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Transition Metals as Probes of Metal Cofactors in Nucleic Acid Biochemistry

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The alkali and alkaline earth metals (Na^+, K^+, Mg^{2+}, Ca^{2+}) are important regulators of nucleic acid biochemistry; however, the kinetic, thermodynamic, and coordination details of their chemical interactions with polynucleotides and enzymes are not well characterized. Magnesium ion is an essential cofactor for many enzymes that digest, cut, ligate or alter the topology of DNA and RNA, or catalyze phosphoryl and nucleotidyl transfer reactions. In this article discussion is focussed on the role of magnesium as a cofactor for enzyme and ribozyme activity. Strategies employing transition metals as chemical probes of metallocofactor reactivity are critically assessed, and newer developments in the use of inert complexes to test the catalytically competent state of Mg^{2+}(aq) are introduced.

Key Words: *nucleic acids, metal cofactors, magnesium biochemistry, enzymes/ribozymes, transition metal probes*

The requirement for divalent metal ions (Mg^{2+}, Ca^{2+}, Zn^{2+}), and to a lesser extent monovalent ions (K^+, Na^+, H^+), as activators of important enzymes in nucleic acid biochemistry has stimulated

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interest in the structural and catalytic roles of these cofactors.[a 1-5] Table I lists some illustrative examples. Of these, only zinc enzymes are metalloproteins in a formal sense, inasmuch as they are isolated with the metal *in situ*. In part this reflects the relatively low concentrations of this ion *in vivo* (Table II); tighter binding is required to harness these scarce metal cofactors to ensure enzyme activation. In contrast the alkali and alkaline earth metals can afford to bind more weakly to proteins since the biological concentrations of these ions are relatively high: Nature has had no reason to refine ligand environments to promote tighter binding of the metal cofactor. Inspection of any catalogue of DNA and RNA processing enzymes demonstrates that most require divalent magnesium for activation, and so this is arguably the most prevalent metal ion in nucleic acid biochemistry.[b] Ribozymes also require metal cofactors for optimal activity, and an increasing number of papers are appearing on this topic.[7-12] It is worth noting that nucleic acids behave as polyelectrolytes at physiological pH. The negatively-charged ribose-phosphate backbone has an affinity for metal ions ($K_a \sim 10^2-10^4\ M^{-1}$),[13,14] and it might be expected that the solution chemistry of protein or ligand interactions with nucleic acids would be intimately connected with the chemistry of the bound counterions. Given the importance of this topic and its fundamental relevance to the understanding of nucleic acid binding proteins, there has been remarkably little activity in this area.[15-19]

A major problem facing researchers pursuing detailed biochemical studies of the alkali and alkaline earth metals relates to the spectroscopic silence of these ions. Although each is NMR active in at least one isotopic form, multinuclear NMR spectroscopy is not as useful as EPR or electronic absorption as a probe of coordination chemistry. Direct NMR studies of exchangeable metal ions can provide useful kinetic and thermodynamic data on metal binding, but these studies offer little in the way of structural in-

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[a]The term nucleic acids will encompass polynucleotides (single- and double-strand), oligonucleotides, and nucleotides.

[b]Zinc is used as a structural factor in many nucleic acid binding proteins. This topic has been extensively reviewed and is not considered here (see Ref. 6) Magnesium tends to be employed in enzymes that catalyze chemical transformations of nucleic acids.

TABLE I

Examples of metal-activated enzymes in nucleic acid biochemistry.

Enzyme	M^{n+}	K_a (M^{-1})[a]	Locn.[b]	Enzyme Function
Eco RI	Mg^{2+}	—	I	Restriction nuclease, specifically cleaves ds DNA at G|AATTC sequences
DNA Polymerase I	Mg^{2+}	—	I	Incorporates deoxyribonucleotides in the complementary strand of a ss DNA template
(3′-5′ exonuclease activity)	Mg^{2+}			Digests ds DNA from the 3′-end, release 5′-phosphomononucleotides
Topoisomerase I	Mg^{2+}	—	I	Relaxes supercoiled DNA by transient cleavage and rejoining of phosphodiester bonds
Staphylococcal nuclease	Ca^{2+}	10^5	E	Phosphomonoesterase, degrades DNA and RNA to mononucleosides
Deoxyribonuclease I	Ca^{2+}	—	E	Digests ss and ds DNA to 5′-phosphooligonucleotides
S1 Nuclease[c]	Zn^{2+}	$>10^9$	I	Digests ss DNA or RNA to 5′-mononucleotides

[a]The lack of data for magnesium binding sites is representative of the state of the field.
[b]Intracellular (I) or extracellular (E) location.
[c]May be isolated with the metal *in situ*.

TABLE II

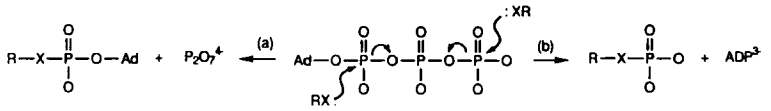
Relative abundance of H^+, Na^+, K^+, Mg^{2+}, Ca^{2+} and Zn^{2+} inside and outside a typical mammalian cell.

Ion	$[M^{n+}]_i$ (mM)[a]	$[M^{n+}]_e$ (mM)[b]
H^+	10^{-4}	10^{-4}
Na^+	10	145
K^+	140	5
Mg^{2+}	30	1
Ca^{2+}	1	3
Zn^{2+}	10^{-6}	$<10^{-6}$

[a]Intracellular concentration.
[b]Extracellular concentration.

formation.[20,21] This problem can be circumvented by use of functionally equivalent transition metals that possess convenient spectroscopic handles to probe and investigate their coordination environment.[c] I emphasize structure here since the probe ion is only infrequently used to test a reaction mechanism.

Magnesium is widely employed by enzymes that catalyze (a) nucleotidyl, and (b) phosphoryl transfer reactions (below), and also by many enzymes that digest, cut, ligate or alter the topology of DNA and RNA.



After magnesium, calcium and zinc are the most prevalent metal cofactors used by hydrolytic (or nuclease) enzymes. There has been widespread use of cobalt(II) as a substitute for zinc(II), and lanthanide(III) ions as probes of calcium(II) sites in metalloproteins.[22-30] The rationale for these choices, in terms of the physical and chemical characteristics of each metal ion, is summarized in Table III. In many cases the similarity in size and coordination geometries for each pair of ions results in *functional* substitution. Both cobalt(II) and several lanthanide(III) ions (Eu^{3+} and Tb^{3+}

[c]We define a metal probe as functionally equivalent if it demonstrates similar structural and, if appropriate, catalytic chemistry.

TABLE III

Physicochemical properties of the alkali and alkaline earth metals, and select transition metals.

Ion[a]	Ionic Radius (Å)[b]	(q/r)[c]	k_{ex} (s^{-1})[d]	Coordination Number	Geometry	Ligand Preference
Na^+	1.16	0.86	$\sim 10^9$	6	octahedral	O
K^+	1.52	0.66	$\sim 10^9$	6–8	flexible	O
Mg^{2+}	0.86	2.33	5×10^5	6	octahedral	O
Mn^{2+}(HS d^5)	0.97	2.07	2×10^7	6	octahedral	O, N
Co^{3+}(LS d^6)	0.69	4.35	–[e]	6	octahedral	O, N
Zn^{2+}	0.74	2.70	7×10^7	4	tetrahedral	N, S
Co^{2+}(HS d^7)	0.72	2.78	–	4	tetrahedral	N, S
(HS d^7)	0.72	2.78	2×10^6	6	octahedral	N, O
Ca^{2+}	1.14	1.75	8×10^8	6–8	flexible	O
Ln^{3+}	1.0–1.2	2.73	2×10^8	6–9	flexible	O

[a]Electron configurations are noted if appropriate.

[b]Relative to the indicated coordination geometry. Values are taken from Cotton and Wilkinson, *Advanced Inorganic Chemistry*, 5th edition.

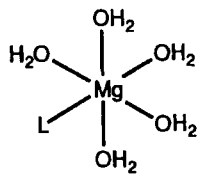
[c]Charge–radius ratio.

[d]Ref. 49. If several values were given, an average was taken.

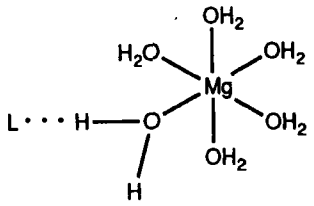
[e]The substitution rates for inert $Co(NH_3)^{3+}$ at pH 7 are $<10^{-12}$ s^{-1}.

in particular) possess useful optical and magnetic properties with which to investigate the coordination environments of the metals. These applications have been widely reported and reviewed.[22-30]

In this article attention will focus primarily on the chemistry of magnesium ion. The use of transition metals as probes of magnesium biochemistry has not been developed as extensively as that of zinc or calcium. The reasons for this are several-fold. First, while the coordination preferences (ligands and geometries) for Zn^{2+} vs. Co^{2+}, and Ca^{2+} vs. Ln^{3+} are similar, the coordination chemistry of Mg^{2+} is variable and, with the possible exception of Mn^{2+}, is not reflected in the chemistry of the transition metals. For example, magnesium may readily adopt either inner or outer sphere coordination modes. It is experimentally difficult to discriminate between these two possible states, and so there is an obvious problem in the choice of appropriate transition-metal analogues.



inner sphere



outer sphere

Second, in contrast to zinc and many calcium-dependent enzymes, which show structurally well-defined metal binding sites that are kinetically and thermodynamically stable, many magnesium binding sites are poorly defined and labile. It is not easy to deduce magnesium binding domains from sequence homologies.

In spite of these caveats, the biological chemistry of magnesium may be probed by judicious choice of transition metal complexes. The aim of this review is to identify the available strategies and critically evaluate their application to biochemical problems.

MANGANESE AS A PROBE OF MAGNESIUM CHEMISTRY

Of all the first row transition metal ions, divalent manganese comes closest to modeling the chemistry of magnesium in terms of ligand

preference and geometry, exchange rates, and a propensity for both inner and outer sphere complexation. As a functional replacement in the study of magnesium-dependent enzymes, Mn(II) has found little use as an optical probe.[d] However, manganese ion has been used extensively as a relaxation agent in NMR studies to determine distances to other magnetic nuclei (^{1}H, ^{31}P, ^{13}C, ^{19}F) on enzyme-bound substrates, substrate analogues, and residues in a protein active site.[31-36] Such measurements may achieve accuracies of up to ± 0.1 Å, however, the method cannot accommodate dynamic changes in the coordination spheres of the enzyme-bound metal, and so the results frequently point to distances that are intermediate between inner and outer sphere values. This may reflect either distorted inner-sphere coordination or a time average of the two coordination modes.[34] Binding constants may also be estimated by use of NMR or EPR spectroscopy.[37] While these measurements provide useful structural information on the metal binding site, the question remains as to whether the inner or outer sphere complex is catalytically competent. Although divalent manganese has been widely and successfully used as a substitute for magnesium ion in studies of enzyme activity, Table IV clearly demonstrates that the story is not quite clear cut. There are frequent changes in substrate and site specificity that must reflect differences in the coordination chemistry of the two ions. There

TABLE IV

Comparison of reactivity for magnesium and manganese activated enzymes.[a]

Enzyme	Summary of Enzyme Reactivity	Ref.
DNA polymerase I	The fidelity of nucleotide incorporation decreases by a factor of 2–20-fold with Mn^{2+}.	36
Eco RI	Highly specific cleavage of dG|AATTCC sequences. Specificity is relaxed in the presence of Mn^{2+}.	38
Calf thymus RNase H	All homopolymeric substrates are hydrolyzed with Mn^{2+} as cofactor. Only poly(rG)·poly(dC) and poly(rA)·poly(dT) are degraded with Mg^{2+}.	39

[a]Many additional examples are described in the chapters of Ref. 47.

[d]High spin d^5 ions possess a $^{6}A_1$ electronic ground state. All optical transitions are therefore spin forbidden and difficult to observe.

is no clear understanding of these effects, in keeping with the general ignorance regarding the molecular details of the chemistry of the metal cofactor. Clearly this is an area that merits more detailed attention.

INERT TRANSITION METAL COMPLEXES AS PROBES OF MAGNESIUM BIOCHEMISTRY

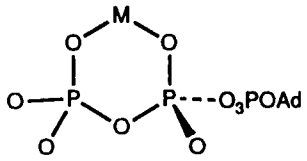
One of the problems inherent to the study of magnesium biochemistry derives from the difficulties in distinguishing the coordination state of the active metal cofactor. To address this issue, we and others have employed inert complexes of defined coordination state to investigate the functional role of magnesium ion.[40-46] In the remainder of the article we highlight some strategies (old and new) that provide some chemical insights into the biochemistry of this ion.

The MgATP Chelate

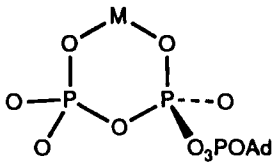
Adenosine triphosphate (ATP) is an important physiological substrate that provides the driving force for many fundamental biochemical reactions in metabolism, transport, and respiration. *In vivo*, ATP is usually found as a chelate complex with magnesium. Relative to the transition metals, alkali and alkaline earth ions are characterized by fast ligand exchange rates, and so MgATP exists as a mixture of rapidly equilibrating isomers of which only one is likely to adopt the proper stereochemistry for reaction. In the early 1970's Cleland introduced the use of inert Co(III) and Cr(III) nucleotide complexes to investigate the mechanistic chemistry of ATP-dependent enzymes.[40-44] By virtue of their electronic configurations (low spin d^6 and d^3, respectively) trivalent cobalt and chromium compounds are exchange inert; thus, the various isomeric forms may be chromatographically separated for use as substrates or inhibitors to test the stereochemical course of enzymatic reaction pathways.[c 40-44] The absolute configurations of many of

[c]Two other methods may also be applied to determining selectivity for the isomeric forms of these coordination complexes: (1) Using the different affinities of Mg^{2+} and Cd^{2+} for O- and S-ligation, respectively, with nucleotide phosphorothioates; (2) EPR studies of paramagnetic Cr(III) complexes with ^{17}O-labeled nucleotides (Refs. 34 and 48).

these isomers have been determined by a combination of crystallographic, enzymatic, and spectroscopic studies.[40,41] The bidentate complex β,γ-MgATP is a common enzyme substrate, and each of the two isomeric $(NH_3)_4$Co(III)ATP analogue complexes may be isolated (below).



(Λ) - β, γ – MATP^{2-}



(Δ) - β, γ – MATP^{2-}

Some common reaction pathways for such a substrate are shown in Fig. 1. When inert transition metal centers are used, the product fragments remain coordinated to the metal. The product complexes may be characterized and useful information established regarding reaction stereo- and regiochemistry. Similar reasoning applies to other nucleotide di- and triphosphates, and pyrophosphate. Since Cr(III) is paramagnetic, CrATP may be used in much the same way as Mn(II) as a relaxation probe.[33-36]

A Comment on the Classification of Magnesium-Dependent Enzymes

Many chemists appear to hold the view that the enzymatic chemistry of magnesium is the chemistry of MgATP. This of course derives from the ubiquity of this chelate in biological systems. However, Table I contains several examples in which magnesium is required for enzyme activity but nucleotide di- or triphosphates are not involved. Magnesium-dependent enzymes can be broadly categorized into one of three groups:

(1) Examples in which magnesium binds primarily to the substrate. Any interaction between the metal and enzyme is secondary. As outlined in Fig. 1, the *primary* reaction chemistry does not necessarily involve rearrangement of the inner coordination sphere of the metal (e.g., in nucleotidyl and phosphoryl transfer). Many ATP-utilizing enzymes fall into this category.

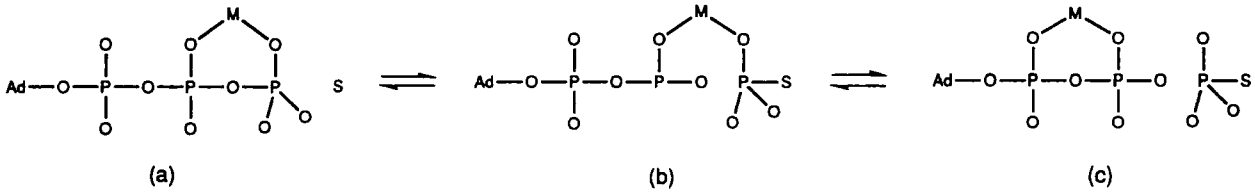
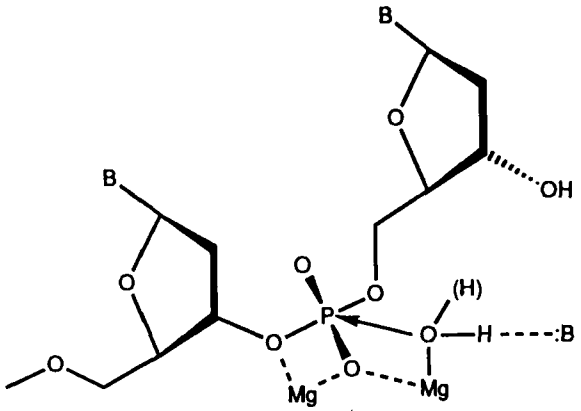


FIGURE 1 Typical sequence of steps in phosphoryl transfer reactions [(a) to (c) via an intermediate (b)] involving a co-substrate S (Ref. 34). Ad = adenosine. Negative charges have been omitted from phosphates. Reaction (b) and (c) is not possible if an inert Co(III) or Cr(III) complex is employed. Similar reaction schemes may be drawn for nucleotidyl transfer.

(2) The active cofactor is a protein-bound Mg^{2+} and the catalytic role of the metal ion may require rearrangement of the inner coordination sphere of the metal, either by activation of nucleophilic water, or by Lewis acid activation of phosphate ester hydrolysis. The crystallographically characterized enzyme-substrate complex in the (3′–5′) exonucleolytic domain of DNA polymerase I Klenow fragment illustrates both of these features (below).[4]



(3) Magnesium is active as an outer sphere complex, binds directly to neither the enzyme nor substrate, but modulates the interactions between the two by electrostatic interactions and hydrogen-bonding.

There is widespread belief that magnesium biochemistry is dominated by the formation of inner sphere complexes with enzyme and/or substrate. However, a brief review of magnesium solution chemistry and crystal chemistry demonstrates that this should not necessarily be the case. Divalent magnesium has one of the largest hydration radii and charge/radius ratios of any metal ion (Table III). In solution it is heavily solvated. This is also reflected in the hydration numbers for crystalline salts of magnesium relative to other alkaline earth metals (e.g., $MgSO_4 \cdot 7H_2O$, $MgCl_2 \cdot 6H_2O$, $BaCl_2 \cdot H_2O$, $CaCl_2 \cdot 2H_2O$, $ZnF_2 \cdot 3H_2O$, $ZnSO_4 \cdot 7H_2O$). It might appear that Mg^{2+} has a certain affinity for H_2O; however, this data best reflects the difficulty for Mg^{2+} to bind counterions that

are much larger than itself.[f] The question of whether a magnesium cofactor is acting as an inner sphere or an outer sphere complex is important and non-trivial, inasmuch as the chemical roles for these two coordination modes are quite distinct. Again, the relatively fast exchange kinetics of Mg^{2+} restricts ready elucidation of the appropriate coordination mode.

Magnesium as an Enzyme Cofactor

Inert cobalt(III) complexes of defined coordination state may be used to differentiate inner and outer sphere reaction pathways for magnesium cofactors. Note that the problems being addressed here are quite distinct from the use of inert metal-ATP complexes by Cleland and Mildvan to test the stereochemistry and regiochemistry of enzymatic reactions employing ATP chelates.[32-36,40-44] Here cobalt(III) complexes, in particular $Co(NH_3)_6^{3+}$, are used as probes of the outer sphere chemistry of $Mg^{2+}(aq)$ in enzymes that do not necessarily have a requirement for ATP; i.e., the focus is on the intrinsic chemistry of the metal cofactor.[g,45] This chemistry is based on the similar sizes of Mg(II) and Co(III), the exchange inertness of Co(III), and the ability of NH_3 to mimic the hydrogen-bonding interactions of H_2O. Since the inner sphere ligands for ammine–cobalt complexes may be readily varied,[53] this offers a wide range of easily accessible derivatives (see below) for fine tuning the chemistry of these systems.[41] Table V defines the role of metal cofactors for several enzymes studied in our laboratory. Even this preliminary survey clearly demonstrates a significant number of metal-activated enzymes that act via outer sphere pathways. If an enzyme is activated by a substitutionally inert metal complex, this immediately precludes the possibility of direct coordination to phosphate (I) and nucleophilic attack by a metal-bound water (II).
However, it is still possible for the metal ion to play a catalytic

[f]The radius ratio rule states that metal–anion interations are maximized when $r(M^{n+}) \sim r(A^{m-})$. This is especially valid for the alkali and alkaline earth metal ions that lack d-orbitals, and ionic (electrostatic) bonding dominates.

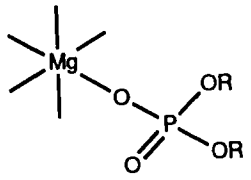
[g]Cobalt requires several N-donor ligands to stabilize the trivalent oxidation state.

TABLE V

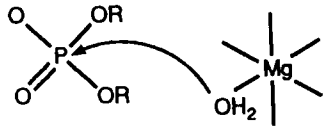
Classification of some metal-activated enzymes according to inner/outer sphere criteria.[a]

Enzyme (M^{n+})	OS	IS (inhib)[b,c]	IS (no inhib)[d]
RNase H (Mg^{2+})	X		
Topoisomerase I (Mg^{2+})	X		
λ Exonuclease (Mg^{2+})	X		
*Eco*RI (Mg^{2+})			X
*Hind*III (Mg^{2+})			X
DNase I (Ca^{2+})		X	
BAL 31 (Ca^{2+})		X	

[a]OS = outer sphere mechanism, IS = inner sphere mechanism.
[b]Inhibition by $Co(NH_3)_6^{3+}$.
[c]The mechanism of inhibition is likely to be by competitive binding at the catalytic site; however, this has not yet been firmly established.
[d]No inhibition by $Co(NH_3)_6^{3+}$

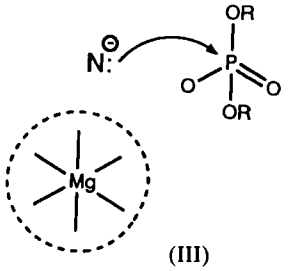


(I)

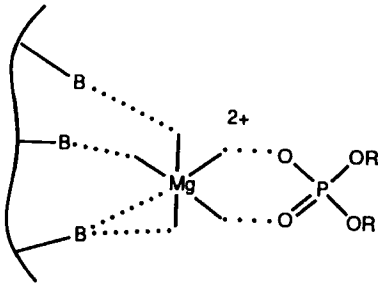


(II)

role by electrostatic stabilization of a developing nucleophile (III) [N = protein residue (e.g., carboxylate, tyrosinate) or outer sphere H_2O/OH^-]. Structural roles (IV) may include hydrogen bonding between the metal-bound water molecules and functionality on the enzyme and substrate.



(III)



(IV)

In those cases where $Co(NH_3)_6^{3+}$ does not promote enzyme activity, this is generally insufficient evidence to prove that the reaction proceeds via an inner sphere pathway. However, it is a strong indicator in favor of such a conclusion.

To obtain further insight into the functional role of the metal cofactor, a series of cobalt-ammine derivatives may be employed, where the inner coordination sphere is used to probe the chemistry of the outer coordination sphere. The problem of differentiating structural from catalytic roles can be approached experimentally by a rational choice of cofactor. Table VI lists inert complexes that have been tested on the enzyme topoisomerase I and some conclusions obtained regarding their likely utility as probes of enzyme mechanism.[46] Rigorous analysis of thermodynamic and kinetic reaction parameters for enzyme-catalyzed reactions employing each complex will provide the most detailed insight into the mechanistic role of the essential metal cofactor. In any kinetic study employing metal cofactors it is important to determine the concentrations of metal ion required to saturate the metal binding site on the enzyme; otherwise a low level of activity may simply result from inactive metal-free enzyme!

Calcium-dependent enzymes are generally inhibited by $Co(NH_3)_6^{3+}$ (Table V). This is in keeping with the inner sphere pathways normally proposed for these enzymes, which in turn reflects the rapid ligand exchange rates of Ca^{2+} (Table III). The importance of exchange rates is clearly demonstrated from pub-

TABLE VI

Inert cobalt complexes for use as probes of enzyme activity.[a]

Structural Parameter	Probe Complexes
Steric Factors	Comparison of M-NH_3 vs. M-en reactivity (e.g., $[Co(NH_3)_6]^{3+}$, $[Co(en)_3]^{3+}$)
Structural Stability (role of H-bonding and electrostatics)	Variation of charge and the number of hydrogen-bond donors (e.g., $[Co(NH_3)_6]^{3+}$, $[Co(NH_3)_5Cl]^{2+}$, $[Co(NH_3)_5(NO_2)]^{2+}$, *trans*-$[Co(NH_3)_4(NO_2)_2]^{+}$

[a]Stabilization of nucleophilic species in the outer coordination sphere may be effectively tested by varying the charge on the metal complex (see above), or by use of ionizable ligands (e.g., $[Co(NH_3)_5(OH)]^{2+}$, $[Co(NH_3)_5(CO_3)]^{+}$), with thoughtful examination of the effect on kinetic parameters.

lished data on deoxyribonuclease I.[50] In contrast to Ca^{2+} and Mn^{2+} activation, the kinetics of DNA digestion by Mg^{2+} and Ni^{2+} are characterized by a lag phase. It was proposed that this reflected two distinct mechanistic pathways; however, the correlation of lag phase with the less labile metal ions suggests that metal binding to the enzyme and substrate may be the key limiting step in the early stages of enzyme activation.[55]

Magnesium Activation of Ribozymes

Several classes of catalytically active RNA molecule have been identified on the basis of structure, reaction chemistry, and cofactor requirements.[h] [51,52] In all cases divalent metal ions (in particular Mg^{2+}) are essential for activation, serving both catalytic and structural roles. Recently the chemistry of these metal cofactors has come under close scrutiny.[7-12] Hammerhead ribozymes have been the vehicle of choice for many of these studies as a result of their relatively small size and ease of synthesis. Both inner sphere and outer sphere roles have been suggested for the magnesium cofactor. The reaction products of these cleavage reactions include a 2′,3′-cyclic phosphate and a 5′-hydroxyl terminus on the two fragments, respectively (Fig. 2a). Computational studies have been reported in support of a pentaaquo-Mg^{2+} bound to the pro-R oxygen (O_R) of the self-cleaving phosphate.[10] The attacking 2′-OH is in line with the leaving 5′-oxygen. The observation of a significant thioeffect when the pro-R_p oxygen is replaced by sulfur at the cleavage site provides further support of direct coordination to the pro-R_p oxygen by Mg^{2+} in the enzyme-substrate complex or the reaction transition state, although the possibility of a change in reaction mechanism is not precluded (Fig. 2).[11]

A fragment of the *Tetrahymena* pre-ribosomal RNA catalyzes the cleavage of appropriate oligoribonucleotide substrates by addition of guanosine to the 5′ end of the downstream cleavage product (Fig. 2b).[i] This has an absolute requirement for Mg^{2+} or

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[h]Group I introns (self-splicing pre-rRNA); plant viruses, virusoids, and linear satellites (commonly containing the hammerhead domain); M1 RNA (RNase P-type RNA).

[i]This is analogous to the first reaction of the self-splicing of pre-rRNA.

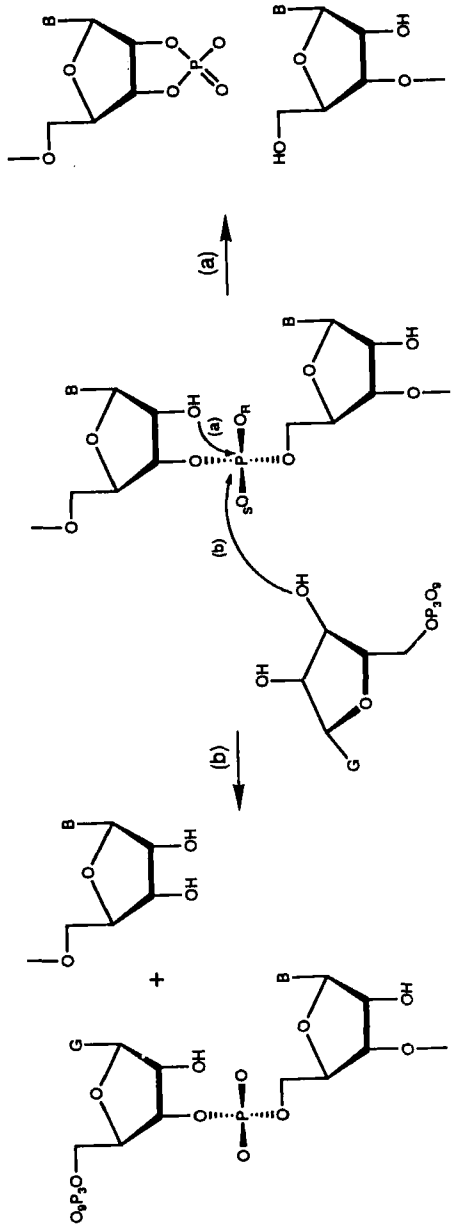

FIGURE 2 Proposed cleavage pathways of an RNA substrate by (a) hammerhead ribozymes, and (b) *Tetrahymena* fragment (Refs. 11 and 12).

Mn^{2+}.[9] In contrast to the hammerhead reaction, substitution of the pro-S_p oxygen (O_S) results in a significant ($\sim 10^3$ fold) decrease in k_{cat} [12]; however, nucleophilic attack is from the opposite direction, relative to the hammerhead ribozyme, and so both results suggest that Mg^{2+} activates phosphate ester cleavage by formation of an inner sphere complex to a specific non-bridging oxygen atom. However, the evidence in support of either an inner or outer sphere pathway has been indirect and no firm conclusions can yet be drawn.

FUTURE DIRECTIONS

Our understanding of the biological chemistry of magnesium ion (and other alkali and alkaline earth metals) remains at a very rudimentary level with regard to fundamental aspects of *structure* and *catalysis*. Several points can be made regarding future research prospects. (1) It is likely that close examination of the few examples of crystallographically characterized magnesium binding sites in proteins may reveal underlying structural principles and correlations with sequence homologies. (2) It is clear that the rational use of transition metal ions and complexes as probes of structure and reactivity can provide insight into the chemistry of essential metal cofactors in enzymes that digest, cut, ligate or alter the topology of DNA and RNA, and phosphoryl and nucleotidyl transferases.[i] Certainly the role of metal cofactors should be explicitly included in mechanistic schemes and analyses of reaction kinetics. Moreover, if firm conclusions are to be drawn from studies using metal ions or complexes (labile or inert) as probes of enzymatic activity, careful consideration should be given to the influence of ion size, exchange kinetics, and coordination chemistry on the reaction pathway. A critical evalution of published literature reveals an alarming number of flaws in experimental design and conclusions resulting from chemically unreasonable substitutions of cofactors. (3) With an increasing number of studies employing site-directed

[i]Much of this discussion is of relevance to magnesium-activated enzymes in general (e.g., enolase, phosphoglucomutase, etc.) (Ref. 54).

mutagenesis to investigate mechanisms of metal-activated enzymes, it is most important to evaluate not only possible changes in enzyme structure that might result from these mutations, but also the effect on the kinetics and especially the thermodynamics of metal cofactor binding at the active site. Changes in enzyme activity, even if the enzyme is structurally similar, may only reflect changes in metal binding affinity with no implications for direct catalytic activity by the mutated residues. (4) It is likely that a close examination of the role of metal ions in the interactions of nucleic acids and proteins/ligands will uncover fundamental regulatory mechanisms in nucleic acid biochemistry.

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