 8-nt long fragment from the modified transcript, confirming that both archaeal enzymes act at position 9 of tRNA. Furthermore, it underlines the dual tRNA m$^1$A9-m$^1$G9 specificity of the T. kodakaraensis TK0422p.

Further characterisation of the unique m$^1$A-m$^1$G MTase activity of T. kodakaraensis TK0422p

To determine whether the formation of m$^1$A and m$^1$G resulted from the use of a too high enzyme/tRNA ratio, various concentrations of the enzyme were incubated with the same amount of E. coli tRNA and [methyl-$^{14}$C]AdoMet (see ‘Materials and Methods’ section). Both m$^1$A and m$^1$G are formed in E. coli tRNA incubated in presence of TK0422p, and the intensity of the spots are related to the concentration of TK0422p. Furthermore, the ratio between m$^1$A and m$^1$G is conserved throughout the dilutions (results not shown).

Interestingly, T. kodakaraensis TK0422p methylates atom N$^1$ of both adenosine and guanosine, whose protonation states are different at physiological pH. Indeed, position N$^1$ of adenosine is unprotonated at physiological pH, whereas position N$^1$ of guanosine is protonated. To get some insight into the catalysis by TK0422p, influence of the pH on the methylation reaction was analysed (Table 1). At pH 4.8, the T. kodakaraensis TK0422p is not active. The MTase is active in a pH range going from pH 5.5 to 9.75, but the intensity of m$^1$A and m$^1$G spots vary greatly as a function of the pH. At pH 5.5, m$^1$A MTase activity of TK0422p is predominant over m$^1$G. At pH 7 or higher, both m$^1$A and m$^1$G were detected, m$^1$G intensity growing with increasing pH.

The T. kodakaraensis MTase TK0422p catalyses m$^1$A and m$^1$G formation in E. coli tRNA in vivo

To get some insight into the in vivo activity of T. kodakaraensis TK0422p, total tRNA was extracted from the E. coli strains expressing or not TK0422p. The tRNA preparations were then hydrolysed by P1 nuclease, snake venom phosphodiesterase and alkaline phosphatase and the resulting mononucleosides were analysed by HPLC (Figure 6). As E. coli does not naturally possess any tRNA (m$^1$A) MTase, no m$^1$A could be detected in tRNA from the control strain. On the other hand, a small amount of m$^1$G could be detected, consistent with the presence of a tRNA m$^1$G37 activity in E. coli. When we then analysed the HPLC profile of the tRNA from the
recombinant strain overexpressing TK0422p, m^1A could clearly be identified (Figure 6), and more m^1G was present in this tRNA preparation compared to the control strain. This result shows that TK0422p can act as a tRNA m^1A and m^1G MTase in E. coli.

**DISCUSSION**

In this work, we have identified and characterised two archaeal orthologues of Trm10p from S. cerevisiae. We have shown that the protein Saci_1677p from the crenarchaeon S. acidocaldarius catalyses N^1 methylation of an adenosine to form m^1A at position 9 of tRNA. Our data also show that Saci_1677p is specific for A9 of tRNA substrates. Interestingly, we have shown that the orthologous protein TK0422p from the euryarchaeon T. kodakaraensis catalyses both m^1A and m^1G formation in tRNA. We have demonstrated that both m^1A and m^1G are formed at position 9 of tRNA. To our knowledge, this is the first description of an MTase with a broadened nucleoside recognition capability. It is interesting to point out that the protonation state of position N^1 of both adenosine and guanosine differs at physiological pH. Indeed, the N^1 atom of adenosine is not protonated at this pH, while the same position of guanosine bears a proton. This difference of protonation state raises questions about the enzymatic mechanism of TK0422p. In this article, we have shown that the pH of the methylation reaction has an influence on the efficacy of m^1A or m^1G formation. Indeed, the relative efficiency of m^1G formation by TK0422p increases with increasing pH, especially at pH values above 9.5 which corresponds to the pKa of N^1 of G (28). This could reflect the need of this enzyme to deprotonate G to be able to catalyse the methyl transfer, although other possibilities are not excluded, such as an influence of pH on tRNA or enzyme structure.

*Figure 4.* TK0422p from T. kodakaraensis methylates atom N^1 of both adenosine and guanosine of *in vitro* transcripts of T. kodakaraensis tRNA^Ala^ and tRNA^Asp^. (A) Nucleotide sequence of *in vitro* transcripts of T. kodakaraensis tRNA^Ala^ and tRNA^Asp^. Position 9 is circled. (B) Autoradiograms of 2D–TLC chromatograms of P1 hydrolysates of [α^32P]ATP- or [α^32P]GTP labelled T. kodakaraensis tRNA^Ala^ transcripts and (C) of T. kodakaraensis tRNA^Asp^ transcripts. Transcripts were incubated in presence of TK0422p for 1 h at 50°C (B3, B4, C3 and C4) or in the absence of enzyme (B1, B2, C1 and C2). Circles of dotted lines show the migration of the four canonical nucleotides used as UV markers. The radioactive nucleotide used to label the transcripts is indicated on the autoradiograms.
analysed in this work. According to structure prediction methods, the catalytic core of the SPOUT domain spans residues 100–260 of TK0422p (and the corresponding positions in the homologous sequences), and includes five β-strands and six α-helices. The N-terminus is conserved only between the archaeal proteins, but not with the eukaryotic protein. Due to uncertain alignments with known SPOUT structures we were unable to generate a confident 3D model of the catalytic domain, nonetheless the mapping of clusters of conserved residues on predicted secondary structures suggests that the active site is composed of nearly invariant (on the level of the whole family) residues D104, Q122 and D206 (numbering for TK0422p). Interestingly, there appears to be no equivalent catalytic residues between the Trm10p family and the bacterial TrmD family of m1G MTases, also members of the SPOUT superfamily that act on the position G37 in tRNA (29). This suggests that the N\(^1\) purine methylation activity has evolved independently at least two times in the SPOUT superfamily. Independent origin of the N\(^1\) purine methylation activity has been also postulated for MTases of the RFM superfamily, i.e. tRNA:m\(^1\)A58 MTase TrmI, tRNA:m\(^1\)A22 MTase TrmK, and 16S rRNA:m\(^1\)A1408 MTase KamB, which also present different active sites despite the common fold of the catalytic domain (30,31). This variety of protein folds and active sites available to carry out the same type of reaction

Table 1. pH dependence of m\(^1\)A and m\(^1\)G formation by TK0422p from T. kodakaraensis

| pH  | m\(^1\)G/m\(^1\)A ratio |
|-----|------------------------|
| 4.8 | 0                      |
| 5.5 | 0.06                   |
| 7   | 0.42                   |
| 9   | 0.18                   |
| 9.75| 1.43                   |

The MTase activity was measured using total (bulk) E. coli tRNA as substrate. tRNA was incubated in presence of [methyl-\(^14\)C]AdoMet and purified TK0422p protein. The different buffers are described in 'Materials and Methods' section. After incubation, tRNA was recovered and the formation of m\(^1\)G and m\(^1\)A was measured as described in 'Materials and Methods' section.
Figure 6. *Thermococcus kodakaraensis* TK0422p MTase displays tRNA (m1A and m1G) MTase activity in *E. coli* in vivo. tRNA from the *E. coli* strain expressing (C) or not (B) TK0422p were extracted and hydrolysed by nuclease P1, snake venom phosphodiesterase and alkaline phosphatase, and then analysed by HPLC. The peaks corresponding to m1A and m1G are indicated by arrows. A standard curve with the reference nucleosides is given in (A).

Figure 7. Alignment of three experimentally characterised members of the Trm10p family, including the eukaryotic founding member of this family and two archaeal proteins analysed in this work. Residues that are conserved and physicochemically similar in >50% of the sequences are indicated by black and grey shading, respectively. Secondary structures predicted for TK0422p are indicated as E (extended) and H (helix).
suggests that N1 purine methylation is probably relatively easy for an enzyme to evolve (e.g. from an MTase of a different specificity).

Unfortunately, we were unable to identify a negatively charged residue conserved between TK0422p (m1G and m1A MTase) and Trm10p (m7G MTase) but absent from Saci_1677p (m7A MTase) that could explain the inability of the latter protein to deprotonate and methylate guanosine. Therefore, the molecular basis of the different specificities in the Trm10p family remains a mystery. This problem may be approached in the future by experimental determination of a high-resolution structure for one of the family members, characterisation of specificities of numerous additional members of the family, or by mutagenesis of individual members. In particular the availability of a crystal structure for a Trm10p family member would enable its comparison with the known structures of evolutionarily related TrmD MTases and would help to evaluate the hypothesis of convergent evolution of their active sites for an analogous reaction, upon homologous protein scaffold.

Interestingly, the distribution of Trm10p orthologues in Archaea is restricted to hyperthermophiles. We found single representatives of this family in Crenarchaeota (S. acidocaldarius, S. tokodaii, S. solfataricus, S. islandicus, Hyperthermus butylicus, Ignicoccus hospitalis, Aeropyrum pernix, Pyrococcus aerophilum, P. arsenaticum, P. caldifontis, P. islandicus, Thermoproteus neutrophilus, Caldivirga maquilingensis, Metallosphaera sedula) and in Euryarchaeota (T. kodakaraensis, T. gammatolerans, T. onnurineus, T. barophilus, T. sibiricus, T. sp. AM4, Archaeoglobus fulgidus, Methanopyrus kandleri, Pyrococcus furiosus, P. abyssi and P. horikoshii), as well as in Nanoarchaeum equitans. On the other hand, Trm10p is absent from the mesophilic archaeon Halobacterium volcanii, which is known to lack m1A or mG at position 9 (32). This suggests that mG or mA modification may contribute to the thermostability of archaeal tRNAs.

A stabilizing function of base methylation has been recently identified for the m1G46 modification in a thermophilic bacterium T. thermophilus (33). In tRNA crystal structures the base A9 is stacked between bases 45 and 46. Modelling of the S. acidocaldarius tRNA with and without modifications (data not shown) suggests that the methyl group of m1G46 would be involved in stacking interactions with the neighbouring phosphate groups of residues 22 and 23. This could increase the surface of interaction between this residue and G46, which may contribute to the increased stability of the tRNA molecule. Furthermore, the positive charge on mG may have a stabilisation function due to electrostatic interactions with the phosphate groups of residues 22 and 23. Thus, it is tempting to speculate that m1A9 may fulfill a similar stabilisatory role in thermophilic Archaea as m1G46 does in thermophilic bacteria.

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