The effect of the 3'-terminal monophosphate group on the metal-ion-promoted hydrolysis of the phosphodiester bonds of short oligonucleotides

Satu Kuusela,* Alex Azhayev, Andrei Guzaev and Harri Lönnberg

Department of Chemistry, University of Turku, FIN-20500 Turku, Finland

The effect of 3'-terminal monophosphate group on the metal-ion-promoted hydrolysis of the phosphodiester bonds of oligonucleotides has been studied. For this purpose, the rate constants for the hydrolysis of the following oligomers in the presence of Zn$^{2+}$ and its 1,5,9-triazacyclododecane chelate, Zn$^{2+}$[12]aneN$_3$, have been determined: (i) ApUpUp(2') and ApUpUp(3'), (ii) Up(Tp)$_n$ (n = 0-4 and 7), (iii) Up(Tp)$_n$Tp (n = 0-4 and 7). The results obtained are used to propose a mechanism for the Zn$^{2+}$ and Zn$^{2+}$[12]aneN$_3$ promoted hydrolysis of polynucleotides.

The metal-ion-promoted cleavage of nucleic acids, DNA and RNA, has received considerable attention during the last two decades. One reason for this is the desire to develop metal-ion-based cleaving agents that would be able to recognize a certain base sequence within the target nucleic acid and cleave a given phosphodiester bond. These kinds of artificial nuclease would greatly enlarge the potential of biotechnology, and they might even offer a tool to combat viral infections and hereditary diseases.1-3 Both hydrolytically4-6 and oxidatively7 functioning cleaving agents have been studied. Hydrolytic cleavage, however, has the advantage in that it produces fragments that can be religated by enzymes.

Since RNA is hydrolytically less stable than DNA, numerous studies on the metal-ion-promoted hydrolysis of various RNA mimics have been published. The model compounds employed range from simple diaryl phosphoesters8-10 to real oligonucleotides.11-14 The mechanism that is widely but not unanimously accepted for the metal-ion-promoted hydrolysis of the phosphodiester bonds of RNA involves coordination of the metal ion to the phosphate group and deprotonation of the attacking nucleophile, viz., the 2'-hydroxy function, by the hydroxo ligand of the metal ion (Scheme 1).12,14-18

Accordingly, acidic metal ions are generally the most efficient catalysts, even though the coordination geometry of the metal ion also influences the catalytic activity of its chelates.14-16,18

Although the mechanism of metal-ion action on the phosphodiester bonds of RNA and on simpler model compounds, such as 3',5'-UpU (1a; B$^1$ = B$^2$ = U), is likely to be similar, it is also known that the adjacent phosphate groups may have a large influence on the rate of the metal-ion-promoted hydrolysis.11,14,18-21 The terminal dianionic 3'-phosphate group, for example, has been shown markedly to accelerate the metal-ion-promoted cleavage of the phosphodiester bond in dinucleoside diphosphates, NpNp (1b), whereas the effects of 2'- and 2',3'-cyclic monophosphate groups or acyclic phosphodiester groups are modest.11 This finding suggests that whenever a 3'-phosphate group is produced in the course of metal-ion-promoted hydrolysis of RNA, it efficiently enhances the hydrolysis of the adjacent phosphodiester bond. Our previous results, according to which the phosphodiester bonds of poly-U are cleaved by metal ions up to 100 times faster than that of 3',5'-UpU (1a; B$^1$ = B$^2$ = U),14 might hence, a priori, be attributed to a rapid stepwise release of 3'-UMP from the 3'-terminus of the polymeric products. However, this does not seem to be the case, since the same studies also showed that the distribution of terminal and non-terminal cleavages is almost statistical when Zn$^{2+}$ aquo ion is used as a catalyst. This result might, at least partly, be accounted for by the observation that the 3'-terminal nucleotides produced by chemical hydrolysis mainly bear a 2',3'-cyclic monophosphate group.14 The same argument cannot, however, be used to explain the effect of Zn$^{2+}$ (1,5,9-triazacyclododecane) (Zn$^{2+}$[12]aneN$_3$). This catalyst cleaves 2',3'-cyclic monophosphates more rapidly than does Zn$^{2+}$, preventing accumulation of 3'-terminal 2',3'-cyclic monophosphate groups. In spite of this, the stepwise depolymerization from the 3'-end was not preferred.

To resolve these ambiguities we now report a more detailed study on the rate-enhancing influence of a terminal 3'-monophosphate group. Firstly, the effect of the position of the
terminal phosphate group was elucidated by studying the reactivity of the two phosphodiester bonds of ribonucleotides bearing either a 3'- or 2'-phosphate function (2a and 2b).

Secondly, the effect of the number of the phosphodiester bonds and the range of the rate-accelerating effect of the 3'-monophosphate group were elucidated by studying the reactions of short oligonucleotides. For this purpose, a series of oligomers that contained only one reactive phosphodiester bond, i.e., one ribonucleoside unit, and several 2'-deoxyribonucleoside units, were prepared. The behaviour of 3'-phosphorylated (3-8) and the corresponding unphosphorylated oligonucleotides (9-14) were compared, and the effect of increasing chain length was studied. The importance of these factors with respect to the metal-ion-promoted hydrolysis of RNA is discussed, and a mechanism for the metal aquo ion and metal-phosphate group at the 3'-position of the dinucleotide di-

Results and discussion

According to the results of Butzow and Eichhorn, the phosphate group at the 3'-position of the dinucleotide di-

<table>
<thead>
<tr>
<th>Table 1</th>
<th>The rate constants for the Zn$^{2+}$-promoted hydrolysis of the phosphodiester bonds of 3'- and 2'-phosphorylated trimers (2a and 2b) at pH 5.5$^a$ and 333.2 K, $[\text{Zn}^{2+}] = 10 \text{ mmol dm}^{-3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>$k/10^9 \text{ s}^{-1}$</td>
</tr>
<tr>
<td>ApUpUp(3') (2a)</td>
<td>$1.7 \pm 0.1$</td>
</tr>
<tr>
<td>ApUpUp(2') (2b)</td>
<td>$1.8 \pm 0.2$</td>
</tr>
<tr>
<td>ApU</td>
<td>$0.037 \pm 0.002$</td>
</tr>
<tr>
<td>UpU</td>
<td>$0.026 \pm 0.001$</td>
</tr>
</tbody>
</table>

$^a$ Adjusted with HEPES buffer; $[\text{HA}] / [\text{A}^-] = 0.1/0.005$, $I = 0.1 \text{ mol dm}^{-3} (\text{NaClO}_2)$. Standard deviation of the mean indicated.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>The rate constants for the hydrolysis of 3'-phosphorylated and unphosphorylated oligonucleotides in the presence of 10 mmol dm$^{-3}$ Zn$^{2+}$ aquo ion at pH 5.5$^a$ and at 333.2 K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>$k/10^6 \text{ s}^{-1}$</td>
</tr>
<tr>
<td>UpTp (3)</td>
<td>$383 \pm 1^b$</td>
</tr>
<tr>
<td>UpTpTp (4)</td>
<td>$33 \pm 1$</td>
</tr>
<tr>
<td>UpTpTp (5)</td>
<td>$44 \pm 2$</td>
</tr>
<tr>
<td>UpTpTp (6)</td>
<td>$44 \pm 2$</td>
</tr>
<tr>
<td>UpTpTp (7)</td>
<td>$18 \pm 2$</td>
</tr>
<tr>
<td>UpTpTp (8)</td>
<td>$23 \pm 3$</td>
</tr>
</tbody>
</table>

* Adjusted with HEPES buffer; $[\text{HA}] / [\text{A}^-] = 0.1/0.005$, $I = 0.1 \text{ mol dm}^{-3}$ (with NaClO$_2$). Standard deviation of the mean indicated.
The complex formation is thus enhanced and the hydrolysis is accelerated. The dianionic monophosphate function does obviously not diminish the catalytic efficiency of Zn$^{2+}$, as the results obtained with the Zn$^{2+}$-phosphorylated compounds show, the 3'-phosphate-bound metal ion may exert its catalytic influence over several nucleotide units.

Significantly different results were obtained with Zn$^{2+}$-[12]aneN$_3$ as a catalyst (Table 3). In the presence of this chelate, a dimer and trimer are hydrolysed at an equal rate, and only a modest rate-enhancement is observed on further increasing the chain length of the substrate. Even the addition of a dianionic monophosphate function does not result in a great rate-acceleration: UpTp (3) is hydrolysed only six times as fast as UpT (9), whereas a 170-fold difference was observed for the Zn$^{2+}$-promoted hydrolysis. This result supports the concept suggested above. In the Zn$^{2+}$-[12]aneN$_3$ chelate, the Zn$^{2+}$-cation is bound to three nitrogen atoms. Thus, to be able to provide a general-base catalyst, a hydroxo ligand for deprotonation of the attacking nucleophile, Zn$^{2+}$ cannot accept more than one phosphoryl oxygen ligand (structure c). Since 3'-phosphorylation still enhances the rate of the Zn$^{2+}$-[12]aneN$_3$-promoted hydrolysis, one may assume that the chelate does not necessarily have to bind to the phosphate group that is cleaved. In fact, our previous studies with poly-U suggest that the phosphodiester bond adjacent to the dianionic phosphate function might be slightly less readily hydrolysed than the non-terminal bonds. This is not, however, clearly supported by the results shown in Table 3.

It is worth noting that the [12]aneN$_3$ ligand as such does not diminish the catalytic efficiency of Zn$^{2+}$[12]aneN$_3$. If the rate constants obtained for the Zn$^{2+}$ and Zn$^{2+}$-[12]aneN$_3$-promoted hydrolysis of UpT (9) are extrapolated to the same conditions, assuming first-order dependence on both the catalyst and hydroxide ion concentration, the chelate is ten times more efficient a catalyst, as expected on the basis of the higher acidity of its aquo ligand. By contrast, UpTp (10) or the longer oligomers that can provide more than one coordination site for the metal ion, are hydrolysed as efficiently by both catalysts. In other words, a substrate that can offer additional coordination sites for the catalyst, enhances the catalytic activity of the Zn$^{2+}$-[12]aneN$_3$ chelate, the ability of which to make use of the additional bonding sites is limited by the presence of the triaza ligand. This explains why Zn$^{2+}$-[12]aneN$_3$ accelerates the hydrolysis of poly-U less efficiently than Zn$^{2+}$, although the situation is reversed when the hydrolysis of an isolated phosphodiester bond of dinucleoside monophosphates is concerned.

The results obtained with poly-U suggest that the phosphodiester bond adjacent to the dianionic phosphate function might be slightly less readily hydrolysed than the non-terminal bonds. This is not, however, clearly supported by the results shown in Table 3.

Table 3 The rate constants for the hydrolysis of 3'-phosphorylated and unphosphorylated oligonucleotides in the presence of 1 mmol dm$^{-3}$ Zn$^{2+}$-[12]aneN$_3$ at pH 6.5 and at 333.2 K

<table>
<thead>
<tr>
<th>Substrate</th>
<th>k/10$^6$ s$^{-1}$</th>
<th>Substrate</th>
<th>k/10$^6$ s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>UpTp (3)</td>
<td>11 ± 1$^a$</td>
<td>UpT (9)</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>UpTp (4)</td>
<td>18 ± 2</td>
<td>UpT (10)</td>
<td>2.0 ± 0.5</td>
</tr>
<tr>
<td>UpTp (5)</td>
<td>11 ± 1</td>
<td>UpT (11)</td>
<td>4.5 ± 0.2</td>
</tr>
<tr>
<td>UpTp (6)</td>
<td>8.5 ± 0.5</td>
<td>UpT (12)</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>UpTp (7)</td>
<td>12 ± 1</td>
<td>UpT (13)</td>
<td>3.8 ± 0.1</td>
</tr>
<tr>
<td>UpTp (8)</td>
<td>3.2 ± 1</td>
<td>UpT (14)</td>
<td>3.8 ± 0.1</td>
</tr>
</tbody>
</table>

$^a$ Adjusted with HEPES buffer; [HA]/[A-] = 0.065/0.035; T = 0.1 mmol dm$^{-3}$ (NaClO$_4$). Standard deviation of the mean indicated.
produces two fragments, one of which contains a 2',3'-cyclic monophosphate function at its 3'-terminus. Since this group is monoanionic, it does not enhance the hydrolysis of the neighbouring phosphodiester bonds. Under the conditions mentioned above, the rate constant for the hydrolysis of the 2',3'-cyclic monophosphate function is $6 \times 10^{-6}$ s$^{-1}$. The opening of the cyclic function gives a mixture of 2'- and 3'-phosphorylated oligomers in the ratio 2:1. The diatomic phosphate functions constitute the most favoured coordination site for the catalyst. In addition to binding to the terminal phosphate function, it coordinates to another phosphodiester bond which then is efficiently hydrolysed by the bifunctional catalysis mechanism. Evidently, the catalyst can bridge nucleotides that are several nucleotide units apart. In other words, the rate-enhancing effect of the 3'-terminal 3'- or 2'-monophosphate group may range over several phosphodiester bonds. The rate constant of the hydrolysis promoted by Zn$^{2+}$ that is bound simultaneously to the terminal monophosphate function is approximately $4 \times 10^{-5}$ s$^{-1}$. However, the phosphodiester bond adjacent to the 2'-monophosphate function is considerably more stable. For this reason, monomeric 2'-UMP is released from oligomers at rates significantly more slowly than 3'-UMP. As a matter of fact, almost no 2'-UMP was detected during the Zn$^{2+}$-promoted hydrolysis of poly-U.14 The rate constant observed for the hydrolysis of phosphodiester bonds of poly-U is 2.5 $\times$ 10$^{-5}$ s$^{-1}$. The rate constant of the hydrolysis promoted by Zn$^{2+}$ is approximately 6 $\times$ 10$^{-6}$ s$^{-1}$. However, the phosphodiester bond adjacent to the 2'-monophosphate function is considerably more stable. For this reason, monomeric 2'-UMP is released from oligomers at rates significantly more slowly than 3'-UMP. As a matter of fact, almost no 2'-UMP was detected during the Zn$^{2+}$-promoted hydrolysis of poly-U.14 The rate constant observed for the hydrolysis of phosphodiester bonds of poly-U is 2.5 $\times$ 10$^{-5}$ s$^{-1}$, which is a reasonable mean value for the parallel and consecutive processes involved.

In the presence of the Zn$^{2+}$-[12]aneN$_3$ chelate, the course of the hydrolysis reaction differs from that suggested above. The metal ion binds to the substrate as a monodentate ligand and promotes the hydrolysis of a phosphodiester bond which is not necessarily the same as that to which the metal ion is bound. The rate constant for the initial cleavage is approximately $5 \times 10^{-6}$ s$^{-1}$ at pH 6.5 and 333.2 K ([Zn$^{2+}$-[12]aneN$_3$] = 1 mmol dm$^{-3}$). The 2',3'-cyclic phosphate group produced by the initial cleavage is opened rapidly to give either a 2'- or a 3'-monophosphate function, the rate constant for the latter process being $1 \times 10^{-5}$ s$^{-1}$. The terminal monophosphate function is again the most favoured coordination site for the catalyst. The metal ion bound to the monophosphate function promotes the hydrolysis of one of the following phosphodiester bonds by providing a general base to assist the nucleophilic attack, but not by coordinating to the bond. The ligand around the metal ion restricts the action of the catalyst, and the catalytic activity of the chelate can range over only about six nucleotide units. Bonds that are further apart from the terminal phosphodiester group cannot be reached by the catalyst, and they react similarly to the bonds of an intact molecule. Previous studies on poly-U show that during the Zn$^{2+}$-[12]aneN$_3$-promoted hydrolysis, the ratio of the molar fractions of 2'- and 3'-monophosphate groups at the 3'-terminal position remains constant during the kinetic run, being the same as that produced by the hydrolysis of 2',3'-cUMP. This suggests that the rate-enhancing effects of the 2'- and 3'-monophosphate functions on the hydrolysis of the adjacent phosphodiester bonds are, in this case, similar.

**Experimental**

**Materials**

Long-chain alkylamine controlled pore glass and 5'-O-(4,4'-dimethoxytrityl)thymidine derivatized CPG (type II) were purchased from Sigma. All the reagents for machine-assisted oligonucleotide synthesis were from Chruachem. The oligonucleotides were assembled on an Applied Biosystems 392 DNA/RNA Synthesizer (ABD, Perkin-Elmer Corp.). Adenyllyl-(3',5')-uridine 3'-monophosphate, uridylyl-(3',5')-uridine and all nucleosides and their monophosphates were commercial products from Sigma, [12]aneN$_3$ as the trihydrobromide salt was from Aldrich. The buffer constituents and the Zn(NO$_3$)$_2$ were of reagent grade.

**Trinucleoside triphosphates (2a, b)**

The completely protected trinucleoside diphosphate, 15, was prepared by the phosphotriester method in solution using 1-hydroxybenzotriazole as an activator.2,3 3'-O-Levulinyl-2'-O-tetrahydropyranylluridine, used to introduce the 3'-terminal nucleotide, was synthesized by levulinating the 3'-OH of 5'-O-(4,4'-dimethoxytrityl)-2'-O-tetrahydropyranylluridine as described earlier,24 and removing the dimethoxytrityl group with dichloroacetic acid in dichloromethane (3%, 10 min, room temperature).

Compound 15 (300 mg) was delevulinated with hydrazine hydrate (0.5 mol dm$^{-3}$) in a mixture of pyridine and acetic acid (4:1, v/v, 20 cm$^3$). After 20 min at room temperature, the mixture was diluted with dichloromethane (100 cm$^3$), washed with saturated aqueous sodium hydrogen carbonate (100 cm$^3$), dried over sodium sulfate, and evaporated to dryness. The residue was additionally co-evaporated with dry pyridine (3 x 30 cm$^3$) and then phosphorylated with phosphororistriazole in dry acetonitrile.23 The reaction was stopped by addition of aqueous pyridine. After 3 h at room temperature, the mixture was evaporated to dryness, and the 3'-O-phosphorylated trimer was deblocked as described previously.23 The deblocked adenyllyl-(3',5')-uridylyl-(3',5')-uridine 3'-monophosphate (2a) was purified by ion-exchange chromatography on Toyopearl DEAE 650 M (HCO$_3$$^-$ form), using a linear gradient of aqueous ammonium hydrogen carbonate from 0 to 0.3 mol dm$^{-3}$. Most of the buffer constituents were removed by coevaporations with water. Prolonged treatment in the buffer resulted in partial isomerization of 2a to 2b. The isomeric mixture was first separated by RP-HPLC (Hypersil ODS column, 250 x 10 mm x 5 μm, acetic acid–sodium acetate buffer, pH 4.3, containing 0.1 mol dm$^{-3}$ NH$_4$Cl and 10% acetonitrile), and then desalted on the same column.

The following enzymatic digestions were used to verify the identity of 2a and 2b: (i) RNase A, a pyrimidine specific endonuclease, to cleave the bond between the two uridine moieties, and (ii) alkaline phosphatase to remove the terminal phosphate function. With both compounds, RNase A digestion
gave adenylyl-(3',5')-uridine 3'-monophosphate which was identified by spiking the product with an authentic commercial product (Sigma). The other product was 3'-UMP from 2a and 2'-UMP from 2b. Comparison with the calibration samples of known concentrations indicated that 2a and 2b gave ApUp and UMP in a molar ratio of 1:1. Dephosphorylation of both of 2a and 2b gave a single product, obviously ApUp.

3'-Phosphorylated oligomers (3-8)

A modified solid support was used to obtain 3'-phosphorylated oligonucleotides on a 10 µmol scale. 4,4'-Dimethoxytrityl-ethyl hydroxyethyl sulfone was succinylated and coupled to the long-chain controlled-pore glass by the method described previously. The solid support, prepared showed a loading ranging from 33 to 34 pmol g⁻¹ when determined by dimethoxytrityl cation assay.

To verify the applicability of the solid support, a model oligonucleotide (Tp)₁₀ was assembled on a 0.2 µmol scale and, upon standard ammonolytic deprotection, analysed by both ion-exchange and RP HPLC. This experiment showed that the step-by-step coupling yield was normal (>98.5%). The product, (Tp)₁₀, co-eluted with an authentic sample prepared by a method described in the literature. The target oligonucleotides, Up(Tp)₂(Tp)₁₀, were then assembled on a 10 µmol scale on support 16 using standard protocols (Scheme 2). After the oligothymidylate sequences were treated with concentrated aqueous ammonia overnight at 55°C. Compounds 29-34 that still bore 5'-O-dimethoxytrityl and 2'-O-Fpmp groups, were isolated by semi-preparative RP HPLC (Hypersil ODS described above), using a linear gradient from 40 to 100% B (A = 0.05 mol dm⁻³ triethylammonium acetate, B = triethylammonium acetate in 70% aqueous MeCN). The final removal of the Fpmp-protections with 0.01 mol dm⁻³ aqueous HCl gave 3-8, which were finally purified by RP HPLC chromatography using a mixture of water and acetonitrile (95:5, v/v) as the eluent. To verify the structure of the oligomers, they were cleaved with a mixture of alkaline phosphatase and phosphodiesterase I. The digestion resulted in a mixture of uridine and thymidine. In every case the molar ratio of these was equal to that expected for the starting material employed.

Oligonucleotides 9-14

Oligonucleotides 9-14 were assembled on a 10 µmol scale on a commercial thymidine-derivated CPG, and purified and characterized as described above for 3-8. The verification of the structure of the oligonucleotides was performed by using the same enzymatic digestion procedure as for 3-8 above.

Kinetic measurements

The principle of the kinetic measurements has been described earlier. The aliquots withdrawn from solutions of UT oligomers were analysed as such. The monomeric products were first separated by using the RP-18 HPLC column (250 × 4 mm × 5 µm) with a mixture of acetate buffer (0.1 mol dm⁻³, pH 4.3, 0.1 mol dm⁻³ NH₄Cl) and acetonitrile (99.75:0.25 v/v). After that, a gradient was applied to elute the dimeric constituents (max. 25% of acetonitrile). The first aliquot of every kinetic run was treated with a mixture of alkaline phosphatase and phosphodiesterase I to obtain a final value for the release of uridine nucleotides. Aliquots containing ribotrimers were analysed by RP HPLC using the gradient program described above.

Calculation of the rate constants

The rate constant for the hydrolysis of compounds 3-14 were obtained by following the concentration of the 5'-terminal uridine nucleotides released by the chemical hydrolysis. The final value for the concentration of the 3'-terminal bond was obtained by monitoring the first aliquot of each kinetic run as described above. The rate constant for the hydrolysis was then calculated by applying the first-order rate-law.

The rate constants for the hydrolysis of the phosphodiester bonds of ribotrimers 2a, b in the presence of Zn⁺₂ aquo ion were also determined by following the formation of the products. The initial products formed did not significantly react further. Thus ApUp bearing a 2',3'-cyclic phosphate function at its 3'-terminus and uridine 2'- or 3'-monophosphate could be attributed to the hydrolysis of the 3'-terminal bond, and 2',3'-cAMP and UpUp to the hydrolysis of the 5'-terminal bond. In both cases, the final value for the concentration of the products was equal to the initial concentration of the starting material, and the integrated first-order rate equation could be applied.

The dimeric products formed were identified with the aid of the following enzyme digests. ApUp bearing a 2',3'-cyclic phosphate function: (i) digestion with phosphodiesterase I and II cleaved the phosphodiester bond yielding monomeric products that were identified by spiking with commercial product; (ii) digestion with RNase A (pyrimidine-specific endonuclease) did not cleave the phosphodiester bond, but opened the cyclic function yielding ApUp(3') that was assigned by coinjection with a commercial product, and (iii) alkaline phosphatase did not dephosphorylate the product. UpUp(2'/
3'), in turn showed the following behaviour: (i) digestion with RNase A gave 3'-UMP from the 3'-phosphorylated molecule and a 1:1 mixture of 2'- and 3'-UMP from its 2'-isomer; (ii) alkaline phosphatase dephosphorylated the molecules to 3',5'-UpU, which was identified by spiking with a commercial product (Sigma).

References