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The non-enzymatic hydrolysis of oligoribonucleotides VI. The role of biogenic polyamines

Arkadiusz Bibillo, Marek Figlerowicz and Ryszard Kierzek*

The Institute of Bioorganic Chemistry, Polish Academy of Sciences, Noskowskiego 12/14, 61-704 Poznan, Poland

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ABSTRACT

Single-stranded oligoribonucleotides containing UA and CA phosphodiester bonds can be hydrolyzed specifically under non-enzymatic conditions in the presence of spermidine, a biogenic amine found in a wide variety of organisms. In the present study, the rate of oligonucleotide and $tRNA_i^{Met}$ hydrolysis was measured in the presence of spermidine and other biogenic amines. It was found that spermine $[H_3N^+(CH_2)_3{}^+NH_2(CH_2)_4{}^+NH_2(CH_2)_3{}^+NH_3]$ and putrescine $[H_3N^+(CH_2)_4{}^+NH_3]$ can replace spermidine $[H_3N^+$-$(CH_2)_4{}^+NH_2(CH_2)_3{}^+NH_3]$ to induce the hydrolysis. For all three polyamines, a bell-shaped cleavage rate versus concentration relationship was observed. The maximum rate of hydrolysis was achieved at 0.1, 1.0 and 10 mM spermine, spermidine and putrescine, respectively. Moreover, we found that the hydrolysis requires at least two linked amino groups since two aminoalcohols, 2-aminoethanol and 3-aminopropanol, were not able to induce the cleavage of the phosphodiester bond. The optimal cleavage rate of the oligoribonucleotides was observed when amino groups were separated by tri- or tetramethylene linkers. The methylation of the amino groups reduced the ability of diamines to induce oligoribonucleotide hydrolysis. Non-enzymatic cleavage of $tRNA_i^{Met}$ from *Lupinus luteus* and $tRNA_i^{Met}$ from *Escherichia coli* demonstrate that both RNAs hydrolyze as expected from principles derived from oligoribonucleotide models.

INTRODUCTION

The biogenic polyamines are simple, aliphatic compounds present in both prokaryotic and eukaryotic cells (1). Three of the most important polyamines are putrescine, spermidine and spermine. In spite of extensive studies, their biological role remains unclear. It has been demonstrated that the level of the polyamines increases remarkably in rapidly growing cells. Gosule and Schellman postulated that the polyamines bind to DNA and RNA, thereby affecting cellular processes (2).

Interactions of the polyamines with single- and double-stranded DNA, RNA and with DNA triplexes have been described (3–15). Sundaralingam *et al.* solved the crystal structure of the complex of spermine and the octamer d(GTGTACAC) (3,4). Surprisingly, there was no direct interaction between spermine cations and phosphate anions. The spermine bound via hydrogen bonds to O6 and N7 of G's and O4 of T's in a GTGT fragment. Interestingly, Williams *et al*. observed different binding interactions of spermine to d(CGATCG) and d(CGTACG) although both DNA hexamers were crystallized in similar conditions (5). Quigley *et al.* demonstrated that spermine binds only to the major groove of d(CGATCG) (6). Robinson and Wang found that spermine can induce DNA conformation to change from B form to A form (9). The authors hypothesize that A form optimizes electrostatic interactions of the DNA with spermine.

Interactions of polyamines with RNA are less well understood than with DNA and studies are almost exclusively limited to tRNA. Frydman *et al*. demonstrated that spermine binds to tRNA stronger than spermidine does (10). Under the same conditions putrescine binds to tRNA very weakly. The authors observed that in all polyamines, internal $-NH_2^+-$ groups bind to tRNA more strongly than terminal $-NH_3^+$ groups. They hypothesize that factors other than the electrostatic interaction are responsible for this phenomenon since the primary amines ($-NH_3^+$) possess a higher density of positive charge than the secondary ones ($-NH_2^+-$). Bolton and Kearns used NMR methods to find that addition of one spermine molecule per 10 phosphate residues of poly(U) eliminates the 2'-hydroxyl group resonances without affecting the resonances of other groups (11). According to Bolton and Kearns this suggests that the polyamines bind non-specifically to RNA.

In this communication we describe a concentration-dependent effect of putrescine, spermidine and spermine on the rate of specific RNA cleavage. Moreover, the influence of the three different polyamine structures on the rate of the oligoribonucleotides hydrolysis is established.

MATERIALS AND METHODS

Synthesis and purification of oligoribonucleotides

The oligoribonucleotides used in experiments were synthesized by the phosphoramidite method on a polymer support and deprotected according to published procedures (16,17). Purification was performed by thin-layer chromatography (TLC) on silica gel plates (0.5 mm, Merck) in propanol-1:ammonia:water (55:35:10 v/v/v). The purity of oligoribonucleotides was analyzed by C-8 high performance liquid chromatography and 20% polyacrylamide gel electrophoresis (PAGE). Oligoribonucleotides were labeled at their 5'-termini with [γ-^{32}P]ATP and T4

*To whom correspondence should be addressed. Tel: +48 61 8528 503; Fax: +48 61 8520 532; Email: rkierzek@ibch.poznan.pl

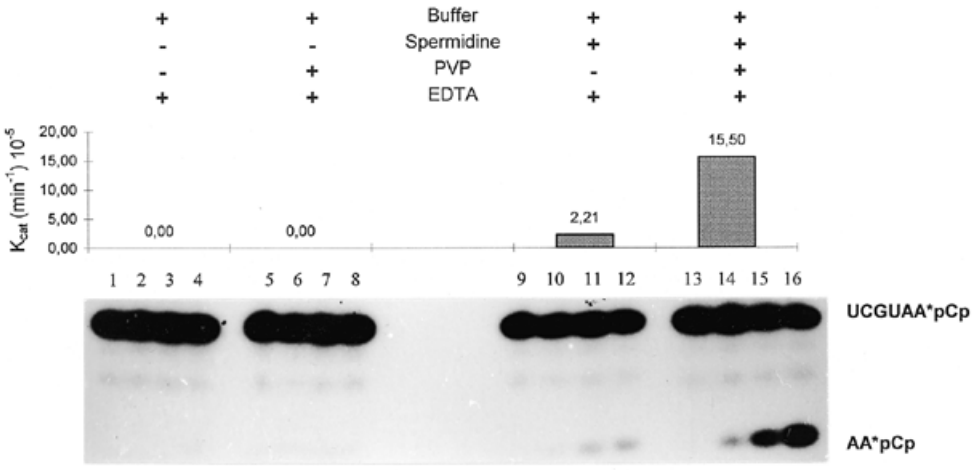

Figure 1. The stability of UCGUAA*pCp in the presence of different components of the standard buffer at 37°C. Lanes 1–4, in 50 mM Tris–HCl (pH 7.5), 2 mM EDTA after 0, 10, 24 and 48 h, respectively; lanes 5–8, in 50 mM Tris–HCl (pH 7.5), 0.1% PVP, 2 mM EDTA after 0, 10, 24 and 48 h, respectively; lanes 9–12, in 50 mM Tris–HCl (pH 7.5), 1 mM spermidine, 2 mM EDTA after 0, 10, 24 and 48 h, respectively; lanes 13–16, in 50 mM Tris–HCl (pH 7.5), 1 mM spermidine, 0.1% PVP, 2 mM EDTA after 0, 10, 24 and 48 h, respectively.

polynucleotide kinase or at 3' termini with [5'-^{32}P]pCp and T4 RNA ligase. The labeled oligomers were purified by TLC or PAGE. The $tRNA_i^{Met}$ were labeled at 5'- or 3'-termini and purified according to general procedures.

Materials

The T4 polynucleotide kinase and T4 RNA ligase were obtained from US Biochemicals, and [γ-^{32}P]ATP and [5'-^{32}P]pCp were from Amersham. Polyvinylpyrrolidone (PVP; mol. wt 360 kDa) was from Sigma. The polyamines, diamines, methylated diamines and aminoalcohols used in the experiments were from Aldrich or Fluka at the highest available purity and additionally purified by vacuum distillation or crystallization. The buffers and glassware were autoclaved or treated with diethyl pyrocarbonate prior to the experiments.

Hydrolysis of oligoribonucleotides

Standard hydrolysis conditions of the oligoribonucleotides include ~0.1 pmol of 5'- or 3'-^{32}P-labeled oligoribonucleotides incubated in 50 mM Tris–HCl (pH 7.5), 1 mM spermidine and 2 mM EDTA in the presence of 0.1% PVP solution at 37°C. Addition of 3' terminal pCp does not affect the reaction. Aliquots were quenched with formamide and analyzed by 20 or 14% (for tRNA) PAGE. To determine the value of K_{cat}, aliquots of the hydrolysis mixture after 10, 30 and 60 min were loaded on a 20% polyacrylamide gel and this was quantitatively analyzed by phosphorimager. To calculate the value of K_{cat}, the first order reaction formula was used since there were eight to nine orders of magnitude excess of the polyamine relative to the oligoribonucleotide during cleavage.

RESULTS AND DISCUSSION

The phenomenon of site-specific, non-enzymatic hydrolysis of single-stranded oligoribonucleotides is well studied in our laboratory (18–22). We have observed that in the absence of enzymes that could cleave RNA (ribonucleases) certain single-stranded oligoribonucleotides, as short as tetramer, undergo a specific hydrolysis (18,19). Typically, cleavage is observed in 50 mM Tris–HCl (pH 7.5), 2 mM EDTA in the presence of 1 mM spermidine and millimolar concentration of PVP, the model cofactor for most experiments. The specific hydrolysis occurs primarily between UA or CA phosphodiester bonds. The products of the hydrolysis are oligomers terminated with 2',3'-cyclic phosphate on the 5'-side and 5'-hydroxyl on the 3'-side of the hydrolyzed internucleotide bond as described previously (18,19). The mechanism of the cleavage was also studied by the use of thiophosphate oligoribonucleotides (20). During the cleavage of oligoribonucleotides no depurination or opening of the nucleobase ring was observed. Two cleavage products accounted for all initial nucleotides.

Identification of the factors necessary for the specific RNA cleavage

To determine which components of the standard reaction mixture are required for the specific UCGUAAp*Cp cleavage, the stability of this heptamer under various conditions was tested. The results in Figure 1 show that without the presence of biogenic amine, there is no cleavage of UCGUAAp*Cp in the reaction buffer [50 mM Tris–HCl (pH 7.5)] with or without 0.1% PVP. Specific hydrolysis is observed in the buffer containing 1 mM spermidine but its rate was about seven times lower than with PVP. This indicates that the presence of the spermidine is

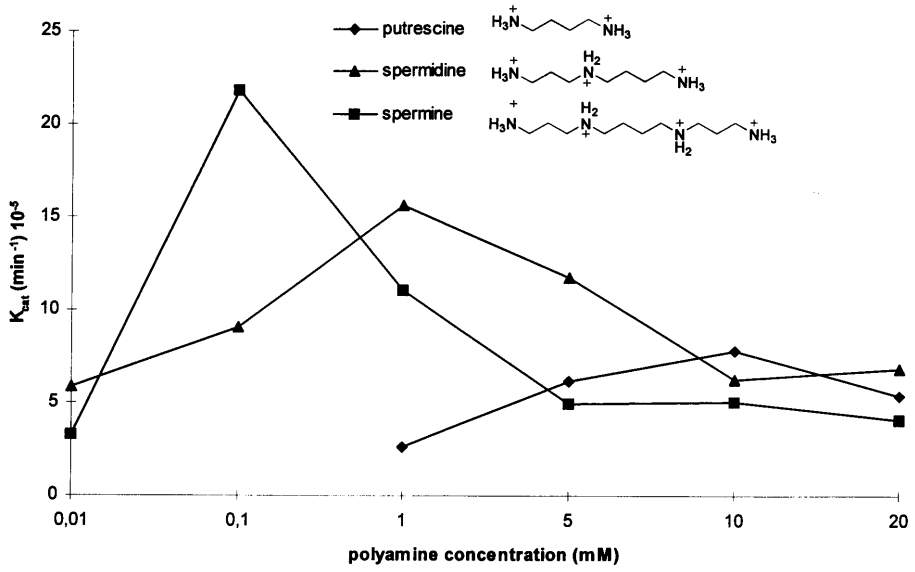

Figure 2. The relationship of UCGUAA*pCp hydrolysis rate and the concentration of putrescine (diamonds), spermidine (triangles) and spermine (squares).

required for the specific oligoribonucleotide cleavage. Great variability of the tested cofactors (some organic polymers and non-ribonuclease proteins) suggests their non-specific interaction with oligoribonucleotide or with the oligomer hydration shell (19). The change in oligoribonucleotide hydration could affect the nucleophilic and electrophilic character of the functional groups involved in the RNA cleavage (i.e. 2'-hydroxyl group, phosphate group and 5'-oxygen).

The observation that spermidine is capable of inducing the selective oligoribonucleotide cleavage raised the question of whether the two other biogenic amines, putrescine and spermine, would display the same activity (18,19). To solve this problem the hydrolysis of UCGUAACp in the presence of putrescine or spermine was carried out. Results show that putrescine and spermine are able to induce the specific oligoribonucleotide cleavage similar to spermidine, but at different concentrations. It was also demonstrated previously that the rate of oligoribonucleotide hydrolysis depends on the spermidine concentration (18). The curve describing this correlation is bell-shaped with maximum cleavage at 1 mM spermidine. We measured the rate of hydrolysis of UCGUAAp*Cp in the presence of putrescine and of spermine at various concentrations from 10 μM to 20 mM. The results obtained for both polyamines were compared with former data concerning spermidine (Fig. 2) (18). Both curves are bell-shaped as was previously observed for spermidine. However, in every case a different concentration of the polyamine was required to reach the maximum rate of the specific cleavage: 0.1 mM for spermine ($K_{cat} = 21.8 \times 10^{-5}$ min^{-1}), 1.0 mM for spermidine ($K_{cat} = 15.6 \times 10^{-5}$ min^{-1}) and 10 mM for putrescine ($K_{cat} = 7.8 \times 10^{-5}$ min^{-1}). This suggests that an active oligomer conformation is present at specific polyamine concentrations (12,23). At higher concentrations, a more ordered, inactive structure may prevail.

The role of polyamine structure

The results described above imply that the rate of specific hydrolysis is influenced by the number of the protonated amino groups present in the polyamine cations. However, even at twice the concentration, putrescine, with two terminal amines, is not able to induce the oligoribonucleotide cleavage as efficiently as spermine which has two terminal and two internal amines. This observation suggests that, in addition to the number of positive charges, other polyamine characteristics affect the cleavage. To explore these factors, different polyamine homologs and derivatives were employed. The rate of hydrolysis in the presence of diamines such as 1,2-diaminoethane (Put2) and 1,3-diaminopropane (Put3) and aminoalcohols such as 2-aminoethanol (Put2OH) and 3-aminopropanol (Put3OH) were compared. In contrast to the diamines, aminoalcohols could not induce oligoribonucleotide cleavage (Fig. 3). This suggests that at least two linked, protonated amino groups are necessary to mediate hydrolysis. We hypothesize that one ammonium group binds to the oligoribonucleotide while the second one participates in the activation of the labile phosphodiester bond (11).

Next, we analyzed whether the length of the methylene chain linking protonated amino groups influences the rate of cleavage. In this experiment putrescine (1,4-diaminobutane; Put4) and its four homologs: Put2, Put3, 1,5-diaminopentane (Put5 or Cad) and 1,6-diaminohexane (Put6 or Cad6) were used (Fig. 4). We found that cleavage rate is maximal with Put3. Hydrolysis is slightly slower for Put2 and Put4. For diamines with longer

Figure 3. The stability of UCGUAA*pCp in the presence of 50 mM Tris–HCl (pH 7.5), 0.1% PVP, 2 mM EDTA at 37°C and different aminoalcohols and diamines. Lanes 1–4, incubated in the presence of 5 mM Put2OH after 0, 10, 24 and 48 h, respectively; lanes 5–8, incubated in the presence of 5 mM Put3OH after 0, 10, 24 and 48 h, respectively; lanes 9–12, incubated in the presence of 5 mM Put3 after 0, 10, 24 and 48 h, respectively; lanes 13–16, incubated in the presence of 5 mM Put2 after 0, 10, 24 and 48 h, respectively.

carbon chains, Put5 and Put6, the rate of specific oligoribonucleotide cleavage was reduced to less than half of the rate with Put3.

In order to confirm that our observations are general, additional studies involving triamines were carried out. Besides the natural spermidine (Spd34) in which the protonated ammonium groups are linked by 3- and 4-methylene chains, we used spermidine 33 [Spd33; $H_3N^+(CH_2)_3{}^+NH_2(CH_2)_3{}^+NH_3$], spermidine 32 [Spd32; $H_3N^+(CH_2)_3{}^+NH_2(CH_2)_2{}^+NH_3$] and spermidine 22 [Spd22; $H_3N^+(CH_2)_2{}^+NH_2(CH_2)_2{}^+NH_3$]. This experiment demonstrated no simple correlation between the results obtained for di- and triamines (data not shown). However, the natural Spd34 induces the hydrolysis the most efficiently.

Earlier observations indicate that the ability of the polyamine to induce the RNA cleavage strongly depends on polyamine–oligoribonucleotide interactions (3–13). These interactions can be achieved by hydrogen bond formation, as we observed previously in structural studies of binding of the polyamine cations with phosphate anions (24–26).

Derivatives of Put3 possessing reduced abilities to form hydrogen bonds were tested including Put3 and the following methylated Put3 derivatives: *N*-methyl-1,3-diaminopropane, *N*,*N*-dimethyl-1,3-diaminopropane, *N*,*N*,*N*',*N*'-tetramethyl-1,3-diaminopropane, 2,2-dimethyl-1,3-diaminopropane and *N*,*N*,*N*,*N*',*N*',*N*'-hexamethyl-1,3-diammonium propane diiodide. The results demonstrated that progressive methylation of the diamine decreased its ability to induce oligoribonucleotides hydrolysis (Fig. 5). The methylation of the one ammonium group slightly reduces the effect of Put3 on hydrolysis. However, a large decrease in rate of hydrolysis was observed when both amino groups were methylated. This indicates that the amino groups perform a different function

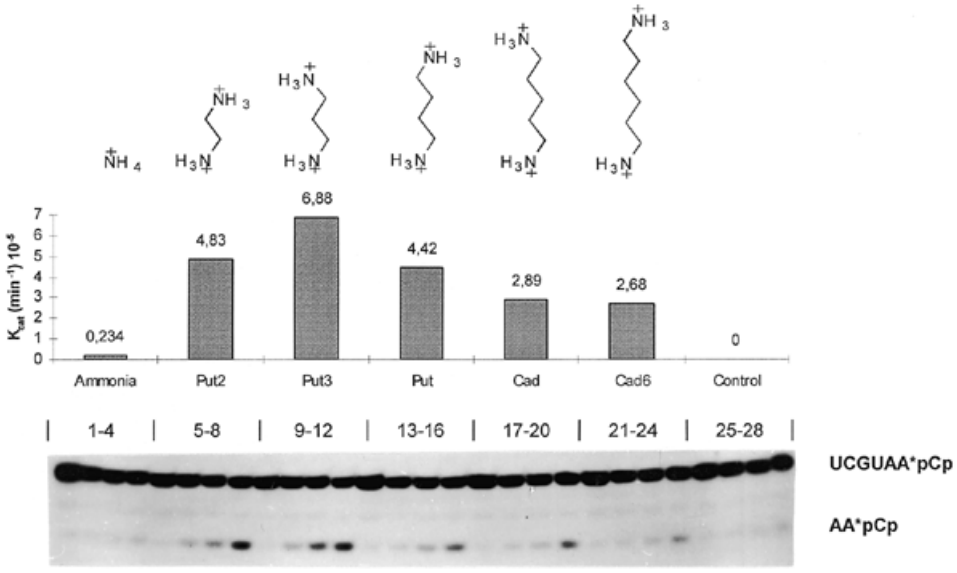

Figure 4. The stability of UCGUAA*pCp in the presence of 50 mM Tris–HCl (pH 7.5), 0.1% PVP, 2 mM EDTA at 37°C and different derivatives of the putrescine. Lanes 1–4, incubated in the presence of 5 mM ammonium chloride after 0, 10, 24 and 48 h, respectively; lanes 5–8, incubated in the presence of 5 mM Put2 after 0, 10, 24 and 48 h, respectively; lanes 9–12, incubated in the presence of 5 mM Put3 after 0, 10, 24 and 48 h, respectively; lanes 13–16, incubated in the presence of 5 mM Put after 0, 10, 24 and 48 h, respectively; lanes 17–20, incubated in the presence of 5 mM Cad after 0, 10, 24 and 48 h, respectively; lanes 21–24, incubated in the presence of 5 mM Cad6 after 0, 10, 24 and 48 h, respectively; lanes 25–28, incubated without any diamine after 0, 10, 24 and 48 h, respectively.

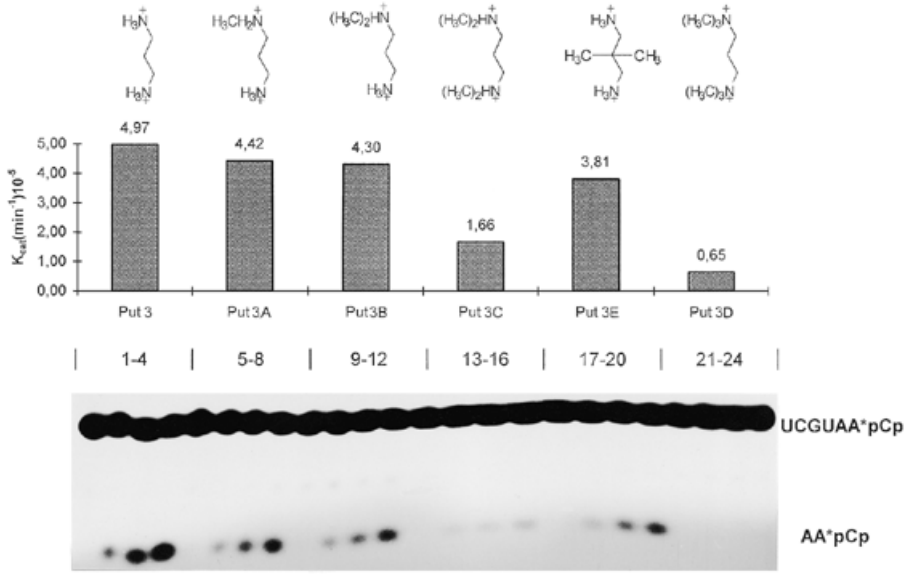

Figure 5. The stability of UCGUAA*pCp in the presence of 50 mM Tris–HCl (pH 7.5), 0.1% PVP, 2 mM EDTA at 37°C and different methylated derivatives of Put3. Lanes 1–4, incubated in the presence of 5 mM Put3 after 0, 10, 24 and 48 h, respectively; lanes 5–8, incubated in the presence of 5 mM *N*-methyl-1,3-diaminopropane (Put3A) after 0, 10, 24 and 48 h, respectively; lanes 9–12, incubated in the presence of 5 mM *N*,*N*-dimethyl-1,3-diaminopropane (Put3B) after 0, 10, 24 and 48 h, respectively; lanes 13–16, incubated in the presence of 5 mM *N*,*N*,*N*',*N*'-tetramethyl-1,3-diaminopropane (Put3C) after 0, 10, 24 and 48 h, respectively; lanes 17–20, incubated in the presence of 5 mM 2,2-dimethyl-1,3-diaminopropane (Put3E) after 0, 10, 24 and 48 h, respectively; lanes 21–24, incubated in the presence of 5 mM *N*,*N*,*N*,*N*',*N*',*N*'-hexamethyl-1,3-diammonium propane diiodide (Put3D) after 0, 10, 24 and 48 h, respectively.

during cleavage; for example, the binding to the oligomer and the activation of the cleaved phosphodiester bond (11). Permethylation of Put3 almost completely abolishes the oligoribonucleotide cleavage although the hexamethyl derivative still possesses two positively charged groups linked with a trimethylene chain. Both Me_3N^+- groups could participate in electrostatic interactions with the phosphate anions but they cannot form hydrogen bonds.

The specific non-enzymatic hydrolysis of biologically active RNA molecules

The data presented heretofore concern only chemically synthesized model RNA oligomers. Based on these data, we tested tRNA for similar patterns of non-enzymatic cleavage. The tRNA was chosen as a suitable object for this study because it is relatively small and well characterized; the secondary structure is well defined. For our experiments we used the initiator tRNA ($tRNA_i^{Met}$) from *Lupinus luteus* (27). The $tRNA_i^{Met}$ was labeled with ^{32}P at the 5'-terminus or [^{32}P]pCp at the 3' terminus. The hydrolysis reactions were performed at 37°C in 50 mM Tris–HCl (pH 7.5), 1 mM EDTA and 0.1% PVP containing 50 mM NaCl. Sodium chloride was included to stabilize native secondary and tertiary structure of tRNA (28). To induce hydrolysis, varying amounts of spermidine and spermine were used (0.1 μM to 10 mM). After 6 h the reaction products were analyzed by 14% PAGE. The cleavage of the phosphodiester bond between C75 and A76, within the aminoacylation stem of the tRNA was the dominating product. Therefore, 3'-labeling did not allow observation of the cleavage of other phosphodiester bonds and consequently 5'-labeled $tRNA_i^{Met}$ was used.

Analysis of $tRNA_i^{Met}$ primary and secondary structures for single-stranded hydrolysis sites suggested that, beside the 3'-end degradation (cleavage between C75 and A76), the hydrolysis within the anticodon loop between C34 and A35 as well as between U36 and t^6A37 (t^6A–*N*^{6}-/*N*-threonylcarbonyl/-adenosine) should be observed. However, we have already demonstrated that the presence of the 6-amino group of adenosine is necessary for the hydrolysis and the modification of adenosine (to t^6A) could prevent the internucleotide bond cleavage [19]. In accordance with our expectations, 5'-labeled $tRNA_i^{Met}$ from *L.luteus* was cleaved within the anticodon loop between C34 and A35 while the bond U36–t^6A37 was resistant for the hydrolysis (Fig. 6). The maximum rate of the tRNA cleavage was observed at 0.1 mM spermine and 1.0 mM spermidine. The $tRNA_i^{Met}$ from *L.luteus* was stable in the buffer without polyamine and in buffer with high polyamine concentrations (5 mM of spermine and 10 mM spermidine, respectively). Other cleavage sites were observed between C13–A14 and U8–m^1G9. For the C13–A14 phosphodiester bond hydrolysis was most efficient in the presence of 0.001 mM spermine or in the presence of 0.1 mM spermidine. Also, 0.001 mM spermine was optimal for mediating hydrolysis of the U8–m^1G9 phosphodiester bond.

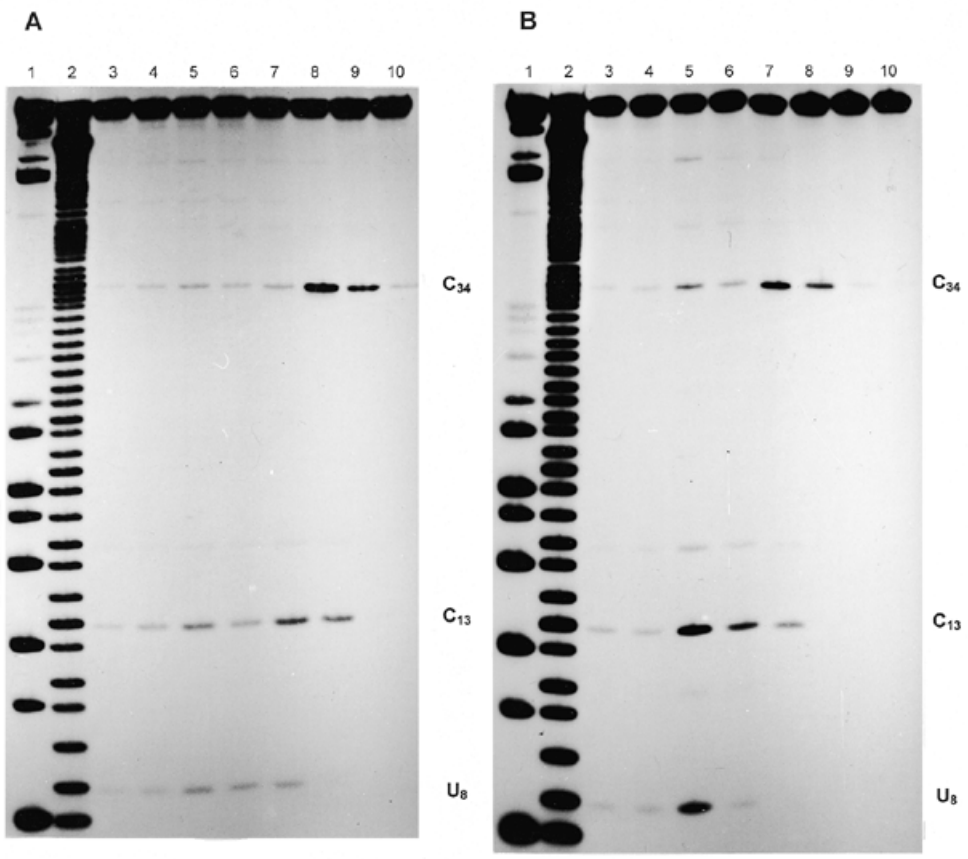

Figure 6. (**A**) The stability of $tRNA_i^{Met}$ from *L.luteus* incubated for 6 h at 37°C in the presence of 50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.1% PVP, 2 mM EDTA and various concentrations of spermidine. Lane 1, incubated in the presence of T1 nuclease; lane 2, the alkaline hydrolysis; lanes 3–10, incubated in the presence of 0, 0.0001, 0.001, 0.01, 1, 5 and 10 mM spermidine, respectively. (**B**) The stability of $tRNA_i^{Met}$ from *L.luteus* incubated for 6 h at 37°C in the presence of 50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.1% PVP, 2 mM EDTA and various concentrations of spermine. Lane 1, incubated in the presence of T1 nuclease; lane 2, alkaline hydrolysis; lanes 3–10, incubated in the presence of 0, 0.0001, 0.001, 0.01, 1, 5 and 10 mM spermine, respectively.

In order to demonstrate that the stability of the U36–t^6A37 phosphodiester bond results from the adenosine modification, we performed hydrolysis reactions on the $tRNA_i^{Met}$ from *Escherichia coli*. It has an anticodon loop sequence very similar to $tRNA_i^{Met}$ from *L.luteus*, except C33 is replaced by U and t^6A37 by A (29). Our data clearly indicate that $tRNA_i^{Met}$ from *E.coli* is efficiently cleaved between C34 and A35 as well as between U36 and A37 (Fig. 7). This confirmed our previous observations that the 6-amino group of adenosine is critical for hydrolysis and confirmed our hypothesis about why the anticodon loop of $tRNA_i^{Met}$ from *L.luteus* is cleaved at only one site [19]. Analysis of the reaction mixtures of $tRNA_i^{Met}$ from *E.coli* revealed the presence of three additional cleavage sites. The first one is located between C13 and A14 and the second between dihydrouridine (D) at position 20 and A21, and the third between C56 and A57. This second cleavage is particularly fast. We hypothesize that the poor stacking of D is responsible for high yield of the D20–A21 phosphodiester bond cleavage (30).

CONCLUSIONS

Because biogenic amines are prevalent in cells of diverse organisms, and our conditions mimic those found *in vivo*, the hydrolysis reaction that we describe may be important biologically. In particular, internal RNA hydrolysis at UA and CA sequences could provide more terminal ends for RNA exonucleases, and faster RNA degradation. Control of hydrolysis could be obtained by providing intracellular compartments with optimal biogenic amine concentrations. Furthermore, RNAs that should not be degraded can form hairpin–loop structures to keep UA and CA sequences in helical regions, which are protected from the hydrolysis (22). Although the hydrolysis presented here proceeds at a slower rate than that catalyzed by endonucleases, it requires only simple chemical compounds, possibly available in a primeval environment. Thus, this mechanism may have been important for RNA degradation in the RNA world.

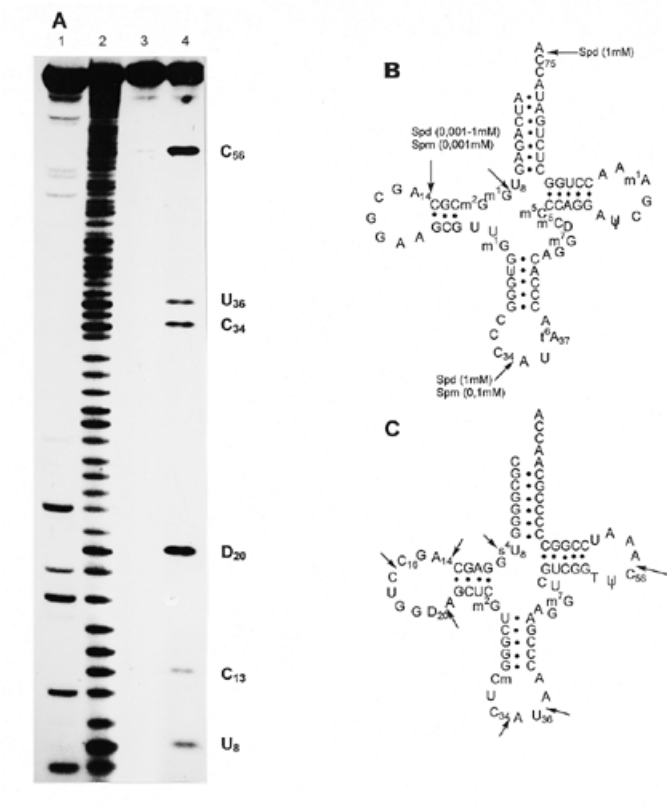

Figure 7. (**A**) The stability of $tRNA_i^{Met}$ from *E.coli* incubated for 6 h at 37°C in the presence of 50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.1% PVP, 2 mM EDTA and various concentrations of spermidine. Lane 1, incubated in the presence of T1 nuclease; lane 2, the alkaline hydrolysis; lanes 3 and 4, incubated in the presence of 0 and 10 μM spermidine, respectively. (**B**) The secondary structure of the $tRNA_i^{Met}$ from *L.luteus.* The arrows indicate the cleavage site and optimal concentration of spermidine and spermine. (**C**) The secondary structure of the $tRNA_i^{Met}$ from *E.coli.* The arrows indicate the cleavage site results by 0.1 mM spermidine.

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