Structural and functional insights into *Archaeoglobus fulgidus* m\(^2\)G\(_{10}\) tRNA methyltransferase Trm11 and its Trm112 activator

Can Wang\(^1\), Nhan van Tran\(^1\), Vincent Jactel\(^2\), Vincent Guérineau\(^3\) and Marc Graille\(^{\circ1,\ast}\)

\(^1\)Laboratoire de Biologie Structurale de la Cellule (BIOC), CNRS, Ecole polytechnique, Institut Polytechnique de Paris, F-91128 Palaiseau, France; \(^2\)Laboratoire de Synthèse Organique (LSO), CNRS, Ecole polytechnique, ENSTA, Institut Polytechnique de Paris, F-91128 Palaiseau, France and \(^3\)Université Paris-Saclay, CNRS, Institut de Chimie des Substances Naturelles, UPR 2301, 91198, Gif-sur-Yvette, France

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ABSTRACT

tRNAs play a central role during the translation process and are heavily post-transcriptionally modified to ensure optimal and faithful mRNA decoding. These epitranscriptomics marks are added by largely conserved proteins and defects in the function of some of these enzymes are responsible for neurodevelopmental disorders and cancers. Here, we focus on the Trm11 enzyme, which forms N\(^2\)-methylguanosine (m\(^2\)G) at position 10 of several tRNAs in both archaea and eukaryotes. While eukaryotic Trm11 enzyme is only active as a complex with Trm112, an allosteric activator of methyltransferases modifying factors (RNAs and proteins) involved in mRNA translation, former studies have shown that some archaeal Trm11 proteins are active on their own. As these studies were performed on Trm11 enzymes originating from archaeal organisms lacking TRM112 gene, we have characterized Trm11 (Aafftm11) from the *Archaeoglobus fulgidus* archaeon, which genome encodes for a Trm112 protein (Aafftm112). We show that Aafftm11 interacts directly with Aafftm112 similarly to eukaryotic enzymes and that although Aafftm11 is active as a single protein, its enzymatic activity is strongly enhanced by Aafftm112. We finally describe the first crystal structures of the Aafftm11-Aafftm112 complex and of Trm11, alone or bound to the methyltransferase inhibitor sinefungin.

INTRODUCTION

Transfer RNAs (tRNAs) are adaptor molecules involved in the translation process of the DNA sequence of a gene into the protein it encodes. They decode the mRNA codon present in the ribosomal A-site and bring the corresponding amino acid into the ribosomal peptidyl transferase center during protein synthesis. Thus, tRNAs directly participate to the accuracy and fidelity of mRNA translation but it is increasingly evident that they also contribute to correct folding of nascent polypeptides by tuning translational speed (1–3). These roles are achieved thanks to the presence of various chemical modifications decorating tRNAs either on bases or on riboses (4,5). Some of these modifications are known to stabilize the tRNA structure, to participate in tRNA binding to the ribosomal A-site or to be important for tRNA recognition by cognate aminocyl tRNA synthetases (4,6,7). Others are present in the tRNA anticodon stem loop and are important for decoding, allowing for instance a single tRNA to recognize several codons (5,8). Although many of the enzymes catalyzing these tRNA modifications are conserved throughout evolution between either two or three domains of life, the importance of these modifications for many organisms emerged only recently. Indeed, growing studies highlight the influence of these tRNA modifications in cell development, protein homeostasis and physiopathologies such as diabetes, cancers and neurological disorders (9–12).

In eukaryotes, several tRNA modifications are written by multi-protein complexes (13). This is indeed the case of the KEOPS complex responsible for the deposition of t\(^6\)A (N\(^6\)-threonylcarbamoyladenosine) at position 37 of tRNAs decoding ANN codons (14) and of the Elongator complex (formed by the Elp1–6 proteins), which catalyzes the formation of cm\(^5\)U (5-carboxymethyluridine) at the wobble position of several tRNAs (15). Interestingly, the human TRMT112 protein (Trm112 in yeast) acts as an allosteric regulator of several S-adenosyl-l-methionine (SAM) dependent MTases, which modify tRNAs, rRNAs or protein factors directly contributing to the protein synthesis process (16). As complexes with WBSCR22 (Bud23 in yeast) and METTL5 (no orthologue exists in *Saccharomyces cerevisiae* yeast), TRMT112 contributes to the biogenesis of

\(^{\circ}\)To whom correspondence should be addressed. Tel : +33 1 69 33 48 90; Email: marc.graille@polytechnique.edu

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the 40S ribosomal subunit by respectively catalyzing the formation of \( N^2\)-methylguanosine (m\(^2\)G\(_{1636}\) in human and m\(^2\)G\(_{1575}\) in yeast) and N\(^6\)-methyladenosine (m\(^6\)A\(_{1832}\) in human) on 18S tRNA (17–21). The TRMT112-HEMK2 complex (Trm112-Mtq2 in yeast) modifies the glutamine side chain of the universally conserved GGQ motif (for Gly-Gly-Gln) found in class I eRF1 release factors, which enter into the peptidyl transferase center to catalyze the release of newly synthesized proteins from ribosomes (22, 23). TRMT112 also contributes to translation elongation by interacting with and activating two tRNA MTases: ALKBH8 and TRMT11 (respectively, Trm9 and Trm11 in S. cerevisiae). The TRMT112–ALKBH8 complex catalyzes the formation of mcm\(^2\)U (5-methoxycarbonylmethyluridine) from cm\(^2\)U, itself formed by the Elongator complex, at position 34 of some tRNAs (24–27). In humans and other metazoans, the TRMT112-ALKBH8 complex further hydroxylates mcm\(^2\)U to form (S)-mcm\(^2\)U (5-methoxycarbonylhydroxymethyluridine; (28, 29)). The yeast Trm11–Trm112 complex (and most probably the human TRMT11–TRMT112 complex, although this has not yet been experimentally determined) methylates some tRNAs at position G\(_{10}\) of the aminoacyl acceptor stem loop to generate m\(^2\)G\(_{10}\) (N\(^2\)-methylguanosine; (30, 31)). Finally, TRMT112 interacts with TRMT9B, a second orthologue of Trm9, whose substrate and biochemical function have not yet been identified (32). Altogether, while S. cerevisiae Trm112 interacts with 4 MTases, human TRMT112 interacts with at least six MTases. Structural studies of Trm112- MTase complexes (only the Trm11–Trm112 complex has been resistant to X-ray crystallography studies so far) have shown that all these MTases compete to interact in a very similar way with Trm112 (18, 20, 23, 27, 31). Cell biology and biochemical approaches have further revealed that in the case of many of these MTases, their interaction with Trm112 was mandatory for their cellular stability and their ability to interact with SAM, but also contributes to sub- strate binding (19, 20, 23, 31, 33–35).

Recently, we have shown that the Trm112 orthologue (HvOTrm112) from Haloferax volcanii, the archaeal model organism, interacts with an even larger number of MTases, including orthologues of eukaryotic HEMK2/Mtq2, METTL5, ALKBH8/Trm9 and probably TRMT11/Trm11 (20, 36). The presence of a protein annotated as Trm11 (also known as TrmG\(_{10}\) in archaea) among the most enriched putative partners of HvOTrm112 was rather unexpected considering former studies conducted on some archaeal Trm11 (37, 38). Indeed, the enzymatic activities of Trm11 from P. abyssi and Thermococcus kodakaren- sis archaea have been characterized, revealing that in these organisms, Trm11 is active on its own and is able to catalyze formation of m\(^2\)G\(_{10}\) but also of N\(^2\)-2,2dimethylguanosine (m\(^2\)G\(_{10}\); (37–39)) on different tRNAs. Such modifications have also been identified at this position in H. volcanii arch- eal tRNAs (40). Trm11 proteins are composed of two essential domains (37, 38, 41): an N-terminal THUMP domain, which has been shown to interact with the aminoacyl acceptor arm of the tRNAs (42), fused to a C-terminal MTase domain. Trm11 proteins are ubiquitously found in eukaryotes and archaea but not bacteria, strongly arguing in favor of an important physiological role in these two do- mains of life, where most factors participating in mRNA translation are very similar (43). This was recently confirmed in T. kodakaren- sis where the TRM11 gene and hence m\(^2\)G\(_{10}\) and m\(^2\)G\(_{10}\) tRNA modifications, proved very important for growth at high temperature (93–95°C; (44, 45)), probably due to the contribution of these modifications to correct tRNA folding.

Our former bioinformatics analysis revealed that Trm112 archaeal orthologues are absent in Thermococcales (to which T. kodakaren- sis and P. abyssi belong) and Methanobacteria, rationalizing that Trm11 proteins from these organisms are active on their own (16). The presence of putative Trm11 within the list of proteins co-purifying with Trm112 in H. volcanii (36) led us to investigate whether in archaea possessing Trm112 proteins, this latter interacts with and activates Trm11-like proteins as previously shown for budding yeast proteins (31). Here, we structurally and functionally characterize a gene product from the Archaeoglobus fulgidus archaeon as a tRNA methyltransferase and demonstrate that it interacts directly with Trm112 protein (hereafter named AfTrm112). We further show that AfTrm112 enhances its enzymatic activity.

**MATERIALS AND METHODS**

**Heterologous expression and purification of A. fulgidus proteins**

A DNA sequence optimized for heterologous co-expression in E. coli was designed to produce A. fulgidus AF_RS01245 (AfTrm112) and AF_1257 (with a C-terminal His\(_{9}\)-tag) gene products. This fragment was obtained by de novo synthesis (Integrated DNA Technologies, Belgium) and cloned into pET21-a between NdeI and XhoI sites to yield plasmid pMG836. To express the AF_1257 protein alone, a DNA fragment encoding a C-terminally His-tagged version of AF_1257 was inserted into a BamHI/XhoI-digested vector pET21a to generate plasmid pMG964. A second plasmid (pMG979) expressing AF_1257 was obtained by inserting the polymerase chain reaction (PCR) product generated using primers oMG682/oMG683 and pMG836 as template, into pET21a between NdeI and XhoI sites. Oligonucleotides oMG676/oMG677 were used to generate the pMG989 plasmid encoding for the AF_1257-D248A mutant and the AfTrm112 proteins by site directed mutagenesis using pMG836 as template (46). Details on plasmids and oligonucleotides are listed in Supplementary Table S1.

The AfTrm112 and AF_1257 proteins were co-expressed in E. coli BL21 (DE3) Gold (Agilent technologies) using plasmid pMG836. Large-scale expression was done in 1 L of auto-inducible terrific broth media (ForMedium AIMTB0260) supplemented with ampicillin (100 µg/ml), first at 37°C for 3 h and then at 18°C overnight. The cells were harvested by centrifugation at 4000 rpm for 30 min and the pellets were resuspended in 30 ml of lysis buffer (0.3 M NaCl, 50 mM Tris-HCl pH 7.5, 5 mM β-mercaptoethanol, 10 µM ZnCl\(_2\) and 10 mM Imidazole). The cells were lysed by sonication on ice and the lysate clearance was performed by centrifugation at 20 000 g for 45 min. The supernatant was incubated with 16 µg of Benzonase at 4°C for 20 min and then heated at 62°C for 30 min. Precipitated proteins were harvested by centrifugation at 20 000 g for 45 min.
were removed by centrifugation at 20 000 g for 30 min. The supernatant was applied on Ni-NTA resin pre-equilibrated with the lysis buffer, incubated at 4 °C on a rotating wheel for 1 h, followed by a washing step with 30 mL of washing buffer (2 M NaCl, 50 mM Tris–HCl pH 7.5, 5 mM β-mercaptoethanol, 10 μM ZnCl2 and 20 mM imidazole). The AF_1257-/Trm112 complex was eluted by 15 mL of elution buffer (0.3 M NaCl, 50 mM Tris–HCl pH 7.5, 5 mM β-mercaptoethanol, 10 μM ZnCl2 and 350 mM imidazole), followed by concentrating up to 1 mL by a 10 kDa cutoff concentrator. The protein sample was then diluted to 5 mL using buffer A (50 mM NaCl, 20 mM Tris–HCl pH 7.5, 5 mM β-mercaptoethanol, 10 μM ZnCl2) and injected on an ion-exchange chromatography Mono Q column. The protein complex was eluted by a NaCl linear gradient from 50 mM (buffer A) to 1 M (buffer B: 1 M NaCl, 20 mM Tris–HCl pH 7.5, 5 mM β-mercaptoethanol, 10 μM ZnCl2). The fractions containing AF_1257-/Trm112 were collected and concentrated up to 5 mL, followed by injection on a Superdex 75-16/100 Increase 10 kDa concentrators (MCWO YM-10) and centrifuged at 16 000 g at 4 °C for at least 10 min. The pellets were then resuspended by 20 μL of 10 mM Tris–HCl pH 7.5. For digestion, 20 μg of in vitro modified tRNA_{Met} were first denatured by heating at 95 °C for 5 min and then quickly transferred into ice for 5 min. Next, tRNAs were incubated with 4 U of nuclease P1 (SIGMA: #N8630–1VL) and 4.4 μL of 100 mM ammonium acetate pH 5.3 at 42 °C for 2 h. 0.02 U of phosphodiesterase (SIGMA: #P3243-1VL) and 4 μL of 1 M ammonium bicarbonate were then added and the reaction was incubated at 37 °C for 1 h. Finally, 2 U of alkaline phosphatase (SIGMA: #P4252-100UN) were added prior to incubation at 37 °C for 1 h. The digested tRNAs were transferred to rinsed Microcon concentrators (MCWO YM-10) and centrifuged at 16 000 g at 4 °C for at least 10 min. The filtrates were stored at −20 °C for further analysis. These reactions were performed in triplicates.

Nucleosides were analyzed by HPLC–MS using an Agilent Technologies 1260 Infinity liquid chromatography (LC) coupled to an Agilent Technologies 6100 Series G6120B electrospray mass spectrometer. Nucleotides were separated on a C18 column (Hypersil GOLD aQ, Thermo Scientific, 3 μm, 150 mm × 2.1 mm) at 36°C and a 0.4 mL/min flow rate as described in (50). Before each run, the column was equilibrated for 30 min with 100% of 0.1% aqueous formic acid. The elution from the HPLC column was continuously analyzed by electrospray ionisation (ESI) mass spectrometry (gas temperature of 350°C, gas flow of 12 l/min, nebulizer gas of 1.5 l/min and capillary voltage of 3000 V). During separation, the ionized adducts of nucleosides were detected by either a positive or negative polarity mode over the m/z range of 100–700. The m^2G and m^1G nucleoside standards were obtained respectively from SIGMA (#M4004) and Berry & Associates (#PR3702).

MALDI-TOF MS-MS to map modifications on tRNAs tRNA_{Met} (15 μg corresponding to 625 pmol) alone or mixed with equimolar quantities of either AF_1257 or AF_1257-/Trm112 complex were incubated overnight in MTase buffer containing equimolar quantities of SAM at 65°C. Modified tRNAs were separated from the reaction system by precipitation with 2 volumes of 100% cold
ethanol. Prior to digestion, in vitro modified tRNA\textsubscript{Met} and RNase A were both washed in 100 mM ammonium acetate solution through several cycles of concentration using Vivaspin 500 (MWCO 500; Sartorius). Next, modified tRNAs (15 µg) were incubated with 10 µg of RNase A at 37°C for 2 h. The samples were then concentrated around 10 µl. One microliter of digest was mixed with 9 µl of 3-hydroxypropionic acid (3-HPA as the matrix for MALDI-TOF analyses; 40 mg/ml in water:acetonitrile 50:50) and 1 µl of the mixture was spotted on the MALDI plate and air-dried (‘dried droplet’ method). 3-HPA (Sigma-Aldrich Co) was of the highest grade available and used without further purification. MALDI-TOF MS and MALDI-TOF/TOF MS/MS analyses were performed using an UltraflexTreme mass spectrometer (Bruker Daltonics, Bremen). Acquisitions were performed in reflector positive ion mode. The laser intensity was set just above the ion generation threshold to obtain peaks with the highest possible signal-to-noise (S/N) ratio without significant peak broadening. For MS/MS experiments, argon was used as collision gas. All data were processed using the program FlexAnalysis (Bruker Daltonics, Bremen). Acquisitions were performed in positive ion mode. These analyses were performed in triplicates.

Crystallization and structure determination

Crystals of the AF\textsubscript{1257}-Trm112 complex were obtained at 24°C by mixing equal volume of protein complex (20 mg/ml) and crystallization condition (1.6 M ammonium sulfate, 0.1M MES pH 6.5; 10% 1,4-dioxane). Prior to data collection, these crystals were transferred into the crystallization solution supplemented first with 15% and then 30% (v/v) ethylene glycerol, followed by flash-freezing in liquid nitrogen. Crystals diffracted up to 3 Å resolution at Synchrotron SOLEIL (Saint-Aubin, France) on beamline Proxima-1A at 100 K. To solve the structure of this complex, we took advantage of the presence of a zinc atom bound to Trm112 zinc-binding domain and collected three datasets around the zinc anomalous edge for Zn-MAD phasing. These datasets were processed with the XDS program and scaled with XSCALE (51). Due to the presence of two copies of the AF\textsubscript{1257}-Trm112 complex in the asymmetric unit, the locations of two zinc atoms found using the SHELXD program (52) were used to obtain experimental phases with SHARP program (53). Density modification and NCS averaging was further performed using the RESOLVE program (54) to improve the quality of the electron density map. The model for the AF\textsubscript{1257}-Trm112 complex was then built in the experimental electron density maps using COOT (55).

In parallel, we solved the structures of AF\textsubscript{1257}-Trm112 bound to sinefungin (SFG; SIGMA: #S8559), a well-known inhibitor of SAM-dependent MTases and of AF\textsubscript{1257} alone or bound to SFG from crystals obtained from experiments aimed at crystallizing the AF\textsubscript{1257}-Trm112-tRNA and AF\textsubscript{1257}-tRNA complexes. Those tRNA–protein complexes were reconstituted by mixing equal amount of each component and then purification by size-exclusion chromatography on a S75-16/60 column (GE Healthcare) using a specific buffer (25 mM Tris–HCl pH 7.5, 50 µM EDTA, 5 mM MgCl\textsubscript{2}, 5 mM NH\textsubscript{4}Cl). Co-crystals of AF\textsubscript{1257}-Trm112-SFG complex were obtained at 24°C by mixing reconstituted AF\textsubscript{1257}-Trm112–E. coli tRNA\textsubscript{Met} complex (20 mg/ml) with 10-fold molar excess of SFG and equal volume of crystallization solution (containing 0.1 M sodium citrate pH 6; 9% PEG 4000, 0.1 M ammonium sulfate). Crystals of AF\textsubscript{1257} were obtained by mixing AF\textsubscript{1257}–E. coli tRNA\textsubscript{Met} complex (20 mg/ml) with 0.1 M sodium citrate pH 6, 18% PEG 4000, 20% isopropanol for the apo-form or with 0.1 M HEPES pH 7, 0.8 M Na/K Tartrate for the SFG-bound form. All crystals were cryo-protected by transfer into crystallization solution supplemented first with 15% and then 30% (v/v) ethylene glycerol, followed by flash freezing in liquid nitrogen. All datasets were collected at synchrotron SOLEIL (Saint-Aubin, France) on beam-line Proxima-2A at 100 K, and processed as described above for the AF\textsubscript{1257}-Trm112 complex. These structures were solved by molecular replacement with the PHASER program (56) and using the structures of AF\textsubscript{1257} and eventually Trm112 as templates. Several cycles of structure building and refinement were accomplished by COOT and BUSTER (57) programs. Statistics for datasets processing and structure refinement are summarized in Table 1.

ITC experiments

ITC experiments were performed at 50°C using an ITC-200 microcalorimeter (MicroCal). Proteins and SAM were prepared in buffer B. For all titration experiments, 20 injections of 2 µl of SAM (500 µM) were added to the target (either AF\textsubscript{1257} or AF\textsubscript{1257}-Trm112 at 50 µM) at intervals of 180 s. The heat of dilution of the titrant was determined from the peaks measured after full saturation of the target by the titrant. A theoretical curve assuming a one-binding site model calculated with the ORIGIN® software gave the best fit to the experimental data. This software uses the relationship between the heat generated by each injection and \(\Delta H\) (enthalpy change), \(K_a\) (association binding constant), \(n\) (the number of binding site per monomer), the total target concentration, and the free and total titrant concentrations.

Thermal shift assay (TSA)

TSA experiments were performed using a CFX96 real-time PCR machine (BioRad). AF\textsubscript{1257} or AF\textsubscript{1257}-Trm112 (25 µg each) were incubated in different buffers (MTase buffer or 16 mM Tris-HCl pH 7.5; 4 mM \(\beta\)-mercaptoethanol, 8 µM ZnCl\textsubscript{2} supplemented with either 0.4 M NaCl or 0.8 M NaCl) in the presence of SYPRO-Orange dye (5×; Invitrogen, #S-6651). After a brief incubation at 20°C (30 sec), the temperature was linearly increased from 20 to 95°C by steps of 0.5°C/s. The Tm values were determined as the inflexion point of the curve depicting the resulting fluorescence intensity as a function of temperature.

RESULTS

Archaeal Trm112 interacts with AF\textsubscript{1257} and enhances its enzymatic activity

In S. cerevisiae, Trm11 catalyzes the formation of N\textsuperscript{2}-monomethylguanosine (m\textsuperscript{2}G\textsubscript{10}) in tRNAs but requires the
assistance of Trm112 (30,31). Unlike yeast Trm11, archaeal TrmG10 enzymes from P. abyssi and T. kodakarenensis (hereafter named PabTrm11 and TkoTrm11, respectively) add both m2G10 and N2,2-dimethylguanosine (m22G10) modifications to tRNAs and are active on their own (37–39). As no gene encoding for a Trm112 orthologue has been detected in P. abyssi or T. kodakarenensis, we have investigated whether when present in an archaeal organism, the Trm112 protein interacts with Trm11 and influences its enzymatic activity. First evidence for an interaction between these two proteins in archaea came from our former investigation showing that Trm11 is one of the most potent partners of H. volcanii Trm112 (36). However, it was not possible to experimentally validate the interaction between these two proteins as H. volcanii Trm11 could not be expressed as a soluble protein alone or upon co-expression with Trm112. We then decided to focus on orthologous proteins from the A. fulgidus archaean. We selected the AF_1257 and AF_RS01245 genes, which encode for proteins sharing 37% and 50% sequence identities with H. volcanii Trm11 and Trm112, respectively. These candidate genes are then very likely to encode for A. fulgidus Trm11 and Trm112 (hereafter AF Trm112) orthologues.

We expressed a His-tagged version of AF_1257 either alone or in the presence of an untagged version of AF Trm112 in E. coli. Interestingly, in both conditions, AF_1257 was expressed as a soluble protein and was retained on NiNTA resin (Figure 1A). This allowed us to further purify AF_1257 and most interestingly, to detect a specific band migrating around 6.8 kDa and co-purifying with AF_1257 only when AF Trm112 was co-expressed with the latter (Figure 1A). The protein corresponding to this band was identified as AF Trm112 by mass spectrometry. Besides, AF_1257 alone was not as stable as when in complex with AF Trm112. Indeed, it had a strong tendency to precipitation at low salt concentration (50–100 mM) while the complex did not. Furthermore, we determined the melting temperature of AF_1257 alone (from 72.8 to 77.1°C in both conditions) revealing that AF_1257 and AF Trm112 form a tight heterodimer of 42.3 kDa in solution (the theoretical molecular weight of this heterodimeric complex is 44.3 kDa) while AF_1257 alone is monomeric in solution (35.8 kDa; Figure 1B). Altogether, these results demonstrate that AF Trm112 interacts physically and stably with AF_1257 and stabilizes the latter, similarly to the effect of S. cerevisiae Trm112 on Trm11 (31).

In order to investigate the biochemical function of AF_1257 alone or in complex with AF Trm112 as tRNA methyltransferase, E. coli tRNA\textsubscript{Met} was used as substrate for \textit{in vitro} enzymatic assays. This tRNA, which contains a G at position 10, was over-expressed in E. coli and due to the absence of the m2G\textsubscript{10} or m22G\textsubscript{10} modifications in bacteria, it should be an appropriate substrate. The enzymatic activity was quantified by measuring the incorporation of radioactivity from [3H]-SAM into tRNAs upon methylation. The AF_1257 protein was able to methylate tRNA by itself...
AF1257 does not require A/Ftm112 to be expressed as a soluble protein in E. coli. AF1257 protein was expressed alone or together with A/Ftm112 in E. coli and subjected to micro-purification on NiNTA-agarose beads. Coomassie Blue stained SDS-PAGE analyses of proteins present in the total (T), soluble (S) and elution (E) fractions. A diffuse band below 10 kDa and corresponding to AF1257 (according to mass spectrometry) is only visible when AF1257 is expressed with A/Ftm112. (B). The AF1257 protein physically interacts with A/Ftm112. Chromatograms resulting from SEC-MALLS analysis of the AF1257 protein (grey) and AF1257-A/Ftm112 complex (black). For the sake of clarity, only the main peak with the refractive index (solid lines, left y-axis) and the distribution of molecular mass calculated from light scattering along this peak (dashed lines, right y-axis) are shown. (C). Enzymatic assay of AF1257 with or without A/Ftm112 on E. coli tRNAiMet in vitro. For each reaction (performed in triplicate), the corresponding proteins (3 pmol) were incubated at 65°C for 2 h with 75 pmol of tRNA in 50 µl of reaction system. (D). HPLC–MS elution profiles depicting the absorbance at 254 nm (left Y axis, solid lines) and the absolute intensity of m/z values of 298+ (corresponding to m2G protonated ion; right Y axis, dotted lines) and 312+ (corresponding to m22G protonated ion; right Y axis, dashed lines) of the nucleosides digested from E. coli tRNAiMet incubated in different conditions. but its activity was around 5 times lower than that of the AF1257-A/Ftm112 complex after 2 h of reaction (Figure 1C). As already observed for several enzymes from hyperthermophilic organisms, the enzymatic activity was higher at 65°C than at 30°C (data not shown). Furthermore, the substitution by Ala of AF1257 Asp248, which is located in a highly conserved signature known to be involved in recognition of the modified nucleotide, results in complete loss of enzymatic activity as previously observed for the corresponding D291A mutant from S. cerevisiae Trm11 (31). Both observations confirm that this enzymatic activity derives from AF1257 and not from an E. coli tRNA MTase contaminant.

AF1257 is an m2G10 tRNA MTase

To determine the molecular function of AF1257, E. coli tRNA10Met was incubated with either AF1257 or the AF1257-A/Ftm112 complex and hydrolyzed into nucleosides prior to HPLC–MS analyses. UV absorbance at 254 nm was recorded and specific ions were tracked by MS (m/z 298° and 312° for m2G and m22G protonated species, respectively). Compared to the negative control (tRNA alone), the tRNAs incubated with either AF1257 or the AF1257-A/Ftm112 complex exhibited a significant absorption peak in the 254 nm UV spectrum with a retention time between 12 and 12.5 min, which coincided with a strong signal at the m/z value expected for m2G (Fig-
The identity of this peak as m2G was further confirmed by a control experiment with digested unmodified tRNA sample spiked with commercial m2G (Figure 1D). Although PabTrm11 and TkoTrm11 were also shown to catalyze formation of m2G at position 10 of some tRNAs (37,38), we did not detect any signal for m2G (elution time around 16 to 17 min; Figure 1D). These experiments indicate that both Af1257 and the Af1257-AfTrm112 complex catalyze the formation of m2G but not m2G on E. coli tRNA_Met in vitro.

Next, due to (i) the presence of Trm14, an m2G6 tRNA MTase sharing the same domain organization as Trm11 in several archaea (58–60) and (ii) the presence of a G at position 6 in E. coli tRNA_Met (Figure 2A), we mapped the m2G modification on this tRNA to clarify whether AF1257 is an m2G6 or an m2G10 tRNA MTase. Control and modified tRNA_Met were first digested by RNAse A (which cleaves after pyrimidines) into oligonucleotides (Figure 2A), which were then analyzed by MALDI-TOF MS. The comparison of the spectra obtained for the samples incubated with either AF1257 or the AF1257-AfTrm112 complex to the spectrum of the control tRNA revealed a decrease in the intensity of the RNA fragment at m/z 1688.1 (unmodified oligonucleotide GGAGC13) concomitantly with the appearance of the m/z value 1702.1 (Figure 2B). This indicates that the methyl group (+14 a.m.u shift) is added to the GGAGC13 oligonucleotide. In contrast, the fragment at m/z 1721.1 corresponding to the GGAGGsU6 oligonucleotide remained unchanged in all these experiments, ruling out methylation at position 6 (Figure 2B). Additional fragmentation by MS/MS further revealed that the c2 (m/z 540.2) and d2 (m/z 558.2) ions lacking one base (either G9 or G10) also existed as a methylated species upon incubation with AF1257 or AF1257-AfTrm112 complex, indicating that the methyl group is added either on the G9 or G10 ring (Figure 2C-D). As no methylated form of a1 (m/z 266.1) or c1 (m/z 346.1) ions (corresponding to G9) were observed in tRNAs incubated with the enzymes, we conclude that the methylated nucleotide maps to position 10 (Figure 2C and E).

Altogether, these results demonstrate that AF1257 (hereafter named AfTrm11) catalyzes the formation of m2G10 on E. coli tRNA_Met and hence, confirm that this protein is the orthologue of the archaeal PabTrm11 and TkoTrm11 enzymes but also of S. cerevisiae Trm11.

The apo-, SFG- and Trm112-bound structures of AfTrm11

To obtain information on AfTrm11 and on the influence of AfTrm112 on its enzymatic activity, we determined the crystal structure of AfTrm11 in three different forms and at various resolutions, i.e. alone (2.75 Å resolution; one AfTrm11 copy in the asymmetric unit), bound to sinefungin (SFG; 1.9 Å; two AfTrm11 copies in the asymmetric unit) and as a complex with AfTrm112 and SFG (2.2 Å; one copy in the asymmetric unit; Table 1). The structure of the AfTrm11–AfTrm112 complex was solved using the multi-wavelength anomalous diffusion technique thanks to the anomalous signal of the zinc atom bound to AfTrm112, as previously done for some eukaryotic Trm11–MTase complexes (23,27). Then, the structure of AfTrm11 as determined in this complex was used to solve its apo- and SFG-bound structures by molecular replacement. All these AfTrm11 structures are virtually identical as revealed by their superimposition (rmsd values of 0.35–0.9 Å).

The AfTrm11 protein is composed of two domains: a N-terminal domain (hereafter named NTD; residues 1–140) separated from a C-terminal MTase domain (residues 154–320) by a short helical linker (residues 141–153; Figure 3A). The NTD domain is made of a curved 7 stranded β-sheet surrounded by three α-helices localized on the outer face of the β-sheet and can be further divided into two subdomains: the NFLD (for N-terminal Ferredoxin-like domain; residues 1–66) and the core THUMP domain (named after THioUridine synthase, MTase and Pseudouridine synthase (61); residues 67–140; Figure 3A and Supplementary Figure S2A). The MTase domain adopts a typical class-I SAM-dependent MTase fold composed of a seven stranded β-sheet surrounded by three α-helices on one side and one on the other side (Figure 3A and Supplementary Figure S2B). This domain harbors the conserved 248DYPY251 motif, which corresponds to the [N/D][S/P][P/I][Y/F/W/H] signature commonly found in MTases modifying planar amino groups from DNA, RNA or proteins (62–64). Overall, this modular organisation is very similar to that of the previously solved TkoTrm11 structure (38).

In the AfTrm11-SFG structure, SFG binds in an unconventional manner within the SAM binding site as revealed by comparison with the structures of TkoTrm11 bound to SAM (38; Figure 3B) and of archaeal P. furiosus Trm14 (59), another tRNA modification enzyme with the same THUMP-MTase modular organisation, bound to SFG. Indeed, while the adenosine rings from SAM and SFG interact in their conventional pocket, the amino acyl group from SFG points in the opposite direction compared to the L-methionine moiety from SAM (Figure 3B). The comparison of AfTrm11 apo- and SFG-bound forms reveals that while the loop containing residues 254–258, which is located just upstream of the 248DYPY251 motif could not be modeled due to intrinsic flexibility in the structure of the AfTrm11 apo-form, this loop is structured in the AfTrm11-SFG complex due to its involvement in crystal packing (Supplementary Figure S3A). Similarly, upon SFG binding, the aromatic ring from Phe206 slightly rotates so as to form a π–π stacking with the adenine base of SFG (Supplementary Figure S3A).

The structure of the AfTrm11–AfTrm112 complex bound to SFG reveals that AfTrm112 is made of a single domain (ZBD for zinc binding domain) composed of an α-helix (α1) packed onto a 4 stranded anti-parallel β-sheet and adopts the same fold as H. volcanii Trm112 (Figure 3A and Supplementary Figure S4, rmsd value of 0.75 Å over 57 Ca atoms and 56% sequence identity; (36)). Similarly to fungal Trm112 proteins, this protein binds one zinc atom through four cysteine residues that are strongly conserved in fungal and to a lesser extent in archaeal Trm112 proteins (Supplementary Figure S4; (18,22,23,27)). In the AfTrm11–AfTrm112 complex, SFG adopts the same conformation as in the structure of AfTrm11. Further comparison of the AfTrm11 and AfTrm11–AfTrm112 SFG-bound forms does not reveal major conformational changes despite the binding of AfTrm112 in the vicinity of the SAM
Figure 2. The AF₁₂₅₇-Af₄Trm112 complex is an m²G₁₀ tRNA methyltransferase. (A) Clover-leaf representation of E. coli tRNAᵢMet with the RNase A cleavage sites of interest for this study shown as red dashed lines. Positions G₆ and G₁₀ are highlighted in blue and red, respectively. Position C₃₂, which is 2'-OH methylated (Cm) in E. coli tRNAᵢMet is highlighted in gray. 4, T and Ψ stand for 4-thiouridine, thymidine and pseudouridine, respectively. (B) MALDI-TOF MS spectrum of 9GGAGC₁₃ oligonucleotide obtained upon digestion with RNase A of E. coli tRNAᵢMet following incubation with either MTase buffer (upper panel), AF₁₂₅₇ (middle panel) or AF₁₂₅₇-Af₄Trm112 (lower panel). (C) Discussed a-, c- and d-type fragment ions for the 9GGAGC₁₃ oligonucleotide upon digestion of the E. coli tRNAᵢMet by RNase A. This nomenclature is based on McLuckey et al. (73). (D) CID Spectrum of the c₂- and d₂-type fragment ions lacking one base observed in E. coli tRNAᵢMet incubated with MTase buffer (upper panel), AF₁₂₅₇ (middle panel) or AF₁₂₅₇-Af₄Trm112 (lower panel). The expected position of methylated ions is depicted by a red asterisk. (E) CID Spectrum of the a₁- and c₁-type fragment ions observed in E. coli tRNAᵢMet incubated with MTase buffer (upper panel), AF₁₂₅₇ (middle panel) or AF₁₂₅₇-Af₄Trm112 (lower panel). The expected position of methylated ions is depicted by a red asterisk.
Figure 3. Structure of the \textit{Af}Trm11-Trm112 complex. (A) Cartoon representation of the \textit{Af}Trm11-Trm112 complex bound to sinefungin (SFG). NFLD, THUMP and MTase domains from \textit{Af}Trm11 are colored in dark blue, blue and light green, respectively. The linker connecting the N-terminal domain (NFLD + THUMP) to the C-terminal MTase domain is colored in gray. \textit{Af}Trm112 is colored in light orange. The zinc atom bound to \textit{Af}Trm112 is depicted as a purple sphere and side chains from the cysteine residues coordinating this zinc atom are shown as sticks. The DYPY signature from the MTase domain is shown as sticks. SFG is colored in magenta. Secondary structure elements from \textit{Af}Trm112 are labeled in italics. With the exception of Figure 4E, all figures illustrating structural aspects have been generated using the Pymol software (74). (B) Sinefungin binds in an unconventional manner. The superimposition of \textit{Af}Trm11 (beige) bound to SFG (magenta) onto \textit{T.kodakarensis} Trm11 (gray; PDB code: 5E72) bound to SAM (grey) reveals that the amino acid moiety of SFG does not bind to the classical pocket involved in the recognition of L-methionine from SAM. The loop corresponding to residues 250–260 from \textit{Af}Trm11 is also not defined in the crystal structure of \textit{T.kodakarensis} Trm11 and is depicted as a black dashed line. Hydrogen bonds are highlighted by dashed black lines. (C) Binding of \textit{Af}Trm112 has no major structural influence on \textit{Af}Trm11 regions involved in SAM recognition or \textit{Af}Trm112 interaction. \textit{Af}Trm11 side chains from the interface adopting different conformations upon \textit{Af}Trm112 binding are shown as sticks. \textit{Af}Trm112 residues facing them are also shown as sticks.

This suggests that \textit{Af}Trm112 does not influence the conformation of the SAM binding site. Next, we compared the affinity of \textit{Af}Trm11 and \textit{Af}Trm11–Trm112 for SAM by ITC experiments at 50°C and found that \textit{Af}Trm112 has little to no influence on the affinity of \textit{Af}Trm11 for SAM, in agreement with our structural observation (Supplementary Figure S5; Table 2). This is in striking contrast with the strict Trm112 requirement of \textit{S.cerevisiae} Trm11 for SAM binding (31).

This first crystal structure of a Trm11-Trm112 complex shows that Trm112 exclusively contacts the MTase domain from Trm11 through an interface area of 900 Å² (Figure 3A and C). This interface relies on the formation of a β-zipper formed by two hydrogen bonds between main chain atoms from strand β3 from the \textit{Af}Trm11 MTase domain and strand β4 from \textit{Af}Trm112 (Supplementary Table S3). A total of 22 amino acids from \textit{Af}Trm11 interact with 17 amino acids from \textit{Af}Trm112, respectively (Supplementary Figure S3B-D). The core of the interface is enriched in hydrophobic residues (Ile202, Val201, Val227, Leu228, Leu229, Leu28, Leu237 and Val242 from \textit{Af}Trm11 and Leu5, Leu9, Ala10, Ile50, Pro51 and Leu53 from \textit{Af}Trm112) while the outer shell is mainly composed of polar residues forming hydrogen bonds as well as three salt bridges (Supplementary Fig-
ure S2, S3B-D and S4; Supplementary Table S3). Furthermore, with the exception of slight side chain rearrangements of A/Trm11 (Supplementary Figure S3E), binding of A/Trm11 has no major consequences on the A/Trm11 structure. Overall, the structure of the Trm11–Trm112 complex, the last Trm112–MTase complex that was resistant to crystallization before this study, shows that this interaction mode is highly reminiscent of previously solved crystal structures of archaeal and eukaryotic Trm112-MTase complexes (18,20,23,27,36,65,66).

From our analyses, A/Trm112 has no structural impact on A/Trm11 or on SAM binding. Therefore, we have characterized the kinetics parameters of A/Trm11 alone or as a complex with A/Trm12 to understand the stimulating effect of A/Trm12 on the A/Trm11 enzymatic activity (Supplementary Figure S5C-F and Table 3). The A/Trm11 protein alone displays stronger affinity (13-fold) for E. coli tRNAiMet but exhibits a lower $k_{cat}$ (almost 7-fold) than the A/Trm11–Trm112 complex. This indicates that A/Trm112 significantly stimulates the catalytic step of the reaction at the expense of tRNA binding. Altogether, combining structural and biochemical characterization, we show that A/Trm12 strongly enhances the enzymatic activity of A/Trm11 by improving its stability (according to the behavior of this protein during purification and to a thermal shift assay) and mostly the catalytic step of the methylation reaction so as to achieve an optimal enzymatic efficiency.

**DISCUSSION**

tRNAs are by far the most post-transcriptionally modified nucleic acids. The biological importance of many of those modifications is becoming increasingly evident for organism development, as mutants affecting the activity of many tRNA modification enzymes are being identified in patients suffering from intellectual disorders or cancers. Most of these enzymes are also conserved between domains of life and in particular between eukaryotes and archaea, emphasizing their functional importance. Here, we have focused on the Trm11 protein from *A. fulgidus* (A/Trm11), an archaeon living in a sulfur-rich environment and whose optimal growth temperature is 83°C. We anticipate that similarly to the *T. kodakarensis*Trm11 gene (44,45), the *AF.1257* gene encoding for A/Trm11 is required for cellular fitness and adaptation at high temperature. We have characterized the enzymatic activity of this protein as an MTase catalyzing the formation of m$^2$G at position 10 of tRNAs (Figures 1D and 2). One major difference between archaeal and eukaryotic Trm11 enzymes is the inability of the latter enzymes to catalyse the formation of m$^2$G$_{10}$ (30,67). In archaea, both m$^2$G$_{10}$ and m$^3$C$_{10}$ have been identified in tRNAs extracted from *Halobacterium salinarum*, *H. volcanii*, *Sulfolobus acidocaldarius* and *T. kodakarensis* (40,45,67,68), while *Pab*Trm11 and *Tko*Trm11 enzymes were shown to catalyse the formation of these two modifications (37,38). In our experimental conditions, coupling LC/MS and MS/MS analyses, we detected m$^2$G$_{10}$ but no m$^3$C$_{10}$ in the *E. coli* tRNA$_{iMet}$ used in our assay upon incubation with the A/Trm11 protein (Figures 1D and 2).

In the absence of information regarding *A. fulgidus* total tRNA modifications, we cannot exclude that tRNAs from this organism exclusively contain the m$^2$G$_{10}$ modification. However, in the case of *Pab*Trm11, the presence of U at position 25 and a four nucleotide long V-loop are required for formation of m$^2$G$_{10}$ in archaeal tRNAs (39). It is then likely that the presence of a C at position 25 (which base pairs with G$_{10}$ in the tRNA structure, Figure 2A) of the *E. coli* tRNA$_{iMet}$ is responsible for the mono-methylation but not dimethylation that we observe with this substrate. We hence do not exclude that this enzyme also catalyzes the formation of m$^3$C$_{10}$ on other tRNA species that have not been tested in our study.

**On the importance of A/Trm112 for A/Trm11 activity**

Here, we demonstrated for the first time that when a Trm112 orthologue is present in the archaeal genome, it interacts directly with Trm11 (Figure 1B), confirming co-immunoprecipitation of *H. volcanii* Trm11 with Trm112 (36). We also showed that Trm112 is important but not mandatory for Trm11 activity. Indeed, similarly to Trm11 enzymes from *P. abyssi* and *T. kodakarensis* archaea (37,38), A/Trm11 was active on its own but its enzymatic activity was significantly enhanced by A/Trm112 (Figure 1C), a well-known allosteric activator of several eukaryotic MTases modifying tRNAs (including Trm11), rRNAs and the translation termination factor eRF1 (16). We observed that A/Trm112 protects A/Trm11 from thermal denaturation by increasing its Tm value (Supplementary Figure S1 and Table S2). Although Tm values measured *in vitro* in a specific buffer imperfectly reflect the stability of a protein in its cellular environment, we anticipate that A/Trm112 might play a crucial role *in vivo* by stabilizing A/Trm11 in the *A. fulgidus* archaeon, whose optimal growth temperature is 83°C. Our detailed analysis aimed at clarifying the role of A/Trm112 in the activation of A/Trm11 reveals that both A/Trm11 and A/Trm11–Trm112 have similar affinities for SAM (Supplementary Figure S5A, B). This correlates with our co-crystal structures of A/Trm11–Trm112 and of A/Trm11, both bound to SFG, a competitive inhibitor of SAM-dependent MTases (Figure 3), which revealed no significant changes in the A/Trm11 SAM binding site upon A/Trm112 binding. In addition, our different crystal structures of A/Trm11 obtained in the absence or the presence of A/Trm112 do not highlight significant changes in the A/Trm11 active site upon A/Trm112 binding. This suggests that the activation role of A/Trm112 is not

| A/Trm11  | $K_d$ (μM) | Stoechiometry (n) | $\Delta H$ (kcal/mol) | $T\Delta S$ (kcal/mol) |
|----------|------------|-------------------|----------------------|----------------------|
| A/Trm11  | 11.4 ± 3.4 | 0.72              | -4.54                | 0.43                 |
| A/Trm11–Trm112 | 8.26 ± 2.43 | 0.83              | -3.77                | 0.58                 |
through the molding of the $Af$Trm11 active site into a form competent for the coordination of G10 (Figure 3C). However, we have clearly shown that $Af$Trm112 significantly enhances the catalytic step of the tRNA methylation reaction at the expense of a decreased affinity for tRNA (Table 3 and Supplementary Figure S5C–F). Hence, contrary to its eukaryotic orthologues, we have no evidence that $Af$Trm112 activation mode relies on an allosteric effect. Several non-exclusive possibilities can explain how $Af$Trm112 activates $Af$Trm11. First, it may contribute to the correct positioning of the substrate in the tRNA binding site so as to orient properly the guanine at position 10 into the enzyme active site. Second, it could help the $Af$Trm11 catalytic subunit to flip out G10 from the tRNA core to increase its accessibility. Indeed, the lower $K_m$ for tRNA measured for $Af$Trm11 ($K_m$ of 88.5 nM) compared to the $Af$Trm11-Trm12 complex ($K_m$ of 1.18 μM) may reflect the formation of a nonproductive enzyme-substrate complex. Unfortunately, despite extensive trials, we could not obtain the crystal structure of $Af$Trm11 or $Af$Trm11-Trm12 bound to a tRNA substrate. Third, our observation that the amount of tRNA modified (2.5 pmol) by $Af$Trm11 is slightly lower than the enzyme quantity (3 pmol) could indicate that in the absence of $Af$Trm12, $Af$Trm11 cannot perform several catalytic cycles and remains tightly bound to its product. Hence, $Af$Trm12 could assist $Af$Trm11 in the release of the reaction product (see below). Finally, another explanation for the stimulating role of $Af$Trm112 on $Af$Trm11 enzymatic activity could be that the latter is much less stable in the absence of $Af$Trm112, $Af$Trm11 cannot perform several catalytic cycles and remains tightly bound to its product. Hence, the guanosine position trapped in this crystal structure is unlikely to be in its correct orientation. Indeed, this structure was obtained by co-crystallizing the enzyme in complex with the corresponding region of $Tko$Trm11 (Supplementary Figure S1 and Table S2; (31)). Indeed, at the temperature (65°C) and buffer condition used in our enzymatic assays, we cannot exclude that in the absence of $Af$Trm12, a significant portion of $Af$Trm11 starts to unfold (Tm of the protein alone is 72.8°C in the MTase buffer), resulting in lower enzymatic activity. Furthermore, the analysis of the $Af$Trm11 surface interacting with $Af$Trm112 reveals the presence of a central area exclusively composed of hydrophobic residues, which is shielded from the solvent upon complex formation with $Af$Trm12 (Supplementary Figure S6, left). In comparison, the corresponding region from $Tko$Trm11 harbors two hydrophobic residues (Thr230 and Lys232) at the heart of this hydrophobic core, thereby probably increasing its solubility and its stability (Supplementary Figure S6, right). Furthermore, the loop connecting strands β3 to β4, which is involved in the interaction between $Af$Trm11 and $Af$Trm12, is longer in $Tko$Trm11, resulting in the presence of a short 3_10 helix that projects Asp241 (Glu241 in $Pab$Trm11) towards the solvent (Supplementary Figures S2B and S6, right). Both observations could explain why $Tko$Trm11 and $Pab$Trm11 proteins do not require a Trm12 orthologue for optimal activity, contrary to $Af$Trm11.

### Table 3. Kinetics parameters

| $Af$Trm11 | $Af$Trm11–Trm12 |
|-----------|-----------------|
| $K_m$ for tRNA (μM) | 0.089 ± 0.013 | 1.18 ± 0.193 |
| $k_{cat}$ for tRNA (min⁻¹) | 0.126 ± 2.25*10⁻³ | 0.883 ± 0.05 |
| $k_{cat}/K_m$ (μM⁻¹.min⁻¹) | 1.42 | 0.75 |

### Implications for tRNA binding

Despite extensive efforts, we could not determine the crystal structure of the complex between $E.$ $coli$ tRNA$_{iMet}$ and either $Af$Trm11 or the $Af$Trm11-Trm12 complex. We then compared our structure of the $Af$Trm11-Trm12 complex to those of two m^2G ($RsmC$) or m^2G$_1$ (TGS1) MTases solved in the presence of a guanosine moiety in their active sites (69,70). Indeed, the structure of human TGS1, an m^2G MTase modifying the m^2G cap present at the 5’ end of small nuclear and small nucleolar RNAs, of the telomerase RNA and of some mRNAs, has been determined in the presence of both m^2GTP and SAH (S-adenosyl-L-homocysteine, the product regenerated upon transfer of the SAM methyl group onto its substrate; (70)). Similarly, the structure of the $T.$ $thermophilus$ $RsmC$ protein, which modifies 16S rRNA on G1207, a nucleotide located near the decoding center in the 30S small ribosomal subunit, to form m^2G1207 (71), has been determined in complex with SAM and guanosine (69). These three enzymes share the [N/D/S]-[P-[P/I]-[Y/F/W/H] active site signature known to coordinate the planar amino group to be modified and their MTase domain are closely related from a structural point of view (rmsd values of 1.5–1.6 Å). Interestingly, in both TGS1 and $RsmC$, the guanosine rings are stacked in a parallel manner onto the aromatic ring of the fourth residue of the active site signature. However, these rings are in orthogonal conformations relative to the [N/D/S]-[P-[P/I]-[Y/F/W/H] signature (Figure 4A, B). In the $RsmC$-SAM-guanosine complex (Figure 4A), the N^2 atom from the guanine ring is 4.7 Å away from the SAM methyl group, which is not compatible with methyl transfer via the SN2 mechanism commonly accepted for this family of MTases. Hence, the guanosine position trapped in this crystal structure is unlikely to be in its correct orientation. Indeed, this structure was obtained by co-crystallizing the enzyme in the presence of both substrates (SAM and guanosine) and hence, if the guanosine ring had been correctly positioned in the active site, one would expect that the methylation would have occurred, resulting in the presence of SAH and m^2G in the active site instead of SAM and guanosine. On the contrary, modeling a SAM molecule in the TGS1-SAH-m^2GTP structure reveals a distance of 3 Å between the SAM methyl group and the N^2 atom from the m^2GTP cap (Figure 4B). Hence, the coordination of the guanine ring as observed in human TGS1 structure is more likely to reflect the correct location of the substrate in the active site of m^2G or m^2G MTases. More detailed comparison of TGS1 and $Af$Trm11 active sites further reveals that the loop preceding helix αZ from $Af$Trm11 might, together with Tyr251 side chain, sandwich the guanine ring as helix αA and Trp766 do in TGS1 (Figure 4B). In addition, the side chain from the strictly conserved Arg313 in $Af$Trm11 is in the same region as Arg807 and Lys836 from TGS1 (Figure 4B, Supplemen-
Figure 4. Implications for tRNA recognition. (A) Comparison of \textit{Af}Trm11 (light green) and RsmC (light pink) active sites. The SAM and guanosine molecules bound to RsmC are shown as pink sticks. In panels A and B, the SAM methyl group to be transferred is shown as a sphere and the distance between this methyl group and the N2 atom to be methylated on guanosine is shown by a dashed black line. (B) Comparison of \textit{Af}Trm11 (light green) and human TGS1 (yellow) active sites. The SAM molecule (magenta sticks) has been modeled by superimposing coordinates of SAM onto those of the SAH molecule bound to TGS1. The m^\text{7}GTP molecule bound to TGS1 is shown as yellow sticks. (C) Sequence conservation mapped at the surface of the \textit{Af}Trm11–Trm112 complex. The conservation score for \textit{Af}Trm11 proteins was calculated using the CONSURF server (75). \textit{Af}Trm112 is colored in beige. Sequence conservation score is represented as a gradient of white (no conservation) to cyan (high conservation) at the surface of \textit{Af}Trm11. The same orientation of the complex is used for panels C to E. (D) Functionally important residues are spread at the surface of \textit{Af}Trm11. Residues important for the enzymatic activity of archaeal Trm11 proteins are mapped in white on THUMP domains and in red wine in the linker region and MTase domain. The NYPY active site signature is colored pink. \textit{Af}Trm11 domains are colored using the same color code as Figure 3A. \textit{Af}Trm112 is colored in beige. (E) A long positively charged groove can accommodate the tRNA. Positively charged (10 kBT/e) and negatively charged (−10 kBT/e) regions are colored in blue and red, respectively. The electrostatic potential was calculated using APBS program as implemented in the Chimera software (76). An aminoacyl acceptor arm (yellow) was modeled by superimposing the structure of the THUMP domain from the 4-thiouridinesynthetase ThiI bound to the aminoacyl acceptor arm of the \textit{E. coli} tRNA^\text{Phe} (PDB code: 4KR6; (42)) onto the structure of \textit{Af}Trm11 THUMP domain.

Further analyses show that the \textit{Af}Trm11 active site, encompassing the 248DYPY251 signature and Arg313, is the most conserved region accessible at the surface of the complex (Figure 4C). \textit{Af}Trm11 residues (Arg148, Arg151, Arg253, Lys256 or Arg306) correspond to \textit{Tko}Trm11 residues that were shown to be important for catalysis and tRNA binding (Lys151, Arg152, Arg266, Lys267, Arg268 and Arg317; Figure 4D; (38)). We then anticipate that those residues are very likely to be important for tRNA binding and in particular to coordinate G10 in \textit{Af}Trm11 active site. A positively charged groove running from the NTD to the active site is very likely to interact with the tRNA backbone. Adjacent to this positively charged region, the surface formed by Cys84, Cys85, Val86, Val112 and Val114 from the \textit{Af}Trm11 THUMP subdomain matches that formed by Phe86, Lys87, Val88, Val118 and Leu120 in \textit{Tko}Trm11, residues that were shown to be important for tRNA recognition (Figure 4D-E). The equivalent regions in the Thi THUMP domain directly interact with the aminoacyl acceptor arm and in particular the CCA motif present at the 3’ end of mature tRNAs. This is in agreement with earlier observations that the enzymatic activity of both eukaryotic and archaean Trm11 proteins is reduced on tRNA sub-
substrates lacking this CCA motif (31,38). Altogether, those observations explain why both domains from PabTrm11 are required for enzymatic activity (41). Very interestingly, A fTrm112 contributes a negatively charged region at the surface of the A fTrm11–Trm112 complex (Figure 4E). As this region is close to the A fTrm11 active site, it is likely to repel the negatively charged tRNA substrate, thereby rationalizing the lower Km of the A fTrm11–Trm112 complex for tRNA compared to A fTrm11 alone (Table 3). This further suggests that one of A fTrm112 functions is to favor product release to allow several catalytic cycles thereby enhancing the k cat of the enzyme (Table 3).

Finally, the structures of four m2G MTases with the same modular organization, i.e. a NTD composed of aNFLD and a THUMP subdomain fused to a MTase domain, are now available: the A fTrm11 and TkoTrm11 m2G/m2,G MTases as well as those of two TrmN/Trm14 m2,Gs MTases from either bacteria (TrmN from Thermus thermophilus) or archaea (Trm14 from P. furiosus; this work and (38,59)). This offers the opportunity to analyse the intrinsic flexibility between these two domains. Interestingly, as observed for the structures of TrmN and Trm14 (overall rmsd values of 0.35–2.0 Å over 286 Ca atoms; (59)), the comparison between several archaeal Trm11 structures (3 for A fTrm11 obtained in different space groups and hence crystal packing as well as one for TkoTrm11) does not reveal striking differences in the orientation of the NTD relative to the MTase domain in Trm11 enzymes (overall rmsd values of 0.5–1.2 Å over 304 Ca atoms), indicating that these two domains most likely behave as a single rigid unit. So, this observation seems to invalidate our former proposal that in the yeast Trm11–Trm112 complex (31), the NTD may move relative to the MTase domain to bring the G10 nucleotide into the Trm11 MTase active site. This new analysis rather suggests that the tRNA may first interact through its aminoacyl acceptor arm with the Trm11 NTD and then kink to bring the region containing G10 into the active site. This might be favored by an incomplete set of modifications within Trm11 tRNA substrates, which may increase their intrinsic flexibility. Indeed, a recent study aimed at monitoring the addition of tRNA modifications using yeast cell extracts revealed that many of those occur in the T-arm, either concomitantly with the activation of the Trm11–Trm112 complex (31), the NTD may move relative to the MTase domain defines the distance between the tRNA CCA extremity and the nucleotide to be modified. In Trm11, the distance between the residues from the region of the NTD supposed to interact with the 3′-OH extremity of the tRNA (Cys85 in A fTrm11 and Lys87 in TkoTrm11) and the SAM methyl group is around 45 Å and hence corresponds to the distance between the N2 atom from G10 and the 3′-OH extremity of a mature tRNA (47 Å). The same is true in the structures of TrmN/Trm14 MTases.

CONCLUSION

In conclusion, we have studied the tRNA modification enzyme Trm11 from the A. fulgidus archaean, revealing that this protein can be considered as a missing link between some archaean Trm11 enzymes, which are active on their own, and eukaryotic ones, which are strictly dependent on the Trm112 activator. Indeed, although A fTrm11 exhibits some enzymatic activity, this activity is strongly enhanced in the presence of A fTrm112. We have also determined the crystal structures of A fTrm11 bound to A fTrm112 and to sinefungin (SFG), a SAM-dependent MTase inhibitor. This structure is the first one of a complex between Trm11 and Trm112, for which so far structural information could only be obtained by a combination of molecular modeling and hydrogen-deuterium exchange experiments coupled to mass spectrometry measurements (31).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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