Probing the importance of electrostatic interactions of Ce(III) with the phosphodiester backbone during transesterification using methyl phosphonates

Todd A. Osiek and James K. Bashkin*†

Department of Chemistry, Washington University, Box 1134, St. Louis, MO 63130-4899, USA

Received (in Gainesville, FL, USA) 10th April 2000, Accepted 13th November 2000
First published as an Advance Article on the web 14th March 2001

This paper investigates aspects of the fundamental mechanism of transesterification and hydrolysis. The ability of remote phosphates to coordinate and deliver metal catalysts to the active site (the 2′-OH) of an RNA residue is reported. Previous literature reports show that ApUp is cleaved much faster than ApU at the internal phosphodiester. This supports the accepted mechanism whereby remote phosphates coordinate and deliver metal catalysts to the active site to promote transesterification of RNA. We report that remote phosphates do *not* recruit metal catalysts from the bulk solution in a productive manner for transesterification. By using a polyanionic substrate, termed embedded RNA, and methyl phosphonates to mask the charge at single positions in the polyanionic backbone, we report rate increases in transesterification with methyl phosphonate substitution adjacent to the cleavage site with aqueous Ce(III) and a Ce(III) hexaaza macrocycle.

We report that Ce(III)-catalyzed RNA cleavage rates increase when phosphodiesters adjacent to the cleavage site are replaced with methyl phosphonates. Methyl phosphonates were chosen because they act as neutral analogs of the typical anionic linkers found in nucleic acids. The observed rate increase was contrary to our expectations, and invalidated a major hypothesis about the contribution of remote coordination to the rapid cleavage of high molecular weight RNA. We had developed that hypothesis to explain the observation that RNA oligomers undergo much faster metal-catalyzed cleavage than RNA dimers. We hypothesized that the polyanionic backbone of larger RNA molecules acts to recruit metal cations from solution, thereby increasing the local concentration of catalysts near the cleavage sites of high molecular weight substrates relative to the concentration found at small RNA substrates such as dimers. This hypothesis was supported by reports that metal catalysts cleaved ApUp much faster than ApU at the internal phosphodiester,[1] leading us to suspect that the terminal phosphate of ApUp recruited metal catalysts to the substrate in a productive manner.

To test our hypothesis, we designed a series of experiments that maintained a constant electrostatic charge on the nucleic acid substrates while replacing specific phosphodiester groups with methyl phosphonates. We then measured the rate of RNA cleavage as a function of methyl phosphonate position.

Fig. 1 shows the major mechanism for cleavage of RNA, called transesterification, which involves the deprotonation of the 2′-OH of RNA by a base catalyst such as a metal-bound hydroxide, to form a nucleophile.[2] This may be part of a concerted process. The alkoxide formed can undergo intramolecular attack on the adjacent phosphate, effecting strand cleavage. The recruitment and direction of metal ions from solution to the 2′-OH, the active site, is an important aspect of the overall transesterification mechanism.

We have developed an assay that allows simple and efficient kinetic analysis of transesterification rates.[3–6] It is called the

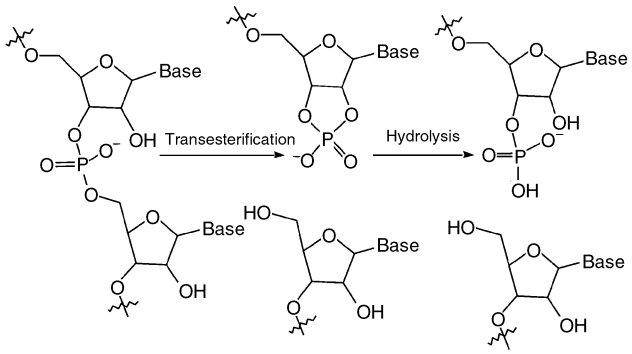

Fig. 1 RNA cleavage mechanism by transesterification and hydrolysis.

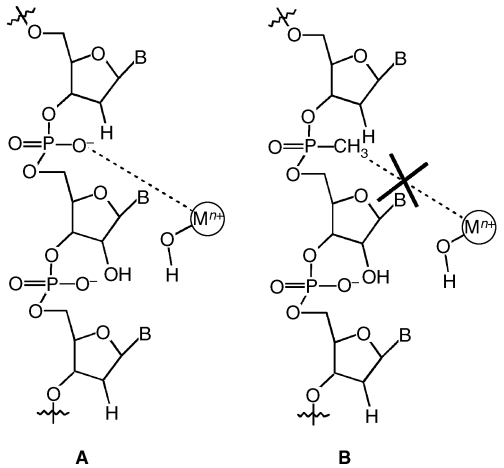

Fig. 2 (A) Possible RNA cleavage mechanism involving remote phosphate electrostatic binding and delivery of metal-bound hydroxide to deprotonate the 2′-OH to effect transesterification. (B) Methyl phosphonate substitution eliminates the possible electrostatic attraction between the metal and embedded RNA backbone.

† Current address: Pharmacia Corporation, Mail Zone R3A, 800 N. Lindbergh Blvd, St. Louis, MO 63167, USA. E-mail: james.k.bashkin@pharmacia.com

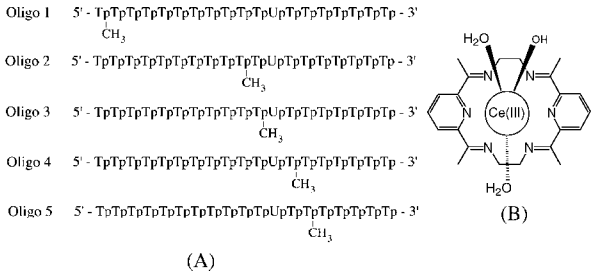

Fig. 3 (A) Embedded RNA oligonucleotides containing methyl phosphonate substitutions along the phosphodiester backbone. (B) Ce(III) macrocycle used during cleavage studies.

embedded RNA (embRNA) assay. EmbRNA, shown in Fig. 2 and 3, is an all-DNA oligonucleotide with one RNA residue embedded within the strand. The embRNA substrate mimics the simplicity of dimeric RNA substrates by having one easily characterized cleavage product, while still maintaining the polyanionic characteristics of a naturally occurring RNA substrate. As has been demonstrated by several groups,[3–6] embRNA maintains many essential features of polymeric RNA while eliminating the competing cleavage reactions that plague studies on RNA biopolymers. We have chosen to cleave embRNA using aqueous Ce(III) and a Ce(III) hexaaza macrocycle [Fig. 3(B)]. The ability of lanthanides to transesterify RNA has been the subject of much investigation.[7,8] Many macrocycles have been developed to become biocompatible counterparts to aqueous metals.[9–13] We have chosen to use neutral methyl phosphonates as replacements for specific phosphodiester linkages, in order to probe the involvement of electrostatic interactions between Ce(III) or Ce(III) hexaaza macrocycle (Ce-mac) and the anionic phosphodiester backbone during transesterification of RNA.

By successively substituting methyl phosphonates up and down the embRNA backbone around the uridine residue, we probed the ability of remote phosphates to bind aqueous Ce(III) and Ce-mac[9,10,14,15] and to promote transesterification.

Fig. 2(A) shows a possible mechanism where the phosphate to the 5′ side of the RNA residue can participate in the transesterification mechanism by electrostatically attracting positively charged metals and delivering them to the 2′-OH, thus allowing the delivery of a metal-bound hydroxide and effecting transesterification. By substituting a neutral methyl phosphonate [Fig. 2(B)] for this anionic phosphate, electrostatic attraction and a coordination site are lost, and thus a change in the observed rate constant for transesterification would be expected.

Five different embRNA oligonucleotides, shown in Fig. 3, were subjected to cleavage by aqueous Ce(III) and Ce-mac. Kinetic analysis of the results during the initial rate regime were performed in triplicate. Fig. 4 illustrates an example of the kinetic data obtained from the gel electrophoresis analysis.

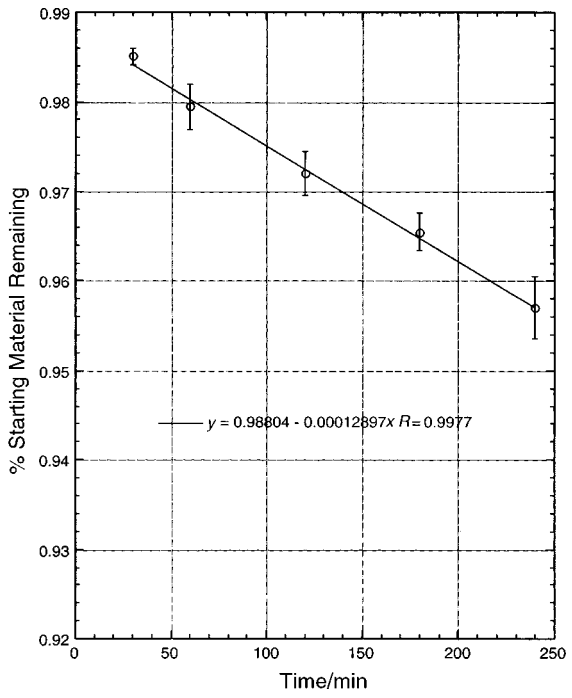

Fig. 4 Data generated from cleavage of oligo 1 with aqueous Ce(III).

Experimental

The five oligonucleotides used for cleavage reactions were bought from Oligos Etc. *N*-(2-Hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) buffer (HEPES), diethylpyrocarbonate (DEPC), Sephadex G-25 column gel, $CHCl_3$, phenol–$CHCl_3$–isoamyl alcohol (25 : 24 : 1), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), and tetra-*n*-butylammonium fluoride (TBAF: 1 M solution in THF) were all purchased from Sigma. T4 polynucleotide kinase and ribonuclease one, used for labeling and RNA cleavage, respectively, were both purchased from Promega. Polyacrylamide gel electrophoresis reagents such as ammonium persulfate (APS), urea, and 40% acrylamide–bisacrylamide (19 : 1) solution were purchased from Fisher. 5X loading buffer was prepared from 50 mg xylene cyanole FF, 50 mg bromophenol blue, 200 L of a 500 mM solution of disodium EDTA dihydrate (pH 8) and 9.20 mL of formamide. All reagents were from Sigma.

Further purification of the oligonucleotides was undertaken after receipt from Oligos Etc. One hundred and fifty picomol of each oligonucleotide was 5′ end-labeled with γ-^{32}P-ATP, bought from Amersham, following normal radiolabeling protocols.[12] The labeling reactions were extracted once with an equal volume of phenol–$CHCl_3$–isoamyl alcohol (25 : 24 : 1) and twice with $CHCl_3$. The aqueous layers were dried by vacuum centrifugation, then reconstituted in 10 μL of 50% ethanol and 100 μL of TBAF, covered with aluminum foil, and shaken overnight to deprotect the TBDMS group from the 2′ position of the RNA residue. These solutions were desalted by gravity Sephadex G-25 columns eluted with DEPC–water. The radioactive fractions were combined, dried down, reconstituted in 10 μL of 1 X loading buffer, and purified by electrophoresing down a 20% polyacrylamide gel. The gel was imaged by autoradiography and the top bands were excised from the gel and eluted overnight in 400 μL of gel elution buffer. The remaining gel fragments were filtered off using G-25 spin filters purchased from Millipore. These solutions were ethanol-precipitated with ammonium acetate, giving the desired labeled and purified oligonucleotides. Concentrations of the oligonucleotides were determined by UV absorption at 260 nm.

Cleavage reactions were carried out in a total volume of 50 μL containing 0.1 M NaCl, 5 mM HEPES buffer at pH 7.6, 100 nM oligonucleotide, and 0.1 mM aqueous Ce(III) or 0.2 mM Ce-mac at 37 °C. Aliquots (4 μL) were removed every 30 min for 4 h and quenched in 1 μL of 5 X loading buffer spiked with 0.5 mM EDTA. Control reactions containing no aqueous Ce(III) or Ce-mac were run along with triplicate runs of reactions containing Ce(III) or Ce-mac. RNase One reactions were run as standards for cleavage products. RNase one is an enzyme that cleaves only at RNA residues, therefore giving a product that co-migrates with our cleavage products from metal-catalyzed transesterification. The 5 μL aliquots were run down 20% denaturing polyacrylamide gels and imaged on a Molecular Dynamics Phosphorimager. The results of the experiments were analyzed using the program ImageQuant. All oligonucleotides were characterized by MALDI-TOF-MS by the Washington University Mass Spectrometry Group.

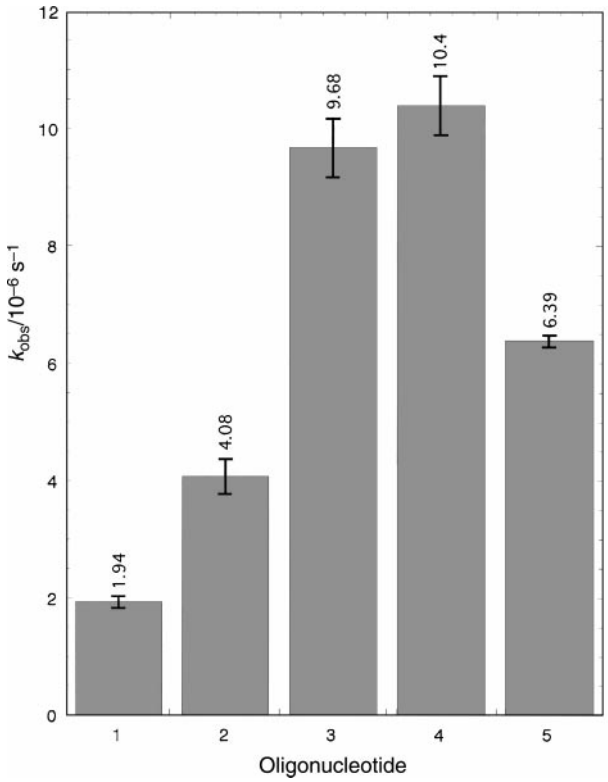

Fig. 5 Values of k_{obs} determined for cleavage of methyl phosphonate oligonucleotides with aqueous Ce(III).

Results

Cleavage results of the methyl phosphonate embRNA with aqueous Ce(III) are shown in Fig. 5. Oligo 1, the control oligonucleotide with the methyl phosphonate substitution at the 5′ end of the oligonucleotide, showed an observed rate constant of 1.94×10^{-6} s^{-1}. Cleavage rate constants of the other four oligonucleotides varied from 4.06 to 10.4×10^{-6} s^{-1}. The observed rate constant for transesterification was large in oligonucleotides 3 and 4 ($k_{obs} = 9.68$ and 10.4×10^{-6} s^{-1}, respectively), which had methyl phosphonate substitutions adjacent to the RNA residue, when compared to oligonucleotides 1, 2 and 5. Oligonucleotides 2 and 5 ($k_{obs} = 4.08$ and 6.39×10^{-6} s^{-1}, respectively), which had methyl phosphonate substitutions two phosphates away from the cleavage site, showed observed rate constants slower than that of oligos 3 and 4, but higher than the control oligonucleotides. A 5.4-fold difference in k_{obs} between the control oligo 1 and oligo 4 was found for cleavage with aqueous Ce(III).

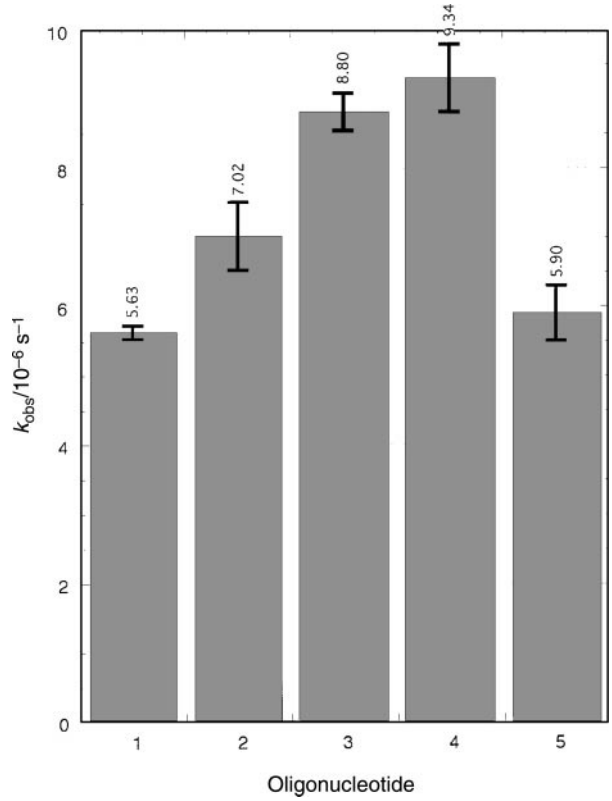

Fig. 6 Values of k_{obs} determined for cleavage of methyl phosphonate oligonucleotides with Ce(III) hexaaza macrocycle (Ce-mac).

Cleavage results performed with Ce-mac are shown in Fig. 6. The control oligonucleotide 1 showed an observed rate constant of 5.6×10^{-6} sec^{-1}. Values of k_{obs} paralleled the results obtained during cleavage with aqueous Ce(III). Cleavage rate constants for oligonucleotides 3 and 4 with methyl phosphonate substitutions adjacent to the RNA residue were higher than those of oligos 2 and 5 with substitutions 2 phosphates away from the cleavage site. However, values of k_{obs} with Ce-mac varied only by a factor of 1.65 between the control oligonucleotide 1 and the most efficient oligonucleotide 4 rather than the factor of 5.4 that was found for cleavage with aqueous Ce(III).

Discussion

The results obtained from the methyl phosphonate study did not show the changes in observed rate constants that we had first expected. Since oligoribonucleotides are cleaved much faster by metal ions than many RNA dimers, we had thought that the remote phosphates might play a key role in recruiting cationic metal catalysts from solution. This study probed the ability of remote phosphates to electrostatically bind aqueous Ce(III) and Ce-mac and to deliver metal hydroxides to the 2′-OH, the active site for RNA transesterification. By eliminating the electrostatic attraction and coordination between cationic metals and the anionic phosphodiester backbone with methyl phosphonate substitution, we expected an observed rate constant decrease when the anionic backbone phosphates were substituted with neutral methyl phosphonates. Contrary to this expectation, the results showed an increase in observed rate constant when anionic phosphates were substituted with methyl phosphonates. This supports a mechanism in which the phosphates do act as strong metal binding sites, but do not direct metal ions to the 2′-OH to increase transesterification rates. We conclude that naturally occurring anionic phosphates bind to metal ions by either an inner- or outer-sphere mechanism, and do not allow the metal to diffuse into the proximity of the 2′-OH. In the methyl phosphonate-substituted example, there is no electrostatic interaction between the metal ion and the backbone, allowing the cation free movement to deliver the metal-bound hydroxide to the 2′-OH. This mechanism accounts for the increase in k_{obs} that is shown with oligonucleotides with methyl phosphonate substitutions adjacent to the uridine residue.

The more pronounced increase in k_{obs} between the control oligonucleotide 1 and oligonucleotide 4 with aqueous Ce(III) compared to Ce-mac also lends further support to this mechanism. Electrostatic attraction is largely dependent on distance. Ce(III) is a cation with the ability to coordinate to the anionic phosphate. Ce-mac, on the other hand, is sterically bulky with far fewer vacant coordination sites. Ce-mac probably has an outer-sphere interaction with the anionic phosphate. Thus, the rate difference caused by elimination of a phosphodiester is larger with aqueous Ce(III) that with Ce-mac, perhaps due to the "free ions" ability to bind more tightly than Ce-mac to the anionic phosphate backbone. This is consistent with the

results showing a 5.4 times range of cleavage rates with aqueous Ce(III) compared to a range of 1.65 times for Ce-mac between cleavage of the control oligonucleotide 1 and oligonucleotide 4.

Conclusion

Substitution of the anionic phosphate backbone with neutral methyl phosphonates results in increases in the observed rate constant for transesterification. The largest increases were seen when substitution occurred adjacent to the RNA residue. The increase in rate was more pronounced for transesterification with aqueous Ce(III) than with Ce-mac. These kinetic results are not consistent with our premise that remote phosphates direct metal ions to the 2′-OH of RNA, the active site in transesterification. However, the data does support a mechanism whereby binding of the cationic metal to the anionic phosphate does not allow activation of the 2′-OH. When the anionic phosphate is substituted with a neutral methyl phosphonate, this binding interaction is nullified, allowing the cationic metal center to effect transesterification.

Acknowledgements

This work was supported by NSF GOALI grant CHE-9802660, and T. A. O. was a GOALI fellow at Monsanto Company.

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