Hydrolysis of phosphomonoesters in nucleotides by cerium(IV) ions. Highly selective hydrolysis of monoester over diester in concentrated buffers

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Phosphomonoesters in nucleotides are efficiently hydrolysed by Ce^{IV} ions under physiological conditions. The half-lives of the residues at pH 7.2 and 50 °C ([Ce^{IV}] = 10 mM) are around 10 min. Phosphomonoester hydrolysis by Ce^{IV} ions is faster than the hydrolysis of phosphodiesters. Significantly, the selectivity for monoester hydrolysis over diester hydrolysis is remarkably increased by using concentrated buffer solutions (TRIS and HEPES). In 500 mM TRIS buffer, pdA and dAp are hydrolysed 500- and 580-fold faster than is d(ApA), whereas the corresponding ratios in 50 mM TRIS buffer are 85 and 90 respectively. Selective removal of the terminal monophosphate from d(pApA) is achieved by Ce^{IV} in these concentrated buffers.

Introduction

Hydrolysis of monophosphates in nucleotides is one of the most fundamental reactions in molecular biology and biotechnology.[1] Currently a natural enzyme, phosphomonoesterase, is used for the purpose. However, non-enzymatic catalysts for the removal of terminal monophosphates, if available, should be useful for various applications.

Recently, remarkable catalyses by lanthanide ions and their complexes of the hydrolysis of biologically important phosphodiesters and phosphotriesters were reported.[2–7] The cerium(IV) ion is the most active for the hydrolysis of both DNA[3] and the 3′,5′-cyclic monophosphate of adenosine,[4] whereas Tm^{III}, Yb^{III}, and Lu^{III} ions are superb for RNA hydrolysis.[5] Lanthanide complexes for phosphotriester hydrolysis were also elegantly designed.[6] However, detailed and systematic studies on the hydrolysis of phosphomonoesters (especially of 2′-deoxyribonucleosides) by lanthanide ions have been rather less well documented, although they should be formed as intermediates in the course of these di- and tri-ester hydrolyses.[8,9] In order to accomplish a phosphomonoesterase-like function by non-enzymatic systems, both the selectivity for the hydrolysis of phosphomonoesters (with respect to phosphodiester hydrolysis) and the catalytic activities must be sufficiently high. However, little is known on these subjects.

This paper reports that the Ce^{IV} ion is quite active for the hydrolysis of monophosphates in nucleotides in neutral solutions.[10] Furthermore, the selectivity of Ce^{IV} for monophosphate hydrolysis, with respect to the corresponding phosphodiester hydrolysis, is greatly promoted by using concentrated TRIS and HEPES buffers as the solvents [TRIS = tris(hydroxymethyl)-methylamine and HEPES = *N*′-(2-hydroxyethyl)piperazine-*N*-ethanesulfonic acid]. The highly selective catalysis is applied to the removal of the terminal monophosphate from a dinucleotide.

Experimental

Materials

2′-Deoxyadenylyl (3′→5′)-2′-deoxyadenoside [d(ApA)], as well as monophosphates of various ribonucleosides and 2′-deoxyribonucleosides, was obtained from Sigma. The 5′-monophosphate of d(ApA) [d(pApA)] was prepared from d(ApA) and adenosine triphosphate by using T4 kinase, and purified by reversed-phase HPLC. HEPES and TRIS were purchased from Aldrich. Lanthanide salts from Soekawa Chemicals were used without further purification. Water was purified by a Millipore Milli-XQ, and sterilized immediately before use. The specific resistance of the water was greater than 18.3 MΩ cm^{-1}, confirming that the amounts of metal ions, if any, were sufficiently minimized. Great care was also taken to avoid contamination by natural enzymes.

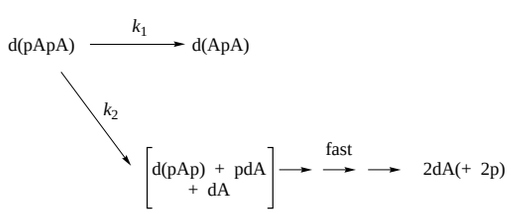
Hydrolysis of nucleotides

The reactions were carried out at 50 °C under air unless otherwise noted, and were followed by reversed-phase HPLC [a Merck LiChrosphere RP-18(e) ODS column; water–acetonitrile = 92:8 or 96:4 (v/v)]. The initial concentration of the substrate was 0.1 mM. At an appropriate interval, 30 μl of the reaction mixture was removed, and 10 wt% aqueous phosphoric acid (3 μl) was added to it. The specimen was treated with a disposable pretreatment filter (Tosoh; W-3-2), and then injected onto an HPLC column. The HPLC peaks were assigned by coinjection with authentic samples.

All the reactions satisfactorily showed pseudo first-order kinetics. The rate constants presented here are the averages of at least duplicate runs which coincided with each other within 5%. The reaction mixtures were homogeneous when the concentration of TRIS buffer was greater than 250 mM. Other mixtures contained some precipitates probably due to the formation of metal hydroxide.

Kinetic analysis of d(pApA) hydrolysis

These reactions were analysed according to Scheme 1. The



Scheme 1

hydrolysis of the terminal monophosphate in d(pApA) (the rate constant k_1) gives d(ApA), whereas either [d(pAp) + pdA +

Table 1 Pseudo first-order rate constants for the hydrolysis of dAp, pdA and d(ApA) at pH 7.2 (50 mM HEPES buffer) and 50 °C [a]

Catalyst	$k/10^{-2}$ min^{-1}		
	pdA	dAp	d(ApA)
$CeCl_3 + O_2$	6.7	6.2	0.20
$Ce(NH_4)_2(NO_3)_6$	6.9	6.5	0.27

[a] [Ce^{IV} salt] = 10 mM.

Table 2 Rate constants for the hydrolysis of nucleotides at pH 7.2 (50 mM HEPES buffer) and 50 °C by $CeCl_3$ (10 mM)

Substrate	$k/10^{-2}$ min^{-1}	Substrate	$k/10^{-2}$ min^{-1}
dAp	6.2	pdA	6.7
dGp	2.4	pdG	2.8
dCp	5.2	pdC	7.3
dTp	4.5	pdT	7.4

dA] or two dpAs should be produced when the internal phosphodiester linkage is hydrolysed (the rate constant k_2).[11] The amounts of d(pApA), d(ApA) and dA during the reactions were determined precisely by HPLC. Thus, the first-order rate constant k_d for the disappearance of d(pApA), which is equal to the sum of k_1 and k_2, was determined from the corresponding time-course. The rate constant k_1 was evaluated from the time-course of d(ApA). Finally, k_2 was obtained by subtracting k_1 from k_d. The d(pAp) and pdA, formed in the k_2 step, were rapidly converted to the final product dA, and not much accumulated in the mixtures.

In the present analyses, the process [d(pApA) ⟶ d(ApA) ⟶ 2dA] was not taken into consideration, since the hydrolysis of d(ApA) to two dAs is much slower than the other reactions (see Table 1) and thus the process did not contribute much to the distribution of the products.

Titration of Ce^{IV} in the reaction mixtures

The amounts of Ce^{IV}, formed *in situ* from $CeCl_3$ in the reaction mixtures, were determined by a back-titration.[3f] Known amounts of $Ce(NH_4)_4(SO_4)_4$ were added to the mixtures, and then the whole solutions were titrated with $FeSO_4$ using 1,10-phenanthroline as an indicator.

Results and discussion

Hydrolysis of nucleoside monophosphates by Ce^{IV} ions

The 3′- and 5′-monophosphates of 2′-deoxyadenosine (dAp and pdA) were rapidly and quantitatively converted to 2′-deoxyadenosine (dA), when they were treated with $CeCl_3$ under air at pH 7.2 and 50 °C (Table 1). The reactions were complete within 1 h (the half-life is around 10 min). The rate constants for the hydrolysis of dAp and pdA (k_{dAp} and k_{pdA}) are virtually identical. The scission is hydrolytic, as confirmed by the absence of by-products. Adenine should be released if the ribose was cleaved by an oxidative pathway. Monophosphates of other deoxyribonucleosides (as well as monophosphates of ribonucleosides) were also hydrolysed (Table 2). The hydrolysis rate is not significantly dependent on the kind of nucleotide, indicating that the catalytic species does not interact with the nucleic acid base in the catalysis.

In addition to $CeCl_3$, $Ce^{IV}(NH_4)_2(NO_3)_6$ is also active in the hydrolysis of monophosphates (Table 1). In the absence of these cerium salts, however, no measurable hydrolysis of the monophosphates occurred (the intrinsic half-lives of the monophosphates are estimated to be 160 000 years).[12] The acceleration achieved here is nearly 10^{10}-fold.

The hydrolysis of d(ApA) by either $CeCl_3$ or $Ce(NH_4)_2(NO_3)_6$ is 30–130-fold slower than is the hydrolysis of dAp and pdA. Thus, phosphomonoesters are hydrolysed by these metal salts far more rapidly than are phosphodiesters.

Table 3 Rate constants for the hydrolysis of the terminal phosphomonoester and the internal phosphodiester in d(pApA) by $CeCl_3$ (10 mM) at pH 8.0 and 50 °C.

Buffer		$k^a/10^{-2}$ min^{-1}		
Kind	Concentration/mM	Terminal k_1	Internal k_2	k_1/k_2 [b]
TRIS	50	15.9	3.0	5.3
		(4.1)	(0.048)	(85)
	500	2.9	0.20	15
		(2.0)	(0.004)	(500)
	50 [c]	5.6	0.70	8.0
HEPES		(6.7)	(0.20)	(34)
	500	4.0	0.52	7.7
		(3.2)	(0.033)	(97)

[a] The numbers in parentheses are the rate constants for the hydrolysis of pdA and d(ApA) [k_{pdA} in the column of k_1 and $k_{d(ApA)}$ in the column of k_2]. [b] The $k_{pdA}/k_{d(ApA)}$ ratios are presented in parentheses. [c] pH 7.2.

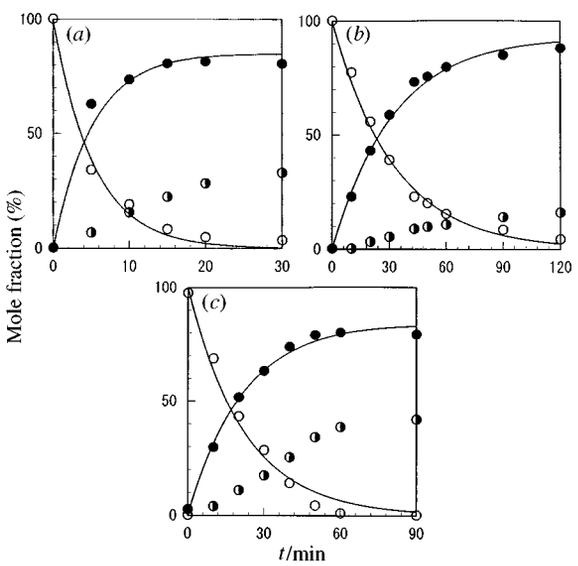


Fig. 1 Time-courses for the hydrolysis of d(pApA) by $CeCl_3$ (10 mM) under air at 50 °C and pH 8.0: (*a*) in 50 mM TRIS buffer, (*b*) in 500 mM TRIS buffer, and (*c*) in 500 mM HEPES buffer. ○, d(pApA); ●, d(ApA); ◑ dA. The solid lines are the theoretical ones calculated by use of Scheme 1 (see Experimental). The attempts to calculate the theoretical lines for dA were not successful, since dA is generated *via* too many pathways.

Removal of terminal monophosphate from d(pApA)

The terminal phosphate of d(pApA) was rapidly removed by $CeCl_3$ at pH 8.0 and 50 °C in 50 mM TRIS buffer [Fig. 1(*a*)]. About 73 mol% of d(pApA) was converted to d(ApA) in only 10 min, during which dA was formed in 17 mol%. The selectivity for the formation of d(ApA) (monophosphate hydrolysis/total hydrolysis) is 80% at the completion of the reaction. Note that two moles of dA are formed when the internal phosphodiester linkage in d(pApA) is hydrolysed. As described above, most of dA in the present reactions is formed by the hydrolysis of the internal phosphodiester linkage. By fitting these time-courses to Scheme 1 (see Experimental section), the rate constants k_1 [for the hydrolysis of the terminal monophosphate in d(pApA)] and k_2 (for the hydrolysis of the internal phosphodiester linkage) are determined to be 15.9×10^{-2} and 3.0×10^{-2} min^{-1}, respectively.

Selective removal of the terminal monophosphate was also achieved in 50 mM HEPES buffer. The selectivity for the formation of d(ApA) is 82% at a conversion of 98 mol% (see Table 3).

Active species for the phosphomonoester hydrolysis by $CeCl_3$

The monophosphate hydrolysis by $CeCl_3$ is ascribed to the

Table 4 Effects of buffer concentration on the rate constants for the hydrolysis of pdA, dAp and d(ApA) by $CeCl_3$ (10 mM) at pH 8.0 and 50 °C

Buffer		$k^a/10^{-2}$ min^{-1}		
Kind	Concentration/ mM	pdA	dAp	d(ApA)
TRIS	50	4.1 (85)	4.3 (90)	0.048
	250	3.5 (120)	4.2 (140)	0.029
	500	2.0 (500)	2.3 (580)	0.004
HEPES	50	7.6 (30)	8.0 (32)	0.25
	50[b]	6.7 (34)	6.2 (31)	0.20
	250	3.6 (52)	3.6 (52)	0.069
	500	3.2 (97)	3.5 (110)	0.033

[a] The numbers in parentheses are the ratios of the rate constant for monophosphate hydrolysis to that for d(ApA) hydrolysis [$k_{pdA}/k_{d(ApA)}$ and $k_{dAp}/k_{d(ApA)}$]. [b] pH 7.2.

catalysis by the Ce^{IV} ion, which is formed *in situ* from $CeCl_3$ and molecular oxygen,[3d–f] based on the following results. (1) The monophosphate hydrolysis did not take place at all when molecular oxygen was removed from the mixture by repeated freeze–thaw cycles (no Ce^{IV} was formed in the reaction mixtures here). (2) About 50% of the Ce^{III} ion was oxidized to Ce^{IV} in the reaction mixtures, according to a titration. (3) The hydrolysis by $Ce^{IV}(NH_4)_2(NO_3)_6$ took place efficiently even in the absence of molecular oxygen. Any reaction mechanism in which the catalytic species are derived from molecular oxygen is rather unlikely.

All these kinetic features are essentially identical with those reported previously for Ce^{IV}-induced DNA hydrolysis.[3f] This strongly indicates that the present phosphomonoester hydrolysis proceeds *via* the nucleophilic attack by Ce^{IV}-bound hydroxide ion towards the phosphorus atom, as does the DNA hydrolysis.[13,14]

Promotion of monoester/diester selectivity using concentrated buffer solutions

Interestingly, the selectivity for the phosphomonoester hydrolysis, with respect to the phosphodiester hydrolysis, notably increases with an increase in the concentration of buffer agent (Table 4). In 500 mM TRIS buffer, for example, pdA and dAp are hydrolysed 500- and 580-fold faster than is d(ApA) (the numbers in parentheses). The corresponding ratios in 50 mM TRIS buffer are only 85 and 90. In HEPES buffers, the monoester/diester selectivity also monotonically increases with increasing buffer concentration. The $k_{pdA}/k_{d(ApA)}$ and $k_{dAp}/k_{d(ApA)}$ ratios in 500 mM HEPES buffer are 97 and 110, respectively.

The increase in the monoester/diester selectivity with increasing concentration of buffers is mostly ascribed to buffer-induced suppression of phosphodiester hydrolysis. As shown in Table 4, the rate constants for d(ApA) hydrolysis are decreased by 12- and 8-fold respectively, when the concentrations of TRIS and HEPES are increased from 50 to 500 mM. However, the values for the hydrolysis of pdA and dAp are decreased by only 1.9–2.3 fold. Presumably, the buffer agents function as competitive inhibitors of the coordination to the Ce^{IV} ions. As a result, the coordination of phosphodiester linkages to Ce^{IV} is drastically suppressed, since they are monoanionic[15] and rather poor as ligands. On the other hand, the coordination of monophosphates to Ce^{IV} is affected to a smaller degree (they are dianionic in the mixtures[15] and are better ligands).[16] Thus, the suppression effect by the buffers is much more significant in the diester hydrolysis. The proposed interactions between Ce^{IV} ions and TRIS are supported by the fact that the metal ions, which easily form metal hydroxide gels above pH 7, are satisfactorily solubilized in concentrated (> 250 mM) TRIS buffers.

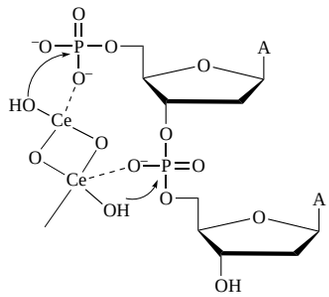


Fig. 2 Proposed mechanism for the hydrolysis of d(pApA). The monophosphate is hydrolysed in preference to the internal phosphodiester.[17]

Improvement of selectivity for removal of terminal monophosphate from d(pApA) using concentrated TRIS buffer solutions

The time-course for the hydrolysis of d(pApA) in 500 mM TRIS buffer is presented in Fig. 1 (*b*). The selectivity for the formation of d(ApA) is notably improved, compared with that in 50 mM TRIS buffer [Fig. 1(*a*)]. Almost 90% of d(pApA) is converted to d(ApA) at 120 min, where only 7% of the starting material is hydrolysed to dA *via* the hydrolysis of the internal phosphodiester. The selectivity for the formation of d(ApA) is 93%. With the use of 500 mM HEPES buffer, the selectivity was 81% at the conversion 98 mol% [Fig. 1 (*c*)], and was not much dependent on the buffer concentration.

These time-courses are analysed according to Scheme 1. The k_1/k_2 ratio in 500 mM TRIS buffer is 15, which is more than 3-fold greater than the value ($5.3 = 15.9 \times 10^{-2}/3.0 \times 10^{-2}$) in 50 mM TRIS buffer. The k_1/k_2 in 500 mM HEPES buffer is 7.7 (Table 3).

Simultaneous coordination of terminal phosphomonoester and internal phosphodiester to Ce^{IV} for d(pApA) hydrolysis

As shown in Table 3, the k_2 values [for the hydrolysis of the internal phosphate in d(pApA)] in 50 and 500 mM TRIS buffers are 63- and 50-fold greater than the corresponding rate constants $k_{d(ApA)}$ for the hydrolysis of d(ApA), which has an analogous internal phosphodiester linkage. Similarly, the k_1 values (for the hydrolysis of the external phosphate) also exceed the rate constants (k_{pdA}) for the hydrolysis of monophosphate in pdA. However, the k_1/k_{pdA} ratios (3.9 and 1.5, respectively, in 50 and 500 mM TRIS buffers) are not so great as the $k_2/k_{d(ApA)}$ ratios presented above. Thus, the k_1/k_2 ratios for the hydrolysis of d(pApA) in TRIS buffers (5.3 and 15 in 50 and 500 mM buffers) are considerably smaller than the $k_{pdA}/k_{d(ApA)}$ ratios (85 and 500: the numbers in parentheses), where large k_2 values are mostly responsible for these facts. In HEPES buffer, the k_1/k_2 ratios are also smaller than $k_{pdA}/k_{d(ApA)}$, as shown in Table 3.

This indicates that coordination of phosphodiester linkage in d(pApA) toward the Ce^{IV} ion is promoted by the terminal monophosphate. One of the most plausible mechanisms is shown schematically in Fig. 2 (the Ce^{IV} ions should at least partially form hydroxide clusters under the reaction conditions). Both the terminal monophosphate and the internal phosphodiester linkage are simultaneously coordinated to the Ce^{IV} ions.[17] With the aid of the monophosphate as a strong ligand, the weakly coordinating diester linkage can efficiently form a complex with Ce^{IV} and be hydrolysed. Otherwise, the catalysis by Ce^{IV} on the diester hydrolysis should be predominantly suppressed (or diminished) due to the competitive inhibition by the buffer agents. The increase in the monoester/diester selectivity with increasing concentration of the buffers (Table 4), which mostly originates from the decrease in k_2, is considerably cancelled out by this effect. In contrast, the coordination of terminal monophosphate (and thus its hydrolysis rate) is affected to a lesser extent by the simultaneous coordination with the adjacent phosphodiester.

The greater monoester/diester selectivity for TRIS buffers than for HEPES buffers is probably associated with stronger binding of TRIS to Ce^{IV}. Consistently, the k_1/k_2 ratio is not much changed when the HEPES concentration is increased from 50 to 500 mM (Table 3). Here, the 'k_2 suppression effect' and 'simultaneously coordinating effect' compensate each other.

A similar coordination was previously proposed on metal ion-mediated RNA hydrolysis.[18] In the proposal, a metal ion is simultaneously bound to both the terminal phosphomonoester and the internal phosphodiester, resulting in the increase of its local concentration at the phosphodiester linkage. Thus, the monophosphate residues accelerate the hydrolysis of the adjacent phosphodiester linkage, exactly as observed here in the Ce^{IV}-induced DNA hydrolysis.

Activities of other lanthanide metal ions and non-lanthanide ions

Other lanthanide(III) ions (La, Pr, Nd, Tb, Dy) also hydrolyse monophosphates of nucleotides. However, their activities are rather small. Even Nd^{III}, which is the most active among them, is about 100-fold less active than Ce^{IV}. All the other lanthanide ions as well as non-lanthanide ions investigated are inactive. The activity of Ce^{IV} is overwhelmingly great.

In summary, Ce^{IV} ions efficiently hydrolyse phosphomonoesters in nucleotides. The terminal monophosphates are preferentially hydrolysed over the internal phosphodiester linkages, especially when concentrated TRIS buffers are used as the media. The present results indicate the potential of Ce^{IV} to be used as the catalytic center of artificial phosphomonoesterases. The selectivity for monophosphate hydrolysis could be promoted still more by designing a complex in which the phosphomonoester cannot significantly assist the binding of the metal ion to the adjacent phosphodiester linkage.

Acknowledgements

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