Discovery and Characterization of an Amidinotransferase Involved in the Modification of Archaeal tRNA*

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The presence of the 7-deazaguanosine derivative archaeosine (G*) at position 15 in tRNA is one of the diagnostic molecular characteristics of the Archaea. The biosynthesis of this modified nucleoside is especially complex, involving the initial production of 7-cyano-7-deazaguanine (preQ0), an advanced precursor that is produced in a tRNA-independent portion of the biosynthesis, followed by its insertion into the tRNA by the enzyme tRNA-guanine transglycosylase (arcTGT), which replaces the target guanine base yielding preQ0-tRNA. The enzymes responsible for the biosynthesis of preQ0 were recently identified, but the enzyme(s) catalyzing the conversion of preQ0-tRNA to G*−tRNA have remained elusive. Using a comparative genomics approach, we identified a protein family implicated in the late stages of archaeosine biosynthesis. Notably, this family is a paralog of arcTGT and is generally annotated as TgtA2. Structure-based alignments comparing arcTGT and TgtA2 reveal that TgtA2 lacks key arcTGT catalytic residues and contains an additional module. We constructed a Haloferax volcanii ΔgtA2 derivative and demonstrated that tRNA from this strain lacks G* and instead accumulates preQ0. We also cloned the corresponding gene from Methanocaldococcus jannaschii (mj1022) and characterized the purified recombinant enzyme. Recombinant MjTgtA2 was shown to convert preQ0-tRNA to G*−tRNA using several nitrogen sources and to do so in an ATP-independent process. This is the only example of the conversion of a nitrile to a formamidine known in biology and represents a new class of amidinotransferase chemistry.

The post-transcriptional processing of tRNA involves a number of functionally distinct events essential for tRNA maturation. Perhaps the most remarkable of these is the phenomenon of nucleoside modification, which can result in the introduction of radical structural changes to the constituent nucleosides. In archaeal tRNA, this is particularly well illustrated by archaeosine (G*, see Fig. 1A), a 7-deazaguanosine-modified nucleoside unique to Archaea (1). Archaeosine is found in the majority of archaeal tRNAs specifically at position 15 of the dihydrouridine loop (D-loop) (2), a position not modified in either eukaryotic or bacterial tRNA. The presence of the positively charged formamide group of archaeosine and its location at the interface of the D-loop/T-stem in the tertiary structure of the tRNA are thought to be important in structural stabilization of the tRNA through electrostatic interactions with the anionic phosphates (1). This is presumably a critical role given the prevalence of thermophiles within the Archaea and the implicit need to protect tRNA from thermal denaturation. Recent computational work suggests that archaeosine can also participate in structural stabilization via strengthening of the hydrogen bonding between the G15-C48 Levitt base pair (3); however, neither mechanism has been tested experimentally.

The archaeosine biosynthetic pathway is complex, and until recently, poorly characterized (4). It had been proposed that like other 7-deazapurines, G* was derived from GTP (5–7), and this hypothesis was recently confirmed when GTP cyclohydrolase I was shown to be the first enzyme of the G* pathway (8). It has been known for almost a decade that 7-cyano-7-deazaguanine (preQ0) is a key intermediate in the pathway and is inserted into tRNA by a tRNA-guanine transglycosylase (TGT,4 EC 2.4.2.29) (9, 10), encoded by the tgtA gene and referred to as arcTGT (see Fig. 1). arcTGT is a modular enzyme comprised of an N-terminal catalytic domain that folds into an α/β8 barrel with a characteristic zinc-binding site and a C-terminal extension of three domains C1, C2, and C3, the last an oligonucleotide/oligosaccharide binding fold-like “PUA (pseudouridine and archaeosine) domain” (see Fig. 1C) (11) that is widely conserved in eukaryotic and archaeal RNA modification enzymes. A crystal structure of the Pyrococcus horikoshii arcTGT in complex with tRNA shows that the C-terminal domains interact specifically with the acceptor stem of tRNA (12). In some Archaea, arcTGT is encoded by two open reading frames, with a split occurring between the C1 and C2 domains (13, 14). Stud-

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4 The abbreviations used are: TGT, tRNA-guanine transglycosylase; arcTGT, archaeal TGT; preQ0, 7-cyano-7-deazaguanine; Arc5, archaeosine synthase; DTT, dithiothreitol; LC-MS, liquid chromatography-mass spectrometry; MS-MS, tandem MS; HPLC, high pressure liquid chromatography; Mes, 4-morpholineethanesulfonic acid; Mj, M. jannaschii.
ies with the enzyme from *Methanosarcina barkeri* have shown that both the catalytic-C1 and the C2-PUA fragments are required to reconstitute the native preQ₀ exchange activity *in vitro* (13).

Although arcTGT was discovered over 10 years ago (9, 10), the enzyme (or enzymes) catalyzing the final step(s) in the biosynthesis of G⁷⁺ has remained elusive. In principle, there are two reasonable routes from preQ₀-modified tRNA to archaeosine; in the first, a nitrile hydratase activity acting on the preQ₀ nitrile would first yield 7-amido-7-deazaguanosine, which in a subsequent step could be converted to archaeosine in an ATP-dependent amidinotransferase reaction. There is good general precedent for both steps, and in fact, a nitrile hydratase has been described in *Streptomyces* that hydrolyzes the nitrile of the structurally similar toyocamycin (7-cyano-7-deazaadenosine) to sangivamycin, the corresponding amide (14, 15). The second path, although mechanistically simpler, lacks any biochemical precedent, and involves an ATP-independent amidinotransferase reaction that delivers ammonia to the nitrile of preQ₀ to generate archaeosine directly.

In this study, we report the use of comparative genomics to identify a candidate gene involved in archaeosine biosynthesis in Euryarchaeota. Using *in vivo* and *in vitro* experimental methods, we demonstrate that it encodes the final enzyme in the archaeosine biosynthetic pathway, a novel ATP-independent amidinotransferase catalyzing the direct conversion of preQ₀ to archaeosine.

**MATERIALS AND METHODS**

**Bioinformatics**—Analysis of the Archaeosine subsystem was performed in the SEED data base (16). Results and protein sequences are available in the "Queuosine and Archaeosine biosynthesis" subsystem on the public SEED; an Annotation/Analysis Tool server. The list of arcTGT and TgtA2 sequences used in these studies is given in *supplemental Text S1*. We also used the BLAST tools and resources at National Center for Biotechnology Information (NCBI) (17). Multiple protein alignments were performed with the ClustalW tool (18) in the SEED data base or the MultiAlign software (available via the Pacific Northwest National Laboratory). The *H. volcanii* genome sequence was accessed through the UCSC Archaeal Genome Browser (19). The three-dimensional models were generated using the protein fold recognition protocols of Phyre (20) based on one- and three-dimensional sequence profiles, coupled with secondary structure and solvation potential information (using the Phyre server available on-line). Structure-based alignment of a subset was performed using the ESPript platform (21) through the web interface.

**General Cloning and Protein Purification Methods**—The general materials as well as the strains and media used are given in *supplemental Text S2*. The constructions of the *H. volcanii* ΔtgtA2 derivatives are also given in *supplemental Text S2* as well as the purification and protein analysis methods.

**H. volcanii tRNA Extraction and Analysis**—Bulk tRNA was prepared, hydrolyzed, and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS-MS) as described in Ref. 8. To evaluate tRNA concentrations, we compared the ratio of the levels of the Ψ-modified base (245 m/z) in each sample by integrating the peak area from the extraction ion chromatograms. The MS-MS fragmentation data were also used to confirm the presence of the nucleosides preQ₀ and G⁷⁺. All tRNA extractions and analysis were performed at least twice independently.

**Glutaminase Assays**—Glutaminase activity was observed indirectly as a coupled assay to glutamate dehydrogenase and directly by measuring the conversion of [U-14C]glutamine to [U-14C]glutamate. The preparation of the preQ₀-tRNASer substrate is described in *supplemental Text S2*. The indirect assay was carried out by incubating recombinant MfTgtA2 (10 μM) with or without preQ₀-tRNASer (50 μM) and with or without ATP (1 mM) in an assay solution containing 50 mM Hepes (pH 8.0), 0.5 mM NaCl, 15 mM MgCl₂, 1 mM DTT, and 2.5 mM glutamine in a total volume of 100 μl at 37 °C for 30 min. The protein and RNA were then removed by centrifugation in a Microcon (Millipore Corp.) for 20 min, 60 μl of the eluant was combined with an equal volume of 50 mM phosphate (pH 7.5), 1 mM NADP⁺, and 4 units of glutamate dehydrogenase, and the assay was incubated at 37 °C. The formation of NADPH was monitored at 340 nm.

Direct assays of glutaminase activity were carried out for 10 min in either 100 mM sodium acetate (pH 5.0) or 100 Hepes (pH 7.0), 20 mM MgCl₂, 2 mM DTT, 12.4 μM [U-14C]glutamine, with or without preQ₀-tRNASer (50 μM), MfTgtA2 (0.38–12 μM), and 0.5 mM NaCl. The reactions were terminated by addition of 0.2 volumes of concentrated NH₄OH. A 6-μl portion of the assay was spotted onto silica TLC plates, and the plates were developed in i-PrOH:NH₄OH (7:3). The plates were then analyzed on the Typhoon 9200. Identical assays were carried out using [3H]asparagine as the nitrogen source, but in this case, after elution the TLC plates were cut into segments and placed in vials, liquid scintillation fluid was added, and the radioactivity was quantified by liquid scintillation counting.

**Amidinotransferase Assays**—Assays of amidinotransferase activity were carried out in 100 mM Hepes (pH 8.0), 0.5 mM NaCl, 20 mM MgCl₂, 1 mM DTT, 10 μM MfTgtA2, 10 μM preQ₀-tRNASer in the presence of the three possible donors: NH₄Cl (100 mM), glutamine (5 mM), or asparagine (5 mM), with and without ATP (1 mM). The assays were run at 40 °C and stopped by the addition of 0.1 volume of 2 mM NH₄OAc followed by one volume of water saturated phenol and 0.2 volume chloroform-isooamyl alcohol (49:1). After vortexing for 20 s, the solution was centrifuged in a swinging bucket rotor at 700 × g for 20 min. The aqueous phase was recovered, and the product G⁺-tRNASer was precipitated with 3 volumes of ethanol and cooling at −20 °C for 2 h. The solution was centrifuged at 14,000 × g for 20 min at 4 °C, the supernatant was removed, and the RNA pellet was washed with 70% cold ethanol. The solution was centrifuged again at 14,000 × g for 20 min at 4 °C, the supernatant was removed, and the RNA was resuspended to 3 μg/μl in H₂O and digested as described previously (22). Nucleosides were subsequently analyzed by both HPLC and LC-MS. HPLC analysis was done using a Discovery HS C18 column (2.1 × 250 mm, Supelco) with a mobile phase comprised of a linear gradient from 100% 5 mM NH₄OAc (pH 5.3) to 85% 5 mM NH₄OAc, 15% acetonitrile developed over 30 min at 0.3 ml/min. LC-MS analysis was performed with the same column and mobile phase on a MicroTOF-Q tandem mass spec-
trometry instrument (Bruker, Billerica, MA) utilizing electro-

RESULTS

Identification of a Candidate for the Missing Archaeosine Synthase Gene—Genomes encoding arcTGT are found in nearly all sequenced Archaea to date, even in *Nanoarchaeum equitans*, which possesses the smallest known archaeal genome. This quasiuniversal distribution confirms the analytical work of McCloskey and colleagues (23, 24), who identified G+H11001 by LC-MS analysis of bulk tRNA extracted from a wide variety of archaeal species. Given that the precursor preQ0 has not been observed in archaeal tRNA, an enzyme (or enzymes) responsible for the conversion of preQ0-tRNA to G+H11001-tRNA should be encoded in most archaeal genomes. This enzyme(s) should be an RNA-binding protein because preQ0-tRNA is the substrate. Finally, with more than 60 archaeal genomes available (see list at Genomes OnLine Database (GOLD) version 3.0), the corresponding gene(s) would likely cluster physically with the *tgtA* gene (encoding arcTGT) in a few genomes because these genes encode consecutive steps in the same pathway (25).

Using the gene clustering tool of the SEED data base, we focused on the gene families that physically clustered with *tgtA* and that encoded proteins with RNA-binding domains. A good candidate was the *tgtA*2 family as members of this family cluster with *tgtA* genes in phylogenetically distant Archaea (Fig. 1B). These genes have been annotated as encoding arcTGT enzymes in most archaeal genomes because the corresponding proteins present around 18% sequence identity with the canonical arcTGT (the identity score was obtained from comparing the *Methanocaldococcus jannaschii* arcTGT (MJ0436) and TgtA2 (MJ1022)).

Detailed analysis of the sequence alignments of the arcTGT and TgtA2 families, presented in supplemental Fig. S1 and summarized in Fig. 1C, revealed that although the C2 and PUA domains are well conserved between the two protein families, the N-terminal and C1 domains are not. The major differences in those two domains are the following. 1) The N-terminal domain in the TgtA2 family is smaller than its counterpart in the arcTGT family by 70–130 N-terminal residues. 2) Active site and known catalytic residues in the N-terminal domain of arcTGT (12) are not conserved in TgtA2; the general acid/base Asp-95 (*P. horikoshii* residue numbers) is absent from most TgtA2 sequences, as are the preQ0 anchoring residues Phe-99, Asp-130, Gln-169, Phe-229, and Met-102, the latter of which stabilizes the cyano group of preQ0 (12). However, Asp-249, which presumably functions as the catalytic nucleophile in the arcTGT-catalyzed reaction in analogy to the bacTGT, is present in TgtA2. 3) The C1 domain of TgtA2 is much larger than its counterpart in arcTGT (200 residues in TgtA2, but only 40 in arcTGT). 4) The C1 domain of TgtA2 contains a TgtA2-specific motif PC3KPYX3S5X2H (Fig. 1C) not found in arcTGT.

Members of the TgtA2 and arcTGT family do not perfectly co-distribute. TgtA2 homologs are found in all sequenced Euryarchaeota but in only a few Crenarchaeota (supplemental Fig. S1), whereas arcTGT proteins are found in all but two archaeal genomes. However, the combination of the clustering evidence and the sequence differences in arcTGT and TgtA2 lead us to hypothesize that the *tgtA*2 gene family encoded an enzyme catalyzing one or more steps in the conversion of preQ0-tRNA to archaeosine-modified tRNA.

*TgtA2 Is Involved in Archaeosine Biosynthesis in Vivo*—If TgtA2 catalyzes a late step in G+ biosynthesis, then tRNA...
extracted from a tgtA2 mutant should lack G+ and accumulate preQ0 or a later intermediate in the pathway. Because H. volcanii is one of the few Archaea that is amenable to genetic manipulations (26) and the only one in which all the tRNA molecules were sequenced and the position of the modified nucleosides was determined (27, 28), we chose to investigate the in vivo function of tgtA2 in this organism. A ΔtgtA2 mutant was constructed in H. volcanii, and the mutant was checked by PCR and Southern blot analysis for the correct deletion of the tgtA2 gene and the absence of any remaining wild-type copy of the gene (Fig. 2A). The tRNA modification profile of tRNA extracted from both the TgtA2− (VDC3225) and the isogenic TgtA2+ (VDC3226) strains is shown in Fig. 2B. The peak at 25.1 min corresponding to G+ (MH+ 325 m/z) detected in the UV trace in the TgtA2+ H. volcanii strain disappeared in the ΔtgtA2 derivative (VDC3225), and a new peak corresponding to preQ0-nucleoside (MH+ 308 m/z) appeared at 25.4 min. The profile of digested tRNA extracted from the ΔtgtA2 strain transformed with plasmid pGPP099 expressing the Halobacterium salinarum tgtA2 in trans (VDC3224) reverted to a wild-type profile (presence of G+), absence of preQ0) (Fig. 2B). Notably, the appearance of preQ0 in the tRNA of the ΔtgtA2 strain establishes that preQ0-modified tRNA is the likely substrate for TgtA2, and although there may be additional enzymatic activities after TgtA2, TgtA2 directly follows arcTGT in the biosynthetic pathway.

In Vitro Glutaminase Activity of MjTgtA2—Expression and purification of MjTgtA2 is described in supplemental Text S2. Several forms of the enzyme were isolated and characterized, including N- and C-terminal affinity fusion proteins, and several forms of protein were produced from a self-cleavage reaction.

Because any route from preQ0 to archaeosine would likely involve an amidinotransferase step, we elected to first screen MjTgtA2 for a glutamine-dependent amidinotransferase activity by monitoring potential glutaminase activity in a coupling assay with glutamate dehydrogenase as well as by direct observation of glutamate formation in radiochemical assays with 1-14Cglutamate. Assays were initially run in the absence of preQ0-tRNA in the presence or absence of ATP for 10 min over a pH range of 4.0–9.0 and a temperature range of 30–80 °C. Glutamate formation in the MjTgtA2 reaction, as deduced from glutamate dehydrogenase activity or from the direct production of 1-14Cglutamate, was dependent on MjTgtA2 (Fig. 3A), did not require ATP, and was observed over a broad pH range (pH 5.0–8.0) (Fig. 3B). Surprisingly, maximal activity occurred at ~40 °C and dropped off significantly above 45 °C at all pH values (Fig. 3C). Based on radiochemical assays, specific activities of 7.7, 11.4, and 16.5 μM/min/mg were calculated for the self-cleaved protein, the full fusion protein, and the C-terminal tagged protein, respectively.

In Vitro Amidinotransferase Activity of MjTgtA2—To probe whether the glutaminase activity of MjTgtA2 was associated with an amidinotransferase activity and the formation of archaeosine, we investigated the ability of MjTgtA2 to catalyze formation of archaeosine under a variety of conditions. In particular, we investigated whether archaeosine formation was ATP-dependent, which would be consistent with archaeosine formation from an amide intermediate. PreQ0-modified tRNA was prepared in an arcTGT reaction as described previously (10) and used for MjTgtA2 assays. LC analysis of MjTgtA2 reactions carried out in the presence or absence of ATP revealed a product peak with a retention time of 17.9 min (Fig. 4A) and a chromatogram (supplemental Fig. S2B) that were distinct from that of preQ0 (17.1 min; Fig. 4A and supplemental Fig. S2B) but consistent with that of archaeosine (1). Subsequent LC-MS analysis of the MjTgtA2-catalyzed reaction provided a parent ion (MH+ 325.1 m/z) for the product of MjTgtA2 (Fig. 4B), consistent with archaeosine, as well as a significant daughter ion (193 m/z), consistent with archaeosine base. Surprisingly, the glutaminase activity of MjTgtA2 was essentially unaffected by the presence of preQ0-tRNA50, indicating that considerable uncoupling of glutaminase and amidinotransferase activities was occurring. Archaeosine formation was also observed when asparagine and NH3 were used in place of glutamine. Using [14]Casparagine, a specific activity of 3.23 μM/min/mg was calculated for asparaginase activity with the fusion form of the enzyme in the absence of preQ0-tRNA.

Structural Analysis of the TgtA2 Family—Analytical gel filtration of both the Trx-His6 fusion and the protein produced from self-cleavage exhibited elution times consistent with the molecular mass of a dimer (154 and 122 kDa, respectively) (supplemental Text S2 and supplemental Fig. S3), demonstrating that like arcTGT (12), TgtA2 also functions as a dimer. To obtain a more comprehensive understanding of TgtA2 proteins, we analyzed TgtA2 sequences in 36 Archaea. Based on ClustalW alignment of 50 TgtA2 sequences, three forms of TgtA2 proteins can be distinguished (supplemental Fig. S1). The first is a long form that contains four domains (N-terminal, C1, C2, and C3) and exists in the majority of Archaea. In some organisms (e.g. Archaeoglobus fulgidus and Methanosaeta thermophila), this form possesses the conserved zinc-binding residues in the N-terminal domain observed in arcTGT. In other organisms (e.g. M. jannaschii and P. horikoshii), the zinc-binding site is absent. The second is a split form found in a small number of Archaea (specifically Methanothermobacter thermostrophicus, Methanobrevibacter smithii, and Methanospaera stadtmanae) in which the enzyme occurs as two separate polypeptides encompassing a small N-terminal domain in one polypeptide and the C1–C3 domains in the other (supplemental Fig. S1). In this form, the N-terminal domain harbors the conserved zinc-binding site. A split form of arcTGT also exists, but in this case, the split occurs between the C1 and C2 domains (13). The third form of TgtA2 is a short form comprised of a standalone C1 domain that contains the TgtA2-specific consensus sequence motif.

To better understand the structural relationship to arcTGT, we generated a structure-based sequence alignment of the three forms of TgtA2 with arcTGT (supplemental Fig. S4). For this alignment, we first generated a three-dimensional model for each TgtA2 form using a representative sequence; the models for the N-terminal and C1–C3 domains were generated based on fold similarity with crystal structures of the respective domains from P. horikoshii arcTGT (Protein Data Bank (PDB) ID 1J2B (12)) and A. fulgidus TgtA2 C-terminal fragment.
Comparison of the crystal structures of the C-terminal fragment of \textit{A. fulgidus} TgtA2 and of \textit{P. horikoshii} arcTGT reveals that the C2 and C3 domains are very similar in the two proteins (root mean square deviation 2.4 Å over 130 C/H9251 atoms, sequence identity 27%), whereas the C1 domains are different (Fig. 5A). The TgtA2 C1 domain contains insertions of four \( \beta \)-helices (\( \beta \)-11–\( \beta \)-14 in \textit{A. fulgidus} secondary structure nomenclature) and three \( \beta \)-strands (\( \beta \)-6, \( \beta \)-9, \( \beta \)-10, Fig. 5 and supplemental Fig. S4) not found in arcTGT. These insertions amount to the creation of a Rossman fold architecture of the TgtA2 C1 domain. In \textit{A. fulgidus} TgtA2, the TgtA2 motif forms a loop and a \( \alpha \)-10-helix followed by one turn from helix \( \alpha \)-11. The motif protrudes from a positively charged surface on one side of the molecule.

To predict the role of the TgtA2 motif, we generated a docking model of the TgtA2 complex with both the \( \lambda \)- and the L-forms of tRNAVal. The docking model with \( \lambda \)-tRNA, which is based on the superposition of our model of full-length \textit{A. fulgidus} TgtA2 with the protein component of the \textit{P. horikoshii} arcTGT/tRNA heterodimeric complex (PDB ID 1J2B (12)), shows that the tRNA binds to the positively charged surface of TgtA2 with the conserved TgtA2 motif located 20 Å from the modification site (position 15) in the D-arm of \( \lambda \)-tRNA (Fig. 5B). However, when TgtA2 is docked onto the L-form of tRNAVal (PDB ID 1IVS (29)), the G15 nucleotide is within contact distance of the TgtA2 motif (albeit occupied in base pairing in the L-form, Fig. 5C). These results suggest that TgtA2 acts on the L-form, not the \( \lambda \)-form of tRNA implicated in arcTGT.
The N-terminal, C1, C2 and C3/PUA domains are colored in yellow, cyan, red and green, respectively. Side chains in the conserved TgtA2 motif are shown in stick or Corey-Pauling-Koltun representation.

Taken together, the in vivo and in vitro data are unambiguous in establishing a role for TgtA2 in the conversion of preQ₆-modified tRNA to archaeosine-modified tRNA. The absence of an ATP dependence suggests that this conversion does not require the initial hydrolysis of the nitrile to an amide and instead involves the direct addition of NH₃, generated from glutamine or asparagine hydrolysis, to the nitrile of preQ₆,a. An ATP dependence suggests that this conversion does not involve the direct addition of NH₃, generated from glutamine or asparagine hydrolysis, to the nitrile of preQ₆,a. Instead, this activity involves the direct addition of NH₃, generated from glutamine or asparagine hydrolysis, to the nitrile of preQ₆,a.

DISCUSSION

TgtA2 is present in the tRNA of a number of Crenarchaeota (Sulfolobus tokodaii, Sulfolobus solfataricus, Ignococcus hospitalis, and Hyperthermus butylicus) possess ArcS homologs (supplemental Fig. S1). Given that it has been demonstrated that G⁺ is present in the tRNA of a number of Crenarchaeota lacking ArcS (33), a different enzyme is clearly responsible for the formation of archaeosine in these organisms, and we are currently investigating several candidates for this activity.

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