Archease from *Pyrococcus abyssi* Improves Substrate Specificity and Solubility of a tRNA m^5^C Methyltransferase*

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Members of the archease superfamily of proteins are represented in all three domains of life. Archease genes are generally located adjacent to genes encoding proteins involved in DNA or RNA processing. Archease have therefore been predicted to play a modulator or chaperone role in selected steps of DNA or RNA metabolism, although the roles of archeases remain to be established experimentally. Here we report the function of one of these archeases from the hyperthermophile *Pyrococcus abyssi*. The corresponding gene (PAB1946) is located in a bicistronic operon immediately upstream from a second open reading frame (PAB1947), which is shown here to encode a tRNA m^5^C methyltransferase. *In vitro*, the purified recombinant methyltransferase catalyzes m^5^C formation at several cytosines within tRNAs with preference for C49. The specificity of the methyltransferase is increased by the archease. In solution, the archease exists as a monomer, trimer, and hexamer. Only the oligomeric states bind the methyltransferase and prevent its aggregation, in addition to hindering dimerization of the methyltransferase-tRNA complex. This *P. abyssi* system possibly reflects the general function of archeases in preventing protein aggregation and modulating the function of their accompanying proteins.

Archease proteins were first annotated in archaeal and eukaryal genomes and were later shown also to be present in the Bacteria (1). According to present data base records, members of the archease superfamily belong to a cluster of orthologue genes (COG1371) and are represented in 28 eukaryal, 18 bacterial, and 25 archaeal species. This suggests that the function of these proteins has been phylogenetically conserved. However, the precise nature of this function has remained undetermined. The structures of two archeases, MTH1598 from the Archaea *Methanobacter thermoautotrophicum* (2) and TM1083 from the thermophile bacterium *Thermotoga maritima* (3), have been solved. The two structures superimpose quite well (root mean square deviation (r.m.s.d.))\(^2\) = 2.6 Å) despite the relatively low identity (14.8%) in their amino acid sequences (1). Intriguingly, the two archease structures are also similar to that of the heat shock protein Hsp33 (r.m.s.d. = 3.1 Å) (4, 5) and the gyrase inhibitory protein GyrI (r.m.s.d. = 3.1 Å) (6). Protein Hsp33 is activated by oxidative stress and protects unfolded proteins against aggregation, whereas GyrI interacts specifically with DNA gyrase to inhibit its supercoiling activity. The structural resemblance to these proteins suggests that archeases might be engaged in similar functions.

Archeases contain two SHS2 modules (also called βαβ2 folds) that are found in other proteins, including the ATPase FtsA, the RNA polymerase subunit Rpb7p, and GyrI (7). The two SHS2 modules in GyrI are arranged in a tandem and opposite orientation, whereas in the MTH1598 and TM1083 archeases the one SHS2 module is inserted within the other. Hsp33 has no SHS2 fold, although it possesses two slightly different SHS2 modules (βαβ2) arranged in the same manner as in the MTH1598 and TM1083 archeases. The S2HS2 modules probably mediate protein-protein interactions, as has been postulated for SHS2 domains (7). Further clues as to the function of archeases come from genome analyses, which reveal that all archease genes are adjacent to an ORF encoding a protein involved in nucleic acid processing, such as a DNA gyrase, a polymerase, or an RNA helicase (1, 7). The structure and the genomic location of archeases suggest that they might interact directly with these proteins. In support of this hypothesis, we additionally note that the C-terminal end of a putative *Thiobacillus denitrificans* tRNA nucleotidyltransferase is an archease sequence (EMBL data base number Q3SDG7).

In the genome of the extreme thermophile *Pyrococcus abyssi*, an archease gene (PAB1946) is located immediately upstream from PAB1947 forming a putative bicistronic operon (8, 9). PAB1947 contains the Sun/NOL1/NOP2 family signature (Prosite profile PS01153) suggesting that it encodes an RNA m^5^C methyltransferase. To date, the specific RNA modifica-

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5. The abbreviations used are: r.m.s.d., root mean square deviation; ORF, open reading frame; BSA, bovine serum albumin; DTT, dithiothreitol; MALDI, matrix-assisted laser desorption ionization; AdoMet, S-adenosylmethionine.
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FIGURE 1. Occurrence of m^5C in archaeal tRNA sequences. The data are a compilation of the 54 halophilic archaeal tRNAs and the five structures from thermophilic and hyperthermophilic archaea that are presently available (15). The conventional nucleotide numbers are encircled. In the boxes, the first number gives the frequency of m^5C, and the second is the number of archaeal tRNAs with cytosine at this position. Nucleotide m^5C is most common at positions 48 and 49 and is found at both sites in nine of the tRNAs. No more than two m^5C are present per archaeal tRNA.

Cloning of the P. abyssi Open Reading Frames PAB1946 and PAB1947—Recombinant versions of the archease (PAB1946) and putative methyltransferase (PAB1947) proteins were constructed with a His_6 tag at their N-terminal ends. The genes were amplified from P. abyssi genomic DNA using the following PCR primers: AAAAAACATGGGTCTACATCATCATTCAACAGATGGAGCCACTATG (5’-primer) and AAAAACTCGAGGTCTAGATGTCGGCCGACAAG (3’-primer) for PAB1946, and AAAAAACATGGGTCTACATCATCATTCAACAGATGGAGCCACTATG (5’-primer) and AAAAACTCGAGGTCTACATCATCATTCAACAGATGGAGCCACTATG (3’-primer) for PAB1947. The 5′-primers created the ATG start codon in the Ncol restriction site followed by six histidine codons. The 3′-primers introduced an Xhol restriction site immediately downstream from the TGA stop codon. The amplified fragments were cut with Ncol and Xhol restriction enzymes, and were cloned into the same sites in the expression vectors pET28b and pET15b (Novagen) for PAB1946 and PAB1947, respectively.

Overexpression and Purification of Recombinant Archease and PAB1947 Proteins—Archease and the PAB1947 enzyme were overexpressed in separate clones of E. coli BL21 (DE3) CodonPlus RIL (Stratagene). Transformed cells were grown at 37 °C in LB medium containing chloramphenicol and were supplemented with kanamycin for the archease plasmid and ampicillin for the PAB1947 plasmid. Recombinant protein expression was induced by 1 mM of isopropyl thiogalactopyranoside when the culture absorbance reached 0.7 at 600 nm. After a further 3 h at 28 °C, cells were harvested and resuspended in 5 volumes of buffer A (50 mM sodium phosphate, pH 7.5, 300 mM NaCl, 10 mM imidazole, 10% glycerol, 5 mM β-mercaptoethanol) supplemented with 1% (w/v) Protease Inhibitor Mixture (Sigma). For the PAB1947 enzyme, NaCl was increased to 1 M to prevent co-purification of nucleic acids. Cells were lysed by sonication and centrifuged at 15,000 × _g_ for 30 min at 4 °C.

The archease supernatant was added to nickel-nitrilotriacetic acid Superflow™ resin (Qiagen) and mixed during 30 min at 4 °C. Special care was required to avoid aggregation of the PAB1947 enzyme, which was loaded in batches onto the resin, and purification was carried out at room temperature. Resin columns were washed with 20 mM imidazole in buffer A, and proteins were eluted with buffer A containing 250 mM imidazole for archease and 150 mM imidazole for the PAB1947 enzyme. The final respective yields were 45 and 35 mg of recombinant protein per liter of culture. After purification, archease was dialyzed against 25 mM Tris-Cl, pH 7.5, 100 mM NaCl, 10% glycerol, 1 mM DTT; for the PAB1947 enzyme, the buffer contained 300 mM NaCl. Both proteins were stored in aliquots at −80 °C.

Cloning of the P. abyssi tRNA_Asp Gene—The P. abyssi tRNA_Asp gene was cloned for transcription by T7 RNA polymerase. Overlapping DNA oligonucleotides were annealed, elongated, and then amplified using Platinum™ Pfx DNA polymerase (Invitrogen). The oligodeoxynucleotide sequences were as follows: (+) primer, CCCCCGCTTATTGCTGACTCACTATAGGGCGGTGTTAGGCCGGGCTATCATGCG-
GGACTGTCAC; and (−) primer, CGCGGATCCTGGCGCC-
CGGCGCCGATTTGACCCGGGTGCGCGGAGTGAC-
AGTCCCGCATGATAAGCCG. The (+) primer contained a
HindIII site (underlined) upstream from the T7 promoter
(italic type), and the (−) primer contained BamHI (underlined)
and Mvai sites (boldface type), positioned to cleave the
template for in vitro transcription. The PCR product was
digested with HindIII and BamHI and was inserted into the same sites in
plasmid pUC18.

T7 in Vitro Transcriptions of RNA—Transcription of the
tRNA genes with radiolabeled [α-32P]CTP were transcribed in vitro
from the T7 promoter as described previously (20). The
plasmid templates were digested with MvaI for P. abyssi
tRNAAsp and E. coli tRNA37 and with BamHI for P. abyssi
tRNA1Leu prior to transcription. Nonradioactive tRNAs were
synthesized by using the RibomaxTM large scale RNA production
system-T7 (Promega). All tRNA transcripts were purified on 6% polyacrylamide gels; yields were around 100 μg of tran-
script from 10 μg of plasmid template. The rRNA fragments
radiolabeled with [α-32P]CTP were T7-transcribed in vitro
from single-stranded DNA templates (21).

Methyltransferase Assays—Methylation reactions were car-
ried out in 50 μl of 25 mM Tris-Cl, pH 7.5, 50 mM KCl, 2 mM
DTT, 80 μM AdoMet (i.e. in large excess), 1 ng/ml RNase-free
bovine serum albumin (Worthington), with 1 μM 32P-radiola-
beled RNA and 200 nM PAB1947 enzyme. In some experiments,
radiolabeled tRNA (1 nm) and unlabeled tRNA (200 nm) PAB1947 enzyme. In some experiments,
radiolabeled RNA (10 fmol) was incubated with the PAB1947 enzyme and/or archease (at a
1:6 molar ratio, respectively, when used together) in 20 μl of 25
mM Tris-Cl, pH 7.5, 50 mM NaCl, 10% glycerol, 0.1 mg/ml
RNase-free bovine serum albumin, 2 mM DTT at 25 °C for 20
min. Samples were quenched on ice, and bromphenol blue
was added to 0.05% prior to loading on 6% polyacrylamide
gels (mono/bisacrylamide, 37.5:1) containing 5% glycerol
and bovine serum albumin (22). The individual RNA oligonucleotides were purified by denaturating 20% polyacrylamide gels, and after extraction as
described (23), they were digested with nucleas P1. The resulting
mononucleotides were analyzed by two-dimensional thin layer chromatography (20).

Gel Retardation Experiments—Radiolabeled RNA (10 fmol)
was incubated with the PAB1947 enzyme and/or archease (at a
1:6 molar ratio, respectively, when used together) in 20 μl of 25
mM Tris-Cl, pH 7.5, 50 mM NaCl, 10% glycerol, 0.1 mg/ml
RNase-free bovine serum albumin, 2 mM DTT at 25 °C for 20
min. Samples were quenched on ice, and bromphenol blue
was added to 0.05% prior to loading on 6% polyacrylamide
gels (mono/bisacrylamide, 37.5:1) containing 5% glycerol
and 1 mM EDTA in 0.5 × TBE at 4 °C. After electrophoresis
and drying, gel bands were scanned with a StormTM system
(GE Healthcare).

Gel Filtration and Stokes Radius Determination—The interac-
tion of PAB1947 enzyme with archease was studied by gel
filtration on a SuperdexTM 75 HR 10/30 column (GE Health-
care) equilibrated with 25 mM Tris-Cl, pH 7.5, 100 mM NaCl,
10% glycerol, 5 mM EDTA. Samples, in 200 μl of the same buffer
containing 150 μg (20 μM) PAB1947 enzyme and/or 450 μg
(130 μM) archease, were incubated for 30 min at ambient tem-
terature (18–20 °C) before loading onto the column and eluting
at a flow rate of 0.5 ml/min. For Stokes radius (Rv) determina-
tion, the column was calibrated with bovine γ-globulin
(158,000 Da; RV = 45 Å), chicken ovalbumin (44,000 Da; RV =
27.5 Å), equine myoglobin (17,000 Da; RV = 20 Å) (Bio-Rad),
and bovine brain tubulin (100,000 Da; RV = 41.5 Å (24)). The
void volume of the column was determined from the elution
volume of blue dextran. The Stokes radii of archease, PAB1947
enzyme, and their complexes were determined using Equation
1, according to Ref. (25),

\[ \sqrt{-\log K_{av}} = f(R_v) \]  

where Kav is the molecular sieving coefficient \((\langle V_{elution} - V_j\rangle) / (V_{gel bed} - V_0)\).

Binding of tRNA to PAB1947 or the archease-PAB1947 com-
plex was followed by SuperdexTM 200 HR 10/30 GL gel filtra-
tion (GE Healthcare) as described above. The PAB1947-tRNA
complex was formed between 16 μM PAB1947 enzyme and 2.8
μM (15 μg) natural E. coli tRNA37 (Sigma); to form the tripar-
tite complex, 100 μM (360 μg) archease was added before the
E. coli tRNA37.
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Sedimentation Velocity and Molecular Mass Calculation—Sedimentation velocity experiments were carried out with a Beckman Optima XL-A analytical ultracentrifuge equipped with a 60 Ti four-hole rotor and cells with two-channel 12-mm path length centerpieces. Measurements were made at 50,000 rpm and at 18 °C with PAB1947 enzyme at 0.3 mg/ml and archease at 0.9 mg/ml in 25 mM Tris-Cl, pH 7.5, 100 mM NaCl, and 10% glycerol. The solvent density was 1.032 g/ml in 25 mM Tris-Cl, pH 7.5, 100 mM NaCl, and 10% glycerol.

The solvent density was 1.032 g/ml in 25 mM Tris-Cl, pH 7.5, 100 mM NaCl, and 10% glycerol. Measurements were made at 50,000 rpm and at 80 °C. Radial scans of the absorbance at 280 nm were taken at 3-h intervals, and equilibrium was reached after 24 h of centrifugation. The base line was recorded at 60,000 rpm, at the end of the experiment. The data were analyzed with XLAEQ and EQASSOC programs (Beckman) to calculate molecular weights.

Aggregation Analyses—The PAB1947 enzyme was adjusted to a final concentration of 0.06 mg/ml in 20 μl of 25 mM Tris-Cl, pH 7.5, 50 mM KCl, 2 mM DTT, with or without 0.18 mg/ml of archease or BSA. Samples were incubated for 10 min at 4, 60, 65, 70, or 80 °C and were then centrifuged at 12,000 × g for 15 min. The pellets were resuspended in 20 μl of the same buffer and were analyzed by SDS-PAGE together with the supernatants.

RESULTS

The P. abyssi ORF PAB1947 Encodes a tRNA m5C Methyltransferase—BLAST analysis (30) of the P. abyssi genome identified five potential RNA m5C methyltransferases. Of these, the sequence

encoded by PAB1947 is most similar to the tRNA m5C methyltransferase from S. cerevisiae, Trm4p (13, 18, 19).

The P. abyssi PAB1947 gene was cloned in E. coli and expressed with an N-terminal His tag. The activity of the purified recombinant PAB1947 protein was tested at 50 and 80 °C against tRNA transcripts containing radiolabeled cytidine. The incubation temperatures in vitro were lower than that for optimal growth of P. abyssi (103 °C) to reduce breakdown of the individual components of the reaction. After methylation, nucleotide monophosphates were derived from the transcripts by nuclease P1 digestion and were analyzed by thin layer chromatography. Analyses of P. abyssi tRNA^Asp^ (Fig. 2A), P. abyssi tRNA^Leu^, and E. coli tRNA^Asp^ and tRNA^Leu^ transcripts (not shown) demonstrated that the recombinant PAB1947 protein is indeed an m5C methyltransferase and uses AdoMet as the methyl donor.

Time courses of the methylation reactions were performed at 80 °C with P. abyssi tRNA^Asp^ and tRNA^Leu^ (Fig. 2B). After 10 min, the PAB1947 enzyme had methylated 1.6 cytosines per tRNA^Asp^ and 2.6 cytosines in tRNA^Leu^. The tRNA substrates at 200-fold higher concentrations were also methylated, and this resulted in a lower final level of m5C incorporation (Fig. 2B).
The mesophilic *E. coli* tRNA$_{Tyr}^2$ was tested only at 50 °C to avoid degradation at the higher temperature. The PAB1947 enzyme is active on all the tRNA substrates at 50 °C (Fig. 2C), although methylation is slower than at 80 °C. Surprisingly, small fragments of ribosomal RNA (Fig. 2D) were also methylated by the PAB1947 enzyme, despite having no obvious structural similarities to tRNAs.

The *Unassisted PAB1947 Enzyme Methylates Multiple Cytosines in* *P. abyssi* tRNA—Pyrococcal tRNAs have been shown previously to contain m$^5$C (31), although the positions of these modifications were not determined. Here we have mapped the methylation sites in *P. abyssi* tRNA$_{Asp}$ (Fig. 3A) after incubation at 80 °C, where 0.3 mol of m$^5$C was incorporated per mol of tRNA. The methylated tRNA was digested with the guanosine-specific RNase T1, and the oligonucleotides were analyzed by MALDI mass spectrometry. In the mass spectra (Fig. 3B), proportions of six oligonucleotides were 14 Da larger than expected from their predicted masses (Fig. 3C), indicating that the recombinant PAB1947 enzyme had methylated at least six different nucleotides in the tRNA$^{Asp}$. The number of methylation sites is potentially higher, as some oligonucleotides (such as the oligonucleotide CCCG) contain several cytosines and could be substoichiometrically modified at more than one cytosine under the conditions used here.

From the compilation of known methylation sites (Fig. 1), only the fragments 35–43 and 47–51 were expected to contain targets for methylation. When tRNA was incubated with the PAB1947 enzyme at 50 °C instead of 80 °C, methylation became more specific for the 47–51 sequence, indicating that this oligonucleotide contained the preferred target. This methylated oligonucleotide (m/z 1622.3) was analyzed by tandem mass spectrometry, and the primary site of PAB1947 methylation was unambiguously identified as the tRNA cytidine 49 (Fig. 4). The methylation site was localized more precisely to the cytosine base of nucleotide 49 from the combination of ions, including y$_3$ that had lost methylcytosine (m/z 863.1), the free methylcytosine ion (m/z 126.1), and the lack of any methylribose ion (at m/z 111.1).

**Nonspecific Methylation in Vitro of Poly(C)RNA by PAB1947**—Our observation that a large variety of RNAs could serve as substrates for the PAB1947 enzyme in vitro was tested further using a poly(C) RNA. The unstructured poly(C) RNA was effectively methylated at 50 °C, with most of the tritiated label from the AdoMet methyl donor becoming incorporated into the RNA (Fig. 5B). However, when the recombinant PAB1947 enzyme was substituted with a *P. abyssi* cell extract, no methylation of the poly(C) RNA occurred. Under comparable conditions, the cell extract efficiently catalyzed m$^5$C formation in *P. abyssi* tRNA$_{Asp}$ transcripts, and retained this activity despite extensive dialysis (data not shown). Clearly, the naturally occurring tRNA m$^5$C methyltransferase activity in *P. abyssi* cells has greater substrate specificity than the isolated recombinant PAB1947 enzyme, and this indicated that at least one essential component was missing from our in vitro assays.

A clue as to what this component might be was given by its resilience to extensive dialysis. The high specificity of methylation exhibited by the *P. abyssi* cell extract was retained after dialysis through membranes with a cutoff at 14,000 Da. This ruled out the hypothetical component being a small molecule,
such as magnesium or a polyamine, which are known to stabilize RNA structures (reviewed in Refs. 32 and 33).

**The Specificity of the PAB1947 Enzyme Is Dependent on Archease—**

An obvious candidate for the missing component was the *P. abyssi* archease, the gene for which (PAB1946) is located immediately upstream in the same operon as the PAB1947 methyltransferase. Because MALDI mass spectrometry is essentially a nonquantitative method, we used an alternative approach to determine the degree to which archease influences the methylation of different tRNA regions. $[^{32}\text{P}]{\text{CTP-labeled tRNA samples that had been modified by the PAB1947 methyltransferase in the absence or presence of archease were digested to completion with RNase T1. Oligonucleotides were purified on gels and subjected to further digestion with nuclease P1 followed by thin layer chromatography to estimate the amounts m$^5$C, as described above. Addition of archease to the methylation reactions with *P. abyssi* tRNA$^{\text{Asp}}$ at 80 °C increased modification within the oligonucleotide ACCCG that contains cytidine 49, while reducing modification at unspecific sites (Fig. 5A). This fits well with the progressive decline in the methylation of the nonspecific poly(C) RNA substrate after addition of archease (Fig. 5B). This effect is specific for the archease, because addition of comparable amounts of bovine serum albumin did not affect the methylation reaction (Fig. 5C).

To investigate further how archease increases the methyltransferase specificity, we determined whether the relative affinities of the methyltransferase for nonspecific RNAs compared with its tRNA substrate are affected by archease. Gel retardation assays for the *P. abyssi* tRNA$^{\text{Asp}}$, together with Scatchard plots to quantify free and bound tRNA, are shown in Fig. 6. The apparent dissociation constant ($K_d$) of the methyltransferase for RNA is $\sim 1 \mu\text{M}$ (Table 1). Upon addition of archease, the $K_d$ of the methyltransferase for the authentic substrate *P. abyssi* tRNA$^{\text{Asp}}$ increased about 3-fold, whereas the $K_d$ for the two nonspecific RNA substrates increased 10-fold.

Archease on its own is unable to bind tRNA (Fig. 6, lane 12) or small rRNA fragments (data not shown), indicating that its effect in reducing the affinity of the PAB1947-RNA interaction is not merely because of substrate sequestration. A more plausible explanation is that archease contributes to specificity by reducing methyltransferase binding and possibly catalytic efficiency at unspecific sites. The interaction between the archease and the methyltransferase was explored further using gel filtration and sedimentation velocity approaches.
The dissociation constants for PAB1947 enzyme on different RNAs in the presence or absence of archease were determined by gel retardation. The ribosomal RNA fragments are represented in Fig. 2D; the domain IV fragment is 72 nucleotides in length; the domain II fragment is 55 nucleotides.

| PAB1947 | PAB1947 + archease (1:6) |
|---------|--------------------------|
| P. abyssi tRNA<sup>Asp</sup> | 1.0 ± 0.1 | 2.8 ± 0.2 |
| tRNA domain IV fragment | 1.3 ± 0.1 | 1.3 ± 1.5 |
| tRNA domain II fragment | 1.7 ± 0.2 | 16.7 ± 2 |

TABLE 1

Effects of archease on the affinity of PAB1947 enzyme for specific and nonspecific RNA substrates

The sedimentation velocity data indicate the solubility state of the proteins. The start and the end of the spectral curves from the isolated PAB1947 enzyme exhibit dispersed points (supplemental Fig. S1A) that are characteristic for a partially aggregated protein. These findings are consistent with the difficulties experienced in keeping the recombinant PAB1947 enzyme soluble during purifi-
cation (see “Experimental Procedures”). The equivalent points of the curve are homogeneous for the archease/PAB1947 mixture (supplemental Fig. S1), showing that the methyltransferase remains soluble under these conditions. These results suggest that the PAB1947 enzyme tends to aggregate, and this tendency is counteracted by archease.

To confirm this observation, the ability of archease to counteract thermally induced aggregation of the PAB1947 enzyme was analyzed using a sedimentation assay coupled with SDS-PAGE analysis (Fig. 9). In the absence of archease, the unprotected PAB1947 enzyme is almost completely aggregated after 10 min at 70 °C (Fig. 9A). However, addition of a 6-fold molar excess of archease enables about half of the PAB1947 enzyme to remain soluble under the same conditions (Fig. 9B). Addition of bovine serum albumin (Fig. 9C) or the thermostable protein stathmin (24) (data not shown) offered no protection at 70 °C. Archease also prevents aggregation of PAB1947 enzyme at 4 °C, whereas BSA does not (Fig. 9D). The archease is itself an extremely thermostable protein and remains soluble up to at least 80 °C.

DISCUSSION

Members of the archease family have been proposed previously to modulate and chaperone the nucleic acid processing activities of the proteins that are encoded immediately downstream (1, 7). Here we provide evidence for this idea after determining the functions of the PAB1946 (archease) and PAB1947 (RNA methyltransferase) genes that are adjacent in the genome of P. abyssi. When the purified recombinant PAB1947 methyltransferase is allowed to function unrestrained, it catalyzes AdoMet-dependent formation of m⁵C at many locations in tRNAs, rRNA fragments, and even unstructured poly(C) RNA. Archease makes the PAB1947 enzyme more selective by reducing its affinity for nonspecific RNA as well as for nonspecific sites within tRNA, raising the respective $K_d$ values by 3- and 10-fold. In gel filtration (Fig. 8B) and in agreement with our gel retardation assays (Fig. 6), we note that a proportion of the PAB1947-tRNA complex remains unbound by archease, and we are therefore probably underestimating the improvement in specificity in the archease-bound fraction.

Thus, without the controlling influence of the archease, the PAB1947 methyltransferase interacts directly with numerous RNAs in addition to its preferred tRNA substrates. Similar cases of broad specificity among tRNA m⁵C methyltransferase have also been reported. For instance in yeast, depletion of the maturation enzyme tRNA guanylyltransferase leads to the methyltransferase Trm4p becoming more promiscuous by adding two extra methyl groups on cytosines at positions 48 and 50 in the tRNAHis, where normally m⁵C is found only at position 49 (34). The human tRNA m⁵C methyltransferase shows greatest activity in vitro on tRNA substrates, although methylation occurs with other substrates such as E. coli rRNA and viral RNA (35). Similar in vitro activity was also observed for the putative murine tRNA m⁵C methyltransferase, Misu (36). Misu expression is upregulated in vivo by the proto-oncogene myc and is connected with myc-induced cell proliferation; arresting Misu expression leads to reduction of some tumors. The broad specificities of these methyltransferases become evident only under unusual conditions, and may be a remnant property of an ancestral RNA m⁵C methyltransferase, which has subsequently evolved along different paths to achieve higher specificity.

In vitro studies with the PAB1947 methyltransferase in the absence of archease can undoubtedly be regarded as aberrant conditions. Such conditions promote the formation of a dimeric complex between the methyltransferase and its canon-
ical tRNA substrate. The PAB1946 archease improves specificity by binding directly to the PAB1947 methyltransferase to form an archease-methyltransferase complex, which then interacts with the tRNA substrates. Under the direction of archease, the resultant methyltransferase-tRNA complex is in a monomeric rather than a dimeric state. Archease on its own is incapable of forming a stable complex with tRNA, and therefore functions by directly interacting with and modulating the methyltransferase, rather than by merely sequestering the reaction components. Two structural features of the archease appear to be important for this mode of action. First, it possesses patches of negative charge on its surface (theoretical pI = 4.6), which could neutralize the predominantly positively charged regions of the PAB1947 methyltransferase and thereby prevent the methyltransferase from making nonspecific interactions with RNA phosphate backbones. Second, in addition to its monomeric state, the archease also forms homotrimers and homohexamers, and only these oligomeric forms bind the PAB1947 methyltransferase.

The structural and functional properties of archease are reminiscent of the molecular chaperone, heat shock protein 33 (Hsp33) (1, 7). Upon activation by oxidative stress, Hsp33 forms dimers, tetramers, and higher oligomers that are able to bind tightly to unfolded proteins and prevent their irreversible aggregation; the monomeric Hsp33 has no comparable function (37, 38). The gel filtration profiles of archease (Fig. 7) resemble those of Hsp33 obtained under oxidation conditions (38). The crystal structure of Hsp33 (4, 5, 39, 40) suggests that a single domain interacts with its identical counterpart to form a dimer, in a process termed domain swapping (41). The crystal structure of the T. maritima archease TM1083 (Protein Data Bank code 1J5U) (3) exhibits domain swapping via the N-terminal strand (supplementary Fig. S2) with a contact area of 1300 Å². This is in the upper range for nonphysiological crystal contacts (42), and it remains to be established whether the contacts are biological relevant and not merely crystal artifacts (1).

Based on theoretical considerations, it has been proposed that the TM1083 archease stabilizes the thermolabile DNA gyrase encoded by the adjacent downstream ORF, TM1084 (1). Such a mechanism is supported here by our experimental findings where the thermostable P. abyssi PAB1946 archease prevents aggregation of its downstream PAB1947 methyltransferase. In this respect, the archease proteins differ from the Hsp33 analogue, as the main archease function involves the
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binding and protection of folded proteins rather than unfolded and aggregated proteins (43). However, there are exceptions among the heat shock proteins, and Hsp90 also binds native p53 to prevent this labile protein from irreversible thermal inactivation (44). It should be added that the full repertoire of pyrococcal archease functions remains to be investigated, and we cannot presently rule out that archeases might also act as classical molecular chaperones to bind and refold denatured proteins.

In conclusion, we show here that the ORF PAB1947 encodes an m^5C tRNA methyltransferase which, under conditions elicited in vitro, shows extensive nonspecific activity. However, it is unlikely that such unrestrained activity would be evident under physiological conditions. The free PAB1947 methyltransferase has a marked tendency to aggregate, and its solubility and specificity require the presence of the PAB1946 archease, which is co-expressed in vivo. Similarly co-operative systems are likely to be functional in other thermophilic genera, where an archease gene is immediately upstream of an ORF encoding a protein that is expected to be functional in other thermophilic genera, where an archease gene is already formed when its chaperone needs to be functional. The study presented here is the first to experimentally test these ideas, and provides credence for the notion that members of the ubiquitous archease family serve to protect and direct the functions of adjacent genes.

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