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Effect of a quaternary pentamine on RNA stabilization and enzymatic methylation

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Abstract

Extreme thermophiles produce unusually long polyamines, including the linear caldopentamine (Cdp) and the branched pentamine tetrakis(3-aminopropyl)-ammonium (Taa), with the latter containing a central quaternary ammonium moiety. Here we compare the interaction of these two pentamines with RNA by studying the heat denaturation, electrophoretic behavior, and ability of tRNA to be methylated *in vitro* by purified tRNA methyltransferases under various salt conditions. At concentrations in the micromolar range, branched Taa causes a considerable increase in the melting temperature (T_m) of yeast $tRNA^{Phe}$ transcripts by >20°C, which is significantly greater than stabilization by the linear Cdp. In non-denaturing gel electrophoresis, strong and specific binding to Taa, but not to Cdp, was clearly observed for $tRNA^{Phe}$. In both types of experiments, polyamines and monovalent metal ions competed for binding sites. Structural probing revealed no significant conformational changes in tRNA on Taa binding. In post-transcriptional *in vitro* methylation reactions, the formation of m^2G/m^2_2G by the methyltransferase Trm1p and of m^1A by Trmlp were not affected or only slightly stimulated by polyamines. In contrast, Taa specifically inhibited Trm4p-dependent formation of m^5C only in $tRNA^{Phe}$, likely by occupying sites that are relevant to RNA recognition by the methyltransferase.

Keywords: biophysical; polyamines; thermal stabilization; tRNA; tRNA methyltransferase; UV melting.

Introduction

Biogenic polyamines are known to carry multiple positive charges at physiological pH and therefore undergo strong ionic interactions with the negatively charged phosphate backbone of nucleic acids. This interaction shields the phosphates from each other, thus affecting the structure of nucleic acids at single-digit millimolar polyamine concentrations. A similar effect is observed for monovalent cations, although comparable effects require much higher concentrations (100–1000 mM), and divalent cations such as Mg^{2+} (1–10 mM) (Tabor and Tabor, 1984).

Early studies on the effect of biogenic polyamines on tRNA (Wildenauer et al., 1974) using UV-melting and post-transcriptional methylation assays suggested that cadaverine (1,5-diaminopentane) can stabilize the tRNA structure under one set of conditions and destabilize the structure under a different set of conditions, such as in the presence and absence of magnesium ions. Indeed, because of their high positive charge density, magnesium ions can compete with polyamines for certain binding sites and this competition may be a key feature in the biological function of polyamines. In discussions on this issue, it is often stressed that the distance between two positively charged amine groups within a linear polyamine fits the average distance between two adjacent negatively charged phosphates in the major groove of B-form DNA or A-form RNA (Ouameur and Tajmir-Riahi, 2004), thus enabling multi-dentate binding to the phosphate backbone of nucleic acids. This is in contrast to the spatially localized charge of divalent cations, whose strongest binding is usually observed in small pockets. This leads to the general notion that an important cellular function of biogenic polyamines might be to confer flexibility to RNA structures by displacing some magnesium ions, thereby weakening their rigidifying effect on RNA (or DNA) structure but still efficiently shielding the negative backbone charges. Such flexibility might be responsible for improving enzyme reactions involving nucleic acids, such as transcription of DNA by T7-RNA polymerase (Frugier et al., 1994).

It was recently observed that thermophilic organisms produce unusual polyamines, in particular those of higher chain length (up to 8 amine groups) and tetrakis(3-aminopropyl) ammonium (the pentamine Taa), the most prominent example of a branched polyamine (Oshima et al., 1987; Oshima, 1989; Ikeda et al., 1992; Hamana et al., 2003). Biophysical experiments have shown that stabilization of RNA structure increases with the number of amino groups in linear polyamines (Terui et al., 2005). Differential stabilization effects were observed for the branched pentamine Taa compared to its linear isomer caldopentamine (Cdp). Indeed, whereas Taa stabilized double-stranded DNA less efficiently than Cdp, the structures of single-stranded DNA and of tRNA harboring a characteristic 3D conformation were more efficiently stabilized by Taa in differential scanning calorimetry experiments (Terui et al., 2005). Since DNA structure essentially comprises a double-stranded helix, it is likely that the *in vivo* binding targets of branched polyamines such as Taa are RNAs, which are known to adopt a plethora of well-defined three-dimensional structures and complicated folds. These observations are in accordance with the requirement for hyperthermophilic organisms to protect RNAs (tRNA and rRNA) from structural denaturation and hydrolytic degradation at temperatures up to 105°C that

are not compatible with RNA function under normal circumstances (Grosjean and Oshima, 2007).

The hypothesis that the quaternary branched Taa uses a binding mode that recognizes particular aspects of tRNA tertiary structure is supported by the findings of Oshima and coworkers, who investigated aminoacylation reactions of various pairs of tRNAs and their cognate synthetases under the influence of polyamines from thermophiles (Uzawa et al., 1994). The effects observed were differential: whereas *in vitro* tRNA charging with glycine, isoleucine, methionine, and proline mediated by an S-100 protein extract was largely insensitive to the presence of Taa, the phenylalanine system showed a drastic decrease in aminoacylation. This strongly suggests that Taa specifically binds to fine details of the tertiary structure of $tRNA^{Phe}$ from *Thermus thermophilus*, thus selectively inhibiting its recognition.

In the present study we investigated in detail the mode of tRNA binding of Taa and, for comparison, its linear counterpart Cdp (Figure 1A). Our RNA models were well-characterized transcripts of yeast $tRNA^{Phe}$ and $tRNA^{Asp}$; the techniques used were UV melting, non-denaturing PAGE, structural probing, and post-transcriptional enzymatic modification reactions to assess binding of proteins to different regions of the tRNA structure.

Results

Strong tRNA stabilization against thermal denaturation by pentamines and competition among cations for RNA binding sites

Differentiated UV melting curves were recorded to determine the influence of polyamines on the thermal denaturation profile of tRNA. Since the polyamines investigated are compounds found exclusively in thermophilic organisms, initial melting experiments measured their effect on a transcript of $tRNA^{Asp}$ from the hyperthermophilic *Pyrococcus furiosus*. However, since the G+C content of this tRNA is high (72%, with almost exclusively G-C base pairs in stems), it naturally exhibited a melting temperature (T_m) of >90°C, which is too high to be useful in monitoring thermal stabilization. Consequently, in experiments at various concentrations and/or combinations of potassium ions, magnesium ions, Cdp and Taa, effects in the temperature range 50–90°C were not significant or not observable (data not shown). We therefore decided to characterize the effect of polyamines using another model transcript, namely that of the well-investigated yeast $tRNA^{Phe}$ (54% G+C, with several A-U pairs in stems).

In the presence of 1 mM $MgCl_2$, the sc-$tRNA^{Phe}$ transcript exhibited a strong well-defined transition with a T_m of 54°C and an additional minor transition visible as a flat shoulder around 65°C (Figure 1C). Addition of Cdp and/or Taa at 5 or 50 μM led to a single transition with an important increase in T_m. The strongest shift of 21°C was evident at 50 μM Taa (T_m 75°C, upper panel of Figure 1C). Even in the presence of 100 mM KCl, polyamines led to strong thermal stabilization (lower panel of Figure 1C). Interestingly, whereas 100 mM KCl stabilized tRNA in the absence of polyamines (increase in T_m from 54 to 59°C), it decreased or even negated the tRNA stabilization by polyamines under certain conditions. Thus, in the absence of potassium ions, addition of 5 μM polyamine led to an increase in T_m of 5°C (Cdp) or 9°C (Taa), whereas in the presence of 100 mM KCl this stabilization amounted to only 1°C (Cdp) or 2°C (Taa) (Figure 1C). The T_m values are summarized in Table 1. These observations support the notion of ionic competition between salts and polyamines for binding sites on the tRNA. Binding of Taa to tRNA appears to be stronger, because in all cases the T_m increase caused by Taa was stronger than that caused by Cdp under identical conditions. Regardless of the presence of potassium ions, 50 μM Cdp led to stronger stabilization of sc-$tRNA^{Phe}$ than 5 μM Taa, which thus represents an upper limit for the relative binding strength of Taa compared to Cdp.

With this limitation in mind, competition experiments were conducted to determine whether tRNA stabilization by Taa could be further increased by addition of Cdp or, conversely, whether Cdp would decrease stabilization by Taa in a competitive manner similar to that described above for KCl. The lower panel of Figure 1D shows that, in the presence of 50 μM Taa, the T_m of sc-$tRNA^{Phe}$ remained constant despite addition of Cdp up to 50 μM. This was true in the presence and absence of 100 mM KCl. However, potassium ions led to a destabilizing effect on T_m, irrespective of the amount and combination of polyamines used. Similar competition experiments were performed at a lower Taa concentration (5 μM). As suggested above, a tenfold Cdp excess (50 μM) can compete with 5 μM Taa and provide further T_m stabilization; again, the presence of KCl decreased all T_m values (Figure 1D).

Similar melting experiments were performed with transcript of another yeast tRNA (sc-$tRNA^{Asp}$; 58% G+C). Although the general trends mentioned above were also apparent, this tRNA exhibited uncooperative melting with several transitions, which made assignment of the T_m values difficult (results not shown).

The quaternary pentamine Taa forms a stable complex with tRNA

The spectacular 21°C increase in T_m for sc-$tRNA^{Phe}$ on addition of the quaternary polyamine Taa suggested a strong and possibly specific interaction between the two components. For a more detailed and comparative characterization, the electrophoretic migration behavior of tRNA on a non-denaturing polyacrylamide gel was investigated. To assess the potential specificity of the polyamine-tRNA interaction, these investigations were extended to sc-$tRNA^{Asp}$. Non-denaturing PAGE is particularly suited to reveal long-lived complexes of RNA and stable alternative RNA structures based on differential migration compared to a standard. The formation of RNA structures and complexes was promoted by an initial protocol of heat denaturation and renaturation of the transcript RNA at room temperature in the presence of magnesium ions, potassium ions, Cdp, and Taa. At the start of electrophoresis in Tris-borate-EDTA (TBE) buffer, the RNA and polyamine and metal cations are immediately separated by migration to opposing directions. Thus, in contrast to native electrophoresis, which is

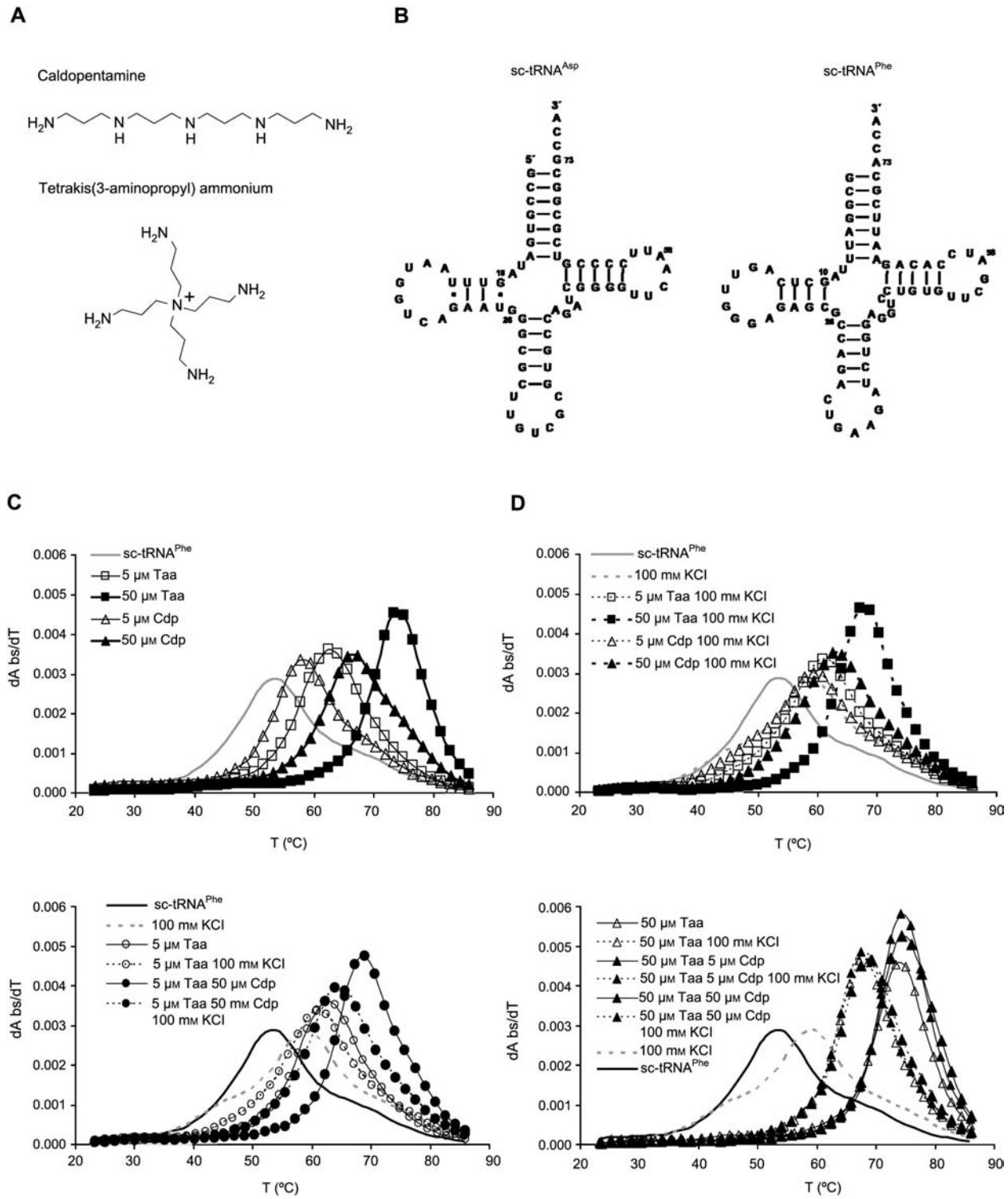

Figure 1 Effects of pentamine on thermal melting of tRNA.
(A) Pentamines used in this study. Caldopentamine (Cdp) is a linear polyamine with five aminofunctions. Tetrakis(3-aminopropyl)ammonium (Taa) is a quaternary amine of the same molecular weight and a permanent positive charge. (B) tRNAs investigated in this study: sc-tRNA^{Asp} and sc-tRNA^{Phe}. (C) Top: differentiated melting curves of sc-tRNA^{Phe} as a function of pentamine concentration in the absence of monovalent ions. Bottom: competition of pentamines in tRNA stabilization in the absence of monovalent ions. Top: differentiated melting curves of sc-tRNA^{Phe} as a function of pentamine concentration in the presence of 100 mM 100 mM potassium chloride. (D) Bottom: competition of pentamines in tRNA stabilization in the presence of 100 mM 100 mM potassium chloride.

typically conducted with circulating buffers, structures and complexes that are stable in the absence of divalent cations and for the duration of the electrophoresis experiment exhibit migration behavior that deviates from that of the control.

As shown in Figure 2, the presence of 1 mM $MgCl_2$ and/or 100 mM KCl in the renaturation mixture did not affect the migration behavior of either tRNA^{Asp} or tRNA^{Phe} (lanes 1–4 and 17–20, respectively). The presence of Cdp (50 μM) caused both tRNAs to slightly smear during

Table 1 Melting point of sc-tRNA^{Phe} as a function of pentamine concentration.

	Tm (°C)					
	0 μM Cdp		5 μM Cdp		50 μM Cdp	
	0 mM KCl	100 mM KCl	0 mM KCl	100 mM KCl	0 mM KCl	100 mM KCl
0 μM Taa	54	59	59	60	67	64
5 μM Taa	63	62	–	–	69	65
50 μM Taa	75	68	75	68.5	75	69

migration (lanes 9–10 and 25–26), indicating that the lifetime of the Cdp-tRNA complex is such that some Cdp is released during electrophoretic migration (apparently faster from tRNA^{Phe} than tRNA^{Asp}). The presence of KCl in addition to Cdp during the initial incubation before gel migration notably decreased this smearing (lanes 11–12 and 27–28), providing further evidence of competition between potassium ions and Cdp for binding sites on the tRNA structure.

The presence of the quaternary pentamine Taa in the mixture caused more drastic effects during PAGE. Samples renatured in the presence of Taa but without potassium ions smeared across the entire lanes (lanes 5–6 and 21–22), indicating that binding of Taa was strong enough to persist throughout the duration of the entire electrophoresis run (~4 h), with persistence of the complex greater for sc-tRNA^{Phe} than for sc-tRNA^{Asp}. Again, this effect was largely overcome in samples preincubated with potassium ions in addition to Taa (lanes 7–8 and 23–24), with distinct bands migrating even slightly faster than for the control samples, possibly indicating a slightly more compact structure of the migrating tRNA than for the control (lanes 1–4 and 17–20). Further addition of Cdp to Taa-containing samples did not change migration behavior (lanes 13–16 and 29–32). These experiments confirm competition between potassium ions and polyamines for apparently overlapping tRNA binding sites and provide evidence of stronger overall binding of the branched Taa compared to the linear Cdp. Of particular interest is the differential behavior of the tRNAs towards Taa. Whereas sc-tRNA^{Asp} migrated as a smear lacking defined bands in lanes containing Taa, two new bands for sc-tRNA^{Phe} were evident in Taa-containing lanes (lanes 21–24 and 29–32), of which only one was subject to efficient competition by potassium ions, whereas the more defined of the two bands (indicated by an arrow in Figure 2) remained stable even after preincubation of pentamine-tRNA in the presence of 100 mM KCl (lanes 23–24 and 31–32). This might indicate the existence of a specific and defined binding mode of Taa to sc-tRNA^{Phe}, which might also be the cause of the spectacular Taa-induced increase in T_m by 21°C.

Structural probing reveals no structural change in tRNA^{Phe} in the presence of quaternary pentamine

The apparent strength of the Taa-tRNA^{Phe} interaction raised the question as to whether it affects the tRNA

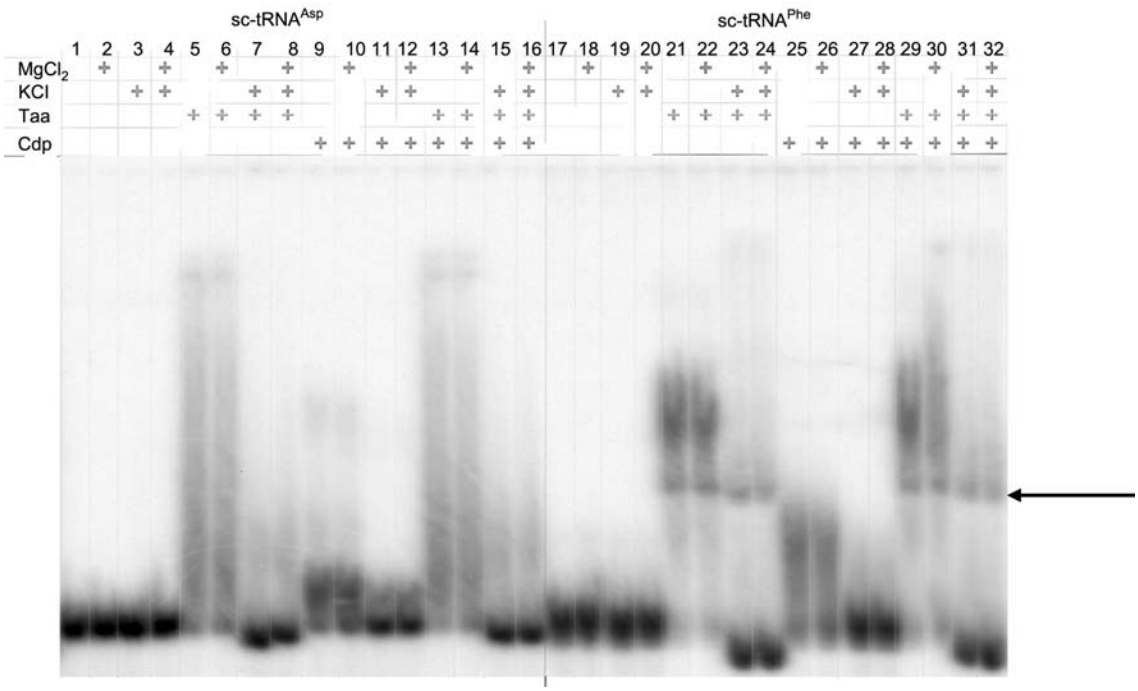

Figure 2 Effect of pentamines on tRNA migration.
[^{32}P]-radiolabeled tRNA (60 nM) was incubated in the presence of 1 mM $MgCl_2$ and/or 100 mM KCl, 50 μM Taa and/or 50 μM Cdp. Samples were heat denatured at 70°C for 5 min and renatured at room temperature for 30 min before loading onto a non-denaturing 8% polyacrylamide gel (29:1). The arrow on the right indicates a distinct new tRNA^{Phe} band arising from the presence of Taa in the renaturation mixture.

structure, which prompted investigation by structural probing. Since lead(II)-induced cleavage of sc-tRNA^{Phe} is exquisitely sensitive to structural perturbations (Behlen et al., 1990), the cleavage pattern of 5′-end-labeled sc-tRNA^{Phe} after exposure to various concentrations of $Pb(OAc)_2$ at several time points was analyzed by PAGE. Neither the major hydrolysis sites at G18 and G19 nor any other cleavage site was affected by the presence of Taa (Figure 3A), indicating that the overall structure was unchanged. This interpretation is supported by a lack of change in the CD spectrum of sc-tRNA^{Phe} on addition of either polyamine to 50 μM (data not shown).

In further investigations, cleavage by nuclease V1 (Ehresmann et al., 1987; Giegé et al., 1999) was slightly altered at only two sites, A33, where cleavage was enhanced in the presence of Taa, and G42, where cleavage was slightly decreased (Figure 3B,C). The low extent of these changes in combination with the unchanged overall tRNA structure suggests potential strong binding of Taa at or close to the variable loop within a canonical L-shaped 3D-architecture.

Effect of pentamines on post-transcriptional modification

Since structural probing experiments did not provide information on the binding of Taa to tRNA, we used selected tRNA modification enzymes to test the efficiency of post-transcriptional methylation by tRNA methyltransferases to determine Taa-related structural changes or potential binding regions. Wildenauer et al. (1974) used a partially purified tRNA methylase fraction from rat liver containing m^2G26, m^1A58 and m^5C49 methylase activities to investigate the effect of magnesium ions in combination with cadaverine (a diamine) on the ability of naturally occurring *Escherichia coli* tRNA^{fMet} to be methylated *in vitro* by eukaryotic enzymes. Here we determined the methylation of sc-tRNA^{Asp} and sc-tRNA^{Phe} transcripts in the presence of various combinations of Cdp, Taa, $MgCl_2$ and KCl by three purified recombinant enzymes. tth-TrmIp, an enzyme from *T. thermophilus*, catalyzes site-specific methylation of N^1-A58 (Droogmans et al., 2003). pfu-Trm1p, an enzyme from *P. furiosus*, catalyzes single or double methylation of N^2-G26 (Constantinesco et al., 1999). sc-Trm4p, an enzyme from *Saccharomyces cerevisiae*, catalyzes the specific formation of m^5C48 and/or m^5C49 (Motorin and Grosjean, 1999). The methylation sites are indicated on the clover leaf and the three-dimensional tRNA structure in Figure 4A.

As shown in Figure 4B, under our experimental conditions tth-TrmIp efficiently methylated sc-tRNA^{Asp} (1 mol m^1A/mol tRNA) but not sc-tRNA^{Phe}, and the methylation yield of yeast tRNA^{Asp} was not affected by the presence of KCl or/and Taa in the reaction mixture. Unexpectedly, linear Cdp alone allowed hypermethylation of sc-tRNA^{Asp} (data under 5 and 6 in Figure 4B) probably at position A57, as does the homolog enzyme from *Pyrococcus abyssi* (pab-TrmIp) (Roovers et al., 2004). Interestingly, this extra methylation was largely decreased in the presence of potassium ions and absent in the presence of Taa.

pab-Trm1p methylated the exocyclic N^2 of G26 in both yeast tRNAs *in vitro* (Figure 4C). For sc-tRNA^{Asp}, monomethylation of G26 (on average 0.8 mol methyl groups/mol tRNA) was detected under all conditions tested. For sc-tRNA^{Phe}, dimethylation of G26 occurred and the yield of radioactive methyl groups incorporated in tRNA was consequently higher than 1 mol per mol tRNA (on average ~1.4 mol/mol tRNA), except when Cdp alone or in combination of KCl was used in the incubation mixture (data under 5 and 6 in Figure 4C). The presence of Taa in the reaction mixture had a mild detrimental effect in both the presence and absence of potassium ions (Figure 4C). Cdp stimulated dimethylation of G26 to levels of up to 1.7 mol equivalents (data under 5 and 6 in Figure 4C). As observed for tth-TrmIp mediated methylation of yeast tRNA^{Asp}, the simultaneous presence of Cdp and Taa led to methylation levels comparable to those obtained under normal conditions (compare data under 7 and 8 and 1–2 in Figure 4B, C).

Yeast Trm4p catalyzes the formation of m^5C at position 49 in both model intronless yeast tRNAs used and at position 48 only in sc-tRNA^{Phe} (Motorin and Grosjean, 1999). As shown in Figure 4D, a strong inhibitory effect of Taa was observed, especially when sc-tRNA^{Phe} was used as substrate, although this was abolished in the presence of potassium ions (compare data under 3 and 4 in Figure 4C). In contrast, the presence of Cdp or Cdp and KCl considerably improved the yield of m^5C formation, which involves both C48 and C49 for sc-tRNA^{Phe} (data under 5–6 in Figure 4C). However, the simultaneous presence of Cdp and Taa or Taa, Cdp and potassium ions led to almost complete inhibition of m^5C formation (data under 7–8 in Figure 4D), reflecting competition among the various cations for common binding sites, with the effect of Taa being dominant. In this case competitive mutual antagonism between Cdp and Taa is evident.

In summary, the general trend for linear Cdp was slight stimulation of all three types of methylation reaction (data under 5–6 in Figure 4B, C), as already reported for shorter biogenic polyamines (Young and Srinivasan, 1971; Wildenauer et al., 1974; Glick et al., 1978; Cartlidge et al., 2005). In contrast, branched quaternary pentamine Taa tended to inhibit methylation, but only in the case of m^5C formation catalyzed by yeast Trm4p. This effect was strikingly more pronounced for sc-tRNA^{Phe} than for sc-tRNA^{Asp} as substrate, supporting the notion of a relatively specific interaction between Taa and tRNA^{Phe}.

Discussion

The interaction of RNA with networks of spatially organized positive charges in the form of amino moieties is of considerable interest in the field of RNA research and has strong implications for public health. For example, aminoglycosides, which can be viewed as a number of amino moieties spatially organized on a sugar scaffold, are known to bind to various structured RNAs and inhibit their interactions with macromolecular partners (e.g. Blanchard et al., 1998). Binding of the aminoglycoside tobramycin to sc-tRNA^{Asp} inhibits aminoacylation by the cognate aspartyl-tRNA synthetase and decreases its T_m in UV melting experiments (Walter et al., 2002). Biogenic polyamines are involved in a wide variety of cellular processes, including regulatory pathways for which inter-

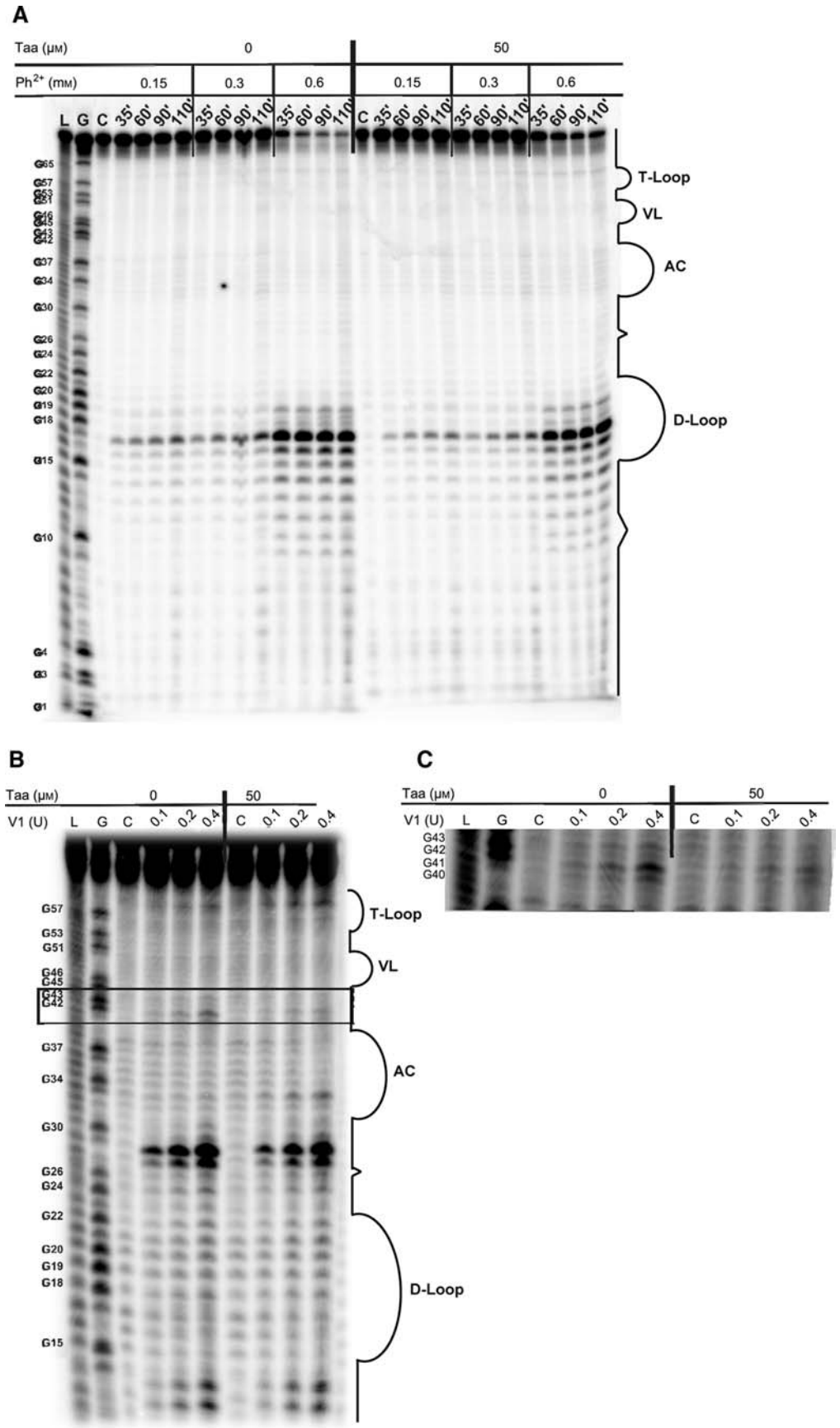

Figure 3 Structural probing of tRNA in the presence and absence of Taa.
(A) Lead(II)-induced cleavage pattern of 5′-labeled $tRNA^{Phe}$ analyzed by 15% denaturing PAGE. (B) Nuclease V1-induced cleavage pattern of 5′-labeled $tRNA^{Phe}$ analyzed by 15% denaturing PAGE. (C) Magnification of the V1 cleavage pattern identified in B.

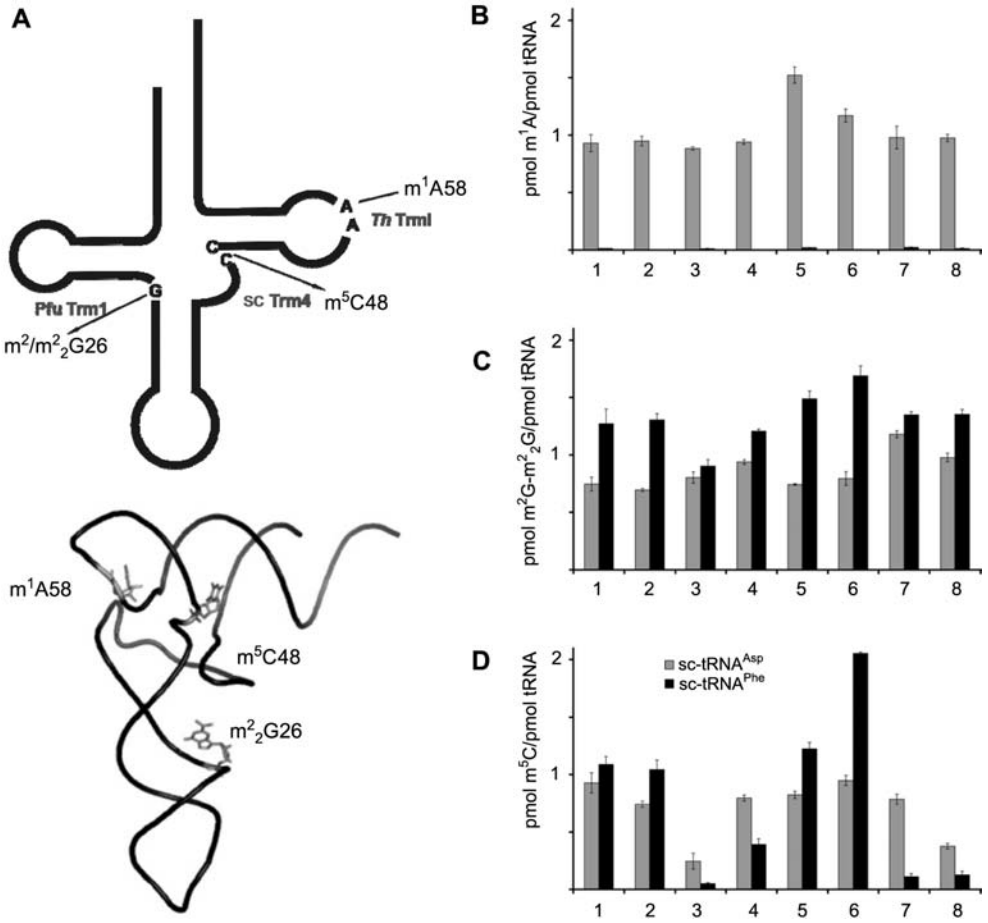

Figure 4 Effect of pentamine on post-transcriptional modification of tRNA.
(A) Methylation sites of TrmI, Trm1 and Trm4 on the clover leaf and the three-dimensional tRNA structure. Modification levels measured after incubation of tRNAs with [^{3}H]-SAM in the presence of (B) TrmI, (C) Trm1, and (D) Trm4. Values for sc tRNA^{Asp} are represented by gray bars and values for sc tRNA^{Phe} by black bars. Salt and polyamine concentrations were as follows: 1, 1 mM $MgCl_2$; 2, 1 mM $MgCl_2$ and 100 mM KCl; 3, 1 mM $MgCl_2$ and 50 μM Taa; 4, 1 mM $MgCl_2$, 50 μM Taa and 100 mM KCl; 5, 1 mM $MgCl_2$ and 50 μM Cdp; 6, 1 mM $MgCl_2$, 50 μM Cdp and 100 mM KCl; 7, 1 mM $MgCl_2$, 50 μM Taa and 50 μM Cdp; 8, 1 mM $MgCl_2$, 50 μM Taa, 50 μM Cdp and 100 mM KCl.

actions between the polycations and nucleic acids, mainly RNA, are central to their biological effects (Igarashi et al., 1980; Yoshida et al., 2001; Higashi et al., 2008). In extreme thermophilic organisms, clear correlation exists between the cellular concentrations of certain unusually long and branched polyamines and optimal cell growth (Oshima, 1989). The binding mode of linear polyamines is commonly viewed as flexible, since it has multiple binding sites with very similar binding energies and low transition barriers between two such modes. Moreover, the overall stability of the complex formed with polymeric nucleic acids is almost proportional to the number of amine groups. In contrast, a branched polyamine might conceivably use differential binding modes that might strongly depend on the type and architecture of the bound nucleic acid.

A central feature of the present study is the observation of a strong increase in tRNA T_m resulting from the binding of two polyamines that are structural isomers, each containing five amino groups. Although Cdp and Taa are of identical size, the differential connectivity among amino groups causes striking differences in their effects on RNA structure. The most obvious difference is the quaternary nitrogen atom of Taa (Figure 1A), which carries a permanent positive charge regardless of the pH of the surrounding medium. The remaining amino moieties in Taa and all the amino groups in Cdp acquire a positive charge only after protonation, with most but not all of these groups being protonated at physiological pH. Previous studies revealed an intriguing and rather selective detrimental effect of Taa on phenylalanylation of *T. thermophilus* tRNA^{Phe}, suggesting a distinct binding mode to this particular tRNA. Since linear polyamines usually bind to RNA rather indiscriminately of its tertiary structure, these findings raised the intriguing question to what extent branched polyamines would specifically interact with RNAs containing a peculiar tertiary architecture. The previous studies were carried out using *T. thermophilus* total tRNA and an S-100 extract of proteins from the same organism, and thus more detailed investigations of selected interaction partners were not possible (Uzawa et al., 1993a,b, 1994). Our methylation studies revealed that such detrimental effects are not limited to biochemical reactions of the protein synthesis itself and occur with other tRNA-interacting proteins. This strongly supports the long-standing notion that polyamines bind strongly to proteins, but to RNA. Our present results establish that the interaction between branched

Taa and sc-tRNA^{Phe} is specific and particularly strong, leading to an increase in T_m of >20°C and visualization of this complex by electrophoresis on non-denaturing polyacrylamide gel at room temperature.

We initiated our studies using a transcript of tRNA^{Asp} from a hyperthermophilic archaeal organism (*P. furiosus*). One aim was to test whether emulation of intracellular conditions such as high intracellular salt content (Scholz et al., 1992) or/and the presence of unusual branched polyamines (Hamana et al., 2003) would allow *in vitro* RNA biochemistry at elevated temperatures (i.e., near 100°C). However, KCl concentrations >300 mM inhibited methylation reactions, and no methylation activity was observable at >80°C. In addition, although the archaeal tRNA^{Asp} is a substrate for both of the hyperthermophilic modification enzymes we tested here, this tRNA was not suitable for the task at hand. The experimental evidence indicates that this tRNA transcript is misfolded *in vitro*, possibly because of a lack of post-transcriptional modification (data not shown). A common feature of tRNA genes in thermophiles is their high G-C content, in contrast to the rest of the genome (Marck and Grosjean, 2002). Typical consequences for tRNA structure include melting points >80°C, leaving little dynamic range in which to investigate structural stabilization by polyamines. In view of the above, it was decided to continue the investigations using well-characterized transcripts from yeast tRNA^{Asp} and tRNA^{Phe}, for which a plethora of biochemical and biophysical data facilitates interpretation. In particular, interaction of native tRNA^{Phe} with a variety of polyamines, including Cdp and Taa, has previously been investigated by differential scanning calorimetry (Terui et al., 2005). Of particular interest in the present investigations are the gel shift data obtained in non-denaturing PAGE experiments. Whereas linear Cdp induced slight smearing only in the absence of monovalent cations, branched Taa resulted in a clearly distinguishable and well-defined band for tRNA^{Phe} (indicated by an arrow in Figure 2C). Such differential migration might represent a metastable alternative folding of lower mobility, which would indicate a metastable alternative conformation with partially denatured features responsible for the retarded migration. However, comparison of hyperchromicity in the UV melting curves clearly shows that Taa led to compaction and stabilization of the tRNA^{Phe} structure rather than to partial denaturation. Therefore, the additional band represents a complex of Taa bound to tRNA^{Phe} for which the interaction is stable enough to remain associated during migration.

Analysis of the binding mode of polyamines to nucleic acids has been the subject of a number of studies (Antony et al., 1999). The emerging view is that of a polycationic 'glue' that essentially follows the phosphate-sugar backbone of a nucleic acid helix. Certain authors attach importance to the distance between amino groups, represented by the number of methylene moieties in the spacers (Koculi et al., 2004; Ouameur and Tajmir-Riahi, 2004). Because of the uniformity of a nucleic helix, polyamine binding is not site-specific; the molecule has numerous binding modes of similar energy. Such a 'linear' binding model might apply to the linear polyamine Cdp investigated here: its moderate stabilization of T_m and slight stimulation of post-transcriptional methylation reactions are in accordance with a number of similar observations for naturally occurring, relatively short linear polyamines such as cadaverine and putrescine (di-amines), spermidine (tri-amine) and spermine (tetra-amine) (Young and Srinivasan, 1971; Wildenauer et al., 1974; Glick et al., 1978; Cartlidge et al., 2005). In contrast, the clear differences in binding of branched Taa to tRNA^{Phe} compared to tRNA^{Asp} strongly point to a peculiar binding mode of Taa to structural features of tRNA^{Phe} that are not present in the similarly folded tRNA^{Asp}, and must therefore be considered as very specific.

Significantly, Taa binding did not disturb Pb^{2+}-induced RNA cleavage, which is known to be very sensitive to structural perturbations, especially for sc-tRNA^{Phe}. From this it can be concluded that the branched pentamine Taa does not prevent m^5C formation by yeast Trm4p by perturbing the tRNA conformation, but more likely by simply blocking access to necessary binding sites of the enzyme on the tRNA. This view is reinforced by the finding that tRNA recognition by bacterial Trm1p and TrmIp, which are known to access the tRNA substrate from a different side, is unperturbed.

In conclusion, we identified an interaction between the branched pentamine Taa and yeast tRNA^{Phe} that is specific for both partners, since interactions with Cdp or yeast tRNA^{Asp} were nowhere near as strong. Although the specificity of the polyamine is attributable to the quaternary ammonium moiety, that of the tRNA species is likely to be associated with the finer details of its structure. Among the implications of the present work is the potential toxicity of quaternary polyamines in thermophiles, which might exert selective pressure against certain RNA structures. However, a number of findings must also be considered. For example, our observation of competition between potassium cations and polyamines for binding to tRNA implies a similar situation in *Pyrococcus* cells, where an intracellular salt concentration of >500 mM (Scholz et al., 1992) would affect any potential selective pressure resulting from polyamine-RNA binding. Of high interest in this context is the fact that certain extreme halophilic archaeons with intracellular potassium ion concentrations of 4–5 M (Brown, 1976) do not synthesize polyamines (Chen and Martynowicz, 1984), possibly because these high salt conditions render polyamines useless.

Materials and methods

Recombinant modification enzymes were prepared as described in the literature for *T. thermophilus* TrmIp (Droogmans et al., 2003), *P. furiosus* Trm1p (Constantinesco et al., 1999), and yeast Trm4p (Motorin and Grosjean, 1999). The plasmid-harboring gene coding for tth-TrmIp was a kind gift from Louis Droogmans. The polyamines caldopentamine (Cdp) and tris(3-aminopropyl)amine (Taa) were a generous gift from Tairo Oshima to H.G. Their preparation is described elsewhere (Oshima et al., 1987).

Preparation of tRNAs

DNA transcription template MH156 (5′-TGG CGC CCG GGC CGG GAT TTG AAC CCG GGT CGC GGG AGT GAC AGT CCC GCA TGA TAG GCC GGG CTA CAC CAC CCG GGC TAT AGT GAG TCG TAT TA-3′) and primers MH159 (5′-TGG CGC CCG GGC CGG GAT TT-3′) and MH53 (5′-CGC GCG AAG CTT AAT

ACG ACT CAC TAT A-3′) were from IBA (Göttingen, Germany). The DNA template was PCR amplified (2 min at 94 °C; 15 cycles of 30 s at 57°C, 45 s at 72°C and 15 s at 92°C; and 10 min at 72 °C). The PCR reaction mixture [3 mM $MgCl_2$, 67 mM Tris-HCl, pH 8.8, 16 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 1 μM each primer, 3 μM template, 1 mM each dNTP, 0.1 U/μl Taq DNA polymerase) was directly used for *in vitro* transcription. Plasmid ptFMA encoding yeast $tRNA^{Asp}$ (Perret et al., 1990) was a gift from Catherine Florentz (Strasbourg, France) and the plasmid for yeast $tRNA^{Phe}$ was from Olke Uhlenbeck (Sampson and Uhlenbeck, 1988). Plasmids were amplified in DH5α cells and isolated using a Qiagen (Hilden, Germany) MaxiPrep kit according to the manufacturer's instructions. Plasmids were linearized by incubation with BstN1 according to the manufacturer's protocol (BioLabs, Frankfurt/Main, Germany).

Non-radioactive tRNA transcripts were prepared by *in vitro* transcription using T7 RNA polymerase. [^{32}P]-radiolabeled tRNA transcripts were prepared by *in vitro* transcription in the presence of [α-^{32}P]-CTP or [α-^{32}P]-GTP (30 μCi in a volume of 40 μl). For transcription, 4 μl of PCR product or 1 μg/μl plasmid was incubated in reaction buffer containing 40 mM Tris-HCl, pH 8.1, 1 mM spermidine, 0.01% Triton-X, 30 mM $MgCl_2$, 10 mM dithiothreitol and, for internal labeling, 1 mM CTP or GTP, 4 mM each ATP, UTP, GTP or CTP and 30 μCi of [α-^{32}P]-CTP or GTP (Hartmann Analytic, Braunschweig, Germany), respectively, and, for cold transcription, 4 mM each NTP in the presence of T7 RNA polymerase in a final volume of 40 μl for 4 h at 37°C. After purification by 10%/8 M urea PAGE, the RNA was visualized by UV shadowing or exposure of a phosphorimaging plate and scanning on a Typhoon 9400 system (GE Healthcare, Munich, Germany). The desired bands were excised and transcripts were passively eluted by agitation in 0.5 M NH_4OAc and subjected to ethanol precipitation.

Non-denaturing PAGE

[^{32}P]-radiolabeled tRNA (60 nM) was incubated in the presence of 1 mM $MgCl_2$ and the amounts of KCl, Taa and Cdp indicated. Samples were heat denatured at 70°C for 5 min and renatured at room temperature for 30 min before loading onto a native 8% polyacrylamide gel (29:1 AA/Bis; 33 cm×40 cm×0.5 cm) run in 1× TBE buffer at room temperature without buffer circulation. Samples were electrophoresed at 20 mA for 4 h. RNA bands were detected by exposure of the gel to a phosphorimaging plate and subsequent scanning on a Typhoon 9400 system (GE Healthcare).

Thermal denaturation of tRNAs

To measure UV melting curves, solutions of tRNAs were prepared at a final tRNA concentration corresponding to an OD_{260} of 0.5 in 50 mM phosphate buffer (NaH_2PO_4 and Na_2HPO_4 at pH 7.0), 1 mM $MgCl_2$, and appropriate concentrations of KCl, Taa and/or Cdp, as indicated in the text. Samples were degassed in an ultrasonic bath for 10 min and transferred to a 1.0-cm cuvette (Hellma QS108B, Hellma, Müllheim, Germany) and a layer of M50 silicon oil (Roth, Karlsruhe, Germany) was added. Melting curves were measured in a Cary 100Bio instrument (Varian, Darmstadt, Germany). As a uniform initial renaturation procedure, the temperature was increased to 90°C at 5 °C/min, held for 5 min, and decreased to 20°C at 5°C/min. After a pause of 5 min, data collection was started for a temperature increase to 90°C at 0.5°C/min, after which the temperature was returned to 20°C at the same rate. T_m values were determined from at least three experiments and showed standard deviation of <0.2°C.

Structural probing of tRNA

Probing with lead was performed as previously described (Helm et al., 1998). Briefly, 5′-labeled transcripts were renatured as described above and incubated under native conditions (40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 40 mM NaCl and 50 μM Taa where indicated) in the presence of 0.15, 0.3 and 0.6 mM $Pb(OAc)_2$ for 35, 60, 90 and 110 min in a final volume of 20 μl. Reactions were stopped by immediate cooling on ice and addition of 20 μl of Pb STOP buffer (8 M urea, 10 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue in TBE buffer).

Probing with nuclease V1 was performed as previously described (Helm et al., 1998). Briefly, reaction mixtures of 20 μl containing 3 μg of total tRNA and 5′-labeled transcripts (approx. 50 000 Cerenkov counts) (and 50 μM Taa where indicated) were incubated for 10 min at 25°C in 20 ml of 40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ and 40 mM NaCl in the presence of 0.1, 0.2 and 0.4 U of RNase V1. Reactions were stopped by immediate cooling on ice and addition of 20 μl of V1 STOP buffer (0.6 M NaOAc, 3 mM EDTA, 0.1 mg/ml total tRNA), followed by phenol extraction and precipitation with 400 μl of 2% $LiClO_4$ in acetone. Aliquots of the mapping reactions were then analyzed by denaturing 12% PAGE using size standards generated by limited digestion with alkali or RNase T1 as described by Helm et al. (1998).

In vitro methylation assay with recombinant enzymes

[^{3}H]-labeled *S*-adenosyl-L-methionine (SAM) was obtained from Hartmann Analytic. tRNA (180 pmol) was diluted in water and denatured at 75°C for 5 min in the presence of appropriate concentrations of KCl, Taa and Cdp as indicated in the text. The assay buffer was added immediately to final concentrations of 100 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$ and 1 mM DTT, and the RNA was renatured by cooling to room temperature for 30 min. [^{3}H]-SAM stock solution (10× containing 70 μM cold SAM and 2 μM [^{3}H]-SAM) was added to a final concentration of 7.2 μM SAM per sample and incubated for 2 min at 37°C for Trm1, at 50°C for Trm4 and at 60°C for TrmI. The reaction was started by addition of the enzyme to a final concentration of 1 μM. Aliquots of the reaction mixture were spotted onto small Whatman filters after 1 or 2 h and precipitated in 5% ice-cold trichloroacetic acid (TCA), followed by two washing steps in 5% TCA for 10 min on ice. The filters were then dried and the incorporated tritium signal was measured by liquid scintillation counting.

For TLC analysis, reactions were run under the above conditions using [α-^{32}P]-NTP labeled RNA and cold SAM. After incubation, the reaction mixture was supplemented with 1 volume of formamide loading buffer [90% (v/v) formamide, 10% (v/v) 1× TBE], heat denatured, and loaded onto a 10%/8 M urea polyacrylamide gel. After electrophoresis, undegraded tRNA was localized by autoradiography and reisolated as described above. After ethanol precipitation, the RNA was redissolved in 20 mM NH_4OAc, 1 mM $ZnCl_2$ and supplemented with total tRNA from *E. coli* to a concentration of 1 μg/μl. Complete digestion to 5′-phosphate mononucleotides was performed by addition of 0.3 U of Nuclease P1 in 1 μl and incubation overnight at room temperature. Aliquots were spotted onto cellulose-coated TLC plates (Merck, Darmstadt, Germany) and developed in two dimensions in solvents A and C (Grosjean et al., 2007). Quantification was performed after reading of exposed imaging plates on a Typhoon system (GE Healthcare) using ImageQuant v5.2 Software.

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