Hydrolysis of Phosphodiester Bonds within RNA Hairpin Loops in Buffer Solutions: the Effect of Secondary Structure on the Inherent Reactivity of RNA Phosphodiester Bonds

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Dedicated to Prof. Dr. *Frank Seela* on the occasion of his 60th birthday

The hydrolysis of phosphodiester bonds of chimeric 2′-*O*-methyloligoribonucleotides was studied in buffer solutions. Pseudo-first-order rate constants for cleavage of phosphodiester bonds within hairpin loops were calculated and compared with those for cleavage of phosphodiester bonds within double-stranded stems and linear single-stranded oligonucleotides. No large differences in reactivity were observed: some of the hairpin structures studied were slightly less and others slightly more reactive than the linear reference. These results suggest that phosphodiester bonds within small hairpin loops are conformationally free to cleave by an in-line mechanism, but also that the secondary structure may influence the reactivity of phosphodiester bonds.

Introduction. – The cleavage of RNA phosphodiester bonds has been extensively studied during the last two decades [1][2]. Efficient catalysts for sequence-specific cleavage are being developed by several research groups [3]. It is hoped that studies of the catalysis mechanisms and factors that enhance the reactivity of phosphodiester bonds will enable rational design of such catalysts. Studies of the chemical cleavage of phosphoesters may also further our understanding of the reaction mechanisms of nucleic-acid-modifying metalloenzymes and, particularly, of ribozymes.

It may be said that the inherent reactivity of all internucleosidic phosphodiester bonds within linear oligonucleotides is approximately the same and depends only on the reaction conditions. The rate constants for cleavage of different dinucleoside monophosphates vary only slightly [4]. The differences observed can, at least in part, be attributed to the different pK_a values of the 2′- and 5′-hydroxy groups in different nucleosides. Phosphodiester bonds within poly-U oligomers also react approximately as fast as those within dinucleoside monophosphates [5]. Only under acidic conditions does poly-U appear to be more reactive, probably because protonation of phosphate groups within a polyanionic molecule is enhanced.

As opposed to the situation with linear oligonucleotides, where the reactivity of phosphodiester bonds depends only on the conditions, the secondary structure of an oligonucleotide strand may significantly influence the reactivity of phosphodiester bonds in some structures. As the most striking example, the phosphodiester bonds within RNA-RNA or RNA-DNA duplexes are relatively stable, their cleavage being very slow compared to the cleavage of phosphodiester bonds within single-stranded

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regions [6] – [9]. This is true in both the absence and the presence of metal ion catalysts. It is believed that the duplex structure forces phosphodiester bonds into a conformation unfavorable for the cleavage. Phosphodiester bonds within bulges or hairpin loops, in contrast, are known to be cleaved by metal ion catalysts [6][8][9].

In the presence of metal-ion catalysts, the observed reactivity of phosphodiester bonds can be regarded as a combination of contributions of the inherent reactivity of the bond and the catalytic properties of the metal-ion species involved. The secondary structure of a substrate oligonucleotide may affect both of these factors in different ways. In addition to the restrictions brought about by the fixed structure of an RNA duplex, the strain induced by the tight bending of a RNA strand at bulged sites or in hairpin loops may destabilize phosphodiester bonds [10]. As for the metal-ion-promoted cleavage, it has been suggested that bending of an RNA strand at an RNA bulge may create strong metal-ion coordination sites that are favorable for the catalysis [11]. In contrast, our previous results show that, in the presence of metal-ion catalysts, phosphodiester bonds within hairpin loops are clearly less reactive than those within linear oligonucleotides [9]. This difference was also attributed to the coordination of metal ions.

We have previously studied the effects of the secondary structure on metal-ion catalysis by studying the cleavage of phosphodiester bonds within small RNA hairpins [9]. In this paper, we set out to study the inherent reactivity of phosphodiester bonds within bent RNA strands. The cleavage of phosphodiester bonds within hairpin loops was studied in buffer solutions, and as in the previous paper [9], the size of the hairpin and the position of the scissile bond within the loop were varied. The rate constants obtained are compared with those for cleavage of a linear single-stranded structure.

Results. – The loop structures studied are shown in the *Figure*. To study the cleavage of one particular phosphodiester bond at a time, all but one of the ribonucleoside units were converted to 2′-*O*-methylribonucleotides. 2′-*O*-Methylnucleosides were chosen, because the ring conformation of a 2′-*O*-methylribose closely resembles that of the parent ribose [12]. Compound **1** is a linear molecule that serves as a reference material. Compounds **2** and **3** constitute a pair of isomers where **2** has a 3′,5′- and **3** a 2′,5′-phosphodiester bond. In **4**, there is a scissile phosphodiester bond within the double-stranded region. In all the other cases, the reactive 3′,5′-phosphodiester bond is located within a hairpin loop.

To verify the presence of a hairpin structure under the conditions of the kinetic experiments, the melting temperatures of the molecules were determined. The hairpin structures studied appear to be very stable; in 10 mM *Tris* buffer at pH 7.0 and ionic strength of 0.1M, all exhibited concentration-independent melting temperatures (T_m) at *ca.* 90°. The T_m values were slightly lower under experimental conditions, where the T_m value of **2**, for example, was 85.5° in 0.1M CHES (= 2-(cyclohexylamino)ethanesulfonic acid) at pH 8.6, and 80.5° in 1M imidazole at pH 7.0. The structure of molecules **8** – **12** has been discussed previously [9].

Kinetic experiments were carried out at 65°, where all of the molecules can safely be assumed to exist as hairpins. Two different buffer systems were employed: 1.0M imidazole buffer at pH 7.1 and 0.1M CHES buffer, at pH 8.6. The mechanism of the cleavage probably differs in these two buffer solutions. In the imidazole buffer at high

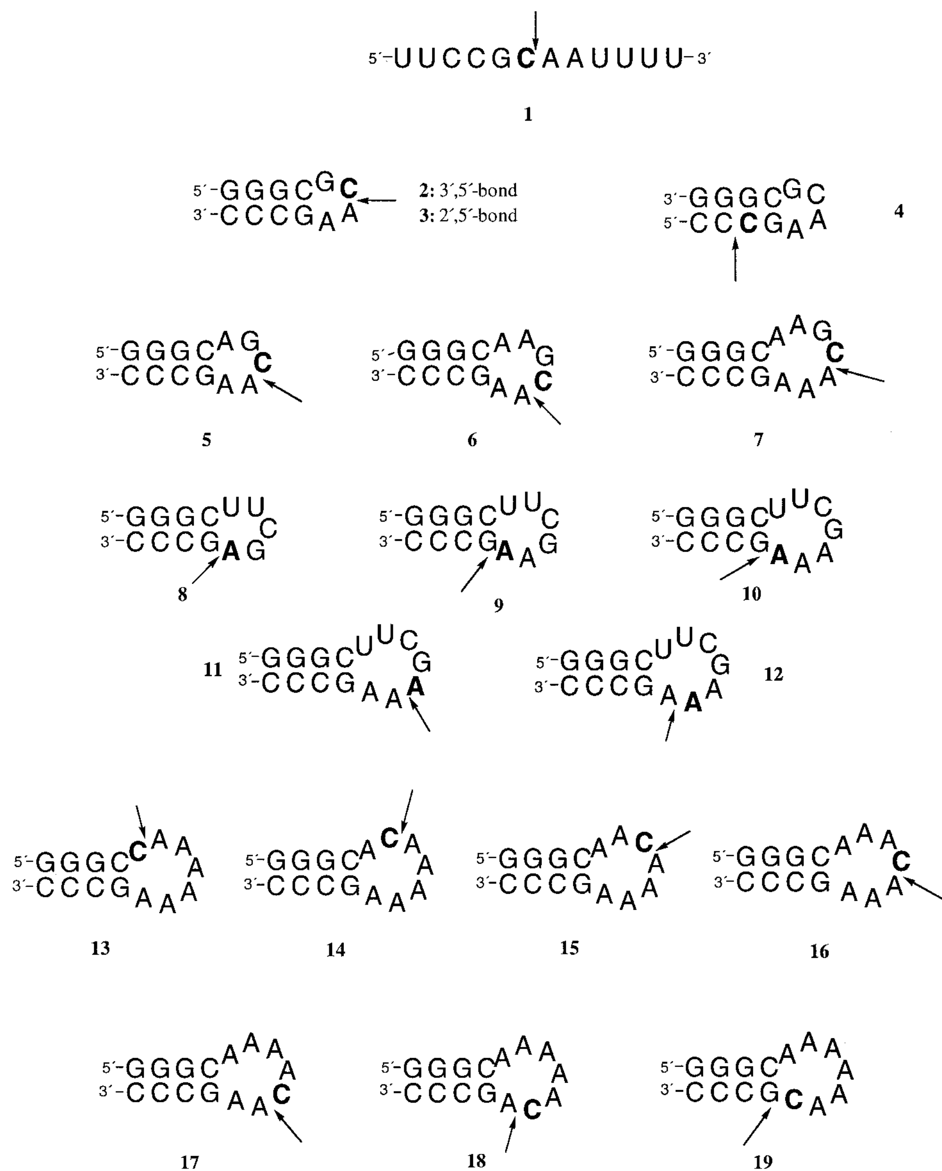

Figure. *Structures of the molecules studied*. The position of the ribonucleoside has been indicated by a bold-case letter and the scissile phosphodiester bond by an arrow.

concentration, the predominant reaction is the buffer-catalyzed cleavage of phosphodiester bonds [13][14]. Isomerization of phosphodiester bonds was insignificant under these conditions, since catalysis of phosphate migration by imidazole buffers is very modest [14]. In the CHES buffers at pH 8.6, the buffer concentration was kept low, and the predominant reaction under these conditions was the OH^{-}-catalyzed cleavage of

the phosphodiester bonds. The alkaline cleavage is not accompanied by the isomerization of phosphodiester bonds [4][5].

The results of the kinetic experiments collected in the *Table* show clearly that the secondary structure of the hairpin loops under study had only a modest effect on the reactivity of phosphodiester bonds. In the CHES buffer, the reactivity of phosphodiester bonds differed only slightly between the linear molecule **1** and the hairpin structures. Similarly, the maximal difference observed between the rate constants for cleavage of 3′,5′-bonds within a hairpin loop was less than 20-fold. A similar comparison between the rate constants obtained in imidazole buffer is more difficult to make, because of the unreactivity of some structures. Replacing a scissile 3′,5′-phosphodiester bond with a corresponding 2′,5′-bond did not appear to have any significant effect when the bond was within the loop. In keeping with the known stability of the 3′,5′-bonds within double-stranded regions [6]–[9], the scissile 3′,5′-phosphodiester bond of **4** placed within the stem was, in both cases, the least reactive. The differences in reactivity observed were, however, rather small: the rate constant for cleavage of **2** in the CHES buffer was only six times larger than that of **4**.

Table. *First-Order Rate Constants for Cleavage of Phosphodiester Bonds within Hairpin Loops in Buffer Solutions at 65°*

Compound	k(CHES)/10^{-6} s^{-1} [a])	k(imidazole)/10^{-7} s^{-1} [b])
1	2.3 ± 0.1	10.6 ± 0.4
2	1.8 ± 0.1	8.0 ± 0.3
3	1.0 ± 0.1	3.1 ± 0.2
4	0.3 ± 0.1	< 0.1
5	20.4 ± 0.9	9.7 ± 0.4
6	4.5 ± 0.2	8.7 ± 0.3
7	3.9 ± 0.1	10.6 ± 0.4
8	1.1 ± 0.1	5.5 ± 0.7
9	1.6 ± 0.1	< 1.0
10	1.5 ± 0.1	< 1.0
11	2.6 ± 0.1	7.9 ± 0.3
12	2.1 ± 0.1	8.2 ± 0.4
13	8.3 ± 0.3	4.6 ± 0.2
14	4.5 ± 0.3	9.0 ± 0.3
15	4.0 ± 0.1	17.1 ± 0.6
16	5.0 ± 0.3	12.8 ± 0.5
17	4.7 ± 0.3	12.2 ± 0.3
18	4.6 ± 0.3	13.0 ± 0.6
19	2.4 ± 0.2	6.3 ± 0.2

[a]) 0.1M CHES, pH 8.6. [b]) 1.0M imidazole, pH 7.1.

The most interesting results were obtained with compounds **2** and **5**–**7**, where the size of the loop was systematically increased by adding adenosine nucleosides. All of the molecules were slightly more reactive than the linear molecule **1**, with **5**, containing the pentaloop, being the most reactive. The rate enhancement was, however, modest; **5** was only about ten times as reactive as **1**. No obvious trend was observed, and a C-A bond in a tetraloop, as in **2**, was not found to be particularly reactive. In imidazole

buffer, no such reactivity differences were observed, and **5**–**7** and **2** appear to be approximately as reactive as **1**. Compounds **8**–**10** form another series of molecules with increasing loop size, in which the scissile phosphodiester bond was placed next to the stem, and in which no large reactivity differences were observed in CHES buffer. In imidazole buffer, however, **9** and **10** were exceptionally unreactive. With all the other molecules, including **8**, the rate constants obtained in CHES buffer were two to four times larger than those obtained in imidazole buffer. The rate of cleavage of **9** and **10** in imidazole buffer was so slow that no rate constants could be obtained at all, which suggests that the rate difference is at least 15-fold.

The position of the scissile phosphodiester bond within the loop seemed to have only a very small effect, if any, on the reactivity of phosphodiester bonds. This is most clearly seen on comparing the rate constants for cleavage of **13**–**19**, where the position of the C-A bond within an adenosine-rich heptaloop is systematically changed. In both the CHES and imidazole buffers, the difference between the most and the least reactive structure was approximately fourfold. In the CHES buffer, molecules **14**–**18** were equally reactive, whereas catalysis by the imidazole buffer appears to be more sensitive to the position of the scissile phosphodiester bond, and a clear trend is observed, although the differences are very modest. In both cases, the bond next to the double-stranded stem seems to be the least reactive (**19**). Similar effects were observed with **10**–**12**: a phosphodiester bond next to the stem, as in **10**, was slightly less reactive than those of **11** or **12**.

The rate constants for cleavage of **5**–**7**, which appear to be more reactive than a linear oligonucleotide, were also determined in the presence of the Zn^{2+}[12]aneN_3 chelate. This catalyst was employed in our previous study on metal-ion-promoted cleavage of hairpin structures [9]. The rate constants obtained were $6.5 \times 10^{-6}\ s^{-1}$, $3.8 \times 10^{-6}\ s^{-1}$ and $3.4 \times 10^{-6}\ s^{-1}$ for structures **5**–**7**, respectively. That reported for cleavage of **1** under identical conditions was $9.2 \times 10^{-6}\ s^{-1}$. It is thus clear that, even if **5**–**7**, in the absence of metal-ion catalysts, exhibit a slightly enhanced reactivity compared to a linear molecule, no such effect is observed in the presence of a metal-ion catalyst.

Discussion. – The results discussed above show that no significant conformational restrictions are involved in the cleavage of the hairpin molecules studied; none of the phosphodiester bonds were cleaved in the CHES buffer significantly less readily than those of the linear oligonucleotide **1**. Furthermore, the 3′,5′- and 2′,5′-phosphodiester bonds within a hairpin loop appear to be equally reactive. This is in contrast to the observation where the reactivity of a 3′,5′-bond within a double helix was found to be significantly reduced compared to a 2′,5′-bond [15], and where the difference was attributed to different conformations around the phosphodiester bond to be cleaved.

There was no significant difference in the effect of secondary structure between the two different reactions studied, although slightly larger reactivity differences were observed in imidazole than in CHES buffers. The clear loss of the reactivity in the case of **9** and **10** in imidazole may well be attributed to the presence of an additional, albeit weak, A-U base pair, as was discussed before [9]. While this base pair is stabilized by the high imidazole buffer concentration at pH 7.1, it is not present, at least not to the same extent, in the CHES buffer at pH 8.6. In the imidazole buffer, the scissile phosphodiester bond lies within the double-stranded region, and could not, therefore, be cleaved efficiently. It would thus seem that the results obtained in the CHES buffer

better reflect the inherent reactivity of phosphodiester bonds, because the high ionic strength of the imidazole buffer may stabilize additional interactions.

Although the reactivity differences observed in this work were small, the results obtained still suggest that secondary structure may enhance the inherent reactivity of RNA phosphodiester bonds. The tenfold rate enhancement between **1**, a linear molecule, and the hairpin structure **5**, can be attributed to the secondary structure, since in both cases a similar phosphodiester bond between C and A residues is cleaved. Since the reactivity differences are very subtle and the structure of the substrates extremely complex, the present data do not permit elucidation of which parameters might contribute to the enhanced reactivity of phosphodiester bonds in certain hairpin molecules studied. On the basis of structural information available for nucleotides within hairpin and bulge motifs, it could, however, be suggested that the conformation of the sugar ring is indicative of the reactivity. It is known that within a hairpin stem, sugar moieties mainly adopt a C(3′)-*endo*-conformation, while those within a single-stranded region exist as an equilibrium mixture of C(2′)- and C(3′)-*endo*-conformers [16]. The proportions of the conformers vary depending on the position of the nucleoside. It is, therefore, tempting to speculate that cleavage occurs only when the nucleoside bearing the attacking nucleophile is in a C(2′)-*endo*-conformation. The observed reactivity would thus be a function of the nucleoside fraction present as the C(2′)-*endo*-conformer.

Experimental Part

The chimeric 2′-*O*-methyloligoribonucleotides were synthesized from commercial 2′-*O*-methylated and 2′-*O*-[1-(fluorophenyl)-4-methoxypiperidin-4-yl]-protected building blocks by means of a conventional phosphoramidate strategy according to the standard RNA-coupling protocol of the *ABI 392* DNA/RNA synthesizer. Melting experiments were performed on a *Perkin Elmer Lambda 2* UV/VIS spectrophotometer equipped with a *PTP-6* temp. programmer. Kinetic experiments were carried out in tightly sealed glass tubes in a water bath kept at $65.0 \pm 0.1°$. The buffer systems employed were imidazole/HCl and 2-(cyclohexylamino)ethanesulfonic acid (CHES)/NaOH. Aliquots withdrawn were cooled immediately in an ice bath, and were analyzed by ion-exchange chromatography. The details of all these procedures were described before [9].

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