



Structural and catalytic roles for divalent magnesium in nucleic acid biochemistry

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Introduction

There is increasing interest in the role of alkali and alkaline earth metals in defining nucleic acid structure and function. While monovalent ions such as Na⁺ and K⁺ tend to act as bulk electrolytes that stabilize surface charge, divalent magnesium binds with higher affinity, often serving a specific structural or catalytic role. The dissociation constants for random and non-specifically bound Mg²⁺ ions are on the order of 1–10 mM, while specific binding interactions lie in the range of ≤ 1 mM. Magnesium ion tends to adopt octahedral coordination, with a marked preference for oxygen-donor ligands or water, and binding to nucleic acids results either from nonspecific electrostatic attractions between the negatively charged phosphate groups and the metal cation, or from specific ligand coordination. Coordination can be either direct (inner-sphere) to base oxygens, nitrogen, ribose sugars or phosphates, or mediated by waters of hydration (outer-sphere). Outer sphere complex formation appears to be the most common binding mode to oligonucleotides, with Mg²⁺ adopting a regular octahedral hydration sphere with a Mg²⁺-H₂O bond distance of ~ 2.0 Å. As a result of their similar electron densities it can be difficult to crystallographically distinguish between Mg²⁺ and the isostructural Na⁺ ion, or to demonstrate direct coordination of the metal ion to the oligonucleotide. This requires X-ray crystallographic data with a resolution greater than 1.5 Å. Beyond this limit, Mg²⁺ and Na⁺ ions cannot be differentiated and each may be mistaken for solvent molecules. General aspects of oligonucleotide-Mg complexes have been reviewed previously and interested readers are directed to a prior review article (Black *et al.* 1994). Herein our

focus will be on other functional roles for magnesium complexes of nucleic acids. First, and primarily, to neutralize the negative charges of phosphates, either electrostatically or by forming hydrogen bonding networks from waters of solvation. Second, Mg²⁺ may serve by lowering the pK_a of coordinated water thereby facilitating phosphate ester hydrolysis. Third, as described below, it is quite clear that many drug molecules interact with DNA in a specific and Mg²⁺-dependent manner. An understanding of the latter may aid in the design of other novel DNA binding drugs.

Mg²⁺ complexes with DNA

Crystallographic characterization of the B-DNA sequence, CGCGAATTCGCG, has been reported at ~ 1.5 Å, with one Mg²⁺ per duplex (Berger *et al.* 1998). Improved structures of this B-form DNA in the presence of Mg²⁺ and Ca²⁺ have been recently solved with atomic resolutions of 1.1 Å and 1.3 Å, respectively (Minasov *et al.* 1999) (Figure 1). Duplexes in the crystal lattice are surrounded by 13 Mg²⁺ and 11 Ca²⁺, respectively. Each cation generates a different DNA crystal lattice and stabilizes different end-to-end overlaps and lateral contacts between duplexes. Mg²⁺ ion allows the two outermost base pairs at either end to interact laterally via minor groove H-bonds, turning the 12-mer into an effective 10-mer. A Mg²⁺ coordinates in the major groove contributing to the kinking of the duplex at one end, while the Ca²⁺ ions reside in the minor groove, coordinating to the bases through their hydration shells. Mg²⁺ ions were also found at the periphery of the minor groove, bridging phosphate groups from opposite strands and contacting the groove at one border of the A-tract.

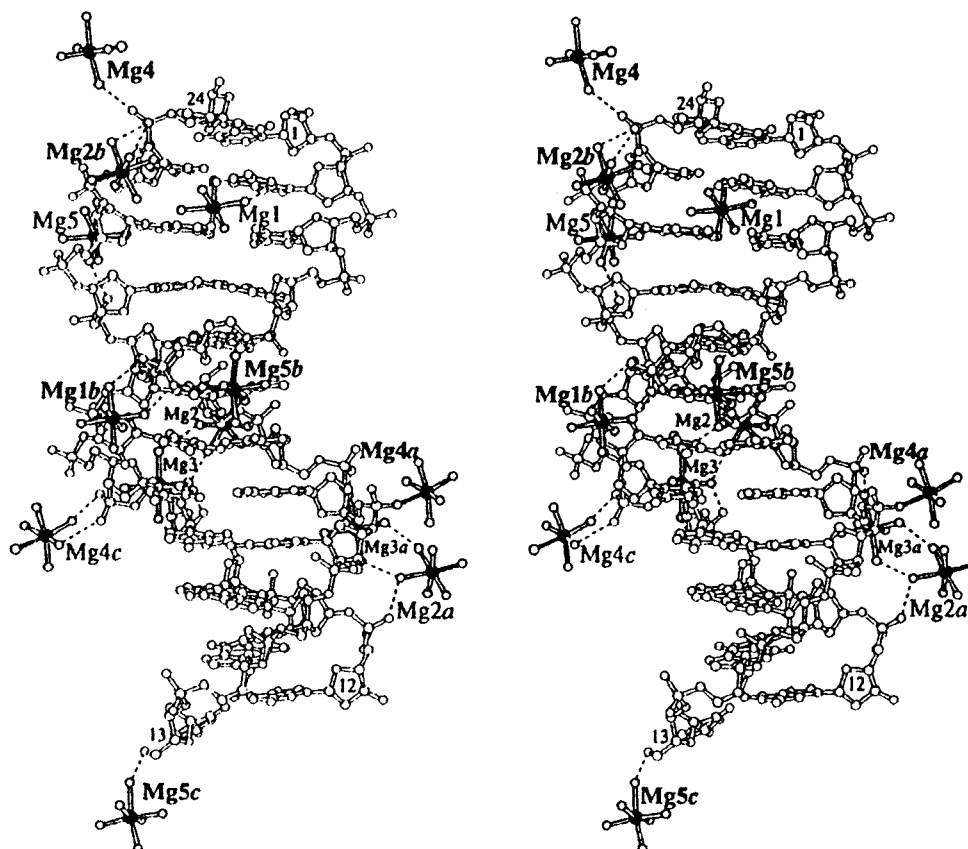


Fig. 1. Stereoview of the B-DNA, CGCGAATTCGCG, in presence of Mg^{2+} . Filled bonds indicate direct ion-DNA contacts, broken lines indicate hydrogen bonds. Adapted from Minasov *et al.* (1999).

Several other Mg^{2+} ions bridge oxygen atoms of bases via coordinated water molecules, forming an extensive network of H-bonds. Only two direct Mg-phosphate oxygen bonds have been identified with a Mg-O distance of ~ 2.1 Å. A comparison of Mg^{2+} and Ca^{2+} coordination modes reveals some important distinctions, with the cations residing in the major and minor grooves, respectively. Further, calcium ions are more likely to form inner-sphere complexes, consistent with the lower hydration enthalpy of Ca^{2+} relative to Mg^{2+} (Cowan 1995). In both cases, the divalent ions mediate contacts between multiple strands. Thus it is possible that Mg^{2+} ions mediate specific contacts between DNA and small molecules (groove binding drugs) or proteins.

Sequence specific binding and groove-specific bending of B-DNA by Mg^{2+} and Ca^{2+} have been observed by crystallography (Chiu & Dickerson 2000). The 1 Å resolution structures of the decamers CCAACGTTGG and CCAGCGCTGG reveal binding

of Mg^{2+} and Ca^{2+} to the major and minor grooves of DNA, respectively, as well as non-specific binding to backbone phosphate oxygen atoms. Minor groove binding involves H-bonds interactions between cross-strand DNA base atoms of adjacent base pairs and the metal-bound water ligands. In the major groove, the hydrated metal ion H-bonds with O and N atoms from adjacent bases.

Divalent Mg interacts with DNA in a more specific manner, since waters of solvation can both donate and accept H-bonds to base atoms. Mg^{2+} is drawn electrostatically to both major and minor grooves of DNA, as well as to the sugar-phosphate backbone. At ds-GGCC sequences, binding of magnesium to the major groove GG residues, and to the minor groove at the GC step, results in a bending of the helix toward the major groove. In addition to having higher affinity for DNA, Ca^{2+} binding to DNA results in greater DNA bending and thermal stabilization than Mg^{2+} .

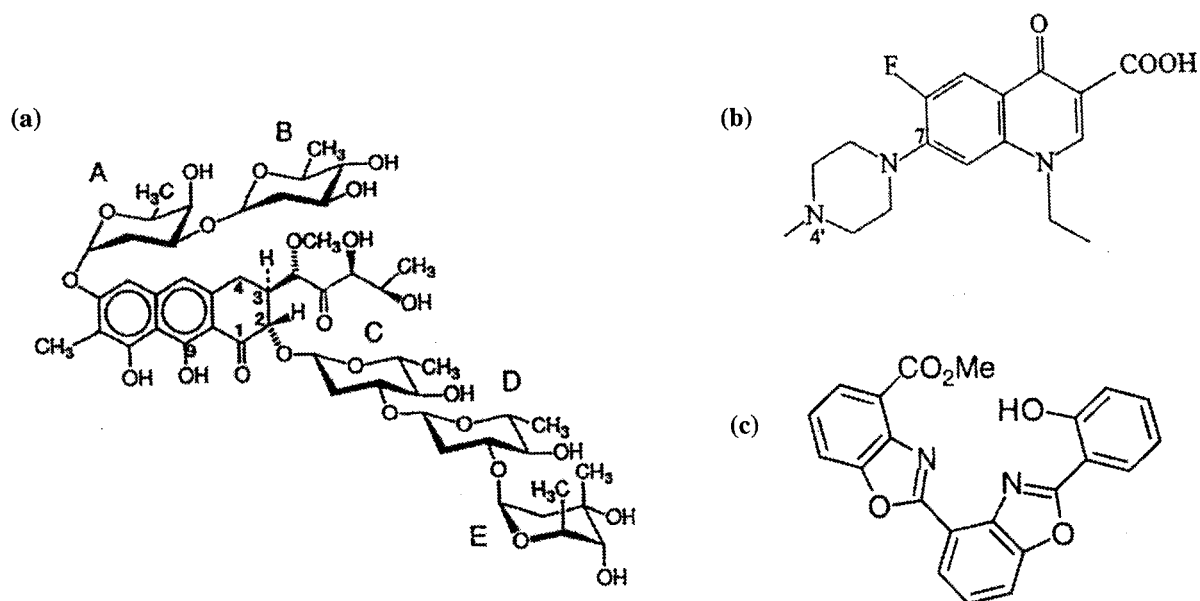


Fig. 2. Chemical structures of (a) mithramycin; (b) pefloxacin and (c) UK-1.

Drug-Mg²⁺-DNA ternary complexes

Mithramycin (MTC) (Figure 2) is a member of the aureolic acid group of antitumor antibiotics. These groups of molecules are characterized by attachment of two pyranose sugar chains and an aliphatic side-chain to a central aureolic acid core. Huang *et al.* (1995) have provided evidence that Mg²⁺ promotes the selective binding of MTC to cognate DNA sequence (5'-XXGCXX-3') by a coupled mechanism requiring local conformation changes in the DNA backbone and a structural rearrangement of the MTC dimer. Molecular modeling suggested that sequence selectivity arises from preferential coordination of Mg²⁺ to d(GpC) domains in the minor groove of Z-type DNA. The authors also found that Mg²⁺ coordinates to both, bridges MTC and DNA and stabilizes a local Z-type conformation, which opens up binding pockets on the DNA for the sugar chains. Additionally, Mg²⁺ was also found to realign and stabilize the MTC dimer in a manner that optimizes the position of both the aureolic acid core and the sugar chains for DNA binding.

Synthetic molecules such as quinobenzoxazine and derivatives have been shown to possess antineoplastic/anticancer activity. This activity is probably due to their interactions with topoisomerase-II, and the DNA binding properties of these molecules parallels their cytotoxic effects (Fan *et al.* 1995). *In vitro* studies

have shown that the antineoplastic activity is dependent upon the presence of divalent metal ions such as Mg²⁺. Fan *et al.* have studied the stoichiometry of the binary Mg-drug complexes and the ternary complex between the drug, Mg and DNA (Fan *et al.* 1995). These studies indicate that a 2:2 drug:Mg²⁺ complex forms a heterodimer complex with respect to DNA in which one drug molecule is intercalated into DNA and the second drug molecule is externally bound, held to the first by two Mg²⁺ ions that are also bound to the DNA phosphate backbone. Based on these studies the authors have proposed several new paradigms to better understand the inhibitory effects of both this family of molecules, and the mechanism of other antibacterial quinolone (Norfloxacin)-Mg²⁺-DNA gyrase complexes. The association constant for binding of the quinobenzoxazine drug A-62176 to poly[d(GC)₂] in the presence of 10 mM Mg²⁺ was determined to be $6.1 \times 10^5 \text{ M}^{-1}$. No binding was observed in the absence of Mg²⁺. This dependence on divalent metal with linear ds DNA is to be contrasted to the antibacterial quinolones, which only bind to supercoiled circular DNA, and not linear ds DNA, in the presence of Mg²⁺. These studies clearly indicate the importance of the ternary complex formed between the drug, Mg²⁺, and the DNA phosphate backbone.

Previously Palu *et al.* have shown, by use of a variety of analytical and spectroscopic techniques that norfloxacin binds to plasmid DNA (Palu *et al.* 1992).

The amount of norfloxacin bound to DNA was modulated by the concentration of Mg^{2+} . No interaction was evident in the absence of the divalent metal ion or in the presence of excess metal ion, and optimal binding was observed with 1–2 mM Mg^{2+} . The data suggested the following complexes: binary complex between the drug and Mg^{2+} , binary complex between the DNA and Mg^{2+} , and a ternary complex between the drug, Mg^{2+} and DNA. The Mg^{2+} ions act as bridges between the phosphate backbone and the carbonyl and carboxyl moieties of norfloxacin.

The interaction of a number of novel 6-substituted quinolone derivatives with DNA in the presence and absence of Mg^{2+} has been evaluated using fluorometric techniques (Sissi *et al.* 1998). All the compounds tested showed a dramatic modification of the fluorescence emission quantum yield upon addition of Mg^{2+} , characteristic of complex formation with the metal ion. Also, binding of the drug to ss-DNA had an absolute requirement for Mg^{2+} and was dependent on an optimal concentration of the divalent metal ion (0.5–15 mM). Higher concentrations of Mg^{2+} (i.e., 20–30 mM) also affected binding, indicating that the concentration of metal ion played a critical role in the affinity of quinolone to ss DNA. Additionally, the authors found that the modified quinolones had a preference for binding to ss DNA over ds B-form DNA. As each of these modified drugs have carbonyl and carboxyl groups, it is reasonable to assume that metal coordination to these sites influences their binding properties to DNA.

Fluoroquinolones, such as pefloxacin (Figure 2), are antimicrobial agents with excellent activity against several bacteria. However, their efficacy seems to decrease in the presence of various metal ions. For a better understanding of the interaction of pefloxacin with DNA, Lecomte & Chenon have performed NMR investigations under conditions of low drug to high Mg^{2+} concentrations (Lecomte & Chenon 1996). C-13 and F-19 NMR investigations of pefloxacin/ Mg^{2+} /DNA interactions show that the first binding sites are the carbonyl and carboxyl groups, and then the N-4' piperazinyl atom. The apparent affinity constants were found to be greater for Mg^{2+} compared to Ca^{2+} . Additionally, under higher drug concentrations, a 2:2 complex is identified that enhances the stacking of pefloxacin. Other studies have shown that Mg^{2+} -complexes of these drugs are involved in binding to DNA (Maxwell 1992; Bazile-Pham Khac & Moreau 1994). The interactions between pefloxacin, Mg^{2+} and DNA have also

been investigated with surface-enhanced Raman spectroscopy (Lecomte & Baron 1997). F-19 NMR was used to evaluate pefloxacin binding to DNA in the presence and absence of Mg^{2+} . In the absence of Mg^{2+} , pefloxacin binds poorly to DNA and preferentially to ss rather than ds DNA (Lecomte *et al.* 1998).

A novel bis(benzoxazole) metabolite of *Streptomyces* species 517-02, UK-1 (Figure 2) is cytotoxic to a variety of cancer cell lines. UK-1 has been found to bind different metal ions (especially Zn^{2+} and Mg^{2+}) forming stable 1:1 complexes. The 2-(2'-hydroxyphenyl)benzoxazole moiety present in UK-1 is analogous to the coordination motif found in microbial siderophores, and in other synthetic metal chelators. The involvement of the phenolate ligand in metal chelation is confirmed by spectroscopic analysis. Both Mg^{2+} and Zn^{2+} bind to UK-1 with sub-micromolar affinities, with ferric ion about one order of magnitude lower and negligible binding from Ca^{2+} and alkali metal ions. Given the available intracellular concentrations of these ions, divalent magnesium is the most likely natural cofactor. In the presence of Mg^{2+} , UK-1 binds to ds DNA 10 times more tightly than in absence of Mg^{2+} (Reynolds *et al.* 1999). Like other antitumor quinobenzoxazines, which also bind Mg^{2+} and bind to DNA in a magnesium dependent manner, UK-1 was found to be an inhibitor of human topoisomerase II, although the issue of natural cellular targets responsible for UK-1 selective cytotoxicity were not addressed.

Role of Mg^{2+} in catalytic polynucleotides – Ribozymes

The discovery that RNA can perform enzyme-like catalysis in cells has resulted in a new field; identifying and characterizing novel catalytic polynucleotides using *in vitro* selection. In addition to challenging the uniqueness of proteins as biological catalysts, both RNA and DNA polynucleotides, ribozymes and deoxyribozymes, respectively, are of fundamental interest. To explain these catalytic properties, several authors have invoked metal ion induced reactions and have pointed out the critical role served by divalent metal ions, especially Mg^{2+} . Divalent metal cations help fold RNA into a well-defined structure and assist in catalysis by functioning either as a Lewis acid, or when coordinated with water, as a general acid base. In the following section we will summarize

the role of Mg^{2+} as a cofactor for various catalytic polynucleotides as characterized by a variety of spectroscopic, analytical and biochemical experiments.

Ribozymes are RNA molecules that adopt three-dimensional structures that allow them to catalyze a variety of chemically important reactions, including, but not restricted to phosphate ester hydrolysis, amide bond formation, ligation etc. One important feature that has been recognized in almost all classes of ribozymes is their exclusive requirement for divalent metal ions. There are at least two functions that metal ions can play in ribozymes: namely, catalysis and structural stabilization of RNA. The RNA molecule is a polyanion in which charge repulsion has to be neutralized between interacting strands. Metal ions bind to RNA by interacting with nearby functional groups, bridging distinct strands and stabilizing folded pockets. Several researchers have discovered the importance of Mg^{2+} in promoting catalytic activity for a variety of ribozymes, and, by analogy to protein enzymes, metal ions are now known to deprotonate a nucleophile, activate an electrophile, stabilize a transition state, or protonate a leaving group. Since metal ions are critical for the correct folding of RNA, the mere requirement of metal ions for RNA to act as a catalyst does not substantiate its involvement in the chemical mechanism. A classical role for a metal ion cofactor is involvement as a general base, in which the conjugate base of the metal hydroxide can deprotonate the active site hydroxyl group (e.g., 2'-OH in RNA). Alternatively, the metal can bind to the active site hydroxyl group, lowering its pK_a , permitting deprotonation and attack by the metal-oxyanion. Additionally, the divalent metal can stabilize the developing negative charge during the trigonal-bipyramidal transition state that is common in phosphate ester transesterification reactions (Figure 3). External transesterification reactions are those reactions that involve nucleophilic attack from outside of the cleavage pocket. This is a strategy employed by known 'large' ribozymes such as group I and II introns and the RNA subunit of RNase P. Each of these ribozymes activates an exogenous nucleophile, including water or nucleotide hydroxy groups, to hydrolyze the phosphate ester backbone and generate a 3'-OH and 5'-phosphate. On the other hand, internal transesterification reactions involve the 2'-OH groups in the active site. Nucleophilic attack at the phosphorous center yields a penta-coordinate transition state or intermediate. This breaks into two fragments, a 2',3'-cyclic intermediate and a 5'-hydroxyl product. The metal ion can play various roles in the

above steps, especially in deprotonation of the 2'-OH, coordination to the nonbridging phosphate oxygen, and neutralization of the charge on the 5'-leaving group.

External transesterification

Involvement of Mg^{2+} in Tetrahymena ribozyme catalyzed reactions: A double metal ion mechanism

Several non-coding DNA (introns) interrupt various coding DNA (exons) in eukaryotic genes. RNA polymerase transcribes the DNA to give large precursor RNA molecules that have to be processed or spliced to give smaller functional RNA molecules. In many cases, RNA itself carries out this splicing reaction, and constitutes a class of RNA catalysts termed Group I intron ribozymes. The well-studied *Tetrahymena* ribozyme is derived from a self-splicing group I intron and is a prototypical metalloenzyme because it requires either Mg^{2+} or Mn^{2+} for activity. The *Tetrahymena* self-splicing reaction consists of two consecutive phospho-transesterification reactions, the first of which involves a 5'-splice site cleavage and the second step is exon ligation. In the first step (splicing process), the ribozyme employs the 3'-hydroxyl group of an external guanosine nucleoside or nucleotide cofactor as a nucleophile. Nucleophilic attack of the target phosphodiester by the 3'-hydroxyl group of the external guanosine proceeds in a S_N^2 type reaction, with an inversion of configuration around the phosphorus atom (McSwiggen & Cech 1989; Rajagopal *et al.* 1989). To identify the Mg^{2+} binding sites that are required both for folding and the later steps of splicing reaction, phosphorothioate interference techniques were utilized. The phosphorothioate analogs contain a sulfur substituent in place of the pro- R_p or pro- S_p phosphate oxygens. These modified nucleotides are known to perturb hydrogen bonding and coordination of Mg^{2+} . Reduction of cleavage rates in the presence of the thioate derivatives and Mg^{2+} (thio-effect) and the observation that Mn^{2+} can promote the reaction as well as the unmodified ribozyme (manganese rescue experiments) have been taken as evidence that support the direct inner-sphere coordination of the oxygen atom in question with a metal ion. This phenomenon can be explained by the hard/soft acid/base (HSAB) rule, wherein a hard acid such as Mg^{2+} preferentially binds with oxygen over sulfur, while softer metal ions such as Mn^{2+} can bind to the softer sulfur atom. In the *Tetrahymena* intron, several different steps have been identified using the above phosphorothioate in-

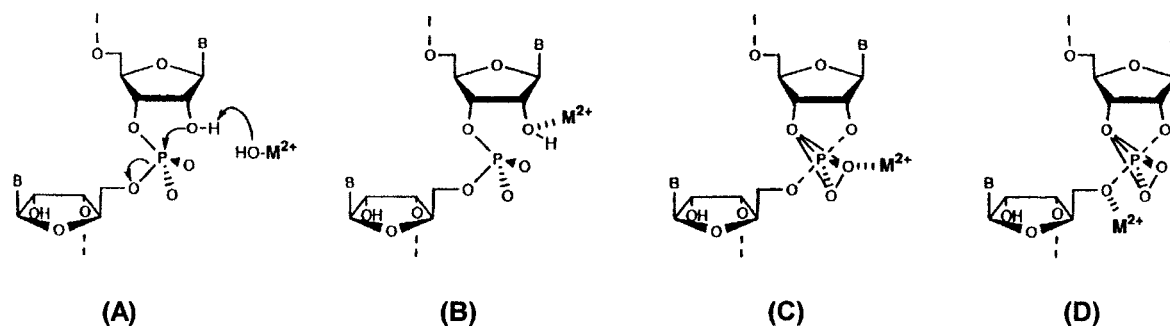


Fig. 3. Possible roles for metal catalyzed transesterification reaction or hydrolysis. (A) metal hydroxide as general base; (B) metal stabilization of hydroxyl (or water); (C) metal stabilization of transition state and (D) metal stabilization of leaving group. Adapted from Kuimelis & McLaughlin (1998).

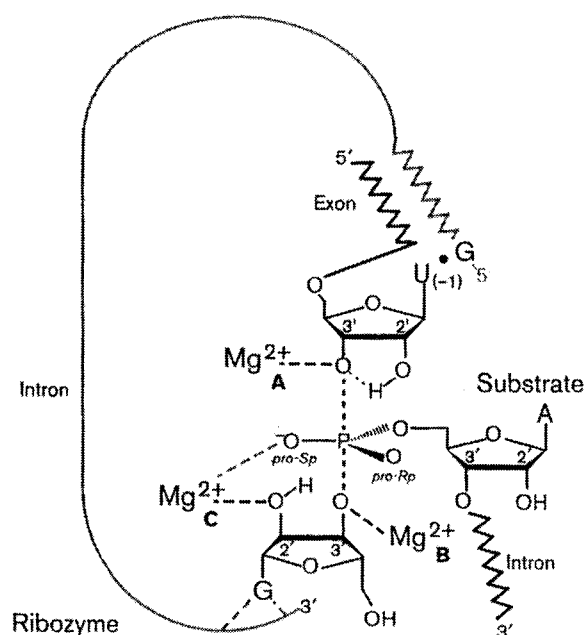


Fig. 4. The transition-state during the splicing reaction mediated by *Tetrahymena* group I intron, showing three metal ion binding sites. Adapted from Yoshida *et al.* (2000).

interference experiments and include: (1) 3'-splice site hydrolysis; (2) guanosine addition; (3) first step of splicing; (4) reverse of the first step of splicing; and (5) folding of the P4-P6 domain (The core of group I intron is composed of two helical domains, P4-P6 and P3-P9. These form a pocket to bind the substrate helix P1 that contains the 5'-splice site).

The effects of divalent metal ions on individual reaction steps has been determined for a Group I ribozyme (McConnell *et al.* 1997). The data provide evidence that Mg^{2+} interacts at four different sites. Mg^{2+} binding to site one is necessary for catalysis

and interaction with the 3'-hydroxyl group of U(-1). The metal ion in site two is proposed to interact with the 3'-OH group of the nucleotide bound to the G-site. The third site facilitates substrate association, while Mg^{2+} binding to site four promotes docking and stabilization of the substrate (Figure 4). Thus, Mg^{2+} ion serves critical roles in Group I intron ribozyme catalysis; first, as a counter ion to assist in the proper folding of the RNA into a specific three-dimensional structure. Second, as a means of forming a substrate helix and in assisting the binding of the helix by the ribozyme for the ensuing reaction. Cech's group has also produced evidence for a second catalytic Mg^{2+} ion in group I introns (Weinstein *et al.* 1997). They argue that the metal ion stabilizing the leaving group during exon ligation would be interacting with the nucleophile (3'-OH of G) in the 5'-splice reaction. Conversely, the metal ion stabilizing the leaving group in the splice reaction must be interacting with the nucleophile in the ligation reaction. Kinetic evidence also seems to support a model for the involvement of two metal ions during the chemical step.

It has been proposed that two divalent ions are required to catalyze the removal of intervening sequences of pre-messenger RNA by spliceosome ribonucleoprotein in two sequential steps. The metal ions, especially Mg^{2+} , are thought to activate the 2'- or 3'-OH for nucleophilic attack and to stabilize the 3'-oxanion leaving groups by direct coordination. Work from Piccirilli's group using 3'-sulfur substitution at the 5'-splice site indicates that interaction between the Mg^{2+} ion and the leaving group is essential for the first step of catalysis to occur (Sontheimer *et al.* 1997). This step is in parallel to the catalytic sequence followed by *Tetrahymena* ribozyme. However, in contrast, substituting a 3'-sulfur at the distinct 3'-splice

site provides no evidence for metal ion binding to this other site. The spliceosome may stabilize the leaving group at the 3'-splice site by some means other than metal binding. For example, this could occur by general acid catalysis, hydrogen bonding through a Mg bound water, other RNA residues with perturbed pK_a 's, or by use of a spliceosomal protein side chain. The difference in metal dependence suggests that the two steps of splicing proceed by different mechanisms and therefore are in distinct active sites.

RNase P ribozyme and a two-Mg²⁺ ion mechanism

The RNA subunit of bacterial ribonuclease P specifically cleaves precursor sequences from the 5'-ends of pre-tRNA molecules to produce mature tRNAs. This unique ribonucleoprotein is catalytically dependent on its RNA subunit for its *in vivo* activity. The protein is necessary for activity, but under high Mg²⁺ and monovalent salt concentrations the RNA portion itself is sufficient for catalysis. RNase P catalyzes the hydrolysis of phosphate esters rather than transesterification. Multiple Mg²⁺ ions appear to be involved in the cleavage reaction, both to labilize the water molecules for attack at the scissile phosphate, as well as to stabilize the resulting transition-state. Experiments using phosphorothioate substitution reactions support an in-line S_N2 displacement at the phosphorus center, with a divalent metal or metals functioning as critical co-factors. The metal ion is probably involved in the orientation of the nucleophilic hydroxide onto the target center. Earlier models by Guerrier-Takada *et al.* (1986) and Smith & Pace (1993) propose the coordination of hydrated magnesium ions to the pro-S oxygen of the scissile P atom (Figure 5A). Other Mg²⁺-coordinated water molecules might be involved in H-bonding interactions, either to the attacking nucleophile and/or to the 2'-OH groups. A second metal ion seems to be coordinated to the pro-R oxygen (Chen *et al.* 1997). More recently, experiments have suggested the interactions of the metal ions with both the attacking hydroxide and the leaving 3'-alkoxide group (Warnecke *et al.* 1996) (Figure B). Thus, RNase P may also follow a two-metal ion model for phosphate ester hydrolysis (Steitz & Steitz 1993).

Internal transesterification

Mg²⁺ catalyzed reactions in hammerhead ribozymes

Hammerhead is one of the most extensively studied ribozymes because of both its importance and its small size. Metal ion rescue experiments have provided im-

portant, yet controversial answers for the functional role of Mg²⁺ in hammerhead ribozymes. Phosphorothioate modifications at specific sites have resulted in great loss of activity in the presence of Mg²⁺ (Scott & Uhlenbeck 1999; Wang *et al.* 1999). Addition of even trace amounts of either Mn²⁺ or Cd²⁺ (thiophilic metal ions) restored catalysis. These results support the hypothesis that cleavage requires direct coordination of a Mg²⁺ to the phosphate oxygen. When a sulfur atom replaces the non-bridging pro-Rp oxygen atom, the ribozyme loses ~4 orders of magnitude of activity in the presence of Mg²⁺ (the so called thio-effect). However addition of Cd²⁺ resulted in normal activity, providing evidence for the direct coordination of Cd to the P-S bond at the Rp position. However, Taira's group has reported that in the presence of other divalents like Ca²⁺, Cd²⁺ does not interact with this S-atom or in the transition-state. They also report that the effect seen in the presence of thiophilic metal ions is probably due to the displacement of the Ca²⁺ bound to the ribozyme (Yoshinari & Taira 2000).

Other metal mediated reaction pathways have also been proposed for the hammerhead ribozyme mediated catalysis. A single metal ion mechanism was proposed in which a Mg²⁺-hydroxide acts as a general base and abstracts a proton from the 2'-OH nucleophile (Dahm *et al.* 1993). In the freeze-trapped crystal structure of the intermediate in hammerhead ribozyme, no metal ion was found close to the 5'-leaving oxygen (Scott *et al.* 1996). This structure seems to support a single metal mediated hydrolysis. However a structure with a modified ribozyme and Co²⁺ indicated the presence of a metal ion close to the 5'-oxygen of the scissile phosphate (Murray *et al.* 1998). Molecular dynamic simulations led to a new and alternative single-metal-ion mechanism which involved one Mg²⁺ coordination simultaneously to the pro-Rp oxygen and the 2'-OH moiety. In addition, the water molecules bound to the metal ion might act as a general acid to donate a proton to the 5'-leaving group.

Based on hammerhead mediated cleavage by La³⁺ ions in the presence of Mg²⁺ ions, von Hippel and coworkers argue for the involvement of a two-metal ion mechanism in the hammerhead ribozyme (Figure 6) (Lott *et al.* 1998). Recent *ab initio* molecular orbital calculations also support the need for two metal ions in the cleavage mechanism (Lyne & Karplus 2000). Taira's group studied the solvent isotope effects and kinetic analysis of a modified substrate with a 5'-mercapto leaving group at the cleavage site. Their results also support the requirement for two metal ions

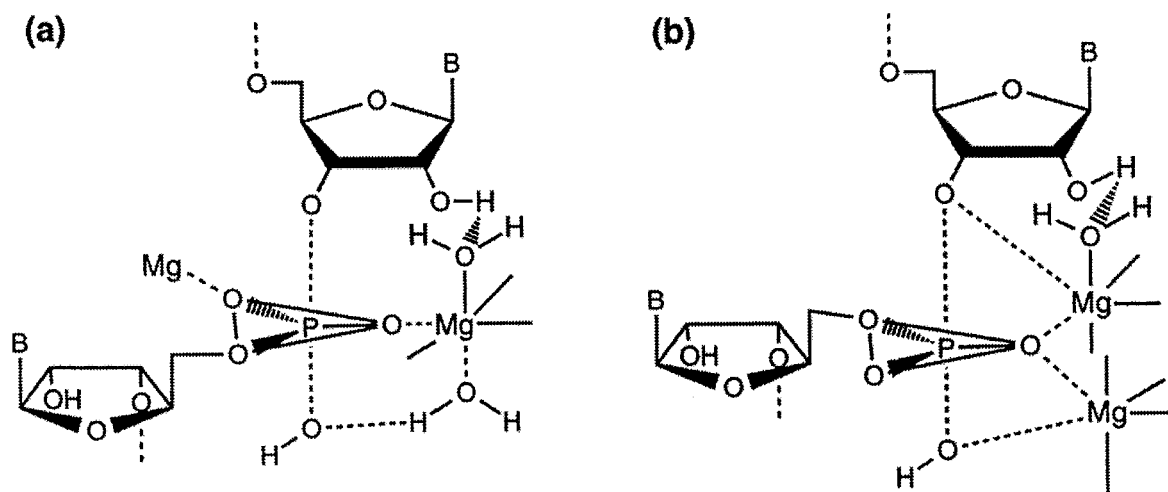


Fig. 5. Possible transition states for a magnesium-promoted reaction via a two-metal mechanism. Adapted from Kuimelis & McLaughlin (1998).

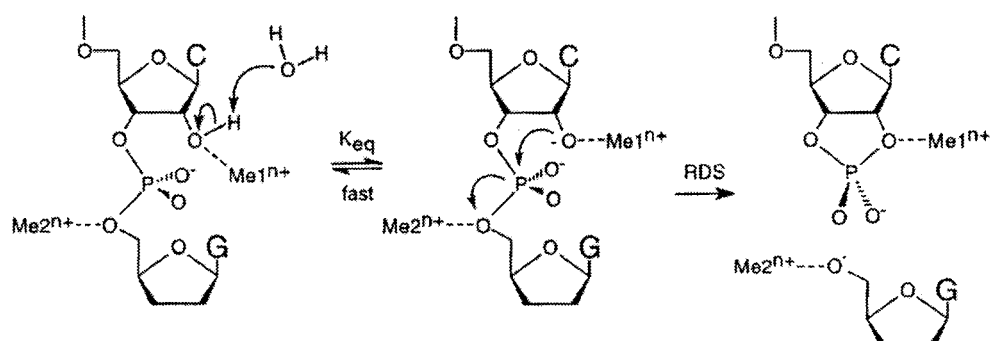


Fig. 6. Modified two-metal ion model for hammerhead ribozyme cleavage based on results from Lott *et al.* (1998). RDS = rate determining step.

in the mechanism of catalysis (Sawata *et al.* 1995; Zhou *et al.* 1997). However, the proposed mechanisms differ in the degree of cooperation of the divalent magnesium ions: from a bridged binuclear core, to two independent metal ions activating distinct oxygen centers.

Mg²⁺-hydroxide and hepatitis delta virus (HDV) ribozyme

HDV is a human pathogen possessing a circular RNA genome that contains a self-cleaving domain. The structural motifs of HDV are different from those found in hammerhead or hairpin ribozymes. The HDV ribozyme requires Mg²⁺ or Ca²⁺ for activity, though, Mn²⁺ or Sr²⁺ work as well. Chemical modification at the atomic level by replacing pro-Rp oxygen with a phosphorothioate interferes with cleavage (Jeoung *et al.* 1994). Moreover, this effect could not be rescued

by adding the more thiophilic Mn²⁺ ion, indicating that the metal ion cofactor is not directly interacting with the pro-Rp phosphate oxygen. Instead the metal ion is important in formation of the active complex consisting of the ribozyme and its substrate. Detailed structural and kinetic characterization by three separate groups have given a detailed mechanism for HDV ribozyme (Ferre-D'Amare *et al.* 1998; Perrotta *et al.* 1999; Nakano *et al.* 2000). It has been suggested that a cytosine, C75, acts as a general acid catalyst in combination with a metal hydroxide that acts as a general base catalyst (Figure 7). The HDV ribozyme has a slope of unity at low pH for self-cleavage and this is consistent with the increase in the concentration of the metal hydroxide as pH increases. Between pH 7 and 9, the slope is zero, and the authors suggest that the increase in metal hydroxide concentration is compensated by an equal decrease in the concentra-

tion of a protonated C75. It is also noteworthy that $[\text{Co}(\text{NH}_3)_6]^{3+}$ inhibited the Mg^{2+} catalyzed reaction in a competitive fashion, suggesting that the Co^{3+} complex might bind to the same site as the functional Mg^{2+} with outer-sphere coordination, but the bound NH_3 do not ionize (c.f. H_2O to HO^- for magnesium-bound H_2O). The finding that both Ca^{2+} and Mg^{2+} show similar rate constants indicate that the hydrated metal ion acts as a Brönsted base rather than a Lewis acid in the HDV ribozyme.

Structural role of Mg^{2+} in hairpin ribozymes

Hampel & Cowan (1997) have reported an approach to differentiate inner sphere and outer sphere pathways in the mechanisms of metal activated ribozyme catalysis. They have emphasized how the hairpin ribozyme falls into a mechanistically distinct category from the *Tetrahymena* and the hammerhead ribozyme. Their strategy is based on use of substitutionally inert cobalt or chromium complexes to probe for outer sphere pathways in the mechanism of metal mediated nuclease enzyme catalysis. In a systematic study of the metal cofactor conditions required for catalysis by the hairpin ribozyme they have observed that in the presence of $[\text{Co}(\text{NH}_3)_6]^{3+}$ the ribozyme activity is 40% of that of Mg^{2+} promoted reaction. The addition of Mg^{2+} in absence of any monovalent ions does not restore the activity to 100%. This leads to an indication that $[\text{Co}(\text{NH}_3)_6]^{3+}$ is indeed the metal cofactor that promotes ribozyme activity in this reaction. Since the complex is a substitutionally inert transition metal complex, activation arises neither from inner sphere binding nor by activation of a bound water molecule. The possibility of interference/contamination has been ruled out by examining the influence of EDTA concentrations on the activity. They have authenticated this by preparing the substrate with non-bridging sulfur at the scissile phosphate by RNA chemical synthesis, which is expected to give equal amounts of the Rp and Sp isomers. They went on to argue that if Mg^{2+} were directly involved in catalysis by an inner sphere mechanism, inhibition of cleavage would be expected for one or the other isomer and therefore the relative activity of $\sim 50\%$ would be expected. On the contrary they found that the extent of cleavage and the cleavage rates, under standard cleavage conditions, were similar for the native and thio-substituted substrates in Mg^{2+} cleavage buffer, except for a small decrease in activity due to the sulfur itself being involved in the reaction. As a control, when cleavage of the thio modified substrate in the presence of $[\text{Co}(\text{NH}_3)_6]^{3+}$ was compared to

that in the presence of Mg^{2+} , no significant differences in cleavage or rate or extent of cleavage were seen. The results from this work provide support for a model of RNA catalysis that does not involve direct coordination of magnesium to the phosphate ester nor activation of a bound water molecule. They proposed a mechanism in which the catalysis is carried out by functional groups on the RNA ribozyme itself; such functional groups are likely to have pK_a values that are appropriate for carrying out this catalysis. The role of the metal cofactor would be that of defining the structural design of the catalytic pocket, resulting in stabilization of the transient species (Hampel & Cowan 1997).

In an independent study, Grasby and co-workers found that neither Rp nor Sp phosphorothioate isomers of the hairpin ribozyme showed any thio-effect. Furthermore, they show that inert cobalt (III) complexes were able to support the hairpin ribozyme reaction with a similar efficiency as Mg^{2+} , even in the presence of EDTA. Variation of the net charge on the Co^{3+} complexes did not alter the rates, ruling out the possibility that metal ions are involved in any electrostatic catalysis by outer sphere mechanisms. An alternate explanation for the role of Mg^{2+} in hairpin ribozyme catalysis is that it is required only for structural purposes. Hairpin ribozyme has two loops (1 and 2) and both the loops have to be associated for catalysis. The association of the looped areas of the RNA molecule must overcome the charge repulsion of the phosphodiester backbone, and the Mg^{2+} ions could facilitate this (Young *et al.* 1997; Yoshinari & Taira 2000).

Conclusions for the role of Mg^{2+} in promoting ribozyme chemistry

It is very clear from the above discussion that Mg^{2+} plays diverse roles in ribozyme chemistry. Apart from being directly involved in the critical catalytic pathways of ribozymes, Mg^{2+} is also involved in structural roles. Also, Mg^{2+} can be involved in a single metal or a double metal mediated catalytic reaction. Binding of the metal ion can involve a bidentate mode utilizing two oxygen atoms of the phosphate group with a compression of the tetragonal O-P-O angle to a trigonal geometry. This could create an apical position susceptible for nucleophilic attack and thus reduce the activation energy necessary for the transition to a bipyramidal intermediate. In general acid catalysis, the metal might interact with the leaving group in the transition-state resulting in the increase of its

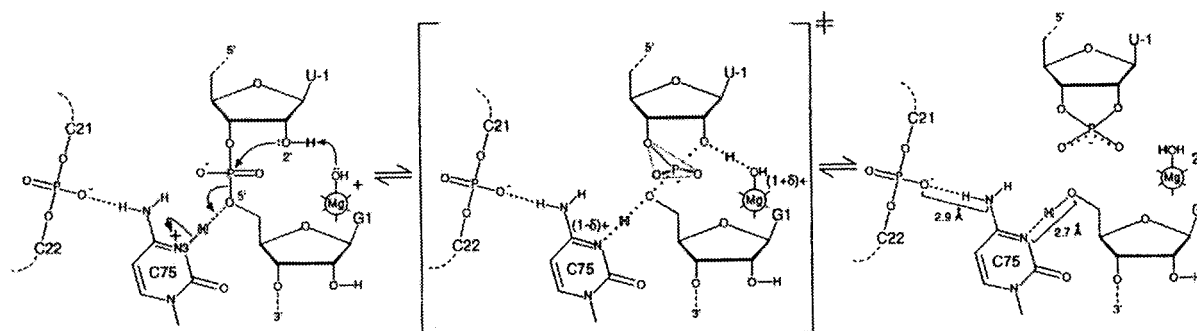


Fig. 7. Proposed mechanism for general acid-base catalysis in HDV ribozyme showing the role of $[\text{Mg}(\text{OH})]^+$ as a general base. Adapted from Nakano *et al.* (2000).

acid strength. This would then stabilize the development of electron density at and a weakening of the bond between the P atom and oxygen of the leaving group. Direct inner sphere coordination of Mg^{2+} to the phosphate oxygens would withdraw electron density and enhance the electrophilicity of the P atom. This would make the P center more susceptible by a nucleophile that is appropriately positioned, e.g., either by a 2'-OH (transesterification) or by a water molecule (hydrolysis).

Role of Mg^{2+} in catalytic polynucleotides - Deoxyribozymes

In natural systems the replicability and relative stability of double helical DNA makes it an ideal candidate for storing genetic information. However, such double stranded DNA has very limited structural flexibility and in a way, very limited opportunity to facilitate substantial rate enhancements. The highly specific and complicated reactions catalyzed by ribozymes have turned attention to single stranded DNA. With the advent of combinatorial biochemical techniques such as SELEX (Tuerk & Gold 1990), random sequences of ss-DNA libraries could be investigated for a variety of catalytic reactions. The first DNA enzymes, also called deoxyribozymes, were isolated by *in vitro* selection strategies and were found to be metalloenzymes in a classical sense in that they required metal ions for catalytic activity. The possibility of an RNA-cleaving catalytic DNA was initially explored in selection constructs containing a single ribonucleotide substrate component.

Role of Mg^{2+} in RNA hydrolysis by deoxyribozymes

While the first deoxy-ribozymes to be isolated were dependent on Pb^{2+} (Breaker & Joyce 1994), other DNA enzymes with Mg^{2+} -dependent RNA phosphoesterase activity have been isolated and characterized (Breaker & Joyce 1995). The structure of the Pb dependent DNA enzymes appears to be quite simple, requiring only six nucleotides of defined sequence and two substrate-binding arms. While the Mg^{2+} -dependent enzyme also uses two substrate-binding arms, there are 15 conserved nucleotides in the catalytic core, suggesting that a more precise active site is required when Mg^{2+} is used as a cofactor. These enzymes perform under cell-like conditions with a maximal catalytic rate of 0.08 min^{-1} , corresponding to about a 10,000-fold increase in RNA cleavage over background rate of hydrolysis. Another DNA enzyme that has been developed is the 10-23 RNA cleaving DNA enzyme. The name is derived from the 23rd clone of the 10th round of *in vitro* selection of the DNA enzyme. These examples and others that might follow, are clear indications that in a role dominated by protein enzymes, DNA, which has been stripped of its complementary strand, is capable of maintaining structural diversity and even catalytic activity in the presence of divalent Mg^{2+} .

Mg^{2+} -dependent phosphodiesterase

Using *in vitro* selections Breaker and Joyce have also developed a family of phosphoester cleaving DNA enzymes that operate in the presence of various divalent metals, focusing particularly on the Mg^{2+} reaction (Breaker & Joyce 1995). They generated a population of $> 10^{13}$ DNAs containing a single ribonucleotide component, followed downstream by 40 random nucleotides to provide molecular diversity. The authors

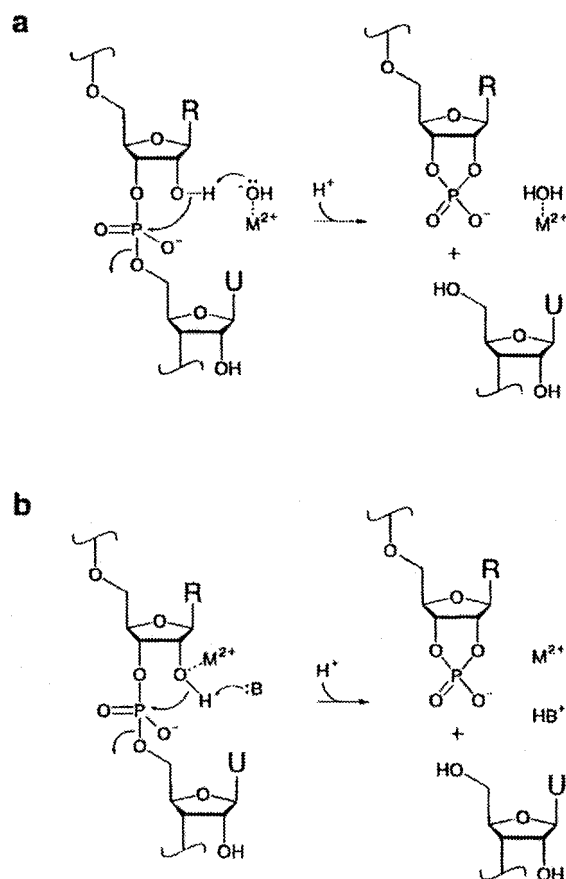


Fig. 8. Proposed mechanism for RNA hydrolysis by the 10-23 deoxyribozyme. (a) Mg^{2+} acting as a general base; (b) Mg^{2+} as a Lewis acid enhancing the acidity of the 2'-hydroxyl group. Adapted from Santoro & Joyce (1998).

carried out repeated rounds of selective amplification, enriching for molecules that cleave a target RNA phosphoester in the presence of different divalent cations. Closer examination of the individual clones from the Mg^{2+} lineage after the sixth round revealed a catalytic motif comprising a three-stem junction. On subjecting this motif to additional rounds of selective amplification, catalysts with a rate of 0.01 min^{-1} were generated. Secondary structure predictions from the above clones were used to configure the trans-cleavage activity of the DNA enzymes using separate substrate and enzyme strands. The Mg^{2+} -dependent cleavage of the RNA phosphoester has a catalytic rate $\sim 10^5$ -fold greater than that of the uncatalyzed reaction, demonstrating that DNA enzymes can use a variety of metal ion cofactors and can catalyze a biologically significant reaction under conditions similar to those that exist within cells.

10-23 Deoxyribozyme

Further development of *in vitro* selections by Santoro and Joyce (Santoro & Joyce 1997) led to the development of Mg^{2+} dependent DNA enzymes capable of cleaving a biologically relevant all-RNA substrate. This enzyme contains approximately 30 deoxynucleotides and can cleave almost any RNA substrate under simulated physiological conditions, recognizing the substrate through two Watson-Crick binding domains (Figure 8A). Under multiple-turnover conditions, the 10-23 DNA enzyme exhibits Michaelis-Menten kinetics, with a k_{cat} of $\sim 0.1 \text{ min}^{-1}$ and K_M of $\sim 1 \text{ nM}$ when measured in the presence of $2 \text{ mM } Mg^{2+}$ and 150 mM NaCl at $\text{pH } 7.5$ and 37°C . Under modified conditions ($50 \text{ mM } Mg^{2+}$, $\text{pH } 8.0$), the enzyme has a better k_{cat} ($\sim 10 \text{ min}^{-1}$) and a catalytic efficiency $k_{\text{cat}}/K_M > 10^9 \text{ M}^{-1} \text{ min}^{-1}$, exceeding that of any known nucleic acid enzyme. RNA cleavage catalyzed by 10-23 enzyme resulted in an upstream fragment terminating in a 2'-3'-cyclic phosphate and a downstream fragment with a 5'-hydroxyl group. Taken together with the metal ion dependence of 10-23 enzyme, these results support a chemical mechanism involving a metal-assisted deprotonation of the 2'-OH located adjacent to the cleavage site. This would produce a nucleophilic 2'-oxyanion that attacks the adjacent P atom giving rise to cleavage of the strand. The Mg^{2+} ion may participate in the chemical mechanism of the DNA enzyme either as a metal-hydroxide that functions as a general base to assist deprotonation of the 2'-OH or as a Lewis acid that coordinates directly to the 2'-OH and enhances its acidity (Figure 8B). The metal ion might also be playing a more structural role, helping to organize the enzyme into its active conformation. Regardless of the role that Mg^{2+} plays in 10-23, the deprotonation of 2'-OH is found to be essential for its RNA cleaving activity. The enzyme displays a high level of substrate specificity, discriminating against RNAs that contain a single base mismatch within either of the two substrate-recognition domains. With appropriate design of the substrate-recognition domains, the enzyme exhibits a potent combination of high substrate sequence specificity and selectivity, high catalytic efficiency, and rapid catalytic turnover.

Conclusions for the role of Mg^{2+} in promoting deoxyribozyme chemistry

Despite the fact that structured single stranded DNA molecules occur rarely in nature, DNAs that act as

receptors, ligands or catalysts can readily be isolated from random-sequence pools using *in vitro* selection. Various studies have made it clear that ss DNA can form intricate structures that are comparable to those found in RNA aptamers and ribozymes. In addition, almost as a general rule, deoxyribozymes are metal dependent for structure and/or for function. While the deprotonation of a 2'-OH by Mg^{2+} is essential for catalytic activity of the 10-23 deoxyribozyme, the challenge remains to create a metal-activated enzyme that promotes hydrolysis of DNA backbones by use of *in vitro* selection techniques.

Conclusions

Clearly divalent magnesium plays a critical role in stabilizing specific structural motifs for both nucleic acids and their complexes with cognate ligands and antibiotics. For the latter the magnesium ion may serve up to triple duty by defining structures for both the nucleic acid and ligand, while also bridging the two. In the context of 'nucleic acid' nucleases, divalent magnesium typically is essential for both structural and catalytic roles. Within these common themes of structure and catalysis there is, however, great diversity, which highlights the rich variation in magnesium coordination chemistry and the propensity toward both inner sphere and outer sphere complex formation. As we have noted on other occasions, while magnesium may be colorless spectroscopically, this cannot be said of its chemistry.

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