Quenching of Guanine Oxidation by Oxoruthenium(IV): Effects of Divalent Cations on Chemical Nuclease Studies

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Reactions of divalent metal ions with the oxidant Ru(tpy)(bpy)O^{2+} (bpy = 2,2′-bipyridine; tpy = 2,2′,2″-terpyridine) in the presence of DNA were studied by gel electrophoresis and optical absorption. The reaction of Mn^{2+} with Ru(tpy)(bpy)O^{2+} produced a transient intermediate with a new absorption at 531 nm and a second-order rate constant of 1300 ± 200 M^{-1} s^{-1}. This rate constant is 100 times greater than that of the reaction with GMP (guanosine 5′-monophosphate), suggesting that Mn^{2+} is a kinetically competent inhibitor of DNA oxidation. A second-order rate constant of 1.1 ± 0.2 M^{-1} s^{-1} was measured for the reduction of Ru(tpy)(bpy)OH^{2+} by Mn^{2+}; this relationship of rate constants for Ru(IV) and Ru(III) by the same substrate is typical for this oxidant. Plasmid gel electrophoresis demonstrated that Mn^{2+} was a competent inhibitor in the conversion of the supercoiled form of ϕX174 plasmid DNA to the circular form. The Mg^{2+} and Ni^{2+} ions, which do not react with the oxidant but do compete for electrostatic binding to the biopolymer, did not inhibit plasmid cleavage. In high-resolution electrophoresis experiments, the extent of quenching of oxidation by Mn^{2+} in the sequences d[5′-TTCAACA $G_{16}TG_{18}TTTG_{22}AA$] and r[5′GUUCUUG$_7$CUUCAACG$_{16}$UG$_{18}$UUUG$_{22}$AACG$_{26}$G$_{27}$AAC] was dependent on the oligomer structure, where cleavage of residues in the hairpin loop was inhibited most efficiently. In contrast, quenching by Mg^{2+}, Ni^{2+}, and Co^{2+} was much less efficient and occurred only in the double-stranded regions. The selectivity of inhibition by Mn^{2+} could be attributed to differential rates of deactivation of the bound Ru(tpy)(bpy)O^{2+} oxidant.

The interactions of divalent metal ions with nucleic acids are responsible for many aspects of nucleic acid folding and catalysis.[1−3] The ability of metal ions to promote hydrolysis at specific sites in folded RNA's is a central feature of ribozyme function,[4,5] and recognition of synthetic and natural nucleic acids by proteins is often mediated by divalent ions.[6] In addition, numerous synthetic systems where nucleic acids promote bimolecular reactions are known, including labeling of GG sites by cationic platinum complexes,[7] binding of intercalators to platinated DNA,[8] bimolecular electron-transfer reactions,[9,10] and bimolecular decomposition of oxidative cleavage agents.[11,12] These observations suggest that chemical nucleases might be used to follow the effects of divalent ions on nucleic acid recognition and chemistry; however, competition of binding between the chemical nuclease and the divalent cation must be considered. In addition, deactivation of the chemical nuclease by the divalent cation is also a concern in the case of redox-active ions such as Mn^{2+}. We report here on the effects of these considerations in chemical nuclease studies of DNA and RNA hairpins.

The oxidation of nucleic acids by complexes based on Ru(tpy)(bpy)O^{2+} occurs by two pathways (bpy = 2,2′-bipyridine; tpy = 2,2′,2″-terpyridine).[11,13] The complex oxidizes ribose and deoxyribose functionalities by activation of the 1′ C−H bond and oxidizes guanine by an inner-sphere reaction that proceeds via a novel Ru−O−G intermediate. The oxidation of guanine is intrinsically about 7 times more efficient than the sugar oxidation as judged by cleavage yields in single-stranded oligonucleotides or by stopped-flow kinetic measurements on mononucleotides.[11] The guanine oxidation is more susceptible to steric hindrance of the reaction either by protection of the oxidation site by the secondary structure of the nucleic acid or by protection of the oxo group by ligands in the metal complex.[14] In RNA, the polar effect of the 2′-OH group destabilizes the product of the 1′ oxidation and decreases the rate of sugar oxidation;[11] therefore, mostly guanine oxidation is observed in RNA.[15,16] We report here that Mn^{2+} efficiently deactivates Ru(tpy)(bpy)O^{2+} in the absence of DNA. Cleavage of DNA and RNA by Ru(tpy)(bpy)O^{2+} is therefore inhibited by small concentrations of Mn^{2+}. In hairpin sequences, a

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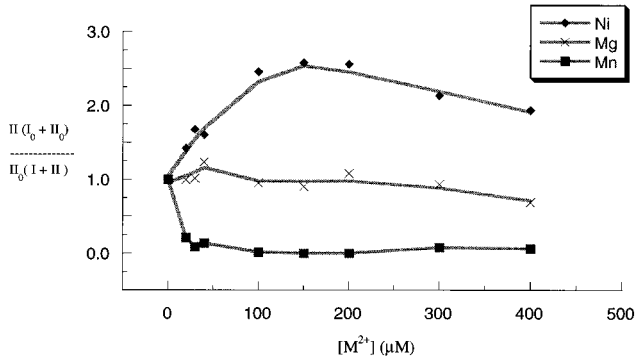


Figure 1. The integrated intensities of supercoiled (I) and circular (II) ΦX174 plasmid after cleavage by Ru(tpy)(bpy)O^{2+} in the presence of Ni^{2+}, Mg^{2+}, and Mn^{2+}. Intensities are given as the fraction of circular DNA (= II/(I + II)) over the fraction of form II at zero metal ion concentration (= $II_0/(I_0 + II_0)$).

selectivity for the inhibition reaction is observed with greater inhibition in single-stranded regions compared to duplex stem regions.

Experimental Section

The oligodeoxynucleotide d(5′-TTCAACAG$_{16}$TG$_{18}$TTTG$_{22}$AA) was purchased from the Oligonucleotide Synthesis Center in the UNC−CH Department of Pathology and purified by gel cutting, as described elsewhere.[14] The RNA transcript (5′-GUUCUUG$_7$CUUCAA CG$_{16}$-UG$_{18}$UUUG$_{22}$AACG$_{26}$G$_{27}$AAC) was synthesized by *in vitro* transcription using T7 polymerase, as described elsewhere.[15] Reaction conditions and electrophoresis procedures are similar to others from our laboratory[14,15] and are described detail in the Supporting Information. For plasmid experiments, a solution of 0.2 μg/μL ΦX174 plasmid DNA (Promega) in 10 mM sodium phosphate buffer, pH 7, metal chloride ($MnCl_2$, $MgCl_2$, $NiCl_2$, or $CoCl_2$), and 45 μM Ru(tpy)(bpy)O^{2+} was reacted for 5 min and then quenched with 95% ethanol. Form I and form II plasmid DNA were separated using an 0.8% agarose gel and visualized by after soaking in an ethidium bromide solution. The cleavage bands were measured using the Apple OneScanner and Ofoto, quantitated using the NIH Image program, and analyzed using KaleidaGraph software.

Results and Discussion

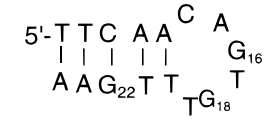
Plasmid Cleavage. We have shown previously that cleavage of DNA by Ru(tpy)(bpy)O^{2+} can be visualized by monitoring the conversion of form I supercoiled plasmids to form II.[17] We therefore used this approach to assess the qualitative effects of divalent cations on the cleavage reaction. We initially chose to contrast the effects of Mn^{2+} and Mg^{2+}, since they exhibit similar binding to DNA,[18,19] but Mg^{2+} cannot reduce Ru(tpy)-(bpy)O^{2+} while Mn^{2+} is redox-active, suggesting possible reduction of the oxidant. As shown in Figure 1, Mn^{2+} inhibits the cleavage reaction while Mg^{2+} has no effect. This result suggests that simple competition for binding of Ru(tpy)(bpy)-O^{2+} to DNA by Mg^{2+} does not dramatically inhibit the reaction while a potential redox reaction between Mn^{2+} and Ru(tpy)-(bpy)O^{2+} does reduce the efficiency of cleavage. Interestingly, the Ni^{2+} ion *increased* the amount of cleavage by Ru(tpy)(bpy)-O^{2+}. This effect was not apparent in high-resolution gels of oligonucleotides where in fact the Ni^{2+} ion was a modest inhibitor of cleavage. The Ni^{2+} result depicted in Figure 1 is likely due to an effect of the ion on the supercoiled plasmid and was therefore not examined further.

Table 1. Rate Constants for Oxidation by Oxoruthenium(IV) and Hydroxoruthenium(III) Complexes

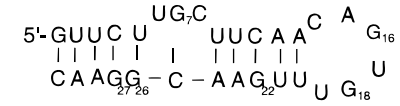
complex	substrate	k (M^{-1} s^{-1})
Ru(tpy)(bpy)O^{2+}	GMP[a]	9[b]
	Mn^{2+}	1500
	dCMP[c]	0.031[b]
	2-propanol	0.067[d]
Ru(tpy)(bpy)OH^{2+}	Mn^{2+}	1.1
	2-propanol	1.1×10^{-4} [d]

[a] GMP = guanosine-5′-monophosphate. [b] Reference 11. [c] dCMP = 2-cytosine-5′-monophosphate. [d] Reference 24.

Chart 1



IRE-D



IRE-R

Reduction of Ru(tpy)(bpy)O^{2+} by Mn^{2+} was observed by optical spectroscopy and followed using stopped-flow methods. Detailed kinetic data are shown in Supporting Information and gave the rate constants for reduction of the Ru(IV)O^{2+} and Ru-(III)OH^{2+} forms shown in Table 1. As with a range of other substrates, the rate constants for Ru(IV) and Ru(III) differ by about 3 orders of magnitude. The kinetic spectra show evidence for an intermediate in the Ru(IV) reduction that absorbs at long wavelengths and might correspond to an Ru−O−Mn oxo-bridged dimer. As shown in Table 1, Mn^{2+} is clearly kinetically competent to inhibit oxidation of guanine by the Ru(IV) form.

Oligodeoxynucleotide Cleavage. The two oligonucleotides studied are shown in Scheme 1. The IRE-R is an RNA 30-mer corresponding to the "iron responsive element", a stem-loop structure responsible for regulating ferritin expression.[20] The IRE-D is a DNA 16-mer corresponding to the analogue of the stem-loop end of the IRE-R where T is substituted for U. We have published the cleavage patterns for Ru(tpy)(bpy)O^{2+} for IRE-D[11,14] and for IRE-R when included in the full-length (500 nucleotides) messenger RNA from ferritin.[16] For IRE-D, we reported previously that Ru(tpy)(bpy)O^{2+} cleaves the guanine residues with intensities in the order G16 > G18 > G22.[14] The relative cleavage intensities are consistent with the relative solvent accessibilities from the recently published NMR studies of similar sequences[21,22] and will be discussed in detail with regard to the IRE structure in a future publication.

When the cleavage reaction of IRE-D is performed in the presence of Mn^{2+}, the cleavage of both base and sugar is quenched. Careful inspection of the cleavage intensities at each nucleotide show that the different sites are quenched with different efficiencies. For the IRE-D sequence, the cleavage is inhibited most strongly for single-stranded G16, which is apparent in Figure 2. *Thus, the extent of quenching by Mn^{2+} is a function of the structural context of the oxidized nucleotide.* The structural selectivity of the quenching efficiency could

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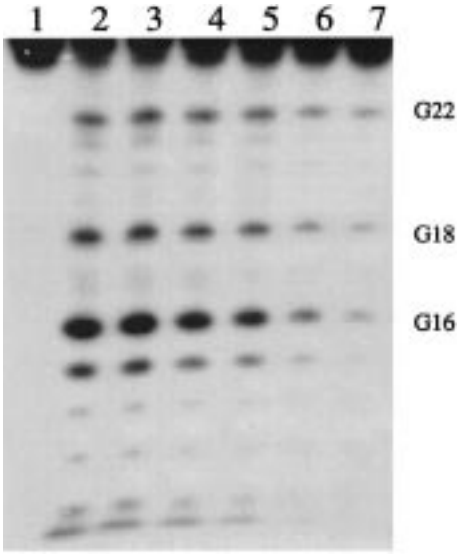

Figure 2. Autoradiogram of the polyacrylamide sequencing gel showing the effect of Mn^{2+} on the cleavage of d[5′-TTCAACAG$_{16}$-TG$_{18}$TTTG$_{22}$AA] by 25 μM Ru(tpy)(bpy)O^{2+}. Lane 1, DNA control; lanes 2 and 3, Ru(tpy)(bpy)O^{2+} only; lanes 4−7, Ru(tpy)(bpy)O^{2+} + 5, 10, 15, and 20 μM Mn^{2+}.

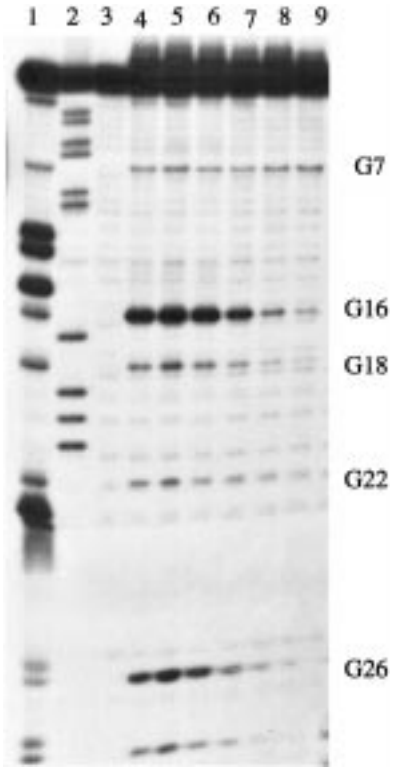

Figure 3. Autoradiogram of the polyacrylamide sequencing gel showing the single-stranded effect of Mn^{2+} on the cleavage of r[5′-GUUCUUG$_7$CUUCAACG$_{16}$UG$_{18}$UUUG$_{22}$AACG$_{26}$G$_{27}$AAC] by Ru(tpy)(bpy)O^{2+}. Lane 1, adenine lane; lane 2, uracil lane; lane 3, RNA control; lanes 4−9: 30 μM Ru(tpy)(bpy)O^{2+} + 0, 3, 6, 12, 24, and 48 μM Mn^{2+}.

therefore result from different binding of the divalent cation at different sites on the oligomer or from different rates of the inactivation of the oxidant, since the inactivation by Mn^{2+} is faster than the rate of DNA oxidation (Table 1).

The first explanation to rule out is simply an effect of the concentration of the oxidant. If Mn^{2+} reacted with all of the Ru(tpy)(bpy)O^{2+} in solution, the bulk concentration of oxidant would be depleted, and if the cleavage pattern was simply dependent on the oxidant concentration in the order G16 > G18 > G22, the result in Figure 2 would be obtained. This explanation was ruled out by comparing the relative cleavage intensities at low Ru(tpy)(bpy)O^{2+} concentration with those at high Ru(tpy)(bpy)O^{2+} concentration with added Mn^{2+}. Under conditions where the Ru(tpy)(bpy)O^{2+} concentration is low enough to give a similar G18 intensity as in the quenched reaction, the intensity of G16 is significantly greater (histogram given in Supporting Information). Thus, the quenching of the oxidation of single-stranded G16 is in fact more efficient as implicated by Figure 2. This experiment rules out a simple concentration effect as a basis for the selectivity of Mn^{2+} quenching.

Two mechanisms for quenching by Mn^{2+} can be envisioned: chemical deactivation of the oxidant or competition for binding of the oligonucleotide by the dication.[13] To rule out the latter explanation for the structural selectivity, quenching reactions were performed with Ni^{2+}, Mg^{2+}, and Co^{2+}, which can compete with Ru(tpy)(bpy)O^{2+} for binding but do not chemically deactivate the oxidant. Quenching by the three cations is much less efficient than for Mn^{2+}, and the sequence dependence is opposite that of Mn^{2+} with the largest inhibition observed at G18 and G22 (gel given in Supporting Information). Therefore, divalent cations that can inhibit Ru(tpy)(bpy)O^{2+} only by competition for binding are poor inhibitors of G16, which is the opposite of the result observed with Mn^{2+}. The special properties of Mn^{2+} both in terms of its efficiency of inhibition and structural specificity therefore appear to result from the chemical reduction of the oxidant and not from competition for binding to the oligonucleotide.

RNA Oxidation. The results of oxidation of the IRE-R oligomer with Ru(tpy)(bpy)O^{2+} are shown in Figure 3. The reactive sites are similar to those in the DNA analogues (G16 > G18 > G22). Addition of Mn^{2+} led to inhibition of all of the cleavage sites; however, inhibition of G16 was much greater than inhibition at G18 or G22, as observed in the DNA sequence. Inhibition was not detected at G7, but strong inhibition was observed at G26. It was further apparent in the RNA studies that the selectivity of Mn^{2+} was not due to a simple change in concentration; for example, G7, G18, and G22 all exhibit roughly the same intensity in the absence of Mn^{2+}; however, G18 and G22 are inhibited by Mn^{2+} while G7 is not.

Mechanism of Inhibition. The divalent Mn^{2+} ion reduces Ru(tpy)(bpy)O^{2+} faster than DNA and is clearly a competent inhibitor of DNA cleavage. The reduction reaction exhibits a specificity for inhibiting cleavage of single-stranded guanines more efficiently than duplex guanines. This selectivity likely arises from a different rate of the Ru−Mn reaction at that site rather than either a simple concentration effect or preclusion of binding of Ru(tpy)(bpy)O^{2+} at the single-stranded site. In fact, the inhibition by binding is more efficient for duplex guanines, as shown with redox-inert divalent cations. A possible explanation for this effect is that when Ru(tpy)(bpy)O^{2+} and Mn^{2+} are bound in the minor groove of duplex DNA, the diffusion of the two metals is restricted, which slows down the chemical reaction and compensates for the increased local concentration of the two species provided by condensation on the biopolymer. On the other hand, single-stranded DNA provides an anionic backbone on which the two cations can condense, but the less structured polymer may allow for more rapid diffusion of the two species. Thus, the single-stranded regions may provide for

a concentration enhancement without the penalty of restricted diffusion imposed by the organized phosphates of duplex DNA.[23]

A second explanation for the specificity might be that the orientation of the oxo complex is affected by binding to the two different regions. To form an inner-sphere intermediate with either Mn^{2+} or guanine, the oxo group of the ruthenium complex must almost certainly be oriented toward the substrate. Thus, orientation of the oxo group of the DNA-bound ruthenium complex toward Mn^{2+} might be more favorable when bound to single-stranded DNA than when bound to duplex DNA. Again, the organized phosphates of the minor groove might dictate an orientation of the ruthenium complex where the oxo group does not collide with bound Mn^{2+}, thereby allowing for nonproductive collisions in duplex DNA that are productive in single-stranded DNA where the metal complex is less likely to be restricted to a single orientation.

Conclusions. Two important points can be gleaned from these studies. The first is that both competition for binding by redox-inactive divalent cations and chemical deactivation by redox-active divalent cations are complicating factors in chemical nuclease studies. The $Ru(tpy)(bpy)O^{2+}$ complex is a rare chemical nuclease in that it does not require a sacrificial oxidant such as hydrogen peroxide or persulfate; nonetheless, chemical deactivation is a significant issue. Oxidation of Mn^{2+} by chemical nucleases is likely to be an issue in general, since Table 1 implies that GMP is oxidized 100 times slower. These points need to be considered in using chemical nucleases to understand divalent cation effects in ribozymes and nucleoprotein recognition. The second point is that if the origins of the quenching mechanisms can be understood, the inhibition patterns might provide even greater information than the direct cleavage pattern in using cleavage sites to indicate novel nucleic acid structures.

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Supporting Information Available: Complete experimental details for cleavage reactions, description of kinetic studies used to obtain the rate constants in Table 1, absorbance changes observed reduction of $Ru(tpy)(bpy)O^{2+}$ by Mn^{2+}, dependences of rate constants on Mn^{2+} concentration, gel showing the effect of Mn^{2+} on cleavage intensities in IRE-D at low $Ru(tpy)(bpy)O^{2+}$ concentrations, gel showing quenching by Mg^{2+}, Co^{2+}, and Ni^{2+}, and histogram of intensities from Figure 3 (10 pages). Ordering information is given on any current masthead page.

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