Transfer RNA modification enzymes from *Pyrococcus furiosus*: detection of the enzymatic activities *in vitro*

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ABSTRACT

The modification patterns of *in vitro* transcripts of two yeast *Saccharomyces cerevisiae* tRNAs (tRNA\(^\text{Phe}\) and tRNA\(^\text{Asp}\)) and one archaeal Haloferax volcanii tRNA (tRNA\(^\text{Hv}\)) were investigated in the cell-free extract of *Pyrococcus furiosus* supplemented with S-adenosyl-L-methionine (AdoMet). The results indicate that the enzymatic formation of 11 distinct modified nucleotides corresponding to 12 enzymatic activities can be detected *in vitro*. They correspond to the formation of pseudouridines (Ψ) at positions 39 and 55, 2′-O-ribose methylations at positions 6 (Am) and 56 (Cm), base methylations at positions 10 (m2G), 26 (m22G), 37 (m1G), 49 (m3C), 54 (m5U) and 58 (m1A) and both the deamination and methylation of adenosine into m1I at position 57. Most of the detected modified nucleotides are common modifications found in other phylogenetic groups, while Am6, Cm56 and m1I57 are specific modifications found exclusively in Archaea. It is also shown that the enzymatic formation of m2C49, m2U54, Ψ55 and m1I57 does not depend on the three-dimensional architecture of the tRNA substrate, since these modifications also occur in fragmented tRNAs as substrate.

INTRODUCTION

Naturally occurring tRNAs always contain a variety of chemically altered nucleotides (at least 80 different structures reported to date; 1), which are formed by enzymatic modification of the primary tRNA transcript during the sequential process of tRNA maturation. Most of these nucleotides bear simple modifications such as ribose or base methylations, base isomerization, base reduction, base thiolation or base deamination. Other naturally occurring modified nucleotides represent more complex modifications such as group thiolation or base deamination. Other naturally occurring modified nucleotides include hypermodified residues, such as 2′-O-methyl derivatives of m2G and ac3C as well as the 2-thiolated derivative of m5U. However, their locations and distribution within individual species of *Pfurioso* tRNA are not known.

The identification and comparison of enzymes that catalyse the modification of the nucleotides in tRNA from different phylogenetic groups should inform on few studies have dealt with tRNA modification enzymes in extracts of archaea (for reviews see 3,4).

In the pioneering work of Best (5), the cell extract of *Methano- coccus vannielii* supplemented with labelled \(^{[\text{1}]}\text{H}-\text{CH}_3\)AdoMet was used to reveal the activities of several tRNA:methyltransferases catalyzing the formation of methylated nucleotides (m1A, m2G, m22G and Cm) in undermethylated bulk *E.coli* tRNA used as substrate. No m7G or m5U were detected, which is consistent with the absence of these modified residues in naturally occurring *M.vannielii* tRNA, implying that the corresponding methyltransferases were absent. In our previous work, the T7 transcript of *Haloferax volcanii* tRNA\(^{\text{Hv}}\) was used to reveal the enzymatic activities of the modification enzymes specific for the formation of m22G, m3C, Ψ, m2Ψ, m1A, m1I and Cm present in the cell extracts of *H.volcanii* (6). Recently, the activity of a novel tRNA-guanine transglycosylase catalyzing the formation of the modified base archaeosine in position 15 of archaeal tRNA has been demonstrated in *H.volcanii* extract using homologous tRNAs (7; see also 8). Also, the gene for the tRNAguanine-26, N2-N2 methyletransferase (Trm1) from the hyperthermophilic archaean *Pyrococcus furiosus* was cloned and successfully expressed in an active form in *E.coli*; this recombinant enzyme was shown to specifically catalyse the *in vitro* formation of m22G at position 26 in the T7 transcript of yeast tRNA\(^\text{Phe}\) (9).

*Pyrococcus furiosus* is a sulphur-metabolizing hyperthermophile of the order Thermococcales which grows optimally at 100°C under strictly anaerobic conditions (10). So far, none of the tRNA species from this archaean organism, or from other hyperthermophilic archaea, have been directly sequenced. However, the occurrence of modified nucleotides in bulk tRNA from *Pfurioso* was demonstrated by high performance liquid chromatography combined with mass spectrometry (LC/ESI-MS; 11). It was shown that *Pfurioso* tRNA contains several typically eukaryotic modified nucleotides, like Am, m1I, m2C, m2G and m22G. In addition, tRNA molecules from this hyperthermophilic archaean also contain a set of unique hypermodified nucleotides, such as 2′-O-methyl derivatives of m2G and ac3C as well as the 2-thiolated derivative of m5U. However, their locations and distribution within individual species of *Pfurioso* tRNA are not known.

The identification and comparison of enzymes that catalyse the modification of the nucleotides in tRNA from different organisms may shed light on the evolutionary origin of the modification machinery. Also, the study of cross-reactions between enzymes and tRNAs from different phylogenetic groups should inform on

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the conservation of the identity elements that are required for modification of a given nucleotide in tRNA. This idea is reminiscent of a similar approach that was successfully used to study aminoacyl-tRNA synthetases and tRNA recognition by these enzymes (reviewed in 12,13).

In this work we used T7 transcripts of two yeast tRNA genes (namely tRNA^{Asp} and tRNA^{Phe}) and of one tRNA gene (tRNA^{Ile}) of H. volcanii to characterize several tRNA modification enzymes from P. furiosus, as well as to locate their potential sites of modification in heterologous tRNAs.

**MATERIALS AND METHODS**

**Reagents**

[α-32P]-radiolabelled nucleotide triphosphates (400 Ci/mmol) were from Amersham (UK). *Penicillium citrinum* nuclease P1 and *Aspergillus oryzae* RNase T2 were from Sigma (St Louis, MO), S-adenosyl-L-methionine (AdoMet) from Boehringer Mannheim (Mannheim, Germany). Chemically synthesized deoxyoligonucleotides were purchased from MWG-Biotech (Ebersberg, Germany) and used without further purification. Thin layer cellulose plates (TLC) (type F1440) were from Schleicher and Schuell (Dassel, Germany). All other chemicals were from Merck Biochemicals (Darmstadt, Germany).

**DNA plasmids and synthetic tRNA substrates**

All plasmids bearing various tRNA genes used in this study are described elsewhere: yeast wild-type tRNA^{Phe} and its variant DNA plasmids and synthetic tRNA substrates [described previously (18)]. The minihelices and most of the TΨC in heterologous tRNA, the transcripts of yeast tRNA^{Phe}, yeast tRNA^{Asp}, and H. volcanii tRNA^{Ile} were used for in vitro transcription using T7 RNA polymerase, following the procedure described previously (18). The minihelices and most of the TΨC stem–loop variants of tRNA^{Asp} were prepared by *in vitro* transcription using T7 RNA polymerase, following the procedure described previously (18). The minihelices and most of the TΨC stem–loop variants of tRNA^{Asp} were prepared by *in vitro* transcription as described (19).

**Preparation of P. furiosus S100 extract**

Cell-free extract of *P. furiosus* (strain DSM 3638) grown at 98°C under strictly anaerobic conditions as described (10; see also 20) was prepared in 25 ml Tris–HCl, pH 7.5, 10 mM MgCl2, 10% glycerol, 2 mM DTT, 1 mM PMSF, 1 mM DFP, 1 mM benzamidine by sonication followed by ultracentrifugation at 100 000 g for 1 h. The S100 extract was stored frozen at −20°C in the presence of 20% glycerol. The protein concentration was determined according to Bradford (21).

**In vitro enzymatic assay**

The standard assay mixture (total volume 50 µl) contained 25 mM Tris–HCl, pH 7.5, 25 mM KCl, 5 mM MgCl2, 2 mM DTT, 75 µM AdoMet, 20 U RNasin and 40 fmol [32P]-radiolabelled tRNA transcript (∼5 × 105 c.p.m.). The reaction was initiated by the addition of the protein extract (1 mg/ml final concentration). Incubations were performed at 50°C for 1 or 2 h as specified in figure legends. To prevent evaporation, the reaction mixture was overlayed with 10 µl of paraffin oil (PCR oil). After incubation, the proteins were removed by phenol/chloroform extraction and the radiolabelled transcript was recovered by ethanol precipitation.

The stability of T7 tRNA transcripts towards endonucleolytic degradation in the P. furiosus S100 extract was also verified. It was noticed that, under the conditions of the test, the tRNA substrates are sufficiently stable since <20% of the transcripts were degraded during the incubation for 60 min (revealed by gel electrophoresis and quantified by trichloroacetic acid precipitation; data not shown).

**Analysis of modified nucleotides**

Modified tRNA transcripts were digested overnight by nuclease P1 (2 µg) in 50 mM ammonium acetate buffer at pH 5.3 or by RNase T2 (0.1 U) in the same buffer at pH 4.6. Identification of the 32P-labelled nucleotides in TNA hydrolysates was performed by two-dimensional (2D) chromatography on TLC plates. To unambiguously identify each modified nucleotide on the TLC plates by comparison with reference maps (22), two chromatographic systems were used: in both cases, the first dimension was developed with isobutyric acid:25% ammonia:water (66:1:33 by volume), while the second dimension was developed in 0.1 M sodium phosphate, pH 6.8:solid ammonium sulfate:n-propanol for the system N/N. Radioactive spots were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) with software ImageQuant. Calculation of the molar amount of modified nucleotide per mol tRNA substrate was done by taking into account the nucleotide composition of the tRNA transcript. For a given batch of enzyme extract, the experimental error was determined by triplicate analysis of identically treated tRNA transcripts and found to be ±0.1 mol/mol tRNA.

**RESULTS**

**Analysis of the modified nucleotides formed in tRNA incubated with P. furiosus cell-free extract**

Figure 1A shows the compilation summarizing the occurrence of modified nucleotides (type, location and frequency) in the 59 archaean tRNAs sequenced so far (23). This compilation derives from 51 tRNA sequences from the halophilic archaeon *H. volcanii* and only 8 tRNA sequences from thermophilic and hyperthermophilic archaea, such as *Methanococcus thermoautotrophicum*, *Thermoplasma acidophilum* and *Sulfolobus acidocaldarius*.

Expecting that *P. furiosus* enzymes are capable of modifying heterologous tRNA, the transcripts of yeast tRNA^{Phe} yeast tRNA^{Asp} and *H. volcanii* tRNA^{Ile} were used to test the formation of modified nucleotides that are normally present in naturally occurring tRNA (they are indicated in grey boxes in Fig. 1A–D). In addition, *P. furiosus* may contain enzymes that are not present in yeast or in *H. volcanii* cells and thus additional modified nucleotides may appear upon incubation of the yeast or *H. volcanii* tRNA transcripts in *P. furiosus* extract. Most of the modifications expected from incubation with *P. furiosus* extract are relatively simple modifications (methylated bases or ribose, pseudouridine and inosine) which do not need cofactors other than AdoMet as a donor of methyl groups. The only complex hypermodified base which may be expected is 5′A7 in *H. volcanii* tRNA^{Ile}.

However, the cofactor of this reaction is not known and this modification was not detected *in vitro* using yeast or HeLa cell extracts (24).
Figure 1. (A) Compilation of the modified nucleotides found in the 59 sequenced archaeal tRNAs indicating type, location and frequency of each modification. Abbreviations of modified nucleotides are according to Sprinzl et al. (23). Numbers in the cloverleaf correspond to location in tRNA. The first number next to each symbol in square boxes indicates the frequency of occurrence of a particular modified nucleotide; the second number corresponds to the frequency of the parent nucleotide U, C, G or A. For example, at position 8 of tRNA, s4U is found only once over a total of 59 archaeal tRNAs bearing a uridine at position 8. Unique modified nucleotides found only in archaeal tRNAs (both by chemical structure and location in the molecule) are shaded. (B–D) Cloverleaf structures of naturally occurring tRNA molecules, the T7 transcripts of which were used as substrates in in vitro tests. Modified nucleotides found in the naturally occurring tRNAs (i.e. homologous modifications) are boxed. Those modified nucleotides that are also found in archaeal tRNA are shaded.

Each of the T7 transcripts corresponding to yeast tRNA\textsuperscript{Asp}, yeast tRNA\textsuperscript{Phe} and \textit{H. volcanii} tRNA\textsuperscript{Ile} was separately radiolabelled with all four \([\alpha-^{32}P]\)nucleoside triphosphates (ATP, CTP, GTP or UTP). Each labelled transcript was then incubated with dialysed cell-free \textit{P. furiosus} extract (S100) supplemented with AdoMet. After incubation, the radiolabelled modified tRNAs were analysed for the presence of modified nucleotides as described in Materials and Methods.

The results derived from the analysis of three tRNA substrates are summarized in Table 1. Figure 2 presents some typical examples of 2D TLC separation of the modified nucleotides.

The results indicate that several modified nucleotides were synthesized in tRNA transcripts upon the incubation with \textit{P. furiosus} S100 extract. Six chemically different modified residues were formed in yeast tRNA\textsuperscript{Asp}, seven in yeast tRNA\textsuperscript{Phe} and nine in \textit{H. volcanii} tRNA\textsuperscript{Ile}. They correspond to the following different types of modified nucleotides: 2′-O-methyladenosine (Am), N\textsuperscript{2}-methylguanosine (m\textsuperscript{2}G), N\textsuperscript{2},N\textsuperscript{2}-dimethylguanosine (m\textsuperscript{2}2G), N\textsuperscript{1}-methylguanosine (m\textsuperscript{1}G), 5-methylcytosine (m\textsuperscript{5}C), pseudouridine (Ψ), ribothymidine (T), 2′-O-methylcytosine (Cm), N\textsuperscript{1}-methylinosine (m\textsuperscript{1}I) and N\textsuperscript{1}-methyladenosine (m\textsuperscript{1}A). In addition to the modifications expected from the sequence of the modified tRNAs derived from homologous systems, all tRNA transcripts bear some additional modified residues specific for the \textit{P. furiosus} modification machinery (indicated in bold in Table 1). The yeast tRNA\textsuperscript{Asp} transcript has four such additional modifications (m\textsuperscript{1}A, m\textsuperscript{1}I, Cm and m\textsuperscript{2}G), tRNA\textsuperscript{Phe} has one (Cm) and tRNA\textsuperscript{Ile} has five (Cm, m\textsuperscript{1}A, m\textsuperscript{2}G, m\textsuperscript{2}2G and Am). It is noteworthy that the Cm residue in tRNA\textsuperscript{Phe} is formed not at the expected position 32 but at position 56, which is a characteristic feature of archaeal tRNAs.

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Table 1. Modified nucleotides detected in the transcripts of yeast tRNA\(^{\text{Asp}}\)\(^{\text{GUC}}\), tRNA\(^{\text{Phe}}\)\(^{\text{GAA}}\) and \(H.\)\(^{\text{volcanii}}\) tRNA\(^{\text{Ile}}\)\(^{\text{GAU}}\) upon incubation with \(P.\)\(^{\text{furiosus}}\) S100 extract.

<table>
<thead>
<tr>
<th></th>
<th>(S.)(^{\text{cerevisiae}}) tRNA(^{\text{Asp}})(^{\text{GUC}})</th>
<th>(S.)(^{\text{cerevisiae}}) tRNA(^{\text{Phe}})(^{\text{GAA}})</th>
<th>(H.)(^{\text{volcanii}}) tRNA(^{\text{Ile}})(^{\text{GAU}})</th>
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<td></td>
<td>(S.)(^{\text{cerevisiae}}) tRNA(^{\text{Asp}})(^{\text{GUC}})</td>
<td>(S.)(^{\text{cerevisiae}}) tRNA(^{\text{Phe}})(^{\text{GAA}})</td>
<td>(H.)(^{\text{volcanii}}) tRNA(^{\text{Ile}})(^{\text{GAU}})</td>
</tr>
<tr>
<td></td>
<td>(P1)</td>
<td>(P2)</td>
<td>(P1)</td>
</tr>
<tr>
<td></td>
<td>Expected</td>
<td>Found</td>
<td>Expected</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>none</td>
<td>(m^1)A (0.7)</td>
<td>none</td>
</tr>
<tr>
<td>CTP</td>
<td>(m^3)C40</td>
<td>(m^3)C (1.1)</td>
<td>(m^3)C70</td>
</tr>
<tr>
<td>GTP</td>
<td>(m^3)G37</td>
<td>none</td>
<td>(m^3)G37</td>
</tr>
<tr>
<td>UTP</td>
<td>(\Psi_{13}^{\downarrow})32(_{55}^{\downarrow})55</td>
<td>(\Psi_{13}^{\downarrow})32(_{55}^{\downarrow})55</td>
<td>none</td>
</tr>
</tbody>
</table>

Incubations were performed for 2 h at 50°C as described in Materials and Methods. The strategy for evaluating the amount of modified nucleotides in mol/mol of tRNA is described in the text. Symbols for modified nucleotides are as in Sprinzl et al. (23). For each digestion, the first column (Expected) refers to the type and location of modifications expected on the basis of known sequence of the naturally occurring tRNA species. The second column (Found) refers to type and molar amount of modified nucleotides detected after incubation with \(P.\)\(^{\text{furiosus}}\) S100 extract. None in the column Found signifies that the corresponding modified nucleotide was not detected. Modifications specific for the \(P.\)\(^{\text{furiosus}}\) modification machinery are indicated in bold. The values are presented with an approximation of ±0.1 mol/mol for a given set of experiments. However, the yield of different modified nucleotides varies depending on the extract preparation and conservation time (±0.3 mol/mol).

*\(m^3\)C\(_{40}^{\downarrow}\)49 is synthesized only in the intron-containing tRNA\(^{\text{Phe}}\) in \(S.\)\(^{\text{cerevisiae}}\).

**\(Cm\) detected in the \(P1\) digest is in fact at position 56 and not at position 32, as detected by \(T2\) digestion of GTP-labelled tRNA\(^{\text{Phe}}\).

***Guanine at position 10 in \(H.\)\(^{\text{volcanii}}\) tRNA\(^{\text{Ile}}\) is most probably modified to \(m^2\)G, but in a given nucleotide context it cannot be distinguished from the \(m^2\)G formed at position 26 as a reaction intermediate of \(m^2\)G synthesis.

n.d., not determined.

The locations of modified nucleotides in the tRNA transcripts were deduced from the known modification patterns of yeast and archaeal tRNAs (Fig. 1A) and from data obtained by ‘nearest neighbour’ analysis (Table 1). For most of the modified residues the location can be easily deduced from the results of \(P1\) and \(T2\) hydrolyses. However, in several cases mutant tRNAs were also used to confirm the location of a given modified nucleoside (see below).

In the case of the \(m^2\)G/\(m^2\)G\(_{2}\) modifications one cannot decide if they are present at positions 10 and/or 26 in yeast tRNA\(^{\text{Phe}}\) and in \(H.\)\(^{\text{volcanii}}\) tRNA\(^{\text{Ile}}\), since both residues have the same 3’-nearest neighbour nucleotide (CMP). In addition, \(m^2\)G is a reaction intermediate of \(m^2\)G\(_{2}\) formation. Optimization of reaction conditions in the case of tRNA\(^{\text{Phe}}\) allowed detection of 1.1 mol/mol of \(m^2\)G and thus indicates the possibility of modification at two independent sites. On the other hand, only formation of \(m^2\)G\(_{2}\) was detected in mutant tRNA\(^{\text{Phe}}\) (\(G_{10} \rightarrow C\), \(C_{25} \rightarrow G\)), where the other potential modification site (\(G_{10}\)) is absent (9; data not shown). Taken together these results allow us to conclude that \(m^2\)G is formed at position 10 and \(m^2\)G\(_{2}\) at position 26 in tRNA\(^{\text{Phe}}\) and tRNA\(^{\text{Ile}}\). This conclusion also agrees with the detection of \(m^2\)G\(_{10}\) in the tRNA\(^{\text{Asp}}\) transcript. A similar situation is found for \(m^3\)C at positions 40 and 49 in \(H.\)\(^{\text{volcanii}}\).
tRNA\textsubscript{Ile} bearing GMP as nearest neighbour nucleotides. Since m\textsubscript{2}G is quantitatively formed at position 49 in yeast tRNA\textsubscript{Asp}, one may expect that the same residue would also be modified in other tRNA transcripts. However, in yeast tRNA\textsubscript{Phe}, no m\textsubscript{2}G was detected (neither at position 40 nor 49).

Analysis of the modification pattern of \textit{H. volcanii} tRNA\textsubscript{Ile} by T2 hydrolysis is more difficult, especially for modified nucleotides at positions 56–58. Depending on the modification efficiency, one can detect several modified dinucleotides after T2 digestion (Fig. 2B). For example, Cm formed at position 56 may be present in three different forms (CmA, Cmm\textsubscript{1}A and Cmm\textsubscript{1}I), since m\textsubscript{1}A is an intermediate of m\textsubscript{1}I synthesis (see below; also 6). In addition, free m\textsubscript{1}A (from both positions 57 and 58) and m\textsubscript{1}I may be detected. Quantification of the different spots indicates that Cm56 is formed in the amount of 0.8 mol/mol tRNA, while at least 0.9 mol/mol of modification at position 57 (the sum of m\textsubscript{1}A and m\textsubscript{1}I) and 0.5 mol/mol of m\textsubscript{1}A at position 58 are found. The quantitative formation of m\textsubscript{1}A (1 mol/mol) in the case of the tRNA\textsubscript{Ile} variant bearing an A\textsubscript{57}G mutation further confirms the assignment of m\textsubscript{1}A to position 58 (Fig. 2G).

The position of Am formation in \textit{H. volcanii} tRNA\textsubscript{Ile} was deduced from the results of T2 digestion, where this modified residue was found 5′-adjacent to A. Four potential AA sites are present in tRNA\textsubscript{Ile} (positions 6–7, 37–38, 57–58 and 58–59). The results of nearest neighbour analysis indicate that the modification occurs in the AAU sequence in tRNA\textsubscript{Ile} (Table 1, UTP/T2 labelling/digestion). This trinucleotide is present at positions 6–8 and positions 58–60. The latter location can be excluded since a considerable amount of another modified base (m\textsubscript{1}A58) is synthesized at this site and almost 0.9 mol/mol of AmA (Table 1) was detected in the tRNA\textsubscript{Ile} transcript. Similar results were also obtained for the A\textsubscript{57}G tRNA\textsubscript{Ile} variant where m\textsubscript{1}I formation is quantitative (see above). On the other hand, the formation of the same AmA dinucleotide was detected in yeast tRNA\textsubscript{Asp} which has the only similar AA sequence at positions 56–57 (the sum of m\textsubscript{1}A and m\textsubscript{1}I) and 0.5 mol/mol of m\textsubscript{1}A at position 58 are found. The formation of m\textsubscript{1}I (Fig. 2B) and m\textsubscript{1}A is an intermediate of m\textsubscript{1}I synthesis (see above; also 6).

\textbf{Modified nucleotides located in the TΨ loop are insensitive to the three dimensional (3D) architecture of the tRNA substrate}

The enzymatic formation of at least two modified nucleotides located in the TΨ loop of \textit{E. coli} or yeast tRNA (ribothymidine 54 and pseudouridine 55) was shown to be independent of the global 3D structure of tRNA and efficiently occurred in the minisubstrates comprised of only the TΨ stem–loop structure (19,25,26). In order to verify whether the same conclusion applies to the enzymes from \textit{P. furiosus}, as well as to test for the enzymatic formation of other modified nucleotides, several tRNA\textsubscript{Asp} mutants with stepwise reduction of the tRNA size (deletion of D stem–loop, minihelix containing the acceptor stem and TΨ stem–loop as well as shorter minihelices) were used as substrates (Figure 4). The results presented in Table 2 reveal that several modified nucleotides are efficiently formed in tRNA with a deleted D stem–loop, except for the formation of Cm56. Progressive reduction of the length of the amino acid stem (substrates 2–5 in Fig. 4) differentially affects formation of T\textsubscript{54}, Ψ\textsubscript{55} and m\textsubscript{1}I\textsubscript{57}. With the shortest minihelix tested (5 in Fig. 4), a trace amount of Ψ\textsubscript{55} was still formed after 1 h incubation at 50°C.

\textbf{m\textsuperscript{1}A\textsubscript{57} is an intermediate in m\textsuperscript{1}I\textsubscript{57} formation}

Incubation of the \textsuperscript{32}P\textsubscript{ATP}-labelled \textit{H. volcanii} tRNA\textsubscript{Ile} with \textit{P. furiosus} extract gave two characteristic dinucleotide diphosphates after hydrolysis with RNase T2, one corresponding to Cmm\textsubscript{1}Ap and the other to the dinucleotide diphosphate Cmm\textsubscript{1}Ip (Fig. 2B). Moreover, no trace of inosine in tRNA was detected when \textsuperscript{32}P\textsubscript{ATP}-labelled tRNA\textsubscript{Ile} was incubated with \textit{P. furiosus} extract without AdoMet (Fig. 2H). On the other hand, the incubation of the \textsuperscript{32}P\textsubscript{ATP}-labelled T7 transcript of the mutant \textit{H. volcanii
Figure 3. Modification patterns of yeast tRNAAsp, tRNAPhe and H. volcanii tRNAile incubated in P. furiosus S100 extract. Potential modification sites are boxed, modified nucleotides formed in tRNA transcripts are shaded. Modified nucleotide m^2G is most probably formed at position 10 in H. volcanii tRNAile (boxed, but not shaded), but due to the same neighboring nucleotide it cannot be distinguished from the reaction intermediate of m^2^2G formation at position 26.

Table 2. Modification pattern of tRNAAsp minisubstrates incubated with S100 extract of P. furiosus

<table>
<thead>
<tr>
<th>Modified nucleotide</th>
<th>m^2G0</th>
<th>m^2C0</th>
<th>m^1A57</th>
<th>m^1A56</th>
<th>m^1A55</th>
<th>m^1C56</th>
</tr>
</thead>
<tbody>
<tr>
<td>tRNAAsp yeast</td>
<td>1.0</td>
<td>1.0</td>
<td>0.5</td>
<td>0.9</td>
<td>0.5</td>
<td>0.0</td>
</tr>
<tr>
<td>deleted D-stem/loop #1</td>
<td>n.a.</td>
<td>1.0</td>
<td>0.6</td>
<td>0.9</td>
<td>0.0</td>
<td>0.5</td>
</tr>
<tr>
<td>minihelix #2 (12 bp)</td>
<td>n.a.</td>
<td>0.0</td>
<td>0.3</td>
<td>1.0</td>
<td>0.0</td>
<td>0.5</td>
</tr>
<tr>
<td>minihelix #3 (9 bp)</td>
<td>n.a.</td>
<td>0.15</td>
<td>0.4</td>
<td>0.0</td>
<td>0.15</td>
<td>0.0</td>
</tr>
<tr>
<td>minihelix #4 (5 bp)</td>
<td>n.a.</td>
<td>0.0</td>
<td>0.35</td>
<td>0.2</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>minihelix #5 (4 bp)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0.0</td>
<td>0.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

n.a., not applicable
The sequences of the five minisubstrates are indicated in Figure 4.

tRNAile gene (A^57→G) with P. furiosus extract yielded no m^1IMP, but only m^1 AMP (1 mol/mol) after hydrolysis with nuclease P1 (Fig. 2G). From these experiments, one can conclude that the biosynthesis of m^1I occurs at position 57 in tRNA via a two-step enzymatic process. The first step corresponds to the formation of m^1A57 by AdoMet-dependent tRNA methyltransferase, followed by the deamination of the 6-amino group of the adenine moiety. The same results were obtained using S100 extract of Sulfolobus shibatae as enzyme source (data not shown).

DISCUSSION

Run-off transcripts of synthetic genes with the primary structure of a natural tRNA but lacking all modified nucleotides have been proven to be valuable substrates for testing the activity of several tRNA modification enzymes in vitro (reviewed in 27). For testing the activity of enzymes present in the cell-free extract of P. furiosus, we used the transcripts corresponding to yeast tRNAAsp, yeast tRNAPhe and H. volcanii tRNAile as a model system. The modification pattern of these tRNAs was extensively characterized under various assay conditions (28–30). Also, the physicochemical properties of the yeast tRNAPhe and yeast tRNAAsp transcripts are known (14,16) and several mutants with disrupted 3D tRNA structure are available (15,17,29). The use of these heterologous tRNAs as substrates for P. furiosus enzymes was based on the expectation that they may also serve as substrates in heterologous reaction. Indeed, the modification pattern of archaeal tRNA (Fig. 1A) has many features in common with eukaryotic tRNAs, with several sites identically modified in both types of substrates. In contrast, the use of homologous P. furiosus tRNA is complicated due to lack of exact information on their natural modifications and to their extremely high GC content (as demonstrated from DNA gene sequences; 23) which has hampered direct RNA sequencing.
In this work, the heterologous transcripts of yeast and *H. volcanii* tRNAs were used to test the activity of *P. furiosus* modification enzymes in *vitro*. The incubation temperature was fixed at 50°C, instead of 70–100°C corresponding to optimal cell growth. This temperature was chosen taking into account the melting of unmodified T7 transcripts of tRNA^Phe^ and tRNA^A^, which begins at 55–60°C (14,16). However, this elevated incubation temperature may nevertheless affect folding of unmodified heterologous tRNA transcripts in the extract and somehow hamper the activity of tRNA 3D structure-dependent modification enzymes. The other major limitation of the *in vitro* approach lies in the lack of knowledge on low molecular mass reaction cofactors that are required for enzymatic formation of certain ‘complex’ modified nucleotides (for example tP^A^).

The use of heterologous tRNA transcripts has allowed us to identify the activities of 11 modification enzymes present in the *P. furiosus* cell-free extract. Most of the detected modifications correspond to AdoMet-dependent methylations of base (m^3^C, m^2^G, m^2^G, m^2^G, m^1^A and m^1^T) or ribose (Am and Cm) and formation of pseudouridine at two distinct positions (Ψ^39^ and Ψ^53^) in tRNA. The locations of most of the synthesized modified residues are similar to those observed for yeast tRNAs (m^5^G^26^, m^2^G^37^, m^5^C^49^, T^54^, Ψ^55^, Ψ^39^ and Ψ^53^). However, some archaea-specific modifications present in the tRNA TV loop (Cm^56^ and m^1^I^57^) and in the aminoacyl acceptor stem (Am) were also detected. Ribose-methylated adenosine has not been found at position 6 (Am^6^) in any tRNA sequenced so far, but the same modified nucleotide was detected at position 4 (Am^6^) in a few eukaryotic tRNA (23).

Several modified nucleotides (Ψ, m^3^C and Cm) can be formed at multiple sites in archaeal mRNA. Two potential sites of m^3^C methylation (positions 40 and 49) are present in *H. volcanii* tRNA^Phe^. Our data suggest that only one m^3^C residue is formed in this transcript upon incubation in the *P. furiosus* extract. Most probably the modification takes place at position 49 while position 40 remains unmodified. In yeast tRNA^Phe^ the formation of m^3^C is a strictly intron-dependent process and the pre-tRNA structure is required for modification (30). In contrast, the tRNA^Phe^ gene of *H. volcanii* has no intervening sequence (R.Gupta, unpublished results). On the other hand, it was recently demonstrated that the formation of Cm^32^ and Cm^34^ in *H. volcanii* tRNA^Phe^ depends on the presence of an intron (C.Daniels, reported at the 98th ASM annual meeting in Atlanta, May 1998). Hence, it is possible that the absence of Cm^32^ formation in *H. volcanii* tRNA^Phe^ is related to the use of an intronless tRNA transcript.

The biosynthesis of Ψ^13^, Ψ^38/39^ and Ψ^55^ is catalyzed by distinct enzymes in *E. coli* and yeast. Our results demonstrate that Ψ^39^ and Ψ^55^ are efficiently synthesized in *P. furiosus* extract. In contrast, the formation of Ψ^13^ in the yeast tRNA^A^ transcript was not detected, while the formation of this residue occurs upon incubation in yeast extract (31). Modification of Ψ^13^ is a characteristic feature of the archaeal tRNAs (out of 30 uridines at position 13 only one remains unmodified; Fig. 1A). The absence of modification in the case of yeast tRNA^A^ transcript may somehow reflect different recognition properties of yeast and archaeal enzymes acting on Ψ^13^. Likewise, m^3^G^37^ is formed in yeast tRNA^Phe^ but not in yeast tRNA^A^. This last situation probably reflects differences in identity requirements between the *P. furiosus* and the yeast enzyme catalysing the formation of m^3^G^37^. A similar situation was described for *E. coli* tRNA^m^G^37^-methyltransferase which catalyses m^3^G formation only in *E. coli* tRNAs bearing a G^36^-G^37^ sequence and not in yeast tRNA^A^ (32,33).

In our *in vitro* study we detected the formation of ribothymidine (m^3^U, T) at position 54 in all three tRNA transcripts. It should be noted that this modified nucleoside is highly conserved in prokaryotic and eukaryotic tRNAs, yet is only rarely present in archaeal tRNA. In the majority of the sequenced tRNAs it is replaced by m^1^Ψ or hypermodified derivatives of T (such as s^2^T or s^2^Tm). However, the HPLC analysis of *P. furiosus* tRNA extracted from cells grown at different temperatures reveals the presence of T in cells grown at 50–60°C, while it is completely absent at 95–100°C (only s^2^T or s^2^Tm are present) (11). The efficient formation of T^54^ in *in vitro* most probably reflects the *in vivo* biosynthetic pathway, but due to the absence of 2-thiolation, the reaction yields only the intermediate which normally does not accumulate.

The absence of detectable amounts of inosine formed at position 57 in tRNA (as an intermediate of m^1^I^57^ biosynthesis) is noteworthy. While inosine is the intermediate of m^1^I formation at position 37 in yeast tRNA^A^ (34), the pathway of m^1^I synthesis in Archaea is different. It has been demonstrated that in *H. volcanii* tRNA, m^1^I^57^ is formed by deamination of m^1^A^57^ and not by the methylation of inosine (57) (6). Here we extend this conclusion to *P. furiosus* and *S. shibatae*. Taken together these results allow us to conclude that the pathway of m^1^I^57^ synthesis in Archaea is different from the one found in Eucaryota.

Numerous studies have clearly established the existence of three major domains of life (Archaea, Bacteria and Eucaryota) (35). However, detailed studies of the archaeal metabolism and enzymes strongly indicate the presence of both eubacterial and eukaryotic features (36–40). The basal metabolism of archaeal cells is more closely related to that of eubacteria, while the metabolism related to gene expression (DNA transcription, mRNA synthesis, mRNA translation and splicing) is definitely more related to that of eukaryotes. The reactions implicated in tRNA modification occupy an intermediate position. These processes are tightly connected to basal cellular metabolism as they use numerous low molecular weight cofactors, synthesized also for other purposes (AdoMet, sulphur-containing precursors, isopentenyl-pyrophosphate,…). On the other hand, since tRNA molecules play a key role in the process of mRNA decoding, one can also expect that the tRNA modification machinery is related to that of Eucaryota, which is indeed the case for formation of m^5^G^10^, m^2^G^26^ and m^3^C^49^.

In addition, the archaeal tRNA machinery has several unique features (the presence of Am^6^, Cm^56^ and m^1^I^57^) which clearly distinguish it from the other two living domains.

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