Enzymatic Manipulation of the Fragments Obtained by Cerium(IV)-Induced DNA Scission: Characterization of Hydrolytic Termini

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Abstract: When a DNA 22-mer (^{32}P-labeled at either the 3′- or the 5′-end) was cut by $[Ce(NH_4)_2(NO_3)_6]$, two types of fragments were formed for each of the internucleotide linkages. These fragments were separated by gel electrophoresis and treated with three natural enzymes (bacterial alkaline phosphatase, T4 polynucleotide kinase, and terminal deoxynucleotidyl transferase). With these enzymatic treatments, all the fragments were quantitatively transformed into the expected forms. Thus the first direct evidence for the hydrolytic scission of DNA oligomers by the Ce^{IV} salt has been provided. Both phosphate termini and OH termini are formed at the 3′- and the 5′-ends of the fragments by the hydrolysis of phosphodiester linkages. This work demonstrates the potential of the metal ion as a tool in molecular biology and biotechnology.

Keywords: cerium · DNA cleavage · hydrolyses · phosphodiester hydrolysis

Introduction

The nonenzymatic hydrolysis of biologically important phosphoesters has recently been attracting interest.[1] It has already been reported that metal ions and their complexes hydrolyze RNA,[2–7] the 3′,5′-cyclic monophosphate of adenosine,[8] and phosphatidyl inositol.[9] Activated mono-,[10] di-,[11] and triesters[12] of phosphate are also hydrolyzed. However, the phosphodiester linkages in DNA are so stable that reports of their nonenzymatic hydrolysis have been rather scarce.[13] A few years ago, the authors showed that lanthanide ions are effective in promoting DNA hydrolysis.[14–16] Cerium was especially prominent; the active species was subsequently shown to be the Ce^{IV} ion.[15,17,18] The hydrolytic character of DNA scission by Ce^{IV} has been firmly established for the scission of dinucleotides, as all the products have been conclusively characterized by HPLC and ^{1}H NMR.[15,17] In the scission of a longer fragment of DNA, however, the evidence for the hydrolytic nature is less abundant. Although the resultant fragments seem to migrate along with the hydrolytic products in polyacrylamide gel electrophoresis,[14,15] the possibility that the Ce^{IV} ion, a well-known oxidant, oxidatively cleaves the deoxyribose is not completely ruled out by these results. The DNA might be chemically damaged somehow during the scission. Furthermore, little is known about the structures of the termini of the fragments formed from longer fragments of DNA. The scope of application of the DNA scission is greatly dependent on these two factors (the hydrolytic character and the terminal structures) and thus unambiguous and straightforward evidence is required.

Herein we show that the fragments formed by $[Ce(NH_4)_2(NO_3)_6]$-induced scission of a DNA oligomer are successfully manipulated by natural enzymes. Since the enzymes discriminate strictly between hydrolytic and nonhydrolytic products, and in addition the present enzymatic transformations are quantitative for both the 3′- and the 5′-termini, the hydrolytic character of the scission is conclusive. Furthermore, the terminal structures of fragments are clarified by the enzymatic treatment. In contrast to the predominant formation of OH termini in dinucleotide hydrolysis[15,17] a considerable proportion of phosphate termini is formed together with OH termini. The results are discussed in terms of the catalytic mechanism.

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Results and Discussion

Scission of a DNA oligomer by $[Ce(NH_4)_2(NO_3)_6]$: When a 22-mer, (5′-GCATCACCA$_9$G$_{10}$CGGTCC$_{16}$T$_{17}$A$_{18}$GCAT-3′), was ^{32}P-labeled at the 5′-end and treated with $[Ce(NH_4)_2(NO_3)_6]$, scission took place almost randomly throughout the DNA chain (Figure 1A, lane 3). Interestingly, and significantly, two pairs of fragments were formed from the scission of a single nucleotide. One of the two fragments had

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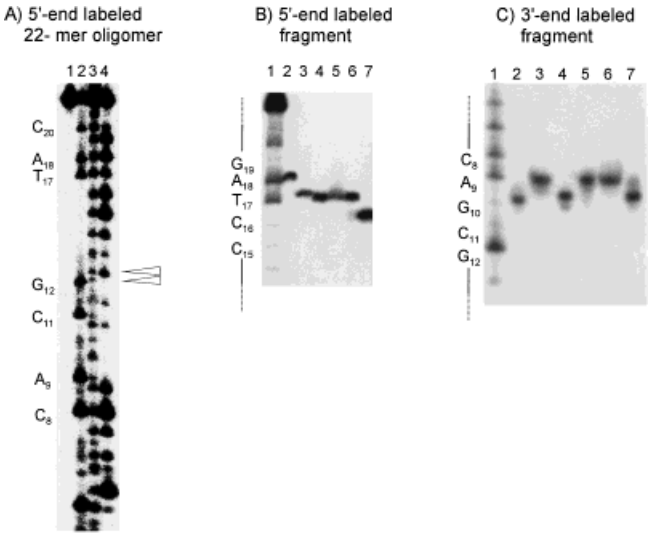

Figure 1. Autoradiographs of a denaturing 20% polyacrylamide gel electrophoresis for Ce^{IV}-induced hydrolysis of the 22-mer DNA (A) and for the enzymatic treatments of the resultant fragments (B and C); the scales on the left-hand side show the nucleotides at the termini (note that one nucleotide is removed in the Maxam–Gilbert reactions). A) The 5′-end ^{32}P-labeled DNA oligomer. Lane 1, control; lane 2, Maxam–Gilbert A + G; lane 3, the Ce^{IV} reaction; lane 4, DNase I. A typical pair of the fast and the slow fragments is designated by open arrows. The reaction conditions for lane 3: $[Ce^{IV}] = 1$ mM and [DNA] = 0.01 mM at pH 7 (HEPES buffer) and 37 °C for 24 h. B) The 5′-end ^{32}P-labeled fragments. Lane 1, Maxam–Gilbert A + G; lanes 2 and 3, 5′P*-T_{17}-3′s; lanes 4 and 5, 5′P*-T_{17}-3′f; lanes 6 and 7, 5′P*-C_{16}-3′s. Lanes 2, 4, and 6 treated with TdT; lanes 3, 5, and 7 untreated. C) The 3′-end ^{32}P-labeled fragments. Lane 1, Maxam–Gilbert C + T; lanes 2–4, 5′f-G_{10}-3′P*; lanes 5–7, 5′s-G_{10}-3′P*. Lanes 2 and 5 untreated; lanes 3 and 6 treated with BAP; lanes 4 and 7 treated with T4 kinase.

virtually the same mobility as the corresponding Maxam–Gilbert fragment (lane 2) and the other co-migrated with DNase I digest (lane 4). On the lower part of the gel, the faster component of a pair of fragments outran the component that was shorter by one nucleotide. The bands on the upper part of the gel were also separated into two components when the migration distance was longer. The formation of two types of termini at the 3′-ends has been substantiated.

Similar binary fragment patterns were obtained in Ce^{IV}-induced scission of the DNA oligomer labeled at the 3′-end. One group of fragments co-migrated with both Maxam–Gilbert fragments and DNase I digests, whereas the other migrated more slowly. The first group had phosphate termini at the 5′-end, whereas the second group had OH termini as shown below.

As the amount of Ce^{IV} was increased, the slower fragments became more dominant for both the 3′- and the 5′-end-labeled DNA. When the concentration of Ce^{IV} was 1 mM, the ratio of the slower component to the faster one was approximately 2:1 and did not much depend on the scission site. With 10 mM Ce^{IV} only the slower fragments were observed.[19] The terminal monophosphates formed by the hydrolysis of phosphodiester linkages are further hydrolyzed by Ce^{IV}, providing OH termini; note that the Ce^{IV} ion is active for the hydrolysis of phosphomonoesters as well as phosphodiesters.[15,20]

Characterization of the structure of the 3′-end termini of the fragments: A pair of 5′-end-labeled 17-mer fragments (for the scission between T_{17} and A_{18}) were isolated from the gel and each of them was examined in terms of the susceptibility to terminal deoxynucleotidyl transferase (TdT) (Figure 1B). The faster and slower components are designated 5′P*-T_{17}-3′f and 5′P*-T_{17}-3′s, respectively. When 5′P*-T_{17}-3′s, in lane 3, is incubated with TdT in the presence of unlabeled 2′,3′-dideoxycytidine-5′-triphosphate (ddCTP) a new band with less mobility than 5′P*-T_{17}-3′s is quantitatively formed (lane 2). It has been definitively shown that the fragment has a 3′-OH terminus and a 2′,3′-dideoxycytidine (ddC) moiety is enzymatically introduced (Figure 2A). In contrast, 5′P*-T_{17}-3′f is not susceptible to TdT since it has a 3′-phosphate terminus (Figure 1B, lanes 4 and 5).

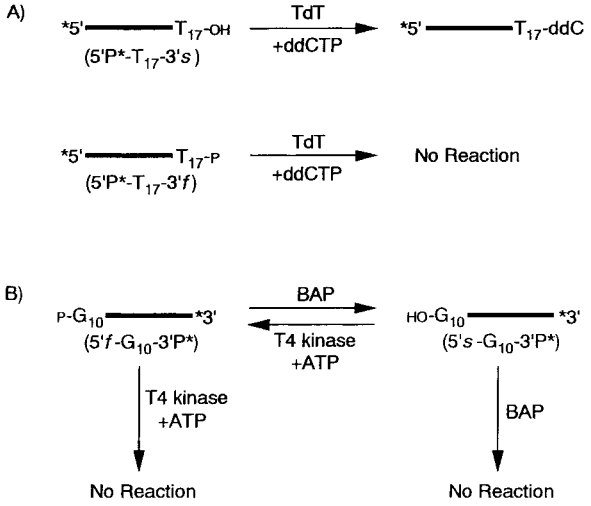

Figure 2. Enzymatic manipulation of the fragments obtained by the hydrolysis of the 22-mer DNA with Ce^{IV}: A) 5′-end ^{32}P-labeled fragments and B) 3′-end ^{32}P-labeled fragments.

In total consistency with this, the slower fragment 5′P*-C_{16}-3′s (lane 7), which is one nucleotide shorter than 5′P*-T_{17}-3′s and has a 3′-OH terminus, is elongated by the enzymatic treatment (lane 6). The mobility of the product is virtually the same as that of 5′P*-T_{17}-3′s.[21]

Characterization of the structure of the 5′-end termini of the fragments: The structure of 5′-end of the fragments prepared by Ce^{IV}-induced DNA scission was examined by means of 3′-end-labeled fragments (Figures 1C and 2B). The faster fragment 5′f-G_{10}-3′P*, which came from the scission between A_9 and G_{10} (lane 2), was treated with bacterial alkaline phosphatase (BAP). A new band with less mobility was quantitatively produced (lane 3). The mobility of the band was identical with that of the slower counterpart 5′s-G_{10}-3′P* (lane 5). In contrast, the mobility of 5′s-G_{10}-3′P* was unchanged on BAP treatment (lane 6). Apparently, 5′f-G_{10}-3′P* has a phosphate residue at the 5′-end, which is removed by BAP. On the other hand, 5′s-G_{10}-3′P* has an OH terminus.

The argument has furthermore been confirmed by the following results. When 5′s-G_{10}-3′P* (lane 5) was reacted with unlabeled ATP in the presence of T4 polynucleotide kinase

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(T4 kinase) a phosphate residue was successfully attached to its 5′-end (lane 7). The mobility of the product is exactly identical with that of 5′f-G_{10}-3′P* in lane 2. As expected, the mobility of 5′f-G_{10}-3′P* was unchanged when it was treated with the combination of T4 kinase and ATP (compare lanes 2 and 4).

If the DNA scission by Ce^{IV} were to proceed by means of the oxidative cleavage of deoxyribose, complicated and unnatural termini should be formed at the ends of the fragments.[22] These termini are not transformed by the enzymes. Thus, the present results unequivocally substantiate the contention that the Ce^{IV}-induced DNA scission occurs by the hydrolysis of phosphodiester linkages.

Comparison of the rate of hydrolysis of DNA oligomer with that of dinucleotide hydrolysis: The half-life for the conversion of the DNA 22-mer to shorter fragments by $[Ce(NH_4)_2(NO_3)_6]$ (1 mM) at pH 7.0 and 37 °C was determined to be about 6 h by gel electrophoresis. This value agrees fairly well with the value estimated on the assumption that each of the phosphodiester linkages is as reactive as the phosphodiester linkage in dinucleotides (8 h). The rate constant of the hydrolysis of TpT by 1 mM of $[Ce(NH_4)_2(NO_3)_6]$ is 0.004 h^{-1} under these conditions,[23] and the hydrolysis rate does not depend on the type of dinucleotide.[15] The DNA oligomer has 21 phosphodiester linkages, and thus its half-life was estimated to be 8 h ($= 0.69/(0.004 \times 21)$) from the above assumption. Apparently, the reactivities of phosphodiester linkages in the DNA oligomer are almost comparable with those in dinucleotides.

Efficient formation of phosphate termini in Ce^{IV}-induced hydrolysis of DNA oligomer: In $[Ce(NH_4)_2(NO_3)_6]$-induced hydrolysis of dinucleotides the nucleoside monophosphates, which are formed by the hydrolysis of the phosphodiester linkage in the first step, are rapidly hydrolyzed and not accumulated in the mixtures.[15] Thus the predominant products are the nucleosides which have OH termini (Figure 3b).

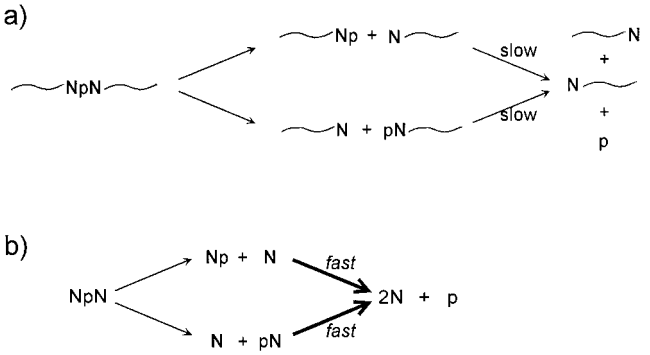

Figure 3. Formation of two kinds of termini in Ce^{IV}-induced hydrolysis of a) DNA oligomer and b) dinucleotide; N = nucleoside.

In the hydrolysis of the DNA oligomer, however, the terminal monophosphates are protected from the subsequent hydrolysis and thus the phosphate termini are formed in considerable amounts (Figure 3b). Apparently, the terminal monophosphates in the oligomeric DNA fragments are much less susceptible to hydrolysis catalyzed by Ce^{IV} than those in nucleoside monophosphates.

We assume that one of the phosphodiester linkages in the DNA oligomer is coordinated to the catalytically active Ce^{IV} ion together with the terminal monophosphate as schematically depicted in Figure 4a. As a result, the catalysis of the

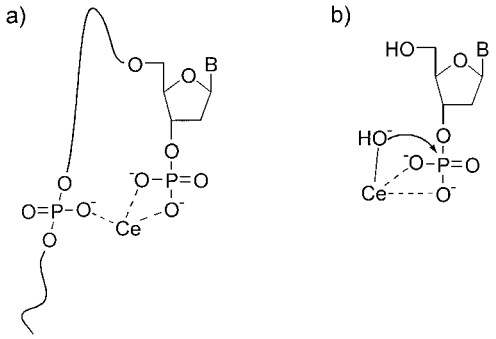

Figure 4. Schematic views of monophosphate termini formed in the course of Ce^{IV}-induced hydrolysis of a) DNA oligomer and b) dinucleotide. In a) the metal-bound hydroxide may be provided by another Ce^{IV} ion in a hydroxo cluster because the phosphodiester is already coordinated to this Ce^{IV} ion.

phosphomonoester hydrolysis by the metal ion would be largely suppressed, mainly because the Ce^{IV} ion cannot provide a metal-bound hydroxide as an intramolecular nucleophile. In dinucleotide hydrolysis, however, the Ce^{IV} ion, which is complexed with the nucleoside monophosphate, is free from other phosphodiester linkages and is available for the hydrolysis of the monophosphate (Figure 4b).[24] A structure similar to that in Figure 4a was previously proposed by Kuusela, Guazev, and Lönnberg for the Zn^{2+}-ion-catalyzed hydrolysis of RNA oligomers.[6]

In order to obtain further information on the reaction mechanism, the hydrolysis of d(pApA) by $[Ce(NH_4)_2(NO_3)_6]$ (10 mM) was examined. The rate constant for the hydrolysis of its terminal phosphate (the conversion of d(pApA) to d(ApA)) was 4.2 h^{-1} at pH 7.0 and 50 °C. This value is almost identical to that (4.1 h^{-1}) for the hydrolysis of pdA to dA (determined with an authentic sample of pdA). Apparently, the rate of monophosphate hydrolysis is little affected by the neighboring phosphodiester linkage. This indicates strongly that the coordination of internal phosphodiester linkages other than the one neighboring the Ce^{IV} is responsible for the protection of the terminal monophosphates in the DNA oligomers.

Conclusion

Both the 3′- and the 5′-ends of the fragments formed by Ce^{IV}-induced DNA scission are quantitatively transformed into the expected forms when they are treated with natural enzymes. Concrete evidence for the hydrolytic scission by Ce^{IV} has been obtained.[25] Other parts of the DNA remain intact during the scission, otherwise some of the fragments would not be susceptible to these enzymatic manipulations.

In contrast with the dominant formation of OH termini in Ce^{IV}-induced dinucleotide hydrolysis,[15] phosphate termini are efficiently formed in the hydrolysis of oligomeric DNA. In the oligomer the terminal monophosphates, produced by the hydrolysis of phosphodiester linkages, are protected from further hydrolysis (probably by the coordination of phosphodiester linkages in the DNA chain). The terminus structure of the DNA fragments might be controlled by the use of appropriate ligands.

The present findings strongly indicate that the Ce^{IV} ion and its complexes are powerful as tools for the fields of molecular biology and biotechnology amongst others. Artificial nucleases for the sequence-selective scission of DNA are prepared by conjugating the metal ion with DNA oligomers.[26] Design of stable and active Ce^{IV} complexes should be of importance for further development of the field.

Experimental Section

Materials: A DNA 22-mer, d(GCATCACCAGCGGTCCTAGCAT), was synthesized on an automated synthesizer and purified by reverse-phase HPLC. The choice of sequence is rather arbitrary. $[Ce(NH_4)_2(NO_3)_6]$ from Nacalai Tesque was used without further purification. d(pApA) was prepared from d(ApA) (Sigma) and adenosine triphosphate with T4 kinase and then purified by reverse-phase HPLC. Terminal deoxynucleotidyl transferase (TdT, from calf thymus), bacterial alkaline phosphatase (BAP, from *E. coli*), T4 polynucleotide kinase (T4 kinase, from *E. coli*), and DNase I (from bovine pancreas) were purchased from Wako. Water was ion-exchanged by a Millipore purification system (Milli-XQ; specific resistance of the water $>18.3\,M\Omega\,cm$) and sterilized in an autoclave immediately before use. Throughout the present study great care was taken to avoid contamination by natural enzymes and other metal ions.

^{32}P-Labeling of the DNA oligomer: The DNA 22-mer was ^{32}P-labeled at the 3′-end by 2′,3′-dideoxyadenosine-5′-[α-^{32}P]-triphosphate (from Amersham) with TdT ($1\,U\,mL^{-1}$) at pH 7.0 in a 100 mM cacodylate buffer containing $CoCl_2$ (1 mM) and dithiothreitol (0.2 mM). The mixture was incubated at 37 °C for 1 h. A loading buffer (tris(hydroxymethyl)aminomethane (Tris)/borate buffer (90 mM) containing ethylenediaminetetraacetic acid (EDTA, 1 mM), urea (7 M), xylene cyanol FF (0.02 %), and bromophenol blue (0.02 %)) was added, and then the whole solution was loaded onto a denaturing 20 % polyacrylamide gel. After the electrophoresis, the labeled DNA was eluted out by water from the gel and purified by ethanol precipitation.

The ^{32}P-labeling of the DNA oligomer at the 5′-end was achieved by [γ-^{32}P]ATP (Amersham) and T4 kinase ($0.5\,U\,mL^{-1}$) at pH 7.6 and 37 °C for 1 h. The Tris–HCl buffer (50 mM) for the enzymatic reaction contained $MgCl_2$ (10 mM), dithiothreitol (5 mM), spermidine (0.1 mM), and EDTA (0.1 mM). The labeled DNA was purified as described above for the 3′-end-labeled one.

DNA scission by $[Ce(NH_4)_2(NO_3)_6]$: The scission of DNA (the initial concentration 0.01 mM) by $[Ce(NH_4)_2(NO_3)_6]$ was performed at pH 7.0 (*N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid (HEPES) buffer, 10 mM) and 37 °C for 10 and 24 h respectively. The concentration of the Ce^{IV} salt was 10 mM for the 3′-end-labeled DNA and 1 mM for the 5′-end-labeled one. The smaller Ce^{IV} concentration was employed for the scission of the 5′-end-labeled DNA in order to minimize the removal of the terminal monophosphate (used for ^{32}P labeling).

After the reactions, the faster and the slower fragments were completely separated from each other by use of a long migration distance (around 30 cm) in denaturing 20 % polyacrylamide gel electrophoresis. The fragments were purified by either ethanol precipitation or ultrafiltration (a Millipore UFC3 LCC filter, made of regenerated cellulose). The fragments used for the present enzymatic treatments were the most convenient for the complete purification by these methods, although virtually the same results were obtained on the enzymatic treatment of other fragments.

Enzymatic manipulation of the fragments obtained by scission with $[Ce(NH_4)_2(NO_3)_6]$: The faster and the slower fragments, removed from the electrophoresis gel and purified as above, were treated with the enzymes under the following conditions:

a) TdT ($1\,U\,mL^{-1}$) at 37 °C for 3 h in the presence of 2′,3′-dideoxycytidine-5′-triphosphate (ddCTP, 10 mM) in a pH 7 cacodylate buffer (100 mM) containing $CoCl_2$ (1 mM) and dithiothreitol (0.2 mM).

b) BAP ($0.03\,U\,mL^{-1}$) at 37 °C for 1 h in a pH 8 Tris buffer with $MgSO_4$ (1 mM).

c) T4 kinase ($0.5\,U\,mL^{-1}$) at 37 °C and pH 7.6 for 2 h in the presence of ATP (0.2 mM) in the Tris buffer which was used for the ^{32}P-labeling at the 5′-end (vide supra).

After loading, Tris/borate buffer was added and the resultant reaction mixtures were subjected to electrophoresis on a denaturing 20 % polyacrylamide gel. The autoradiographs were quantified on a Fujix BAS-1000 II system.

Hydrolysis of nucleotides: The hydrolysis of d(pApA) and d(ApA) at pH 7.0 (HEPES buffer, 50 mM) and 50 °C was followed by reverse-phase HPLC (a Merck LiChrosphere RP-18(e) ODS column; water/acetonitrile = 94:6 (v/v)). The initial concentration of the substrate was 0.1 mM. The HPLC peaks were definitively assigned by co-injection with authentic samples. In the hydrolysis of d(pApA) the amounts of d(pApA), d(ApA), and dA in the reaction mixtures were determined by the HPLC.[27] Thus, the rate constant for the hydrolysis of the terminal monophosphate, the conversion of d(pApA) to d(ApA), was evaluated by fitting the time-course for each of d(pApA), d(ApA), and dA to the theoretical line. Experimental error in the rate constant is estimated to be smaller than 10 %. The rate constant for d(ApA) hydrolysis was directly determined according to pseudo-first-order kinetics.

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[20] The ratio of the slower fragment to the faster one in the product is governed mainly by the following three factors: i) the amounts of the phosphodiester linkage and the terminal monophosphate in the reaction mixture, ii) the ratio of the rate constants of their hydrolysis, and iii) the manner of the scission of the phosphodiester linkage (P–O(3′) cleavage vs. P–O(5′) cleavage) in the DNA oligomer. Evaluation of these parameters from the time-course of the product distribution was unsuccessful.

[21] When fragments that were 5′-end-labeled were treated with bacterial alkaline phosphatase (BAP), the expected transformation took place at the 3′-termini. However, the ^{32}P-labeling monophosphates were gradually removed by the enzyme, making a quantitative analysis difficult.

[22] Structures of the termini for the oxidative DNA cleavage depend mainly on the kind of protons that are abstracted by the radical-like species, ref. [18]. At least half of the termini involve the moieties derived from the deoxyribose cleavage. Although a natural monophosphate terminus is in some cases produced, an unnatural terminus is simultaneously formed and thus the argument presented by us here still holds.

[23] The rate constant was evaluated by use of the value at pH 7 in the presence of 10 mM $[Ce(NH_4)_2(NO_3)_6]$ (0.19 h^{-1}), the activation energy (23 kcal mol^{-1}), and the linear increase of the rate constant with $[Ce^{IV}]$ in ref. [15].

[24] The rate of phosphodiester hydrolysis in the oligomer is less affected by the coordination of other phosphodiester linkages to Ce^{IV} than is the corresponding value for phosphomonoester hydrolysis. We assume that the net positive charge on the Ce^{IV} ion is large enough to achieve the catalysis, even when two phosphodiester linkages are coordinated to the metal ion. In addition, the binding of the phosphodiester to Ce^{IV} is promoted by the chelate effect, which compensates for the decrease in the catalytic activity of the Ce^{IV}.

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