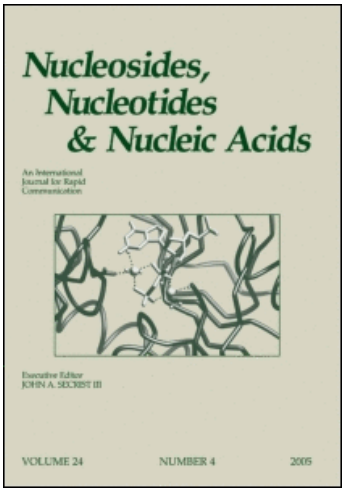

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

The Nonenzymatic Hydrolysis of Oligoribonucleotides VII. Structural Elements Affecting Hydrolysis

Arkadiusz Bibillo[a]; Marek Figlerowicz[a]; Krzysztof Ziomek[a]; Ryszard Kierzek[a]
[a] Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznan, Poland

To cite this Article Bibillo, Arkadiusz , Figlerowicz, Marek , Ziomek, Krzysztof and Kierzek, Ryszard(2000) 'The Nonenzymatic Hydrolysis of Oligoribonucleotides VII. Structural Elements Affecting Hydrolysis', Nucleosides, Nucleotides and Nucleic Acids, 19: 5, 977 — 994
To link to this Article: DOI: 10.1080/15257770008033037
URL: http://dx.doi.org/10.1080/15257770008033037

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 19(5&6), 977–994 (2000)

THE NONENZYMATIC HYDROLYSIS OF OLIGORIBONUCLEOTIDES VII. STRUCTURAL ELEMENTS AFFECTING HYDROLYSIS.

Arkadiusz Bibillo, Marek Figlerowicz, Krzysztof Ziomek and Ryszard Kierzek*
Institute of Bioorganic Chemistry, Polish Academy of Sciences,
Noskowskiego 12/14, 61-704 Poznan, Poland

ABSTRACT

Several elements of oligoribonucleotide structure are important for efficient hydrolysis. We have found that the following factors influence oligoribonucleotide hydrolysis: (i) single-stranded structure of RNA flanking the scissile phosphodiester bond, (ii) the substituent on atom C-5 of the uridine adjacent to the cleaved internucleotide bond, (iii) the position of the scissile UA phosphodiester bond within a hairpin loop, (iv) the concentration of formamide, urea, ethanol and sodium chloride.

INTRODUCTION

The nature of specific, nonenzymatic hydrolysis of oligoribonucleotides and tRNA's has been an object of our interest for several years (1-6). Cleavage occurs primarily at phosphodiester bonds in pyrimidine-purine steps, and is fastest at UA steps. We have studied the reaction with the model oligoribonucleotide,

UCGUAA (nucleotides linked by the scissile bond are underlined). There are several factors required for the oligonucleotide cleavage which can be divided into two groups. The first comprise the necessary environment of the RNA molecule and include polyamines and nonspecific cofactors, as described previously (1, 2, 6). The second group consists of requirements of the RNA structure: (i) single-stranded RNA, (ii) the presence of the labile phosphodiester bond, (iii) the position of the scissile internucleotide bond within the oligoribonucleotide, (iv) the presence of certain functional groups on the nucleotides flanking the cleaved phosphodiester bond.

The crystal structures of hammerhead and group I introns ribozymes demonstrate the complex patterns of interactions required for the catalytic activity (7-9). In contrast, single-stranded oligoribonucleotides, which are the objects of our investigation, are far less sophisticated. Their autocatalytic function is limited to a specific cleavage of a phosphodiester bond.

In the present paper, we extend our observations of the structural factors affecting the oligoribonucleotide hydrolysis. In particular, the effect of the following variables on hydrolysis are addressed: (i) partially single-stranded oligoribonucleotides, (ii) 5-substituents of the uridine-4 (in UCGU^{R}AACp), (iii) position of the UA step within a hairpin loop, (iv) functional groups of the nucleobases adjacent to the cleaved phosphodiester bond, (v) denaturing reagents.

METHODS AND MATERIALS

Synthesis and purification of oligoribonucleotides

The 5-modified nucleosides were synthesized on two ways - the substitution at the 5-position of the uridine (chloro-, bromo-, iodouridine derivatives) (10) or by condensation of a 5-substituted uracil and 1-O-acetyl-2,3,5-tri-O-benzoyl-β-D-ribose using the silyl Hilbert-Johson method and $SnCl_4$ as catalyst (fluoro-, methyl-, ethyl- and n-propyluridine derivatives) (11). 6-

Methyluridine (N3 isomer) was obtained by condensation of the 6-methyluracil and 1-O-acetyl-2,3,5-tri-O-benzoyl-β-D-ribose in presence of $SnCl_4$ (12). The thermodynamically more stable N3 isomer was almost exclusive product of the synthesis. 6-Methyluridine (N1 isomer) was synthesized by condensation of 6-methylcytosine and 1-O-acetyl-2,3,5-tri-O-benzoyl-β-D-ribose in the presence of $SnCl_4$. The product - 6-methylcytidine – was transformed into 6-methyluridine by chemical deamination of the cytidine derivative.

The isocytidine and isoguanosine were synthesized by multistep reactions starting from uridine and 2,6-diaminopurine riboside, respectively according to published procedures (13, 14).

The oligoribonucleotides used in experiments were synthesized by the phosphoramidite method on polymer support and deprotected according to published procedures (6, 15-19).

Purification of the oligoribonucleotides 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) (20). The purity of oligoribonucleotides was analyzed by C-8 high performance liquid chromatography (HPLC) and 20% polyacrylamide gel electrophoresis (PAGE). Oligoribonucleotides were labeled at their 5'-termini with ^{32}P γ-ATP and T4 polynucleotide kinase or at 3'-termini with (5'-^{32}P) pCp and T4 RNA ligase. The labeled oligomers were purified by TLC or PAGE.

Hydrolysis of oligoribonucleotides

Standard hydrolysis conditions of the oligoribonucleotides include ~ 0.1 pmol of ^{32}P 5'- or 3'-labeled oligoribonucleotides, incubated in 50 mM Tris-HCl (pH 7.5), 1 mM spermidine and 2 mM EDTA in the presence 0.1% PVP solution at 37^0C. Addition of 3'-terminal p*Cp does not affect the reaction. Aliquots were quenched with formamide and analyzed by 20% polyacrylamide gel electrophoresis.

Digestion oligoribonucleotides by nuclease S1

The digestion was performed in 40 mM sodium acetate (pH 4.5), 10 mM zinc acetate, 280 mM sodium chloride at 15^0C. The concentration of the oligomer

GCUCUAA was 0.5 μM and the complementary strand 1μM and 0.25 U of S1 nuclease (Promega) was used for single digestion. The aliquots were taken after 0, 5 and 15 minutes and analyzed by 20% polyacrylamide gel electrophoresis.

Ultraviolet melting experiments

Oligoribonucleotide duplexes were melted in 1.0 M NaCl, 20 mM sodium cacodylate and 0.5 mM Na_2EDTA, pH 7.0. Strand concentrations were calculated from absorbance at 80^0C and single-strand extinction coefficients approximated by a nearest-neighbor model (21). Absorbance vs. temperature melting curves were measured at 260 or 280 nm with a heating rate of 1^0C/min from 0 to 90^0C on a Gilford 250 spectrometer controlled by a Gilford 2527 thermoprogrammer. The results were analyzed with the Meltwin computer program (22, 23).

RESULTS AND DISCUSSION

The hydrolysis of single stranded oligoribonucleotides in the presence of partial complements.

As described previously, oligoribonucleotides must be single stranded for specific, nonenzymatic hydrolysis (1, 6). However, we did not establish the length of the single stranded fragment required to undergo specific cleavage (1, 5). To answer this question, the stability of the octamer, GCUCGUAA, was tested in the presence of four different complementary or partially complementary oligoribonucleotides. The first complement, UUACGAGC, can form a perfect Watson-Crick duplex with the octamer. The three other complementary oligomers were one, two and three nucleotides shorter at their 5'-ends (see Table 1). This exposed the 3'-end of GCUCGUAA as a single-stranded region.

To ensure hybridization between the octamer and its complements during cleavage, thermodynamic parameters of the duplexes formed were established by ultraviolet melting (Table 1). The stabilization effect (ΔG^0_{37}) of the duplex was the largest when 3'-dangling end was formed by one nucleotide. Fewer base pairs resulted in decreased duplex stability. Each additional base pair lost reduced the

Table 1. Thermodynamic parameters for RNA duplex formation.

RNA duplex	Average of curve fits				T_M^{-1} vs log C_T plots					
	$-\Delta H^0$ (kcal/mol)	$-\Delta S^0$ (eu)	$-\Delta G^0_{37}$ (kcal/mol)	T_M (^{0}C)	$-\Delta H^0$ (kcal/mol)	$-\Delta S^0$ (eu)	$-\Delta G^0_{37}$ (kcal/mol)	T_M (^{0}C)	$\Delta\Delta G^0_{37}$ (kcal/mol)	$\Delta\Delta G'^0_{37}$ (kcal/mol)
GCUCGUAA CGAGCAUU	72.82±6.05	201.38±18.86	10.36±0.21	54.2	66.35±2.17	181.18±6.75	10.15±0.08	54.9	0	-
GCUCGUAA CGAGCAU	70.81±3.42	194.70±10.20	10.42±0.28	55.0	64.66±1.37	176.16±4.16	10.03±0.08	54.7	0.12	-0.83
GCUCGUA CGAGCAU					61.4*	168.1*	9.2*	51.1*	-	0
GCUCGUAA CGAGCA	58.03±4.91	158.17±15.31	8.97±0.17	50.6	54.23±0.98	146.52±3.07	8.79±0.03	50.5	1.36	-1.09
GCUCGU CGAGCA					53.3*	147.4*	7.7*	46.4*	-	0
GCUCGUAA CGAGC	50.31±8.53	137.51±26.81	7.66±0.23	44.1	44.26±3.08	118.53±9.89	7.50±0.09	43.9	2.63	-1.90
GCUCG CGAGC					43.1*	121.2*	5.6*	36.0*	-	0

* calculated from the nearest neighbor model (29). Solutions are 1 M NaCl, 20 mM sodium cacodylate, and 0.5 mM Na_2EDTA, pH 7, b - calculated for 10^{-4} M oligomer concentration

Tm by ca. 5^0C and ΔG^0_{37} by ca. 1.2 kcal/mol relatively to the 3'-mononucleotide dangling end duplex. All Tm values of the model duplexes were above 43^0C, suggesting that under our reaction conditions (15^0C), the complements were bound significantly to the oligomer. The effect of overhanging 3'-dangling end presented herein is similar to that reported by Sprinzl for aminoacyl stems of tRNA (24).

Additionally, to check whether duplexes were present during the experiments the stability of model duplexes was tested in the presence of S1 nuclease, which catalyzes hydrolysis of unpaired region of RNA (25). When used alone, GCUCGUAA was effectively degraded by S1 nuclease, while the complementary octamer (UUACGAGC) and heptamer (UACGAGC) conferred full protection. For the shorter complementary oligomers (ACGAGC and CGAGC) the digestion was limited to the single stranded 3'-end (data not shown).

The nonenzymatic hydrolysis of the model duplexes was performed in 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM spermidine, 2 mM EDTA and 0.1% PVP at 15^0C. Reactions were performed with 0.5 μM labeled oligomer p*GCUCGUAA in the presence of 1 μM complement. After 0, 24, 48, and 90 h, reaction mixtures were analyzed by PAGE (Figure 1). The results indicate that the specific hydrolysis of GCUCGUAA does not take place when the terminal UAA is involved in the Watson-Crick helix and cleavage occurred only in the presence of the shortest complementary oligomer, CGAGC. Thus, effective hydrolysis requires both nucleotides flanking the scissile phosphodiester bond to be unpaired. We hypothesize that helix formation with these nucleotides precludes the in-line orientation of functional groups (2'-hydroxyl, phosphorous and 5'-oxygen) involved in chemical cleavage UA phosphodiester bond.

The effect of C5 substituents of uridine-4 in UCGU^{R}AACp on hydrolysis rate.

Several spectroscopic methods have shown that stacking interactions of nucleobases depend on the DNA or RNA sequence (26, 27). Less stacking was observed for Y-R than R-Y dimers (Y-pyrimidines, R-purines). For example, UA and CA dinucleotide monophosphates are 15% and 24% stacked, while AU and AC are 34% and 38% stacked, respectively. Previously, we showed that R-Y

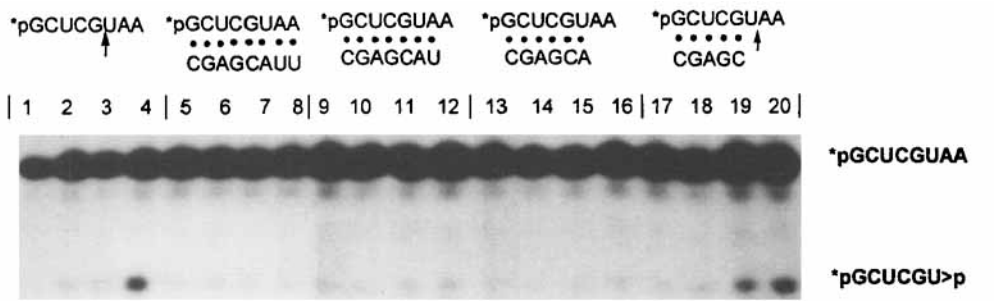

FIGURE 1. The stability of the partially complementary duplexes incubated in the presence of 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1% PVP, 1 mM spermidine, 2 mM EDTA at 15^0C. Lanes 1-4: incubation of *pGCUCGUAA for 0, 24, 48 and 90 h respectively. Lanes 5-8: incubation of *pGCUCGUAA/CGAGCAUU for 0, 24, 48 and 90 h, respectively. Lanes 9-12: incubation of *pGCUCGUAA/CGAGCAU for 0, 24, 48 and 90 h, respectively. Lanes 13-16: incubation of *pGCUCGUAA/ CGAGCA for 0, 24, 48 and 90 h, respectively. Lanes 17-20: incubation of *pGCUCGUAA/CGAGC for 0, 24, 48 and 90 h, respectively

diester bonds are stable under conditions that promote the cleavage of UA and CA bonds (2). We assume that the weak stacking interaction allows conformational rearrangement of the cleaved phosphodiester bond (to place in-line 2'-hydroxyl, phosphorous and 5'-oxygen) while strong stacking limits conformational flexibility of the diester bond.

To further establish the correlation between the hydrolyzability of the phosphodiester bond and stacking of the flanking nucleobases, we modified uridine-4 to produce a series of oligomers of the sequence UCGU^{5R}AA*pCp, where R = F, Cl, Br, I, H, Me, Et, n-Pr. Figure 2 shows that the alkylated derivatives of UCGU^{5R}AA*pCp (where R is either 5-methyl, 5-ethyl or 5-n-propyl) have affected the rates of hydrolysis. The 5-alkylation of uridine changes the stability of U^{5R}-A internucleotide bond in the following order: methyl> ethyl> n-propyl. Presumably the larger alkyl substituents donate electrons to the base, strengthening the stacking interaction, however, hydrophobic interactions can be consider as well.

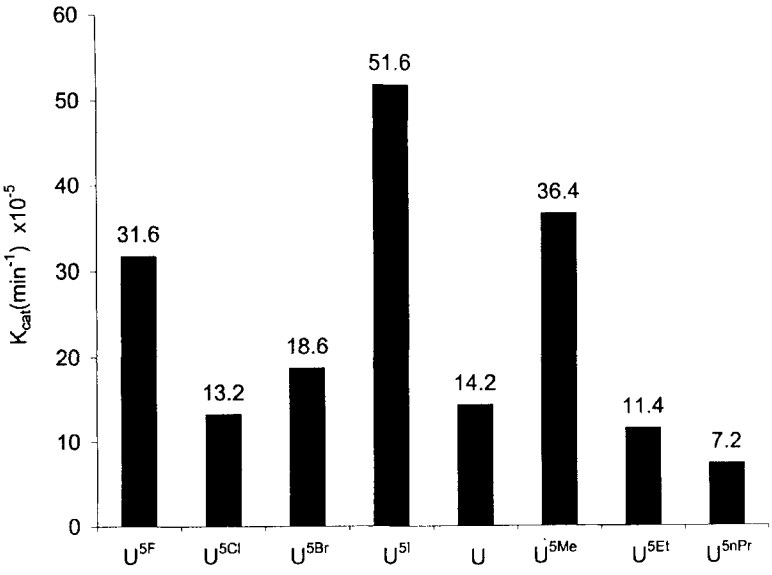

FIGURE 2. The comparison the stability of UCGU^{R}AA*pCp (where, R = F, Cl, Br, I, H, Me, Et and n-Pr) in the presence of 50 mM Tris-HCl (pH 7.5), 0.1% PVP, 1 mM spermidine, 2 mM EDTA at 37^{0}C.

Interpretation of the 5-halogenation of uridine on the hydrolyzability is more complex. The two contrary effects, electronic and steric, can influence the stacking interactions and in consequence the cleavage rate of the phosphodiester bond U^{R}A within UCGU^{R}AA. The results summarized in Figure 2 show that the rate of cleavage of the U^{5Hal}–A phosphodiester bond is in the order: chloro<bromo<fluoro<iodo uridine derivatives. The halogens, mostly F and Cl withdraw electrons and reduce the stacking interaction of U^{5Hal} and A(5), thereby enhancing cleavage. Similarly, the large sizes of Br and I may disturb the parallel orientation of U^{5Hal} and A5 to further reducing stacking interactions. In consequence the overlapping of the both effects changes the stability of UCGU^{Hal}AA in the observed manner. Moreover, the results suggest that the steric effect of the 5-substituent of uridine has a greater effect on the cleavage of the phosphodiester bond than the electron-withdrawing effect of the 5-substituent (K_{cat} U^{5I} - 51.6 x 10^{-5} min^{-1}, K_{cat} U^{5F} - 31.6 x 10^{-5} min^{-1}).

These data support the hypothesis that weak stacking enhances the population of the active conformation.

Specific nonenzymatic hydrolysis of a hairpin loop.

In native RNA molecules such as tRNA or the L-21 Sca I fragment of the group I intron from *Tetrahymena thermophila,* it has been shown that the hydrolysis occurs within a single stranded fragment of RNA, including hairpin loops (1, 5, 6, 28).

To evaluate the importance of the position of the UA phosphodiester bond within a hairpin loop, the following hairpins were tested for hydrolysis: H1, A**GGC**UACCCCCCC**GCC**; H2, A**GGC**CCCCUACCC**GCC**; and H3, A**GGC**CCCCCCCUA**GCC** (where stem nucleotides are bold). Thus the UA diester bond was placed at the 5'-end, in the center, and at 3'-end in hairpin loops H1, H2 and H3, respectively. The calculated free energy (ΔG^0_{37}) is –2.1 kcal/mol and the predicted Tm is 63.5^{0}C (29, 30). Hydrolyses of the ^{32}P-labeled hairpins (at 5'-termini) were performed in 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1% PVP, 2 mM EDTA at 37^{0}C using various concentration of spermine (0.0001 mM, 0.001 mM, 0.01 mM, 0.1 mM, 1 mM, 5 mM and 10 mM). After 6 h incubation the aliquots of the oligomers were analyzed by polyacrylamide gel electrophoresis.

For the hairpin H1, where the UA phosphodiester bond was placed just 3' to the hairpin stem, the cleavage was maximal at 0.0001 – 0.01 mM spermine (data not shown), which is a 10-100 fold lower concentration than for single stranded oligoribonucleotides (6). In H2, where the UA phosphodiester bond was located in a central position of the loop, the hydrolysis was maximal at 0.1 mM, the same concentration of spermine as for the model oligoribonucleotide, UCGUAA (6). In the hairpin H3, the UA phosphodiester bond was placed just 5' to the stem and the maximum hydrolysis was observed at 0.01-0.1 mM spermine.

This experiment demonstrates that the position of the UA diester bond within the hairpin loop is not crucial for cleavage. However, different concentrations of spermine are necessary to reach the maximum rate of hydrolysis. This may reflect the ability of the spermine to bind stronger to double-stranded RNA than to single-stranded RNA (31).

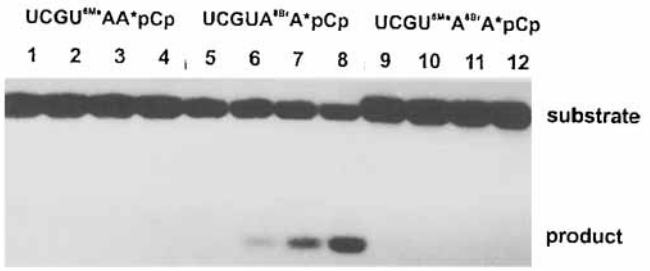

FIGURE 3. The stability of the substituted oligoribonucleotides incubated in the presence of 50 mM Tris-HCl (pH 7.5), 0.1% PVP, 1 mM spermidine, 2 mM EDTA at 37^0C. Lanes 1-4: incubation of *pUCGU^{6Me}AA for 0, 10, 24 and 48 h, respectively. Lanes 5-8: incubation of *pUCGUA^{8Br}A for 0, 10, 24 and 48 h, respectively. Lanes 9-12: incubation of *pUCGU^{6Me}A^{8Br}A for 0, 10, 24 and 48 h, respectively.

The influence of the functional groups of the nucleobases flanking the cleaved phosphodiester bond.

Our previous experiments showed that some Y-R phosphodiester bonds are hydrolyzed much faster than others. For example, the UA bond is cleaved 50-100 fold faster than UG (2). As discussed earlier, an important factor for the hydrolysis is a weak stacking interaction of the nucleobases flanking the cleaved phosphodiester bond. However, comparison of the stacking of dimers UA and UG demonstrates that the stacking interactions are similar for these: 15% and 10%, respectively (26, 27). Thus factors other than stacking are also important for the cleavage.

One example is that replacement of uridine-4 in UCGUAA by 6-methyluridine (U^{6Me}) inhibits hydrolysis (Figure 3). A likely explanation is that 6-methyluridine has a *syn* orientation around the glycosidic bond, rather than the *anti* orientation, which is preferred for natural nucleotides (32, 33). In the *syn* conformation, oxygen-2 of uracil is located near the 2'-hydroxyl group. A *syn* conformation in 6-methyluridine would move oxygen-2 far away from the 2'-hydroxyl. We speculate that oxygen-2 and the 2'-hydroxyl group interact via a hydrogen bond to increase the hydroxyl nucleophilicity.

Replacement of oxygen-2 with an amino group also eliminates hydrolysis. When cytidine-4 in UCGCAA, where hydrolysis occurs, is replaced by isocytidine (iC, 2-amino-4-oxo-pyrimidine riboside) hydrolysis is not observed (data not shown). Thus the substituent at C-2 of the pyrimidine ring is crucial for hydrolysis. We assume that a hydrogen-bond acceptor is required at this position.

We also tested the influence of modifications of the purine base on the hydrolysis reaction. Previous experiments demonstrated the importance of an unprotonated N-1 and the amino group at C-6 of the purine for oligoribonucleotide cleavage (1, 2). Presently, we find that a bulky bromine atom at position 8 of adenosine-5 (UCGUA^{8Br}A) does not affect the hydrolysis (Figure 3), although, a bromine at this position changes the glycosidic bond from *anti* to *syn* (34). In another experiment of this series, we observed that UCGUiGA is stable under conditions of nonenzymatic hydrolysis (iG is isoguanosine; 6-amino-2-oxo-purine riboside). Previously we observed the high stability of UCGUGA (1, 2). This is consistent with our hypothesis that effective cleavage is ensured if N1 of the purine is available as a hydrogen bond acceptor and the C-6 substituent is either a hydrogen-bond donor or acceptor. The replacement of guanosine at position 5 by isoguanosine does not restore hydrolysis, even though isoguanosine and adenosine contain similar substituents at N1 and C6. Similarly, hydrolysis is inhibited for UCGU2APA (where, 2AP is 2,6-diaminopurine riboside) in spite of the similarity of the N1-C6 system with adenosine and isoguanosine (2). We assume that the amino group at position 2 of isoguanosine and 2,6-diaminopurine riboside sterically hinders formation of the active oligomer conformation.

The effect of the denaturating factors.

It has been reported by several groups that denaturing reagents affect cleavage specificity of the ribozymes from Hepatitis delta virus (HDV) and *Tetrahymena thermophila* (35-39).

We examined the effect of formamide, urea, ethanol, and sodium chloride on the rate of the oligoribonucleotide hydrolysis. The hydrolysis reaction of *pUCGUAA was performed in the standard conditions at 37^{0}C with various concentrations of the denaturants. The curves reflecting correlation between the

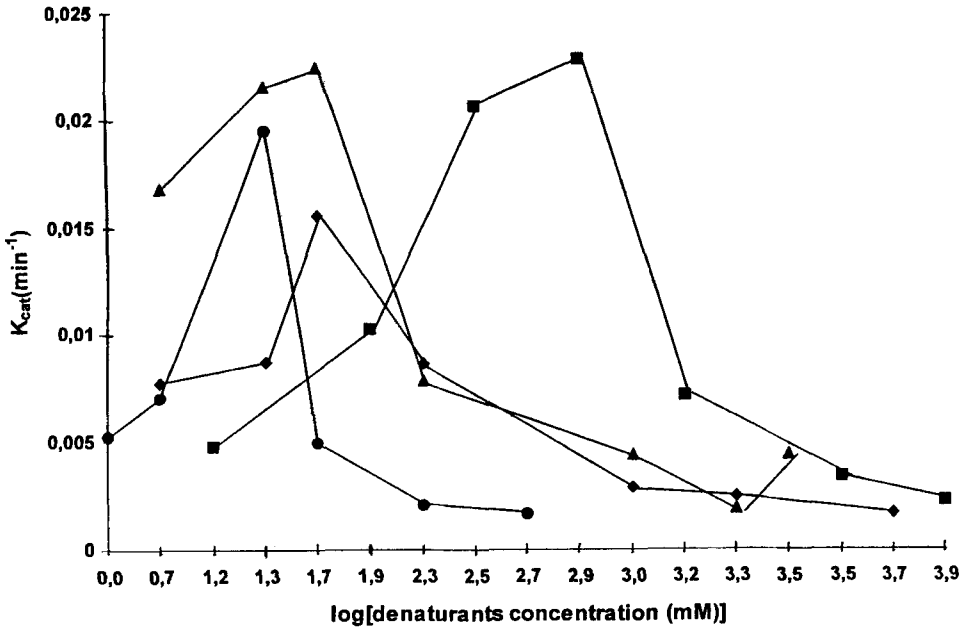

FIGURE 4. The correlation between the rate of the hydrolysis of *pUCGUAA and the concentration of the formamide (⇩), urea (◆), ethanol (■) and sodium chloride (●).

rate of the hydrolysis of *pUCGUAA and concentration of denaturants are shown in Figure 4.

The stability of *pUCGUAA was examined at seven concentrations of the formamide in the range from 5 mM to 3.2 M. The maximum rate of hydrolysis was observed at 20 mM formamide. For 1 M formamide or higher, the hydrolysis loses specificity. In urea, the optimum cleavage occurs at 50 mM. Above 200 mM the urea hydrolysis rate is reduced significantly and hydrolysis is completely inhibited in 1 M urea. Ethanol displayed an effect similar to formamide and urea. The hydrolysis of *pUCGUAA at 5% v/v (ca. 0.85 M) ethanol was the most rapid. Finally, the stability of *pUCGUAA in seven different concentrations of sodium chloride (1 mM to 0.5 M) was analyzed. The maximum rate of hydrolysis of *pUCGUAA was observed at 5 mM of sodium chloride and the cleavage was significantly inhibited above 50 mM of sodium chloride.

All the studied denaturants showed a similar influence on hydrolysis. The denaturants presumably affect the hydration network of the cleaved oligoribonucleotide and change the nucleophilic and electrophilic characters of functional groups directly involved in the cleavage of the phosphodiester bond. As with other hydrolysis mechanisms, there is likely to be a particular active conformation required for hydrolysis (35-39). The active form is likely to have an in-line arrangement of the functional groups involved in cleavage of the phosphodiester bond (3). Very low concentrations of denaturants result in ordered structures with a very small population of the active conformation while very high concentrations promote the population of numerous inactive forms, also limiting active form population and in consequence phosphodiester bind cleavage.

Mechanism of phosphodiester bond cleavage.

Based on these experiments, we can postulate the hypothetical mechanism of cleavage of the phosphodiester bond (Figure 5). Earlier studies involving Sp and Rp thiophosphate diastereoisomers demonstrated that phosphodiester bond hydrolysis results in an inversion of configuration at the phosphorous atom. This inversion requires an in-line arrangement of the participating atoms: 2'-oxygen, phosphorous and 5'-oxygen (3). However, this arrangement is not consistent with A-form conformation. It is necessary to change the ζ or ε torsion angles to achieve the active conformation. Such a conformation breaks the stacking interaction of the nucleobases flanking the scissile phosphodiester bond. This is consistent with our observation that nucleobases flanking cleaved phosphodiester bonds cannot be involved in hydrogen bonding with the complementary strand to undergo cleavage. Moreover, hydrolysis of the single-stranded oligoribonucleotide is sensitive to modifications on both sides of the cleaved diester bond; such changes may often promote stacking of their nucleobases. Weak stacking promotes flexibility of the scissile internucleotide bond, allowing functional groups to fall in line thereby allowing cleavage.

We also postulate that some substituents of the nucleobases flanking the scissile phosphodiester bond participate in hydrogen-bond interactions which promote reactivity of the functional groups crucial to cleavage. The present data

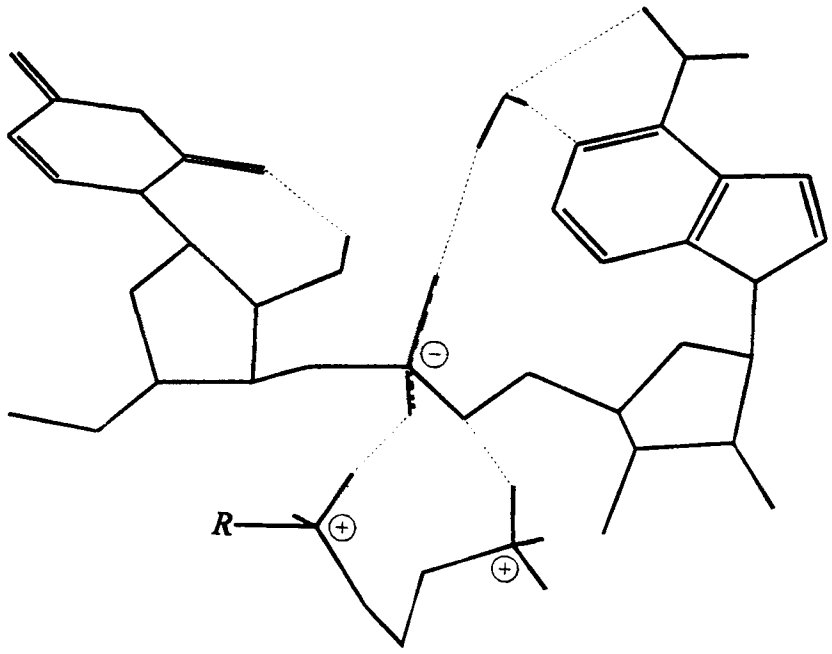

FIGURE 5. The postulated interactions leading to cleavage of the phosphodiester bond. On the figure, R represents the remaining part of the polyamine, for example for spermidine R = $NH_3^+(CH_2)_4$. Dotted lines show the hydrogen bonding interactions important for cleavage.

suggest a hydrogen bond interaction between the C-2 substituent (oxygen or sulfur) of the pyrimidine and its 2'-hydroxyl. This can increase the nucleophilic character of the 2'-hydroxyl and accelerate the cleavage. This is analogous to the role of divalent metal cations in many ribozymes; the hydrated cation interacts with the 2'-hydroxyl group (7-9). The present results also suggest that the purine nucleobase (for example, A5 within UCGUAU) is involved in hydrogen bonding. We observed that N1 has to be a proton acceptor and the substituent at C-6 can be either a hydrogen bond donor or acceptor. However, these are not sufficient for successful cleavage if a substituent at position C-2 of the purine is present. According to the model, hydrogen bonding between the purine base with its 5'-phosphate (directly or bridged by water) is likely. This interaction should increase the electrophilic character of the scissile internucleotide bond.

Previous experiments showed that polyamine is necessary for cleavage (6). Direct interactions of polyamines and RNA and DNA have been demonstrated (1-6 and references therein). In particular, secondary ammonium groups of polyamines interact electrostatically with phosphate groups (31). Moreover, we found that for the phosphodiester bond cleavage at least two ammonium groups are necessary. The optimal rate of the hydrolysis was observed when they were linked via a 3-4 carbon chain (6). We postulate that secondary ammonium groups of polyamines interact with phosphate anion while the primary ammonium form hydrogen bonds with the 5'-oxygen to make it a better leaving group (Figure 5). Two histamines are involved in catalysis of RNA hydrolysis by ribonuclease A (40, 41). The His 119 forms a hydrogen bond with a 5'-oxygen while the second, His 12, activates the adjacent 2'-hydroxyl. In our postulated model of oligoribonucleotide hydrolysis, another possibility is that the primary ammonium activates the 2'-hydroxyl group. We believe that this interaction is unlikely for three reasons. First, we observed that the distance between the proton acceptor at the C-2 position and the 2'-hydroxyl is correlated with hydrolysis rate. Next, the polyamine ammonium can only act as a hydrogen-bond donor; if there is a hydrogen bond between a primary ammonium group and a 2'-hydroxyl, the hydroxyl oxygen has to be an acceptor. This interaction can reduce the nucleophilic properties of the 2'-hydroxyl and limit the phosphodiester bond cleavage. Finally, the formation of a polyamine bridge between a phosphate group and the adjacent 2'-hydroxyl will reduce the flexibility of the cleaved phosphodiester bond. Thus it is difficult to achieve the proper configuration of atoms involved in cleavage if the primary ammonium activates the 2'-hydroxyl.

Another factor in the RNA hydrolysis is the function of the requisite cofactor: organic polymers or nonspecific, nonribonuclease proteins. The wide variety of molecules that can act as cofactors (1, 2) effecting the cleavage suggests their nonspecific interactions with RNA or the RNA hydration shelf. This interaction can change the hydrogen bond network of water molecules surrounding the RNA, thereby affecting reactivity of the RNA functional groups. Alternatively, the cofactor can exclude water from the RNA molecule since some known cofactors, including polyethylene glycol and its derivatives, are useful for crystallization of nucleic acids and proteins.

CONCLUSIONS

As described above we determined several factors affecting specific, nonenzymatic cleavage of oligoribonucleotides including the flexibility of the cleaved phosphodiester bond and the interaction of the C-2 substituent with the 2'-hydroxyl. These data allow us to postulate the mechanism of the RNA cleavage.

Furthermore we found that the presence of denaturing reagents affects the rate of the oligoribonucleotide hydrolysis. The optimal concentration of the denaturing reagents ranged from 5 to 50 mM. For formamide, urea, ethanol and sodium chloride we observed a bell-shaped correlation between the concentration of reagent and the rate of the hydrolysis (1, 6). The correlation between the rate of $tRNA_i^{Met}$ hydrolysis and the concentration of spermine or spermidine was also bell shaped (6). This suggests a delicate balance among all the factors influencing the RNA hydrolysis, which provides the opportunity for cellular regulation of the reaction. Therefore the specific, nonenzymatic hydrolysis of RNA is a general phenomenon that can be regulated and does not depend on the size of the RNA molecule.

ACKNOWLEDGEMENTS

The work was supported by the State Committee for Scientific Research (KBN), grants 3 T09A 023 11 (to RK) and 6 P04A 027 12 (to MF). We would like to thank Mark E. Burkard (University of Rochester) for comments and suggestions on this manuscript.

REFERENCES

1/ Kierzek, R. *Nucleic Acids Res.* **1992 20**, 5079-5084.

2/ Kierzek, R. *Nucleic Acids Res.* **1992** 20, 5073-5077.

3/ Kierzek, R. *Collect. Czech. Chem. Commun.*, **1996 61**, 161-163.

4/ Kierzek, R. *Collect. Czech. Chem. Commun.*, **1996 61**, 253-257.

5/ Bibillo, A., Ziomek, K., Figlerowicz, M., Kierzek, R. *Acta Biochimica Polonica*, **1999 46**, 145-154.

6/ Bibillo, A., Figlerowicz, M., Kierzek, R. *Nucleic Acids Res.*, in press

7/ Pley, H.W., Flaherty, K.M., McKay, D.B. *Nature*, **1994 372**, 68-74.

8/ Cate, J.H., Gooding, A.R., Podell, E., Zhou, K., Golden, B.L., Kundrot, C.E., Cech, T.R., Doudna, J.A. *Science*, **1996 273**, 1678-1684.

9/ Ferre-D'Amare, A.R., Zhou, K., Doudna, J.A. *Nature*, **1998 395**, 567-574.

10/ Asakura, J-I. And Robins, M.J. *J. Org. Chem.*, **1990 55**, 4928-4933.

11/ Niedballa, U., Vorbruggen, H. *J. Org. Chem.*, **1976 41**, 2084-2091.

12/ Niedballa, U., Vorbruggen, H. *J. Org. Chem.*,**1976 39**, 3660-3667.

13/ Strobel, S.A., Cech, T.R., Usman, N., Beigelman, L. *Biochemistry*, **1994 33**, 13824-13835.

14/ Seela, F., Wei, C.F. *Hel. Chim. Acta*, **1997 80**, 73-85.

15/ McBride, L.J. and Caruthers, M.H. *Tetrahedron Lett.* **1983 24,** 245-249.

16/ Kierzek, R., Caruthers, M.H., Longfellow, C.E., Swinton, D., Turner, D.H. Freier, S.M. *Biochemistry*, **1986 25**, 7840-7846.

17/ Markiewicz, W.T., Biala, E., Kierzek, R. *Bull. Acad. Polon. Sci., ser., chim.,* **1984 32**, 433-451.

18/ Wincott, F., DiRenzo,A., Shaffer, C., Grimm, S., Tracz, D., Workman, C., Sweedler, D., Gonzalez, C., Scringe, S., Usman, N. *Nucleic Acids Res.,* **1995 23**, 2677-2684.

19/ Asakura, J-I., Robins, M.J. *J. Org. Chem.,* **1990 55**, 4928-4933.

20/ Chou, S.-H., Flynn, P., Reid, B. *Biochemistry*, **1989 28**, 2422-2435.

21/ Borer P.N. (1974) in Handbook of Biochemistry and Molecular Biology: Nucleic Acids (Fasman, G.D., ed.) 3rd ed., Vol. I p 597, CRC Press, Cleveland.

22/ McDowell J.A., Turner D.H. *Biochemistry*, **1996 35**, 14077-14089.

23/ Petersheim, M., Turner, D.H. *Biochemistry*,**1983 22**, 256-263.

24/ Limmer, S., Hofman, H.P., Ott, G., Sprinzl. M. *Proc. Natl. Acad. Sci. USA*, **1993 90**, 6199-6202.

25/ Vogt, V.M. *Eur. J. Biochem.*,**1973 33**, 192-196.

26/ Ezra, F.S., Lee, C-H., Kondo, N.S., Danyluk, S.S., Sarma, R.H. *Biochemistry*,**1977 16**, 1977-1987.

27/ Brahms, J., Maurizot, J.C., Michelson, A.M. *J. Mol. Biol.*, **1967 25**, 481-495.

28/ Dock-Bergeon, A.C., Moras, D., (1987) Cold Spring Harbor Symposia on Quantitative Biology. Vol. LII, Cold Spring Harbor Laboratory Press, p. 113-121.

29/ Xia, T., SantaLucia, J., Burkard, M.E., Kierzek, R., Schroeder, S.J., Cox, C., Turner, D.H. *Biochemistry*, **1998 37**, 14719-14735.

30/ Serra, M.J., Axensen, T.J., Turner, D.H. *Biochemistry,* **1994 33**, 14289-14295.

31/ Frydman, L., Rossomando, P.C., Frydman, V., Fernandez, C.O., Frydman, B., Sameijima, K. *Proc. Nat. Acad. Sci. USA*, **1992 89**, 9186-9190.

32/ Suck, D., Saenger, W. *J. Am. Chem. Soc.*, **1972 94**, 6520-6526.

33/ Suck, D., Saenger, W., Vorbruggen, H. *Nature*, **1972 235**, 333-334.

34/ Tavale, S.S. Sobell, H.M. *J. Mol. Biol.*, **1970 48**, 109-123.

35/ Rosenstein, S.P., Been, M.D. *Biochemistry,* **1990 29**, 8011-8016.

36/ Belinsky, M., Dinter-Gottlieb, G. *Nucleic Acids Res.,* **1991 19**, 559-564.

37/ Smith, J.B., Dinter-Gottlieb, G. *Nucleic Acids Res.*,**1991 19**, 1285-1289.

38/ Smith, J.B., Gottlieb, P. A., Dinter-Gottlieb, G. *Biochemistry*, **1992 31**, 9629-9635.

39/ Zaug, A.J., Grosshans, C.A., Cech, T.R. *Biochemistry*, **1988 27**, 8924- 8931.

40/ Richards, F.M., Wickoff, H.W. (1971) In Boyer, P.D. (ed.) The Enzymes, Academic Press, New York and London, pp. 647-806.

41/ Witzel, H. (1963) In Davidson, J.N., Cohen, W.E. (eds), Progress in Nucleic Acids Research, Academic Press, New York and London, pp. 221-258.

Received 9/23/99
Accepted 3/13/00