Nonenzymatic hydrolysis of oligoribonucleotides

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Received July 9, 1992; Revised and Accepted September 7, 1992

ABSTRACT

Selective cleavage of phosphodiester bonds in RNA is important in the processing of large RNA molecules. This paper reports specific cleavage at UA sequences in single stranded oligoribonucleotides as short as hexamers. The hydrolysis between U and A leaves a 2',3'-cyclic phosphate on the 5'-side and a 5'-hydroxyl group on the 3' side of the cleavage. The hydrolysis is promoted by a wide range of cofactors, including polymeric organic compounds such as polyvinylpyrrolidone (PVP) and by proteins. A variety of experiments suggests the cleavage is not due to contamination by ribonuclease. The rate of cleavage is a function of oligoribonucleotide, PVP and spermidine concentrations. Mg^{2+} is not required. The phenomenon described here can potentially provide a relatively simple way of coding chemical stability into single stranded RNA based on its sequence and structure. This process seems to be similar to that involved in post-transcriptional degradation of mRNA.

INTRODUCTION

The chemical stability of individual phosphodiester bonds in RNA is important for many biological processes, including RNA degradation¹, site specific cleavage^{2–4}, and splicing^{5,6}. The discovery of the transformation of phosphodiester bonds without participation of any proteins changed our understanding of the stability and function of RNA. This paper reports the observation that some single stranded RNAs have phosphodiester bonds that are unusually susceptible to hydrolysis when in the presence of certain cofactors. The results suggest RNA sequences may sometimes encode chemical stability in a relatively simple way.

MATERIALS AND METHODS

Synthesis and purification of oligoribonucleotides

The oligoribonucleotides used in this study were synthesized chemically by a phosphoramidite method and deprotected according to a published procedure⁷. Purification was performed 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 (HPLC) and 20% polyacrylamide gel

electrophoresis (PAGE). Oligoribonucleotides were labeled with ^{32}P at their 5' termini with T4 polynucleotide kinase; the labeled strands were purified by polyacrylamide gel electrophoresis and isolated by standard methods⁸.

Materials

The reagents used in this work come from the following suppliers: T4 polynucleotide kinase (New England Biolabs or Pharmacia or Boehringer), reverse transcriptase (Life Sciences), T7 RNA polymerase⁹, Eco R1 endonuclease (New England Biolabs), proteinase K (Sigma), RN-ase (from human placenta, Sigma), Inhibit-ACE (5'–3'), ribonuclease A (Worthington), lysozyme (Worthington), pepsin A (from porcine stomach, Worthington), trypsin (TRL3, from bovine pancreas, Worthington), polyvinylpyrrolidone (PVP) (MW 360kD, Sigma), Brij 58 (Sigma), dextrin (Aldrich), polyethylene glycol (PEG) (MW 200 or 400 or 1000, Fisher or Serva) and ^{32}P -ATP (New England Nuclear).

Hydrolysis of oligoribonucleotides

In typical hydrolysis reactions, ca. 0.1 pmole ^{32}P 5'-phosphorylated oligoribonucleotides were incubated in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM spermidine and 1 mM EDTA (conditions for proteins) or 50 mM Tris-HCl (pH 7.5), 1 mM spermidine, 1 mM EDTA (conditions for PVP, PEG, Brij 58 and dextrin) at 37°C. Aliquots were quenched with formamide and analyzed by electrophoresis on 20% polyacrylamide gels⁸.

RESULTS AND DISCUSSION

It was observed that several UA containing oligoribonucleotides (for example: UCGUAA, AGAUGUAUUCU, UCGUAAAA-CGA, GUCGUAGCC, UCGUAAACUCU and GUUUCGUA-CAAAC) are cleaved during kination reactions in the presence of T4 polynucleotide kinase. This cleavage required T4 polynucleotide kinase. This same cleavage occurs when 5'-labeled oligoribonucleotides are incubated in the presence of several other proteins (see below). Since such cleavage could be due to ribonuclease contamination of the proteins, special attention was paid to find a non-protein compound that could substitute for T4 polynucleotide kinase and other proteins. Several such compounds were found, including: dextrin, polyethylene glycol (PEG), Brij

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58 and polyvinylpyrrolidone (PVP)¹⁰⁻¹³. PVP was selected for intensive studies because of the simple structure of the monomeric unit and its high efficiency in hydrolysis. Site specific cleavage, mainly between U and A, was observed when certain oligoribonucleotides, for example UCGUAA, were incubated at 37°C with 0.1% (wt, 10.3 mM monomer) PVP, 1 mM spermidine, 1 mM EDTA, 50 mM Tris-HCl at pH 7.5 (see Figure 1, lanes 3-5).

Figure 1. The kinetics of hydrolysis of p*UCGUAA in PVP. Lane 1: p*UCGUAA. Lane 2: p*UCGUAA incubated in buffer (50 mM Tris-HCl pH 7.5, 1 mM spermidine and 1 mM EDTA) for 25 h at 37°C. Lanes 3-5: Incubation of p*UCGUAA in buffer containing 0.1% PVP for 1, 5 and 25 h, respectively.

Nonribonuclease type of hydrolysis

The oligoribonucleotide sequence requirement observed for the hydrolysis described above is similar to that for ribonuclease A¹⁴⁻¹⁶, and suggests ribonuclease contamination may be responsible. Several experiments were done to prove that ribonuclease is not responsible for this hydrolysis. It was demonstrated that in the absence of cofactors UCGUAA and AGAUGUAUUCU are stable (see Figure 1, lane 2 and Figure 5, lanes 1-3). The hydrolysis of AGAUGUAUUCU over a wide range of PVP concentrations shows a bell-shape, not a linear correlation between rate of hydrolysis and PVP concentration. This is inconsistent with PVP as a source of ribonuclease A contamination (see Figure 6). Moreover, it was demonstrated that silica gel column chromatography of PVP or purification by chloroform extraction does not change the ability of PVP to cleave UA containing oligoribonucleotides.

Another set of experiments involving PVP, dextrin, pepsin and lysozyme also suggests hydrolysis is not due to ribonuclease (Figure 2). It was observed that preincubation of 0.1% PVP and 1% dextrin solutions with proteinase K¹⁷ (50 g/mL) for 12 h at 37°C does not eliminate hydrolysis of UCGUAA and AGAUGUAUUCU. However, inhibition of cleavage by 5-10 fold as a function of preincubation time was observed. The reason for this phenomenon is not clear, but could involve slow hydrolysis of the 'peptide bond' of the pyrrolidone ring and/or binding of PVP to proteinase K.

When lysozyme and pepsin were preincubated with proteinase K under similar conditions, no hydrolysis was observed (Figure 2, lanes 11-18; pepsin data not shown). Complete hydrolysis of lysozyme and pepsin by proteinase K under these conditions was demonstrated by analyzing the mixtures by 15% polyacrylamide gel electrophoresis and coomassine blue staining. It was also shown that the presence of PVP does not prevent hydrolysis of ribonuclease A (20 ng/mL) by proteinase K (50 µg/mL) (data not shown). The situation that proteinase K can digest pepsin and lysozyme (both at concentration of 0.4 mg/mL)

Figure 2. The chemical stability of p*AGAUGUAUUCU in the presence of PVP and lysozyme before and after preincubation of these cofactors with proteinase K. Lanes 1 and 2: p*AGAUGUAUUCU incubated in the buffer for 0 and 24 h, respectively. Lanes 3-6: p*AGAUGUAUUCU incubated in buffer containing 0.1% PVP for 1, 4, 8 and 24 h, respectively. Lanes 7-10: Oligomer incubated in 0.1% PVP and buffer which was preincubated with proteinase K (50 g/mL) for 12 h at 37°C. Lanes 7-10 correspond to 1, 4, 8 and 24 h incubation, respectively. Lanes 11-14: p*AGAUGUAUUCU incubated in the presence of lysozyme (0.4 mg/mL) for 1, 4, 8 and 24 h, respectively. Lanes 15-18: Oligomer incubated in lysozyme (0.4 mg/mL) after the lysozyme solution was preincubated with proteinase K (50 g/mL) for 12 h at 37°C. Lanes 15-18 correspond to 1, 4, 8 and 24 h incubation, respectively.

plus potential ribonuclease contamination but is not able to digest ribonuclease contamination in PVP and dextrin is very unlikely. This suggests that hydrolysis of AGAUGUAUUCU is due to PVP, dextrin, pepsin and lysozyme rather than to ribonuclease contamination.

Especially strong suggestions for nonribonuclease hydrolysis come from experiments with ribonuclease inhibitors. Two of them, RN-asin¹⁸ and Inhibit-ACE¹⁹, promote UCGUAA hydrolysis (Figure 3, lanes 5–7 and 11–13). However, preincubation of both inhibitors with proteinase K (50 g/mL) for 12 h at 37°C eliminates their ability to hydrolyze UCGUAA (Figure 3, lanes 8–10 and 14–16). Moreover, experiments with

Inhibit-ACE prove that preincubation of this inhibitor for 25 h at 37°C does not denature this protein (Figure 4, lanes 12–14 and 18–20) since it still inhibits ribonuclease A at a concentration of 20 ng/mL (Figure 4, lanes 5–8). It was also demonstrated that Inhibit-ACE does not require Mg^{+2} to participate in hydrolysis of UCGUAA (Figure 4, lanes 15–20). Proteinase K by itself does not promote hydrolysis of UA-containing oligoribonucleotides (Figure 3, lanes 2–4).

Additionally, experiments with hydrolysis of UA oligoribonucleotides in the presence of polyethylene glycol demonstrate that distillation of polyethylene glycol-400 (at 250–270°C/10⁻² mm Hg) or heating 50% aqueous solution of

Figure 3. Chemical stability of p*UCGUAA in the presence of ribonuclease inhibitors. Lane 1: p*UCGUAA. Lanes 2–4: Incubation of p*UCGUAA with proteinase K (50 g/mL) for 1, 5 and 25 h, respectively. Lanes 5–7: Incubation of p*UCGUAA with RN-asin (170 U/mL) for 1, 5 and 25 h, respectively. Lanes 8–10: Incubation of p*UCGUAA with RN-asin after prior preincubation of RN-asin with proteinase K (50 g/mL) for 12 h at 37°C; lanes 8–10 are 1, 5 and 25 h time points, respectively. Lanes 11–13: Incubation of p*UCGUAA with Inhibit-ACE (38 U/mL) for 1, 5 and 25 h, respectively. Lanes 14–16: Incubation of p*UCGUAA with Inhibit-ACE after prior preincubation of Inhibit-ACE with proteinase K (50 g/mL) for 12 h at 37°C; lanes 14–16 correspond to 1, 5 and 25 h time points, respectively. Lane 17: Alkaline hydrolysis of p*UCGUAA (NH₄OH pH 12 for 15 min at 90°C).

Figure 4. Chemical stability of p*UCGUAA in the presence of Inhibit-ACE (38 U/mL) at different experimental conditions. The lanes represent the following conditions: Lanes 1–4: Inhibit-ACE was preincubated with ribonuclease A (20 ng/mL) for 10 min at 37°C before p*UCGUAA was added. Time points are 5 min, 1, 5 and 25 h, respectively. Lanes 5–8: Inhibit-ACE was preincubated for 25 h at 37°C after which ribonuclease A (20 ng/mL) was added. Following preincubation for 10 min at 37°C p*UCGUAA was added. Time points are 5 min, 1, 5 and 25 h, respectively. Lanes 9–11: p*UCGUAA was added to Inhibit-ACE. Time points are 1, 5 and 25 h, respectively. Lanes 12–14: p*UCGUAA was added after preincubation of Inhibit-ACE in buffer for 25 h at 37°C. Time points are 1, 5 and 25 h, respectively. The experiments presented in lanes 1–14 were done in 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM spermidine and 1 mM EDTA, whereas those shown in lanes 15–20 were without MgCl₂. Lanes 15–17 present results of incubation of p*UCGUAA in the Inhibit-ACE without any preincubation whereas lanes 18–20 are results from incubation of substrate with Inhibit-ACE after Inhibit-ACE preincubation for 25 h at 37°C. Time points are 1, 5 and 25 h, respectively.

Figure 5. Stability of p*AGAUGUAUUCU in the presence of different cofactors. Lanes 1–3: in the buffer after 0, 1 and 4 h, respectively. Lanes 4 and 5: endonuclease Eco R1 after 1 and 4 h, respectively. Lanes 6 and 7: RN-ase after 1 and 4 h, respectively. Lanes 8 and 9: pepsin A after 1 and 4 h, respectively. Lanes 10–12: trypsin after 5 min, 1 and 4 h, respectively. Lanes 13 and 14: T7 RNA polymerase after 1 and 4 h, respectively. Lanes 15 and 16: T4 polynucleotide kinase after 1 and 4 h, respectively. Lanes 17 and 18: 0.1% polyvinylpyrrolidone after 1 and 4 h, respectively. Lanes 19 and 20: 0.5% Brij 58 after 1 and 4 h, respectively. Lanes 21 and 22: 1% dextrin after 1 and 4 h, respectively.

this polyglycol for 14 h at 150°C does not eliminate its ability to hydrolyze. Moreover, treatment of a 10% aqueous solution of polyethylene glycol-400 with diethylpyrocarbonate (DEPC) (0.2% final concentration of DEPC⁷ for 16 h at 37°C) followed by autoclaving leaves polyethylene glycol able to stimulate hydrolysis of UA-containing oligoribonucleotides (data not shown).

The products of hydrolysis

The products of the cleavage reaction are a 2',3'-cyclic phosphate on the 5' fragment and a 5'-hydroxyl on the 3' fragment. This was determined by comparison with the products from alkaline hydrolysis. As shown in Figures 1 and 3, the ³²P labelled product of the cleavage of p*UCGUAA induced by PVP or RN-ase or Inhibit-ACE runs at the same position as the p*UCGU > p product from alkaline hydrolysis. Incubation of p*UCGU > p in 0.001 M HCl for 3 h at 50°C partially converted this fragment to a species that runs slightly faster on a denaturing gel (data not shown). These observations are consistent with the product being p*UCGU > p, where the 3' terminus is a 2',3'-cyclic phosphate that is partially opened to 2'- and 3'-phosphates upon incubation with HCl²⁰. The 2',3'-cyclic phosphate is the most common terminus for hydrolysis of RNA²¹. The expected 3'-fragment from such hydrolysis of UCGUAA is AA with a 5'-hydroxyl group. This was confirmed by comparing the mobilities of the products of the cleavage reaction of UCGUAA with the mobility of AA on a C₈ column (HPLC) and on a silica gel TLC plate (propanol-2/ammonia/water- 7:1:2 v/v/v). Thus the products of cleavage of UCGUAA are UCGU > p and AA with a 5'-hydroxyl group. These additional methods of the analysis (HPLC and TLC) confirm primary (PAGE) observations. That is particularly important due to analytical limitation of 5'-labeling technic. Moreover, the sequence of the product of the hydrolysis of AGAUGUAUUCU was also determined to be AGAUGU > p by digestion with RNA sequencing enzymes (data not shown).

The variety of active cofactors

The effects of several other proteins and polymers on the chemical stability of AGAUGUAUUCU were also studied (see Figure 5). Complete hydrolysis between U and A was observed within 4 h

Figure 6. The rate of hydrolysis of p*AGUGUAUUCU as function of polyvinylpyrrolidone (○), spermidine (●) and oligoribonucleotide concentrations (▲) (insert).

with reverse transcriptase (700 U/mL), T7 RNA polymerase (5000 U/mL), lysozyme (0.4 mg/mL), trypsin (0.4 mg/mL), and within 8 h with Eco R1 endonuclease (650 U/mL), pepsin (0.4 mg/mL), T4 polynucleotide kinase (330 U/mL), 0.1% (wt) PVP, 1% (wt) dextrin, and 1% (wt) polyethylene glycol-1000. Less than 50% cleavage at UA after 24 h was observed with human placental RN-ase (170 U/mL), Inhibit-ACE (38 U/mL), 1% (wt) polyethylene glycol-200 and 1% (wt) polyethylene glycol-400. In the experiment with trypsin, additional hydrolysis between U(4) and G(5) was observed (see Figure 5, lanes 10–12 and Figure 1 in accompanying paper for more data). No hydrolysis of AGAUGUAUUCU was observed with polyglutamic acid, hexaglycine, L-arginine, RNA dinucleotide monophosphates, poly A, poly U, alfa -cyclodextrin, imidazole, sucrose and proteinase K.

The function of oligoribonucleotide and PVP in the hydrolysis process

The resistance to hydrolysis of several oligoribonucleotides demonstrates the importance of the oligomer sequence for this process. The hydrolysis proceeds for single-stranded oligoribonucleotides only. It was found that: (i) the self-complementary oligoribonucleotide, UCGUACGA, is stable to hydrolysis at 37°C at a concentration of 10^{-6} M but hydrolyzes at 50°C (calculated T_m at this concentration is 43.8°C)²². (ii) GUCGUAGCC cleaves to GUCGU>p, but the presence of GGCUCGAC stops this hydrolysis. UV-melting curves of GUCGUAGCC in the absence and presence of GGCUCGAC show single and double stranded melting behavior, respectively²³. The UV-melting experiments were performed in the standard conditions²² and compared to UV-melting in the hydrolysis buffer in the presence and absence of 0.1% PVP solution. Several research groups have reported limited base stacking within pyrimidine-purine single-stranded oligoribonucleotides based on ¹H and ¹³C NMR, CD and X-ray data²⁴⁻²⁸. Some reports^{24,26} extend this sequence to purine-pyrimidine-purine and propose a bulge-out of the pyrimidine base with purine bases stacking on top of each other.

The function of factors, like PVP, for hydrolysis is not known. The rate of hydrolysis is a bell-shaped function of the concentrations of both PVP and spermidine. That suggests formation of some cooperative structure (see below for more data). The interaction of PVP with a wide range of aromatic compounds, drugs, dyes and toxins has been reported¹⁰⁻¹². Moreover, application of PVP cross-linked to support for separation of nucleotides was demonstrated¹³. The wide range of active factors (polymeric organic compounds including PVP and proteins) could mean that nonspecific interactions are involved in this process. These interactions of cofactor and oligoribonucleotide could cause conformational changes accelerating hydrolysis. Alternatively, volume and/or water exclusion effects may be important. Such effects could change the pattern of hydration and hydrogen bonds within an oligoribonucleotide and make hydrolysis possible.

Hydrolysis of AGAUGUAUUCU, fragment of the self-splicing intron from *Tetrahymena thermophila*

To determine the effects of conditions on the hydrolysis reaction, a naturally occurring sequence, AGAUGUAUUCU, was synthesized. This represents nt 284-294 of the self-splicing LSU group I intron from *Tetrahymena thermophila*⁵, and contains a UA site that is a strong stop when the circular form of the intron is reverse transcribed³⁰ (see Figure 3 of reference 30). Optical melting experiments show that this sequence is single stranded in 1 mM spermidine, 1 mM EDTA, 50 mM Tris-HCl at pH 7.5 in the presence or absence of 0.1% PVP. This sequence is cleaved between U(6) and A(7) in the presence of the melting buffer with 0.1% PVP. Addition of Mg^{+2} did not affect cleavage. The rate of cleavage as a function of oligomer, PVP, and spermidine concentrations is shown in Figure 6. The linear dependence of log (rate) with log [AGAUGUAUUCU] up to 10 μ M is consistent with a pseudo-first order reaction. The saturation in rate between 10 and 100 μ M oligomer occurs in a region where the monomer pyrrolidine concentration is only about 10 times the nucleotide concentration. Thus a noncovalent intermediate between oligomer and PVP may be the reactive species. The dependence of rate on both PVP and spermidine is sharply peaked, suggesting formation of some cooperative structure. Under some conditions,

a lag was observed in plots of hydrolysis product vs time, suggesting formation of some ordered structure.

Biological implications

The results described above suggest an internal single stranded UA sequence is unusually susceptible to hydrolysis. There have been previous observations of cleavage of large RNAs in the presence of detergents³¹: Brij 58, Nonidet P40 and Triton X-100, and during transcription with RNA polymerase³². These may reflect a similar phenomenon. Because the cleavage patterns observed here are similar as for ribonuclease A, it is difficult to definitively prove that these observations are not due to trace contamination by ribonuclease. However, simultaneous analysis of all the experiments and properties of the hydrolysis makes it seem unlikely that it is due to contamination by ribonuclease A. The similarity between the observed hydrolysis activity of these cofactors and of ribonuclease A could, however, reflect evolutionary processes rather than contamination. If RNA originally cleaved at sites intrinsically susceptible to cleavage, then it is likely this same cleavage would be facilitated as proteins evolved. Literature data^{14,15} does not report a requirement for any interactions of the pyrimidine and purine bases with ribonuclease A for hydrolysis. Thus it may be that ribonuclease A 'recognizes' the most labile phosphodiester bond of the oligoribonucleotides just as do the cofactors used in this paper. The phenomenon of RNA encoded cleavage could be important for regulating the stability of RNA in vivo. For example, the circular RNA formed from the LSU intron and containing one of the sequences studied here has a lifetime of only 6 sec in *Tetrahymena thermophila*³³. The cleavage sites important for degradation of the polycistronic lac mRNA in *Escherichia coli* have been reported to be pyrimidine-A^{1,34}. In this case, $t_{1/2}$ for degradation is between 0.5 and 8 min at 37°C. Moreover, the spontaneous hydrolysis of different tRNA molecules occurs most often at UA sequences³⁵. The phenomenon described here can potentially provide a relatively simple way of coding a base level of degradation into any RNA sequence and thus be important in post-transcriptional degradation of mRNA.

ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health Grant GM25149 (for Professor Douglas H. Turner). The author thanks Professor Douglas H. Turner for stimulating discussions and financial support during this work.

ABBREVIATIONS

PVP, polyvinylpyrrolidone; PAGE, polyacrylamide gel electrophoresis; R, purine nucleoside; Y, pyrimidine nucleoside

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