Principles of Nucleic Acid Cleavage by Metal Ions

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1 Introduction

Nucleic acids are responsible for the storage, transmission, and expression of genetic information in all living organisms. These macromolecules tend to behave as salts in solution since the negative charges of the polynucleotide phosphodiester backbone are usually neutralized by metal solute ions. Therefore, for practical purposes, it is difficult if not impossible to separate the behavior of DNA and RNA from their interactions with metal ions. We must also take into account water molecules specifically bound to nucleic acid residues since they frequently mediate interactions between polynucleotides and metal ions (Prive et al. 1991). Often molecular biologists dealing with DNA and RNA believe that the properties of metal ions are simpler than the properties of nucleic acids due to their smaller size. In fact, both nucleic acids and metal ions exhibit considerable complexity in their interactions, and such interactions could affect the chemical and biochemical properties of both parties.

Metal ions are usually required to promote and stabilize functionally active or native conformations of nucleic acids, but they can also trap polynucleotides in inactive conformations (Heilman-Miller et al. 2001). In addition to structural roles, most polyvalent metal ions ($M^{\geq 2+}$) can induce cleavage (i.e., breakage, scission, fragmentation, depolymerization, or rupture) of nucleic acids. These reactions can be either non-specific or dependent on the chemical nature of nucleotide residues, nucleic acid sequence, or secondary and/or tertiary structure. The specificity of these reactions is dependent on both the nucleic acid conformation and metal binding modes as well as the properties of the metal ions.

Metal ions can catalyze the cleavage of nucleic acids through different mechanisms, which are briefly discussed below. These mechanisms may involve direct interaction of metal ion catalysts with either the atoms flanking

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the cleavable bonds or the nucleic acid functional groups participating in the cleavage chemistry. In aqueous solutions, metal ions are hydrated and can act as either general acids ($M–OH_2$) or general bases (M–OH). Alternatively, metal ions can stabilize the transition states of cleavable bonds. In addition, metal ions can promote cleavage through modification of nucleic acid base (nucleobase) and/or sugar residues, resulting in destabilization of the N-glycosidic bond between these moieties. Oxygen radicals generated by some metal ions also can cleave both RNA and DNA.

However, not all cleavages of nucleic acids promoted by metal ions occur through direct involvement of metal ions in cleavage chemistry. For example, metal ion cofactors stabilize the catalytically active conformations of several ribozymes, but do not participate directly in catalysis (Hanna and Doudna 2000). Metal ions added to solutions may also indirectly promote nucleic acid cleavage through activation of proteins having nuclease activity (Beresten et al. 1992).

Here, we critically review the fundamental factors that influence the efficacy and specificity of nucleic acid cleavage promoted by metal ions. These (frequently disregarded) factors can have dramatic effects in experiments or might result in artifacts or misinterpretation of experimental data.

2 General Mechanisms for the Cleavage of Nucleic Acids

Cleavage of nucleic acids refers to a reaction that results in the breakage of bonds in the phosphodiester backbone of a polynucleotide chain. There are two types of reactions resulting in the cleavage of either P–O (Fig. 1) or C–O bonds (Figs. 2, 3) in the nucleic acid backbone (Kochetkov and Budovskii 1972). Cleavage of the P–O bond occurs as a result of nucleophilic attack on the phosphorus atom via either an *inter*molecular reaction with a H_2O molecule (hydrolysis) (Fig. 1A) or an *intra*molecular reaction involving the ribose 2′-OH (transesterification) (Fig. 1B). Of these two reactions, transesterification is specific to RNA (since DNA lacks a 2′-OH moiety) and can be catalyzed by alkali, acid, and some M^{2+} ions, generating 5′-OH and 2′,3′-cyclic phosphate ends. The same catalysts promote subsequent fast hydrolysis of 2′,3′-cyclic phosphate (Kochetkov and Budovskii 1972) resulting in an equimolar mixture of 2′- and 3′-phosphates (Fig. 1B). Otherwise, 2′,3′-cyclic phosphates are moderately stable at neutral pH, and can be obtained in high yield by RNA cleavage with ribozymes (Pan et al. 1993) and ribonucleases (Vlassov 1998).

It is a common mistake to use the term "hydrolysis" when referring to RNA cleavage by the transesterification mechanism. Only the post-cleavage opening of a 2′,3′-cyclic phosphate proceeds via a hydrolytic mechanism. However, direct hydrolytic cleavage of RNA, yielding 5′-phosphate and 3′-OH ends (Fig. 1A) can be catalyzed by some nucleases, such as RNase V1 (Lockard and

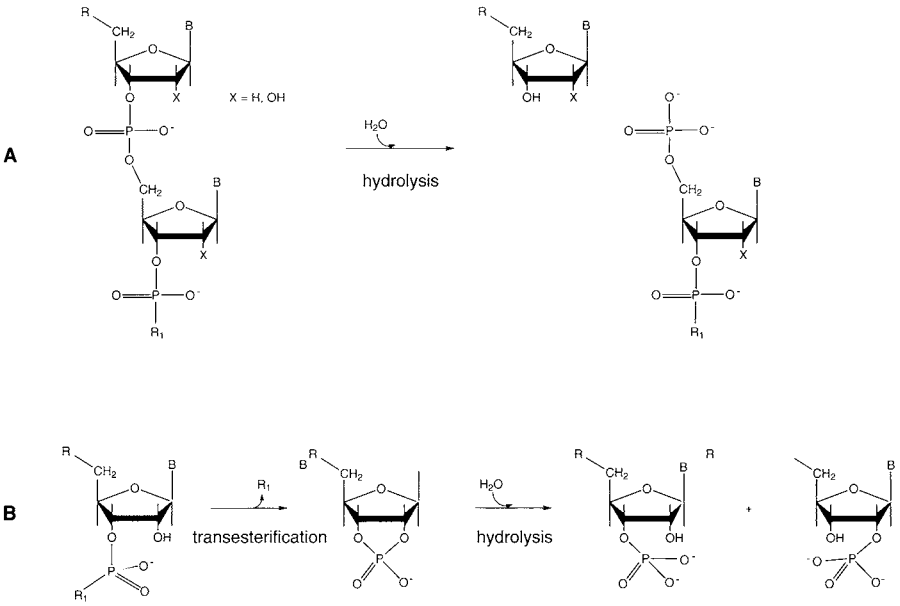

Fig. 1. Reactions that result in direct cleavage of the phosphodiester backbone. **A** Hydrolysis (DNA, RNA) and **B** transesterification (RNA only)

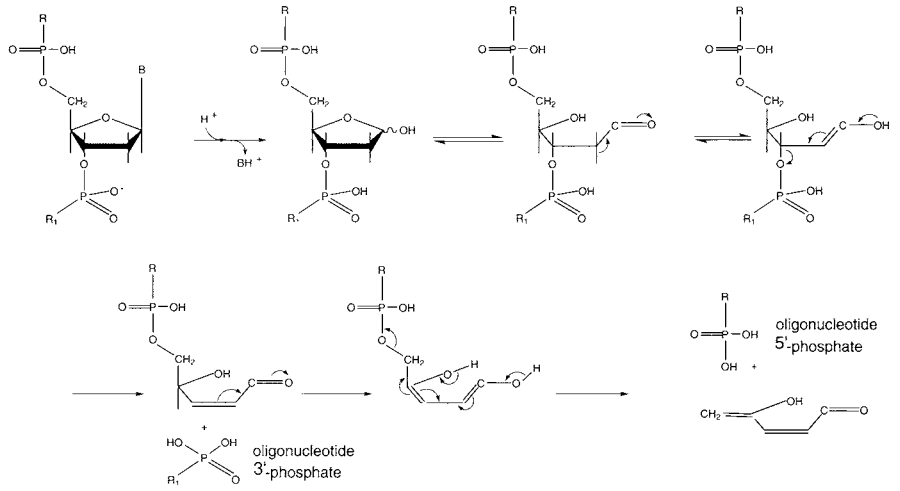

Fig. 2. Mechanism of acid catalyzed hydrolysis of DNA

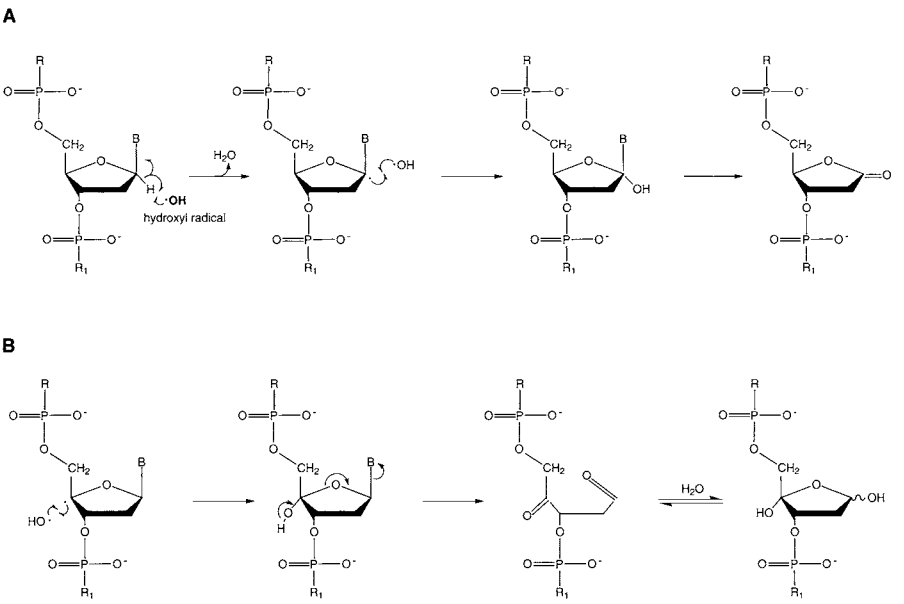

Fig. 3. Possible pathways of nucleobase removal induced by hydroxyl radicals (•OH) that involve hydrogen atom abstraction from either the **A** C1′ or **B** C4′ positions of the deoxyribose moiety

Kumar 1981), RNase H (Caserano et al. 1995), and the catalytic RNA subunit of RNase P (Cedergren et al. 1987; Guerrier-Takada et al. 1986).

RNA cleavage catalyzed by alkali and metal-hydroxides is well known. The catalytic role of the HO^- is to deprotonate the ribose 2′-OH, thereby increasing its nucleophilicity, which then attacks the electrophilic phosphorus atom in the phosphodiester (Kochetkov and Budovskii 1972). Surprisingly, H^+ and general acid catalyze RNA cleavage also via the transesterification mechanism even though the undissociated 2′-OH is a weak nucleophile (Kochetkov and Budovskii 1972). The catalytic role of the H^+ is to protonate the internucleotide phosphate oxygen, thereby increasing the electrophilicity of the phosphorus atom, which then can be attacked by 2′-OH. In contrast to general base catalysis, acid catalysis may also result in phosphodiester isomerization – from 3′-5′ to 2′-5′ (Morgan et al. 1995; Oivanen et al. 1998).

Both RNA and DNA can be cleaved by acid hydrolysis albeit by different mechanisms. Surprisingly, the chemical stability of RNA at pH 5–6 may be even higher than that of DNA because DNA can be depurinated and cleaved at pH 8 (Ugarov et al. 1999). While all internucleotide P–O bonds in RNA have the potential to be cleaved through transesterification, acid-catalyzed DNA hydrolysis occurs through cleavage of internucleotide C–O bonds exclusively at positions of purine nucleotides as shown in Fig. 2 (Kochetkov and Budovskii 1972). The reaction products include pyrimidine mono- and oligodeoxynucleotides phosphorylated both at their 3′- and 5′-ends, which

are resistant to acid hydrolysis. The first step of this reaction is hydrolysis of the N-glycosidic bond that can be catalyzed by acids and some metal ions in high oxidation states (see below). The abasic sites in DNA, which are otherwise stable at neutral pH, can then be cleaved via the "β-elimination" reaction (Fig. 2) catalyzed by acid, alkali, or amines (Kochetkov and Budovskii 1972).

Alternatively, nucleobases can be removed indirectly through degradation of base or sugar moieties by oxidation or attack by radicals (Burrows and Rokita 1998; Pogozelski and Tullius 1998). Oxidation and opening of the sugar rings in DNA and RNA may be achieved through different mechanisms. One of these mechanisms includes generation of hydroxyl radicals (•OH) that can be induced by metals ions (e.g., Fe^{2+} and Cu^{+}) through the Fenton reaction with hydrogen peroxide. These free or metal-bound radicals are believed to abstract H atoms from sugar residues at the C1′ and C4′ positions in a reaction leading to the removal of a nucleobase (Fig. 3) (Burrows and Rokita 1998), and ultimately leading to scission of the sugar-phosphate backbone. The mechanistic details for cleavage of the ribose moiety of RNA by •OH radical may differ from Fig. 3, but the final result is the same.

DNA and RNA differ sharply in the stability of their phosphodiester bonds in aqueous solutions. In general, DNA is thought to be more stable than RNA (Li and Breaker 1999; Thorp 2000; Williams et al. 1999). Indeed, RNA is easily cleaved even in mild alkali solutions while DNA is stable under these conditions. Contamination of solutions by RNases and M^{2+} ions may result in fast degradation of RNA even at neutral pH and low temperatures. However, in M^{2-} and RNase-free solutions at slightly acid pH, RNA molecules are cleavage-prone even at room temperature (Ambion 1998). Interestingly, although RNA is hydrolytically less stable than DNA, there is evidence that RNA can be more stable to oxidative cleavage than DNA (Thorp 2000).

3 Basic Properties of Metal Ions in Solution

In order to better understand how experimental conditions influence the efficacy and specificity of metal-assisted cleavage of nucleic acids, it is important to take into account the chemical behavior of metal ions in solution and the principles that govern their interactions with different ligands (see Feig and Uhlenbeck 1999; Kazakov 1996; Pan et al. 1993, and references therein). In aqueous solutions, most metal ions exist as cationic aquo-complexes $[M(H_2O)_x]^{n+}$, which tend to form aquo-hydroxo complexes $[M(H_2O)_{x-1}(OH)]^{(n-1)+}$ according to their pK_a (Table 1). The pK_a of metal-bound H_2O is lower than the pK_a of a free water molecule because the positive charge of the metal ion facilitates the loss of a water proton. In addition to H_2O and hydroxyl anion (^{-}OH), other neutral (e.g., NH_3, imidazole, and nucleobases) or negatively charged ligands (e.g., RS^{-}, Cl^{-}, $CH_3CO_2^{-}$, or PO_4^{3-}) can be bound simultaneously to the same metal cations. The maximum

Table 1. Basic features of hydrated metal ions involved in metal-nucleic acid interactions. (Kazakov 1996)

Ion	Ionic radius	Coordination numbers	Typical geometry of inner-sphere complex	First pK of * $[M(H_2O)_n]^{M+a}$
Ag^+	1.26	2>4	Linear	9.8–11.7
Al^{3+}	0.51	6>4	Octahedral	4.3–5.0
Ba^{2+}	1.34	8	Square antiprism	13.4–14.0
Be^{2+}	0.35	4	Tetrahedral	5.7–6.7
Bi^{3+}	0.96	6	Octahedral	Unstable
Ca^{2+}	0.99	8	Square antiprism	12.6–13.4
Cd^{2+}	0.97	6>4	Octahedral	7.6–10.2
Co^{2+}	0.72	6	Octahedral	7.6–9.9
Co^{3+}	0.63	6	Octahedral	0.9–2.0
Cr^{2+}	0.89	6	Octahedral	8.7–11
Cr^{3+}	0.63	6	Octahedral	3.8–4.4
Cu^+	0.96	4	Tetrahedral	Unstable
Cu^{2+}	0.72	4>6	Square planar	6.8–8.5
Eu^{3+}	0.95	9	Variable	4.8–8.5
Fe^{2+}	0.74	6	Octahedral	5.9–6.7
Fe^{3+}	0.64	6	Octahedral	≈2.5
Hg^{2+}	1.10	2>4,6	Linear	2.4–3.7
In^{3+}	0.81	6	Octahedral	≈3.7
Mg^{2+}	0.66	6	Octahedral	11.4–12.8
Mn^{2+}	0.80	6	Octahedral	10.6–10.9
Ni^{2+}	0.69	6	Octahedral	6.5–10.2
Pb^{2+}	1.20	6>9	Octahedral	6.5–8.4
Sn^{2+}	0.93	3>6	Pyramidal	3.7–6.8
Sr^{2+}	1.12	6	Octahedral	13.2–13.8
Th^{4+}	0.99	8–12	Variable	2.4–5.0
UO_2^{2+}	0.80 (U^{6+})	6>8	Octahedral	≈5.7
VO^{2+}	0.63(V^{4+})	6	Octahedral	≈5.4
Zn^{2+}	0.74	4>6	Tetrahedral	8.2–9.8

* Dependent on ionic strength

number of ligands that can bind directly (inner-sphere) to a metal ion with single bonds is indicated by its coordination number. The geometry of the inner coordination sphere, including the number of ligands and their spatial arrangement around the central metal ion, depends on the ionic radius and oxidation state of the metal ion (Table 1). Besides inner-sphere complexes, metal ions can form outer-sphere complexes in which a ligand is localized near the complexed metal ion via interaction with an inner-sphere ligand (e.g., by hydrogen bonds with H_2O molecules directly coordinated to a metal ion). In fact, most interactions between metal ions and nucleic acids observed in crystal structures are outer-sphere coordination. An example of inner- and outer-sphere binding between $tRNA^{Phe}$ and Mg^{2+} is shown in

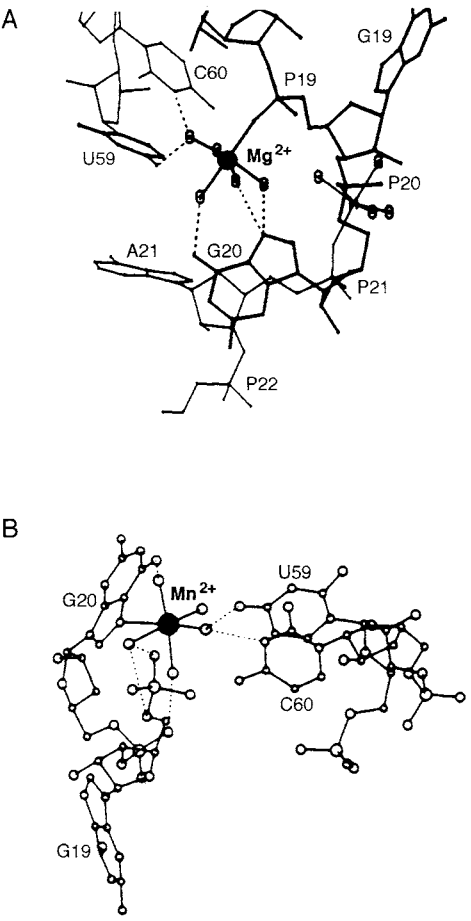

Fig. 4. The binding modes of metal ions with the D loop of yeast $tRNA^{Phe}$. **A** $[Mg(H_2O)_5]^{2+}$ is bound directly to the phosphate-19 oxygen, whereas metal-coordinated H_2O molecules are involved in a number of hydrogen bonds (reprinted from Teeter et al. 1980, with permission). **B** $[Mn(H_2O)_5]^{2+}$ is bound directly to the N7 atom of G20, whereas five H_2O ligands form hydrogen bonds with neighboring (in the tertiary structure of the tRNA) atoms (reprinted from Jack et al. 1977, with permission)

Fig. 4A. The number of specific bonds (both inner- and outer-sphere) that can be formed by hydrated metal ions (e.g., Mg^{2+}) surprisingly exceeds the number of potential bonds that can be formed by nucleic acid bases (see Fig. 5).

The behavior of particular complexes with metal ions depends mainly on the kinetic lability and thermodynamic stability of the corresponding metal-ligand bonds (Kazakov 1996 and references therein). In dynamic equilibrium, metal complexes can be formed and dissociated as a result of ligand (or donor atoms) exchange reactions where inert metal-ligand bonds are retained while labile ones are replaced. Some anions such as ClO_4^- and NO_3^- have no tendency to bind to the hydrated metal ions even in concentrated solutions, but many others such as Cl^-, $CH_3CO_2^-$ or PO_4^{3-} anions can compete with other metal-bound (coordinated) ligands. Some commonly used biological buffers (e.g., polyamines, Tris, and phosphates) also can form metal-ion complexes of varying degrees of stability. Moreover, cationic buffer components may also

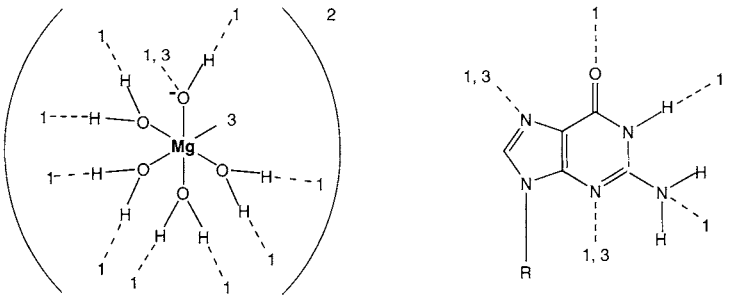

Fig. 5. Types of specific bonds that can be formed by hydrated metal ion (*left*) as compared to guanine (*right*). The bond types are designated as follows: *1* hydrogen, *2* ionic, and *3* coordinative (donor–acceptor)

compete with metal ions for electrostatic and outer-sphere metal binding sites in nucleic acids. It has also been reported that under certain conditions, common buffers such as Tris (pH>7.5) and sodium borate (pH 7.0) when in the presence of Mg^{2+} can promote cleavage of RNA (AbouHaidar and Ivanov 1999).

The ligand exchange reactions rarely proceed directly. Instead, the leaving ligand is first replaced by water; the entering ligand then substitutes the metal-bound H_2O (M-OH_2). The reactivity of the aquo-metal complexes correlates with the rate of the H_2O substitution in the inner sphere of the corresponding metal ions: $Cs^+ > Ba^{2+} > Hg^{2+} > K^+ > Cr^{2+} \sim Cu^{2+} > Na^+ > Sr^{2+} > Li^+ > Ca^{2+} > Cd^{2+} > Mn^{2+} \sim Zn^{2+} > Fe^{2+} > Co^{2+} > Mg^{2+} > Ni^{2+} > Pd^{2+} > Pt^{2+} > Cr^{3+}$ (Cotton and Wilkinson 1988). In most cases, the interactions between hydrated metal ions and nucleic acids are fast with $t_{1/2}$ in the range 10^{-9}–10^{-3} s (Williams and Crothers 1975). However, if the limiting step of the exchange reaction is dissociation of a relatively stable bond (e.g., metal-hydroxyl, M-OH), the rate of reaction could be quite slow. While pH <pK_a (Table 1) favors aquo-complex (M-OH_2) formation, pH >pK_a favors formation of M–OH, which is resistant to ligand substitution and also decreases or even neutralizes the positive charge of the metal complex, resulting in precipitation of metal hydroxides (Martin 1976). For instance, both $Pb(OH)_2$ and $Fe(OH)_2$ begin to precipitate at pH 7.5 and are almost completely insoluble (~10^{-5} M) at pH 9.0 and 9.7, respectively. In contrast, $Mg(OH)_2$ only begins to precipitate from pH 10.4 (Dean 1985). Therefore, it is important to optimize the solution pH depending on metal ions to be used. The formation of hydroxy-forms also can result in oxidation of metal ions as, for example, Mn^{2+} and Co^{2+} at pH ≥8.5 (Dahm et al. 1993).

Metal ions and ligands (including the donor atoms of nucleic acids) can be classified as "hard" or "soft" according to the thermodynamic stabilities of their complexes, and these designations govern trends of their affinity for each other (Feig and Uhlenbeck 1999; Kazakov 1996 and references therein). In general, "hard" metal ions (e.g., alkali monovalent ions, M^+) prefer "hard"

ligands such as O-donor atoms (OH^-, H_2O, and phosphate oxygens), whereas "soft" metals (e.g., Ag^+, Cu^+, Hg^{2+}, Pd^{2+}, and Pt^{2+}) prefer "soft" ligands such as N-donor atoms of the nucleobases and S-donor atoms in nucleic acid derivatives (e.g., thiouridine and phosphorothioates). Intermediate (or "borderline") metal ions have ambivalent properties and have the ability to bind to both "hard" and "soft" donor atoms. The tendency to favor N- and S- over O-donors for selected hard and "borderline" metal ions has the following trend: Cu^{2+}>Ni^{2+} >Cd^{2+} >Co^{2+} >Zn^{2+} >Fe^{2+} >Mn^{2+} >>Mg^{2+}. However, one should not be fooled by the "higher" thiophilicity of Mn^{2+} over Mg^{2+} since the affinities of these ions for S atoms are still not higher than for O atoms (Feig and Uhlenbeck 1999). It is also important to keep in mind that nucleic acids are complex ligands and their interactions might not be completely consistent with the "hard-soft" classification (Martin 1986).

4 Metal Ion Binding to Nucleic Acids

The initial interaction between positively charged metal ions and negatively charged nucleic acids involves a fast, electrostatic interaction that is weak and nonspecific. For many metal ions, this electrostatic interaction can be followed by stronger and more specific binding with nucleic acids via the formation of inner- and outer-sphere complexes (Egli 2002). Because nucleic acids are polyanions, the concentration of cations in the vicinity of nucleic acids will be far greater than that in bulk solution. For a low ionic strength solution of DNA, an estimate of the ratio of concentrations of metal ions proximal to and distant from the polynucleotide (polyelectrolyte effect) for Na^+ and Mg^{2+} is about 3×10^3 and 9×10^6, respectively (Daune 1974), which reflects the greater affinity of divalent cations over monovalent ones. However, M^+ can compete for ionic binding with nucleic acids when present in higher concentrations than M^{2+} ions. Since the negative charge density is smaller for single-stranded than for double-stranded nucleic acids, so is the polyelectrolyte effect. In addition, the polyanionic electric field also increases the pK_a values of nucleobases and promotes polarization and deprotonation of water molecules directly bound both to the nucleic acid residues and to metal ions (Guerrier-Takada et al. 1986).

"Hard" *mono*valent cations (M^+) usually interact with nucleic acid polyanions only in a diffuse "ion atmosphere" manner, whereas "soft" and "borderline" *poly*valent (M^{2+}) cations can form both outer- and inner-sphere complexes. "Soft" metal ions tend to form inner-sphere complexes, however. Some M^{2+} ions that can promote cleavage of RNA or DNA also can form strong complexes with chelating agents such as EDTA with formation constants varying over many orders of magnitude (10^8–10^{36} M^{-1}). Here, we list formation constants (log K_a) for selected metal complexes with EDTA in descending order: Co^{3+} (36) >Fe^{3+} (24.2) >Cr^{3+}(23) >Hg^{2+} (21.8) >Cu^{2+} (18.7)

$>Ni^{2+}$ (18.6) $>Pb^{2+}$ (18.3) $>Eu^{3+}$ (18.0) $>Cd^{2+}$ (16.4) $\approx Zn^{2+}$ (16.4) $\approx Co^{2+}$ (16.3) $>Al^{3+}$ (16.1) $>Fe^{2+}$ (14.3) $>Mn^{2+}$ (13.8) $>Cr^{2+}$ (13.6) $>Ca^{2+}$ (11.0) $>Mg^{2+}$ (8.6) (Dean 1985). Addition of a small amount of EDTA (e.g., 0.1–1 mM) to buffer solutions can prevent binding of these potential cleaving agents that frequently contaminate buffers to nucleic acids and, therefore, decrease background cleavage.

Since nucleic acids are polyanionic, some metal-RNA complexes can survive even in the presence of negatively charged chelating agents such as EDTA because of electrostatic repulsion and steric inaccessibility of some metal ions bound in internal pockets of RNA tertiary structure (Wacker et al. 1963). For this reason, to most effectively remove M^{2+} ions bound to RNA, the RNA should be heat-denatured in the presence of chelating agents and high concentrations of M^{+} ions to accelerate ligand-exchange reactions if possible (Vary and Vournakis 1984a).

At neutral pH, the principal metal binding sites (donor atoms) in nucleosides are believed to be as follows: termini phosphate oxygens, internucleotide phosphate oxygens, endocyclic atoms in guanosine N7, adenosine N7 and N1, and cytidine N3 (Marzilli 1981). The purine atom N3 is sterically hindered by the sugar, but changes in the relative orientation of the nucleobase and the sugar could make this site available to bind metal. The O2 and N3 atoms of cytosine are well-established exocyclic metal binding sites at neutral pH while the carbonyl groups of other nucleobases are relatively weak donors and usually only can bind a metal ion if reinforced by chelation with another ligand. Unhindered nucleobase donor atoms can be involved in octahedral (coordination number 6) complexes while sterically hindered atoms prefer to form complexes with metal ions with coordination number of 4 or 2 (see Table 1). As a rule, the order of relative stability of nucleotide base-metal complexes is given as $G > A \geqslant C >> T$ (or U) (Eichhorn 1973). Despite the ability of metal ions to compete with protons for the same binding sites that have been used for adenine-specific DNA cleavage (Iverson and Dervan 1987; Pulleyblank 1982), the relative affinities of donor atoms toward metal ions differ from that for protons (Martin 1985).

The metal binding sites of non-structured, single-stranded polynucleotides are similar to those of the bases in mononucleotides. Generally, the affinity of a metal ion for structured or multistranded polynucleotides is weaker than for denatured ones because some potential metal-binding sites are sterically inaccessible or are masked by internucleotide H-bonds (Sissoeff et al. 1976). However, a favorable steric arrangement of nucleotide metal-binding atoms in structured polynucleotides could also result in a metal-binding mode that is less possible for the corresponding mononucleotides. Alternatively, metal binding to several nucleotide residues could bridge them in a newly formed tertiary structure, thus creating novel metal binding sites that result in cooperative metal binding to polynucleotides such as tRNA (Misra and Draper 2000).

The formation of kinetically and thermodynamically stable bonds with specific group(s) on the nucleoside, nucleotide or polynucleotide molecule results in specific metal-nucleic acid binding. There are different kinds of metal binding and nucleic acid cleavage selectivities: (1) atom-specific (oxygen in phosphate or sulfur in thiophosphate groups; N-donor atoms in nucleobases); (2) group-specific (phosphate, sugar and nucleobases or their alternative combinations); (3) nucleobase-specific in polynucleotides; (4) dinucleotide-sequence-specific; (5) secondary structure specific; and (6) tertiary structure specific.

In general, the higher the complexity of the secondary and tertiary structure of polynucleotides, the stronger the specificity of metal binding is possible due to the higher probability of forming unique ligand environments. An example of the exquisite specificity of how different metal ions coordinate to the same binding pocket is in the crystallographic studies of yeast $tRNA^{Phe}$ (Shi and Moore 2000 and references therein). Both Mg^{2+} and Mn^{2+} have been observed binding specifically at the interface of the D loop and TΨC loop. Mg^{2+} or $[Mg(H_2O)_5]^{2+}$ makes one inner-sphere coordination to the G19 phosphate oxygen and five outer-sphere coordinations with H_2O molecules that are hydrogen bonded to the tRNA (Fig. 4A). Mn^{2+} or $[Mn(H_2O)_5]^{2+}$ also is coordinated to 5 H_2O molecules, but its one direct coordination is shifted from the G19 phosphate to the N7 of neighboring G20 (Fig. 4B). This difference in direct coordination preferences is consistent with Mg^{2+} being a "harder" metal than Mn^{2+} and thus prefers the "harder" phosphate oxygen ligand to the nucleobase nitrogen.

An interaction of metal ions with phosphates usually leads to stabilization of polynucleotide secondary and tertiary structures, whereas nucleobase binding or simultaneous nucleobase and phosphate binding by the same metal ion may result in destabilization and denaturation of the native polynucleotide structures (Kazakov 1996 and references therein). The destabilization effect is usually negligible if the concentrations of the metal ions having high affinity to the nucleobases is 10^3 times less than the concentrations of the "hard" monovalent ions in the same solutions (Eichhorn 1973). In such solutions, the role of the M^+ ions is to protect polynucleotides against the "borderline" and "soft" metal ions by creating an electrostatic barrier or to compete with them for the phosphate binding. These effects may both inhibit and promote the metal-induced cleavage of polynucleotides. Inhibition may occur because binding of the metal ion catalyst is prevented or the dynamic flexibility of the internucleotide bonds required for the cleavage reaction is decreased. Cleavage can be promoted, on the other hand, as a result of the formation of a specific binding site for catalytic or redox-active metal ions in polynucleotide structures.

5 Transesterification and Hydrolytic Cleavage of Nucleic Acids Catalyzed by Metal Ions

5.1 Efficacy of Cleavage of Nucleic Acids by Metal Ions

A large variety of metal ions catalyze the cleavage of RNA through the transesterification mechanism, and some of them also catalyze hydrolysis of cyclic phosphates (e.g., Pb^{2+}, Zn^{2+} and Cd^{2+}) and nucleoside phosphomonoesters (e.g., Pb^{2+}, La^{3+}, and Th^{3+}) (Kochetkov and Budovskii 1972). The most rapid cleavage of RNA in aqueous solutions by free metal ions is achieved by the action of rare metal ions (e.g., Eu^{3+}, La^{3+}, and Tb^{3+}), Pb^{2+}, and Zn^{2+} (Kochetkov and Budovskii 1972; Pan et al. 1993). Zn^{2+} is only about 4% as active as Pb^{2+} (Farkas 1968), but other catalytically active metal ions (e.g. Al^{3+}, Cd^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, or Mg^{2+}) are 1–2 orders of magnitude less active than Zn^{2+} (Butzow and Eichhorn 1965). Surprisingly, insoluble metal hydroxides, e.g. $Bi(OH)_3$ and $Al(OH)_3$, can also promote depolymerization of RNA at high temperature presumably via a heterogeneous reaction (Eichhorn 1973). As a rule, RNA cleavage has a maximum rate at pH values around the pK_as of the first metal-bound H_2O (see Table 1), i.e., when the hydroxo-metal species still carry positive charge (Polacek and Barta 1998). Besides hydroxyl anions, some other ligands may dramatically facilitate RNA cleavage by stabilizing high positive charge on the metal ion and the specific ligand-nucleic acid interactions (Bashkin and Jenkins 1994; Hurst et al. 1996; Linkletter and Chin 1995; Morrow 1996).

Some metal ions and metal complexes can directly cleave DNA via hydrolytic mechanisms (Basile et al. 1987; Franklin 2001; Ott and Kramer 1999; Williams et al. 1999). Hydrolysis of both NTPs and dNTPs is accelerated in the presence of M^{2+} ions especially at elevated temperatures (Bose et al. 1985; Cooperman 1976; Sigel et al. 1984; Utyanskaya et al. 1989). It has also been reported that Zr^{4+}, Th^{4+}, and Cu^{2+} ions are capable of hydrolyzing the pyrophosphate bridge selectively in 5′-capped RNAs without cleaving phosphodiester bonds (Baker 1993; Visscher and Schwartz 1992).

5.2 Possible Mechanisms of Metal Ion Catalysis

Due to the labile nature of nucleic acid-metal ion complexes and the rapid equilibrium between alternative structures of these complexes, it is difficult to experimentally identify metal binding modes in catalytically active structures. Although it is uncertain how metal ions catalyze phosphoryl transfer reactions in each case, there are putative mechanisms that can accommodate experimental observations. These possible catalytic roles of metal ions in the cleavage of phosphodiester bonds have been reviewed elsewhere (Brown et al. 1985; Cedergren et al. 1987; Cooperman 1976; Hendry and Sargeson 1990;

Kazakov 1996; Pan et al. 1993; Pyle 1993; Yarus 1993; Zhou and Taira 1998) and are discussed below briefly.

The cleavage reactions are the result of a phosphoryl transfer reaction of metaphosphate (PO_3^-) from one nucleophilic atom to another, presumably through a pentacoordinate trigonal bipyramidal transition state (see Fig. 6A, B). In these transition structures, there are several sites at which hydrated

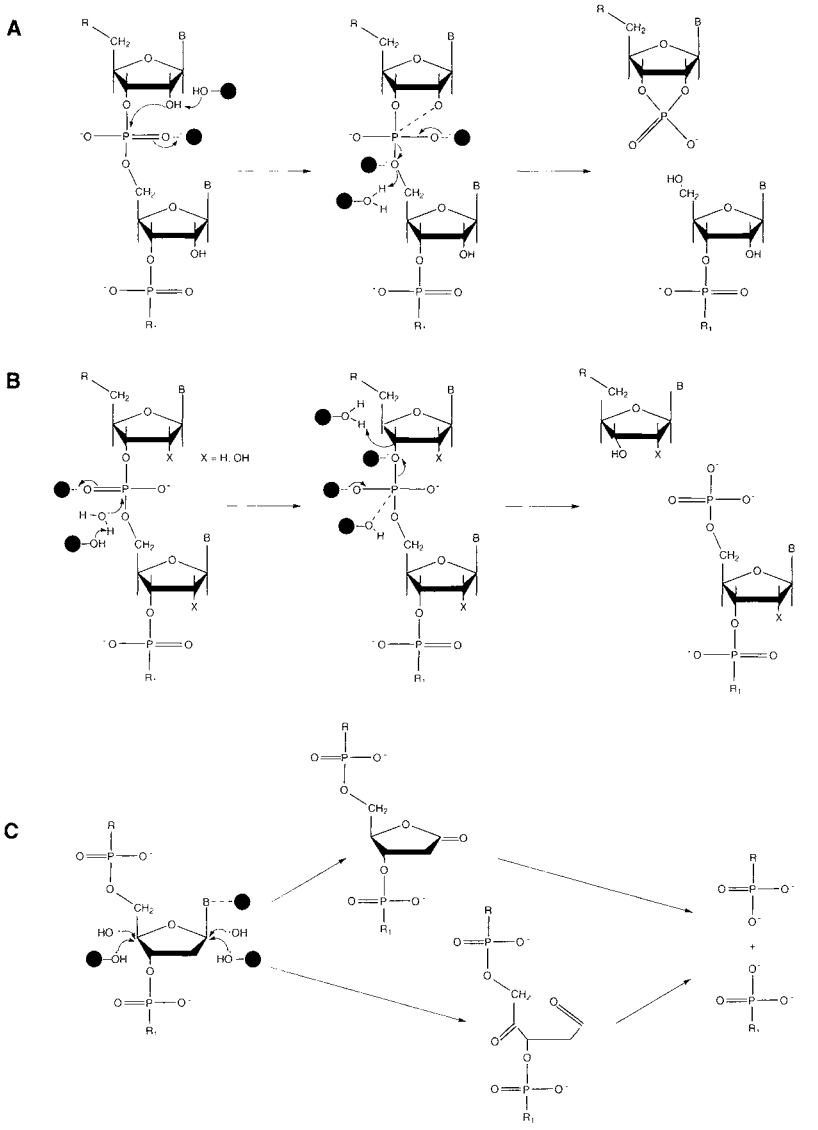

Fig. 6. Possible roles of hydrated metal ions (*filled circles*) in promoting phosphodiester bond cleavage through **A** transesterification, **B** hydrolysis, and **C** oxidation reactions involving free and metal-bound hydroxyl radicals. (**A** and **B** reprinted from Pyle 1993, with permission)

metal ions can interact: the phosphate moiety, the leaving groups and the entering groups. Generally, catalysis takes place when metal ions bind more tightly to the presumed transition state than to the ground state of reactants. Below, we discuss a variety of specific mechanisms for cleavage reactions that have been proposed in the literature.

One possible mechanism could involve bidentate binding of two oxygen atoms of a single phosphate to the metal ion, compressing the tetragonal O-P–O angle to the trigonal geometry necessary for transition to the bipyramidal intermediate, in which one apical position is free and, therefore, susceptible to the intramolecular (transesterification) or intermolecular (hydrolysis) nucleophilic attacks (Hendry and Sargeson 1990). By shielding the negative charge of the backbone phosphates, metal cations can favor the rearrangement of negatively charged oxygens of the phosphate group from normal tetrahedral geometry toward the bipyramidal transition state where the distance between the oxygen atoms is shorter and, therefore, repulsive charge interactions are stronger (Cooperman 1976).

Alternatively, the cleavage mechanism involves metal-ion catalysis by the interaction with the anionic leaving group and is an example of general acid catalysis (Cooperman 1976). Both electrostatic and coordinative binding of the metal ion to the leaving group in the transition state would increase the acidity of this group. This, in turn, would stabilize the development of electron density on the leaving group in the transition state and, thus, weaken the bond between the phosphorus and the oxygen of leaving group, accelerating the cleavage reaction.

Also, there is possible involvement of highly reactive metal-bound hydroxyl groups in cleavage mechanisms (Hendry and Sargeson 1990). The nucleophilicity of the bound hydroxide would be expected to be lower than free hydroxide, but higher than free water (Martin 1976). A metal ion-bound hydroxyl anion, oriented via appropriate H-bonding at the proper distance and orientation relative to the sugar 2′-OH group or outer-sphere H_2O molecule, would enhance the nucleophilicity of these two species via proton abstraction. The activated hydroxyl groups would be better able to perform a nucleophilic attack on the neighboring internucleotide P atom, forming either 2′,3′-cyclic phosphate and 5′-OH termini (Fig. 6A; Brown et al. 1985) or 3′-OH and 5′-phosphate ends at the cleavage site (Fig. 6B; Guerrier-Takada et al. 1986).

Another mechanism would result from direct (inner-sphere) binding to a phosphate oxygen, thus withdrawing electron density and enhancing the electrophilicity of the phosphorus atom (Cooperman 1976). This would make the phosphorus more susceptible to nucleophilic attack by the neighboring 2′-OH (transesterification) (Fig. 6A) or by an external nucleophile, e.g., a water molecule (hydrolysis) (Fig. 6B). However, such polarization might have little or no catalytic effect. This is because the increasing positive character of the phosphorus atom could be offset by a corresponding inhibition of leaving-

group expulsion. Also, the withdrawal of electron density from the oxygen bound to metal ion can be compensated by increased π-bonding between the phosphate O and P atoms (Cooperman 1976). The aqua-hydroxy complexes (H_2O–M–OH) can, in theory, transfer protons between the incoming nucleophile and leaving group attached to the internucleotide phosphate undergoing cleavage (Guerrier-Takada et al. 1986). Similar mechanisms could involve some other inner-sphere ligands, e.g., amines (Hendry and Sargeson 1990) or imidazole (Breslow and Huang 1991), which are capable of accepting or donating protons and can function similarly to metal ion-coordinated water molecules.

5.3 Nucleic Acid Structure and Specificity of Metal-Catalyzed Cleavage Reactions

Just as metal ions can interact with nucleic acids both specifically and non-specifically, metals can promote both specific and non-specific cleavage of nucleic acids. However, not every specifically bound metal ion can catalyze site-specific cleavage due to either insufficient proximity to or unfavorable spatial orientation between the metal ion and the relevant internucleotide bond.

The rate and specificity of the cleavage reactions varies markedly with the identity of both metal ions and nucleic acid structures as well as with the conditions of experiments. High temperature and high concentrations of the catalytic metal ions enhance the rate of the polynucleotide cleavage but decrease its specificity. For example, Mg^{2+} (Forster and Symons 1987) and Mn^{2+} (Pieken et al. 1991) ions at millimolar concentrations can produce a ladder at 95–100 °C by random cleavage of RNA molecules. In contrast, moderate conditions including short reaction time and low concentration of the metal ion catalysts favor specific cleavage of nucleic acids. In most cases, the specificity of cleavage reactions may be dramatically increased via optimization of the reaction conditions.

Numerous studies of metal-induced cleavage of RNA provide considerable insight into the relationship between nucleic acid structure and the specificity of the polynucleotide cleavage. A higher rate of phosphodiester bond cleavage promoted by metal ions is observed for polynucleotides than for short oligonucleotides (Butzow and Eichhorn 1971). In RNA oligomers, the nature of the adjacent phosphate group can affect the rate and primary position of metal-dependent cleavage of internal phosphodiester bonds. For example, the relative rates (shown in parentheses) of Zn^{2+}-dependent cleavage (marked by '↓') of short adenosine oligomers were found to decrease in the following order (Butzow and Eichhorn 1971): Ap↓A3′-p (125) >>Ap↓ApA (7) >Ap↓A2′-p (3) >Ap↓A2′,3′-cyclo-p (2.5) >ApAp↓A (2) >Ap↓A (1). The fact that the 3′ terminal phosphate can selectively enhance the rate of cleavage of

the neighboring internucleotide phosphodiester bond suggests that metal ions may cleave RNA both in an endonuclease and exonuclease-like fashion.

The nucleotide composition and the nature of the nearest-neighbor base of RNA molecules also affects the rate of metal-promoted cleavage (Eichhorn 1973). For example, the rates of metal ion-dependent cleavage of polyribonucleotides at pH 7 were found to decrease in the following order (Butzow and Eichhorn 1965; Farkas 1968): poly(A) ⩾poly(U) >poly(C) >poly(I) – for Pb^{2+}; poly(C) >poly(U) >poly(I) ~poly(C) for La^{3+}; and poly(U) ~poly(A) >bulk yeast RNA ~poly(C) ~poly(I) for Zn^{2+}. The reactivity of the dinucleotides toward Zn^{2+} ions decreases in the order of UpUp >ApAp >CpCp >GpGp, which is exactly opposite to the order of the affinity of the base residues for this metal ion (Ikenaga and Inoue 1974). The differences in reactivity found for the isomeric pairs of dinucleotides (ApGp >GpAp, CpAp >ApCp ~CpGp >GpCp, ApUp⩾UpAp and GpUp⩾UpGp) indicates that RNA cleavage by Zn^{2+} depends on both the base composition and sequence of the dinucleotides (Ikenaga and Inoue 1974). To explain this sequence dependence, it is necessary to consider the specific interactions (e.g., stacking, H-bonds) between adjacent bases which determine the local conformation of the ribose-phosphate backbone at the site of cleavage (Dock-Bregeon and Moras 1987). These interactions might be introduced, modified or diminished by the binding of metal ion(s). The zinc-dependent cleavage of polyribonucleotides is inhibited dramatically in the presence of metal species having a high affinity for nucleobases, such as Ag^{+} (Eichhorn 1973). RNA duplexes are more resistant to cleavage by metal ions than single-stranded RNAs (Ciesiolka 1999; Farkas 1968; Kolasa et al. 1993). One of the principal reasons for this could be that base pairing interactions stabilize the conformation of the dinucleoside phosphate moiety such that the phosphorus atom is moved away from the sugar 2′-OH group and rotation about the C3′-O bond is restricted, which can interfere with the conversion of the polynucleotide to a conformation suitable for the transesterification reaction (Kierzek 1992; Soukup and Breaker 1999; Tereshko et al. 2001).

Some metal ions bound to suitable "pockets" within the RNA tertiary structure can induce very specific cleavage of the RNA near the binding sites. Several metal ions, e.g., Eu^{3+} (Ciesiolka et al. 1989a,b; Marciniec et al. 1989a; Michalowski et al. 1996; Rordorf and Kearns 1976), Pb^{2+} (Brannvall et al. 2001; Brown et al. 1985; Ciesiolka et al. 1989, 1994; Dorner and Barta 1999; Kufel and Kirsebom 1998; Marciniec et al. 1989a; Matysiak et al. 1999; Olejniczak et al. 2002; Polacek and Barta 1998; Pan et al. 1994; Rordorf and Kearns 1976; Streicher et al. 1993, 1996; Sundaralingam et al. 1984; Winter et al. 1997; Zito et al. 1993), Zn^{2+} (Hertweck and Mueller 2001; Kazakov and Altman 1991; Rordorf and Kearns 1976), Fe^{2+} (Berens et al 1998; Vary and Vournakis 1984b), and Mn^{2+} (Hertweck and Mueller 2001; Kuo and Herrin 2000; Wrzesinski et al. 1995) can induce specific non-enzymatic cleavage of different RNAs at neutral pH. Other metals, such as Mg^{2+}, Ca^{2+}, Ba^{2+}, Sr^{2+}, and Cu^{2+}

need higher pH (8.5–9.5) to promote the specific cleavages of the RNA molecules effectively (Ciesiolka et al. 1999b; Kazakov and Altman 1991; Lafontaine et al. 1999; Marciniec et al. 1989a, b; Matsuo et al. 1995; Michalowski et al. 1996; Matysiak et al. 1999; Olejniczak et al. 2002; Polacek and Barta 1998; Rordorf and Kearns 1976; Streicher et al. 1996). Aside from ribozymes, the most well-known example of this phenomenon is the site-specific cleavage of $tRNA^{Phe}$ by Pb^{2+} ions (Brown et al. 1985; Sundaralingam et al. 1984). Only one of the three tightly bound Pb^{2+} ions observed in crystallographic studies provides effective cleavage whereas the other two are inactive, presumably because of an unfavorable structure of these Pb^{2+} sites (i.e., the distance and spatial orientation between the metal ion and the relevant internucleotide bond). The latter sites must also lack flexibility within the phosphodiester bond, restricting its ability to undergo conformational changes conducive to cleavage. A study of several different tRNAs indicates that the specificity and efficacy of lead-induced cleavage depend more on the conformation of the substrate than on its sequence (Ciesiolka 1999, and references therein).

6 Oxidative Cleavage of Nucleic Acids Induced by Metal Ions

6.1 Cleavage Reactions Promoted by Metal Ions in High Oxidation States

Some metal ions in high oxidation states can indirectly promote nucleic acid cleavage via modification of nucleobases, resulting in the destabilization of the N-glycosidic bond between the modified base and sugar residue. There are two distinct types of base modifications: (1) electrophilic attack of guanine residues, and (2) oxidation of pyrimidine nucleotides.

Complexes of platinum that are stable in high oxidation states such as those containing Pt^{4+} can act as alkylation agents. These complexes kinetically prefer binding at the N7 atom of guanine residues (Astashkina et al. 1988). A transfer of positive charge from the metal cation to the platinated guanine residue results in the formation of abasic sites in which DNA can be specifically cleaved via β-elimination (Astashkina et al. 1988). Interestingly, formation of the abasic sites is highly specific for single-stranded DNA and does not occur in duplexes. In contrast, the complexes of Pt^{2+}, which have substantially less electron acceptor capacity than Pt^{4+}, cannot promote the removal of guanine residues.

Amino complexes of Ru^{3+}/Ru^{4+} act similarly to their platinum relatives, Pt^{2+}/Pt^{4+}. Although coordination of $(NH_3)_5Ru^{3+}$ to N7 of guanine residues in DNA does not promote efficient depurination, after disproportionation of Ru^{3+}, the corresponding complex of Ru^{4+} is able to carry out this reaction (Clarke and Stubbs 1996). Thorp and coworkers have shown that oxo-Ru^{4+} complexes also can promote DNA cleavage by different mechanisms through

the C1′-sugar or C8-guanine atoms by oxygen-radical modification (Carter et al. 1996). Since Ru^{4+} species can be easily generated electrochemically, it may provide a convenient way to cleave single-stranded DNA or DNA duplexes with mismatched guanine bases at electrode surfaces.

The specific N7-guanine modification by tetramine-Ni^{2+} complexes in the presence of $KHSO_5$ results in Ni^{3+}-mediated oxidative alkylation and removal of the modified guanine residue (Burrows and Rokita 1998). A possible "template" role for nickel ions is to bridge guanine and oxidant species. This reaction is highly specific for single-stranded or mismatched regions in polynucleotides, and has been successfully used as a structural probe for both DNA and RNA molecules (Burrows and Rokita 1998).

DNA can also be specifically cleaved at thymidine residues after treatment with either MnO_4^- or OsO_4^{2-}, where the formal metal oxidation states are Mn^{7+} and Os^{6+}. These anionic metal species can oxidize the pyrimidine C5-C6 double bond, which results in formation of *cis*-5,6-dihydroxy-5,6-dihydro-pyrimidine. This is followed by opening of the pyrimidine ring and its subsequent removal from the polynucleotide chain (Kochetkov and Budovskii 1972). Permanganate anion can oxidize and remove guanine residues (but not adenine) in addition to pyrimidines while osmium tetraoxide acts much more specifically on pyrimidine residues with the following kinetic preferences (relative rate shown in parenthesis): T (45) >>U (4.5) >dU (2.8) >C (1.8) >dC (1) (Kochetkov and Budovskii 1972). Although these reagents can attack both DNA and RNA pyrimidine residues, the necessity for alkaline treatment to open the oxidized pyrimidine rings makes these reagents unsuitable for the specific cleavage of RNA.

The relatively bulky permanganate and osmium tetraoxide anions are very sensitive to secondary structure and react preferentially with pyrimidines in single stranded regions, single-base mismatches, bulges, loops, unstacked nucleobases in four-way junctions, curved $(A\text{-}T)_n$ tracts, B-Z and Z-Z junctions, H-DNA, and pre-melting of AT-rich sequences in supercoiled DNA (Bui et al 2002; Palecek 1992). This feature makes them very useful probes for detection of local distortions in DNA molecules under different conditions and upon binding of other molecules including small drugs and proteins.

6.2 Cleavage Reactions Involving Metal-Induced Oxygen Radicals

Metal ions that can exist in multiple oxidation states (e.g., $Cu^{+/2+}$, $Fe^{2+/3+}$, $Ni^{2+/3+}$, $Co^{2+/3+}$, $Cr^{2+/3+}$, $Ce^{3+/4+}$, $Ru^{3+/4+}$, $Pd^{2+/4+}$, and $Pt^{2+/4+}$) undergo redox reactions in the presence of oxidizing (e.g., H_2O_2) and/or reducing (e.g., ascorbic acid) agents (Burrows and Rokita 1998; Pogozelski and Tullius 1998). These reactions may occur either with or without generation of free oxygen radicals. Binding of these metal ions to chelating agents can inhibit the redox

activity of such metal ions because binding may preclude interactions of the chelated metal ions with both nucleic acid and oxidizing agents or because the redox process might be prevented through stabilization of one particular oxidation state of the metal ion. However, some chelating agents can preserve (e.g., EDTA) or even enhance (e.g., 1,10-phenanthroline, OP) metal ion redox activity (Burrows and Rokita 1998; Pogozelski and Tullius 1998). Their complexes, e.g., [Fe-EDTA] (Draganescu and Tullius 1996) and [Cu-OP] (Sigman et al. 1996) are widely used to probe nucleic acid structures and to elucidate details of site-specific recognition of nucleic acids by other molecules.

The most common mechanism of oxidative cleavage of nucleic acids involves the oxidation of transition metals such as Fe^{2+} or Cu^{+} in the presence of H_2O_2 and reducing agent (Fenton reaction; see Burrows and Rokita 1998 for details).

$$M^{n+} + H_2O_2 \rightarrow HO\text{-}M^{(n+1)+} + HO^-$$
$$HO\text{-}M^{(n+1)+} \rightarrow M^{(n+1)+} + HO\bullet$$

These reactions generate neutral hydroxyl radicals ($HO^{\bullet}$) that can modify and cleave nucleic acids in a sequence and secondary structure-independent manner. The generated hydroxyl radicals can either remain bound to the metal ion such as in the case of $Cu^{+/2+}$ or may be a diffusible species such as that produced by $Fe^{2+/3+}$ (Burrows and Rokita 1998).

Most reagents that cleave polynucleotides via metal-dependent redox mechanisms exhibit little preference for the degradation of DNA or RNA, and some do not discriminate between single- and double-stranded structures (Celander and Cech 1990; Pogozelski and Tullius 1998).

Metal-dependent redox-reactions can cause cleavage of polynucleotides both directly and indirectly via a modification that results in cleavage only after additional treatment (e.g., high temperature, treatment by alkali or amines) (Brosalina et al. 1988; Kazakov et al. 1988; Vary and Vournakis 1984b). These direct and indirect reactions may result in the site-specific cleavage of the polynucleotide chain near the sites of the metal ion or metal-complex binding. The proximity of the cleavage site to the site of metal binding depends on the nature of the oxygen radicals produced by metal ions and complexes. "Free" radicals such as $^{\bullet}OH$ can travel substantial distances – up to 50 Å (Joseph and Noller 2000) before attacking nucleic acid residues. In contrast, metal-induced radicals that remain bound to the metal ion (i.e., nondiffusible) can attack only residues located in spatial proximity and in favorable orientation relative to these bound radical species (Kazakov et al. 1988; Vary and Vournakis 1984b; Wang and Van Ness 1989). However, if the redox-active metal ion (or its complex) is not strongly bound (or tethered) to a particular site in polynucleotide chain, then cleavage occurs randomly. This is observed in the case of free $^{\bullet}OH$-radicals produced by [Fe-EDTA], which cleave at any site that is not sterically protected by polynucleotide tertiary structure or by

other molecules bound to the polynucleotide (see Latham and Cech 1989; Pogozelski and Tullius 1998 and references therein).

It was demonstrated that some redox-active metal ions could cleave polynucleotides very specifically, presumably at sites where these metal ions are strongly bound. These reactions involve metal-induced oxygen radicals. Such redox reactions induced by Cu^{2+} ions were reported for the site-specific cleavage of single-stranded DNA (Kazakov et al. 1988), supercoiled DNA duplex (Wang and Van Ness 1989), and M1 RNA, the catalytic subunit of *E. coli* RNase P (Kazakov and Altman 1991). Vournakis and co-workers pioneered the use of Fe^{2+} ions to detect metal binding sites in naturally occurring RNA molecules (Vary and Vournakis 1984b). They showed that low micromolar concentrations of ferrous ammonium sulfate in the presence of DTT cleaves $tRNA^{Phe}$ only at nucleotide positions G18 and G19 within the dihydrouracil loop and that these residues are identical to the bases involved in Mg^{2+} coordination observed in the crystal structure. However, no iron-catalyzed cleavages were observed at another known strong Mg^{2+} binding site. These results may indicate that there are different binding modes for the different metal ions. At higher concentrations of Fe^{2+} ions, more cleavages could occur although they would generally be less specific.

Berens and colleagues demonstrated that the location of multiple cleavages of the catalytic core of group I intron RNA induced by the optimized Fe^{2+}-H_2O_2-ascorbate cocktail correlates well with the divalent metal ions binding sites observed by x-ray crystallography (Berens et al. 1998). Millimolar concentrations of Fe^{2+} ions in the presence of oxidizing and reducing agents have also been used as cationic probes to detect the surface accessibility of RNA molecules (Zhong and Kallenbach 1994). Interestingly, iron-specific cleavage sites in RNA molecules may be observed even in the presence of the chelating agents such as EDTA-derivatives if the chelating agent is not in at least two-fold excess over Fe^{2+} (Wang and Cech 1992).

Many RNAs and RNA-protein complexes require Mg^{2+} ions for their formation and structural stability, and so divalent ions must be included in solutions to map their interactions with each other. While in one study it was observed that site-specific cleavage of DNA using oligodeoxyribonucleotides with [Fe-EDTA] attached to a single position was inhibited by inclusion of $MgCl_2$ in the reaction mixture (Moser and Dervan 1987), other researchers have not found an overall inhibition of cleavage when comparing cleavage patterns in the presence and absence of Mg^{2+} ions (Celander and Cech 1990; Latham and Cech 1989; Zhong and Kallenbach 1994). Rather, they have reported site-specific protections upon addition of $MgCl_2$ that correlate with the higher-order folding of RNA molecules in the presence of divalent cations, and one group even observed enhancements in cleavage, which are attributed to conformational rearrangements of the RNA in the presence of Mg^{2+} ions (Zhong and Kallenbach 1994). It should be noted that these experiments were carried out using free [Fe-EDTA] rather than these complexes tethered to an

oligonucleotide probe. Nevertheless, RNA-RNA and RNA-protein interactions are mapped routinely and successfully in buffer solutions that typically include 1 mM to 20 mM $MgCl_2$ in hydroxyl radical footprinting experiments with free [Fe-EDTA] (Powers and Noller 1995) and by site-directed probing experiments where [Fe-EDTA] is tethered to a unique location in a complex (Joseph and Noller 2000).

7 Probing Metal Binding Sites in RNA by Metal-Induced Cleavage

Because metal ions can play a crucial role in the folding and stabilization of an RNA structure, which, in turn, affects its function, it is difficult to separate the structural and functional role of metal ions. As discussed above, certain metal ions can induce efficient and structure-specific cleavages of RNA molecules through different mechanisms. This allows, in principle, the use of such metal ions as probes of nucleic acid structure.

Pb^{2+} ions are used routinely along with some other chemical and enzymatic probes to investigate RNA structure (Ciesiolka 1999; Lindell et al. 2002 and references therein). In optimized conditions, lead-induced cleavages preferentially occur in single-stranded regions of RNA, which include loops, bulges, and single-base mismatches while stable RNA stems and highly structured tetraloops are partially resistant. However, cleavages may also occur in destabilized base-paired regions. In general, the conformational flexibility of an internucleotide bond determines its susceptibility to lead-catalyzed cleavage. Therefore, lead-cleavage can be a sensitive method to detect RNA conformational changes upon interaction with other molecules. Similar to Pb^{2+}, lanthanide metal ions (e.g., Eu^{3+}, Tb^{3+}) have been used as a probe of RNA folding as well as to map metal binding sites in RNA molecules (Ciesiolka 1999; Dorner and Barta 1999; Gast et al. 1996; Hargittai and Musier-Forsyth 2000; Sigel et al. 2000; Walter et al. 2000).

A major challenge in studying RNA structure is to locate the binding sites for functionally important divalent metal ions such as Mg^{2+} and Ca^{2+}. Since site-specific RNA cleavage may occur where a specific metal is bound, it opens up the possibility of mapping such sites in the RNA tertiary structure. The metal-dependent cleavage of RNA in a particular site does not directly implicate, however, the involvement of these nucleotides in the binding with this ion. Moreover, some specifically bound metal ions may not be able to induce cleavage at their binding sites because of the restricted flexibility or steric accessibility of the surrounding polynucleotide backbone. There are three general approaches based on metal-induced cleavage of RNA to identify metal binding sites.

In the first approach, the location of strongly bound Mg^{2+} and Ca^{2+} ions can be revealed by inducing cleavage at pH 9–9.5, which dramatically increases

the ability of these metals to catalyze RNA cleavage than at neutral pH (Ciesiolka et al. 1989; Dorner and Barta 1999; Kazakov and Altman 1991; Lafontaine et al. 1999; Streicher et al. 1996). One potential drawback of this approach is the high, non-physiological pH that could alter the stability and, thus, the native RNA conformation. However, it has been demonstrated that the functionally essential structures of tRNA (Ciesiolka et al. 1989), the catalytic M1 RNA subunit of RNase P (Kazakov and Altman 1991), and ribosomal RNA (Polacek and Barta 1998) have not been affected at the high pH.

The second approach, which has been used in the studies of many RNAs, is based on the assumption that certain metal ions (e.g., Fe^{2+} and Pb^{2+}) can replace Mg^{2+} at the same binding sites and induce RNA cleavage upon binding (Berens et al. 1998; Ciesiolka 1999; Polacek and Barta 1998; Streicher et al. 1996). A serious problem with this approach is the fact that different metals most probably will have different binding patterns. For example, although Mn^{2+} has been thought to mimic Mg^{2+}, their binding coordination preferences are different. In $tRNA^{Phe}$ crystals, only one of three 'strong' Mg^{2+} was substitutable by Mn^{2+} (Jack et al. 1977; Teeter et al. 1980). Moreover, it should be noted that even though the metals both occupied the same 'binding pocket', their RNA coordination partners were different (Fig. 4). To overcome this uncertainty, Kazakov and Altman (1991) used a functional test to determine that specific cleavage sites in M1 RNA induced by different metal ions correspond to specific magnesium binding sites. It was demonstrated that there were different cleavage patterns for metal ions that can cleave M1 RNA but cannot support its catalytic activity and for metal ions that can do both.

The third technique is based on the same assumption of substitutable metal binding sites but uses an opposite approach. It takes advantage of the fact that metal-induced cleavage of RNA (e.g., by Pb^{2+} or Zn^{2+}) can be inhibited when the reaction is chased with increasing concentrations of Mg^{2+} due to substitution of the cleaving metal at the particular sites with Mg^{2+} (Ciesiolka 1999; Labuda et al. 1985; Streicher et al. 1996). A potential problem with interpreting the results of such an experiment is that Mg^{2+} binding can alter and stabilize a different conformation of the RNA molecules (Latham and Cech 1990; Markley et al. 2001; Vary and Vournakis 1984a), which may also result in a different pattern of metal-induced cleavage.

Currently, high-resolution x-ray crystallography is the best method for mapping metal ion binding sites in RNA, whereas metal-induced cleavage techniques are useful tools to confirm published metal ion binding sites. When no structural information is available for a particular RNA molecule, some useful information about the binding sites of functionally important metal ions can be obtained by the metal-cleavage methods described above. However, careful comparison of results obtained from multiple approaches using several different metal ions is recommended.

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