Metal Activation of Enzymes in Nucleic Acid Biochemistry

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Received August 15, 1997 (Revised Manuscript Received January 15, 1998)

Contents

James A. Cowan was born in Cleland, Scotland, in 1961. He earned a B.Sc. Chemistry with first class honors from the University of Glasgow in 1983, and a Ph.D. in Chemistry from the University of Cambridge in 1986, where he worked with Professor J. K. M. Sanders. His work with Professor Sanders included the synthesis and study of complex porphyrin derivatives designed as models for the electron-transfer events in the photosynthetic reaction center. At the same time he worked with Professor A. D. Buckingham to develop a theory of chiral NMR. After receiving his doctorate, he was granted a NATO fellowship to study with Professor H. B. Gray at the California Institute of Technology. In 1988, he accepted a position as an Assistant Professor in the Department of Chemistry at The Ohio State University. He was promoted to Associate Professor in 1993 and Full Professor in 1996. His research spans the fields of chemistry and biology, including the reaction mechanisms of oxidoreductase enzymes, the assembly of complex metal cofactors, the biological chemistry of the alkali and alkaline earth ions, the chemistry of viral and regulatory RNA response elements, and immunochemistry.

I. Introduction

Most enzymes that participate in the biochemistry of nucleic acids require divalent metal ion cofactors to promote activity.[1,2] Magnesium is, with few exceptions, the metal ion of choice, and its central role as a metal cofactor in nucleic acid biochemistry emphasizes the importance of understanding its mechanistic chemistry at a molecular level. This review will focus attention on those enzymes that use free divalent magnesium as a cofactor. There exists a large number of enzymes that utilize magnesium as a chelate with nucleotidyl di- or triphosphates (especially ATP and ADP), where the metal cofactor serves as a mediator of phosphoryl or nucleotidyl transfer chemistry, or hydrolytic elimination of phosphate or pyrophosphate.[3−6] In such enzymes the role of the metal is fairly well understood, while association of the metal to the enzyme is a consequence of nucleotide binding. Such enzymes are of only passing relevance in this review, although nucleotidyl transferases are the subject of greater focus since this activity is relevant to the chemistry of RNA and DNA polymerases. The polymerase domains of such enzymes also possess an additional labile metal-binding site in addition to the metal ion chelated to the nucleotide di- or triphosphate. Acidic active-site residues can interact with this metal−phosphate center and contribute to active-site chemistry, as exemplified by adenylate kinase.[7] These kinds of binding interactions have been previously reviewed.[8,9]

In this paper I will consider the functional role of the magnesium cofactor in a number of enzyme families. As a prelude to this discussion, I will summarize pertinent facts relating to the suitability of Mg^{2+} for its role as enzyme activator, required metal ion stoichiometry, methods for characterizing its solution chemistry, and mechanistic details of function. This will provide an essential background for the subsequent review of specific enzyme families.

S0009-2665(96)00436-0 CCC: $30.00
Published on Web 04/07/1998

II. Physicochemical Properties of Magnesium

The selection of magnesium as an enzyme metallocofactor reflects the high natural abundance of this divalent cation, and a favorable combination of physical and chemical properties (Table 1). These include its redox inertness and small ionic radius, resulting in high charge density, transport numbers, and slow solvent exchange rates.[1] There is a tendency to bind water molecules rather than bulkier ligands in the inner coordination shell, which leads to extensive hydration.[1] In terms of these properties, the biological chemistry of divalent magnesium differs significantly from the other alkali and alkaline earth metals. The distinction is clearly made by comparing the binding modes of magnesium and calcium ions to biological macromolecules. There exists a large body of crystallographic data (summarized in ref 3) that demonstrates outer-sphere complexation by hexahydrated magnesium to be the normal binding mode for divalent magnesium to oligonucleotides (Figure 1). Similarly, binding sites on proteins normally show three or fewer direct binding contacts by protein side chains in order to minimize steric congestion around Mg^{2+},[1,8,10] while Ca^{2+} binding proteins typically show fewer bound water molecules (≤ 2) and an expanded coordination number of seven (Figure 2).[11,12] This reflects the larger ionic radius of Ca^{2+} and reduced steric barriers to direct binding of larger protein-derived ligands.

Table 1. Physicochemical Properties of Biologically Relevant Alkali and Alkaline Earth Ions[165]

	coordination no.	k_{ex} (s^{-1})	radius (Å)	transport no.[a]
Na^+	6	10^{10}	0.95	7−13
K^+	6−8	10^{10}	1.33	4−6
Mg^{2+}	6	5×10^5	0.65	12−14
Ca^{2+}	6−8	10^9	0.99	8−12

[a] Number of outer-sphere water molecules loosely associated and transported with the mobile cation in solution.

Primarily, it is the tendency of Mg^{2+} to maintain a moderate to high hydration state, and the involvement of these metal-bound H_2O in mediating the binding and catalytic chemistry of the magnesium cofactor, that characterizes and is the hallmark of the chemical interactions of this metal ion with nucleic acid substrates.

III. Protein Binding Constants

The design of metal binding sites on proteins is not ad hoc, but rather must satisfy certain constraints that relate to the physiological availability of the metal species. Clearly, proteins must bind metal ions with binding affinities (K_Ds) that are smaller than the available concentration of these species in their respective in vivo environment—otherwise the metal cofactors would not bind. Creation of high-affinity metal binding pockets on proteins requires more extensive design than low-affinity sites and is therefore more demanding of evolutionary development. If a metal cofactor is available in high concentrations (greater than or equal to millimolar) there is no need for a high-affinity binding site since the metal binding pocket would be populated under less stringent conditions, and so proteins typically bind metal ions with K_Ds that match physiological availability. Selection between metal ions of comparable cellular concentrations can be made on the basis of ligand type and coordination geometry.[13] In the case of intracellular free calcium ion, the concentration is sub-micromolar and Ca^{2+} binding proteins show $K_D \leq 1\ \mu M$, while extracellular Ca^{2+} proteins show $K_D > 1$ mM, reflecting the higher extracellular concentrations of calcium. As a result of the high cellular concentration of free Mg^{2+} (~0.5 mM free ion),[14] typical magnesium binding sites on nuclease enzymes and ribozymes are of only moderate affinity ($K_D \approx 0.1-1.0$ mM). Later it will be seen that this simple fact provides a powerful constraint for analysis of metal-binding stoichiometry. Despite the similarity

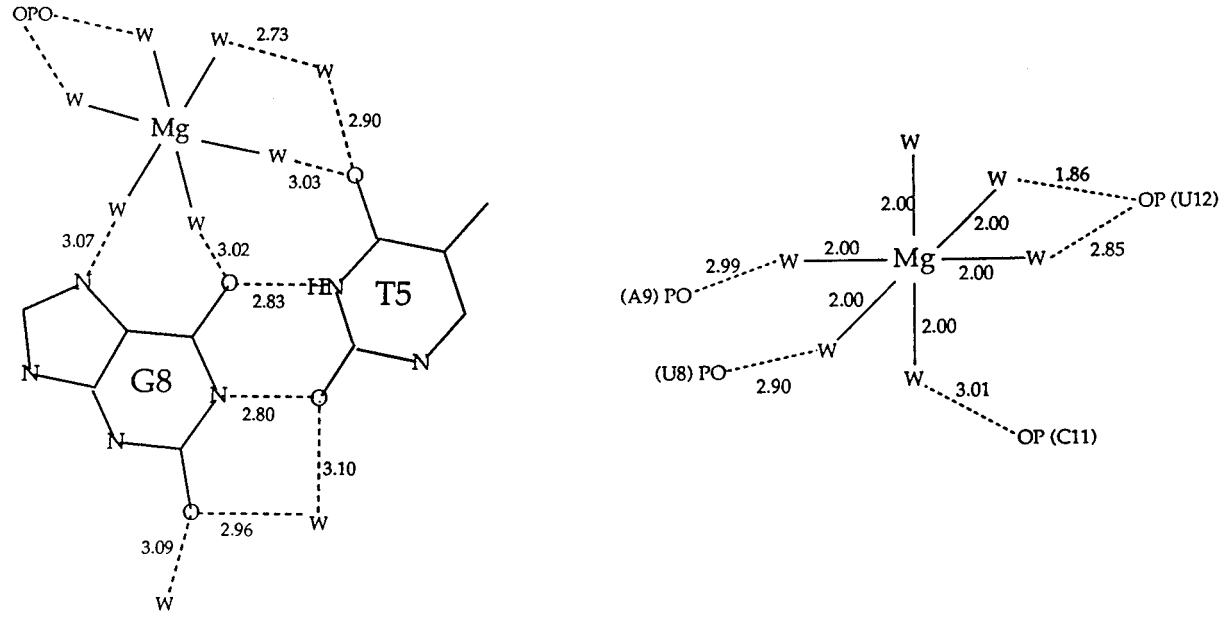

Figure 1. Hexahydrated Mg^{2+} binds to the wobble base pair GT in the DNA strand 5′-CGCGTG-3′ (left) and a site on $tRNA^{Phe}$ (right).[161,162] Hydrogen bonds are shown by the dotted lines, and the distances (in Å) are oxygen−oxygen internuclear spacings. Key: W, water molecule; OPO, a backbone phosphate.

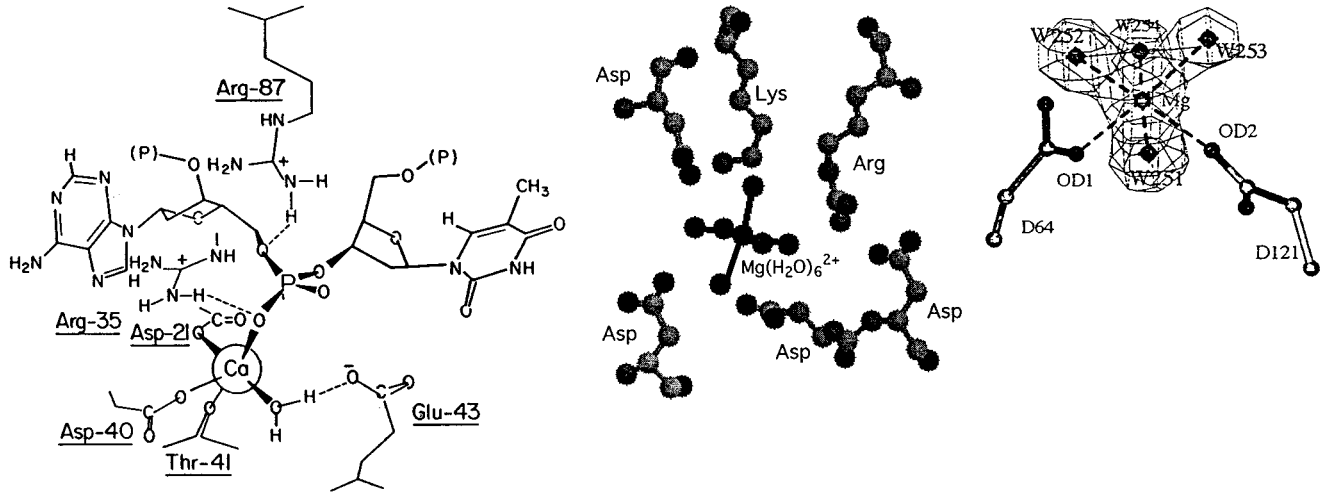

Figure 2. Typical protein coordination modes for magnesium- and calcium-binding proteins, illustrating the tendency for extensive hydration for the former. The Ca^{2+} site in staphylococcal nuclease (left) shows four direct binding contacts from Asp21, Asp40, the backbone carbonyl of Thr41, and a phosphate from the nucleotide substrate mimic.[163,167] One of two additional bound H_2O molecules is also shown. For magnesium-dependent nucleases the hexaaquo Mg^{2+} site in bacteriophage *E. coli* T4 ribonuclease H (middle),[102] and the Mg^{2+} site in avian sarcoma viral integrase (right) are shown.[49]

Table 2. Magnesium Binding Motifs

motif[a]	enzyme	ref(s)
-NA**D**F**D**G**D**-	RNA polymerase	19
-G**DD**-	RNA polymerase	21
-**D**-NSLYP- and -K-NS(L/V)YG-	DNA polymerase φ29	129, 130
-YG**D**T**D**S-	DNA polymerase α	22
-YX**DD**-	reverse transcriptase	147
-LX**DD**-	telomerase	166

[a] Letters in bold are metal binding ligands.

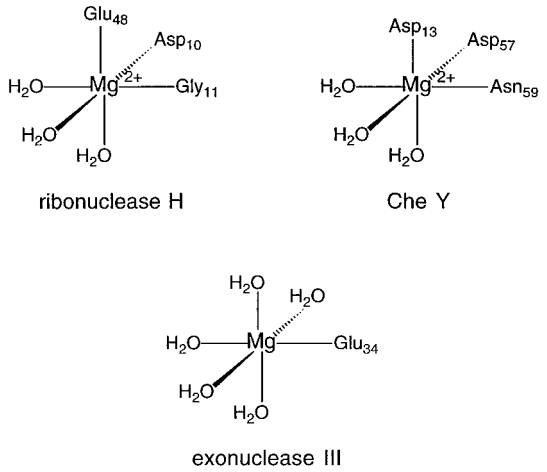

Figure 3. Comparison of magnesium coordination spheres for selected magnesium proteins (the metal binding domains of *E. coli* ribonuclease H, the chemotaxal protein CheY, and the DNA repair enzyme exonuclease III).

in K_D, many metal-binding proteins, and magnesium-dependent enzymes in particular, show a rich variety of coordination motifs (Table 2 and Figures 3 and 4).[8,10] These variations tailor the K_D as described, but also accommodate the diverse functional roles of the metal cofactor. For magnesium, the importance of metal-bound H_2O in the catalytic mechanism of many enzymes has already been illustrated[8,15,16] and can extend to a requirement for specific coordination geometries for bound waters (Figure 2). Consequently, the coordination mode for the protein-bound metal must also vary, while maintaining a K_D at a physiologically balanced level. The availability of high-resolution structural data on several metal-dependent nuclease enzymes allows a critical analysis of the coordination chemistry of the bound cofactor, and the implications for an understanding of the functional mechanism.

In contrast to the situation for Ca^{2+} binding proteins, where metal-binding motifs can be identified from primary protein sequences,[17,18] few such general motifs are known for Mg^{2+} (Table 2, and Figures 3 and 4). Some of these include the homologous -NADFDGD- motif found in RNA polymerases isolated from *E. coli*, *M. thermoautotrophicum*, *S. oleracea* chloroplast, and *S. cerevisiae* Pol I, II, and III. By use of Fe^{2+} as a probe with hydroxyl radical footprinting, and studies of Asp mutants, Mustaev and co-workers have provided evidence that the conserved triad of carboxylates illustrated in Figure 4 is involved in Mg^{2+} coordination.[19] Such residues are also observed in DNA Pol I, and HIV-RT.[20] A -GDD- motif has been observed for poliovirus RNA-dependent polymerase, and it has been proposed that the Asp−Asp pair is involved in metal binding.

Overall, magnesium binding sites appear to show a greater homology in their three-dimensional structure, rather than in primary sequence. This is especially so in the case of the restriction enzymes, and the family of bacterial and viral RNase H domains and viral integrases, illustrated in Figure 5.[1,8,10]

IV. Magnesium Analogues

Divalent magnesium is essentially spectroscopically invisible and has such a low electron density that it is difficult to distinguish by most spectroscopic and crystallographic experiments. For this reason, the chemistry of magnesium-dependent enzymes has been studied with the use of transition metal probes and analogues. The underlying assumption in many studies of metal cofactor requirement in nucleic acid

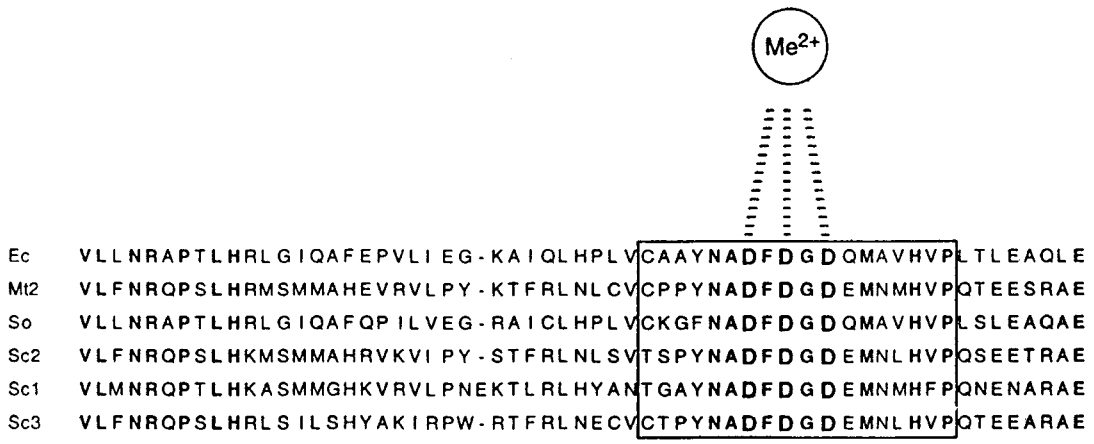

Figure 4. The conserved -NADFDGD- metal-coordination motif from a selection of RNA polymerases from *E. coli*, *M. thermoautotrophicum*, *S. oleracea* chloroplast, and *S. cerevisiae* Pol I, II, and III (adapted from ref 19). The three aspartate residues most likely form a metal binding core. Note the different sequence spacing of the three binding residues relative to the three binding residues shown for *E. coli* RNase H and Che Y in Figure 3.

biochemistry is that magnesium analogues (especially manganese) show similar chemistry to that displayed by Mg^{2+}. It will be seen that this is not a good general assumption. In fact, the stoichiometry and coordination mode of other metals may, and frequently do, differ from Mg^{2+}. In particular, it is usually observed that Mn^{2+} (or other transition metals such as Co^{2+}, Fe^{2+}, or Zn^{2+}) confers higher levels of activity for both native enzyme, and Asp or Glu mutants (since these typically coordinate more tightly than the natural Mg^{2+} cofactor). For example, the plant-stimulated nuclease from *Fusarium solani* is stimulated by $Mn^{2+} > Ca^{2+} > Co^{2+} > Mg^{2+}$, but is inhibited strongly by Zn^{2+}.[24] These transition metal ions may also show changes in coordination geometry, or induce conformation perturbations in active-site residues that influence substrate specificity.

The basis for metal ion mutagenicity and nucleotide selectivity in human DNA polymerase β has been examined.[25,26] It has been demonstrated that distinct nucleotidyl reactivity patterns arise with a variety of divalent metal ions, while these also promote a change in side-chain position of Asp192 relative to Mg^{2+}. *E. coli* DNA polymerase I shows reverse transcriptase ability; however, the fidelity is lower with Mn^{2+} as the catalytic cofactor,[27] and so Mn^{2+} and other divalent analogues such as Be^{2+} are mutagenic and carcinogenic.[28] Mn^{2+} mutagenicity of T7 DNA polymerase and *E. coli* DNA polymerase I arises as a result of reduced nucleotidyl discrimination, even though the activity levels are higher.[29]

In keeping with the aforementioned trends for polymerase enzymes, loss of specificity for the *Taq*I restriction endonuclease is observed with Mn^{2+} relative to the natural Mg^{2+} cofactor.[30] Evidence for three types of ternary complex with Mn^{2+} are presented, presumably each reflecting distinct Mn^{2+} coordination modes. Such loss of sequence specificity with the use of metal cofactors other than Mg^{2+} is common for *Eco*RI and other restriction nucleases.[31]

It has been earlier noted that Mn^{2+} will often promote higher levels of enzyme activity than Mg^{2+}. This should not be misconstrued as an indication that Mn^{2+} is in fact the natural cofactor, but simply reflects the distinct chemistry of this transition metal ion which promotes activity levels that are greater than the requirements for metabolism. For example, Myers and co-workers have explored the metal dependence of the ribonuclease H activity of *Thermus thermophilus* DNA polymerase and have shown that Mn^{2+} promotes higher levels of RNase H activity than Mg^{2+}.[32] This activity is manifest by 5′-3′ exonuclease digestion of RNA/DNA hybrids. Mn^{2+} activates optimally over Mg^{2+}; however, Mg^{2+} is most likely the natural cofactor. In fact, many class I RNases H are activated by Mn^{2+} and can utilize Mn^{2+}, but class II RNases H are inhibited by this metal ion.[33]

In summary, while the use of analogues can provide insight on function, there are differences in coordination chemistry that can lead to changes in substrate selectivity and metal ion stoichiometry. Such problems need to be recognized and accounted for during data analysis.

V. Kinetics/Inhibition

Measurement of enzyme activity as a function of solution conditions provides an effective means of characterizing the metallobiochemistry of magnesium-dependent enzymes. The dependence of activity on solution pH and cofactor concentration can be used to report directly on the ligand environment in the active site, and on metal cofactor stoichiometry.[34−36] This is particularly pertinent to the issue of the metal cofactor requirement to effect catalysis, where there is inconsistency between crystallographic and solution measurements.[10] This issue will be addressed later, but can be readily approached in solution studies by a combination of kinetic and thermodynamic measurements.

Several methods for monitoring activity have been reported. Often these involve the use of radiolabeled substrates and evaluation of the rate of formation of labeled fragments.[37−39] A more convenient method, when applicable, is direct monitoring of the change in absorbance that results from hydrolysis of a nucleic acid oligo- or polynucleotide substrate.[16,34,36,40]

A variety of mechanistic models have been proposed and used to rationalize the metal dependence

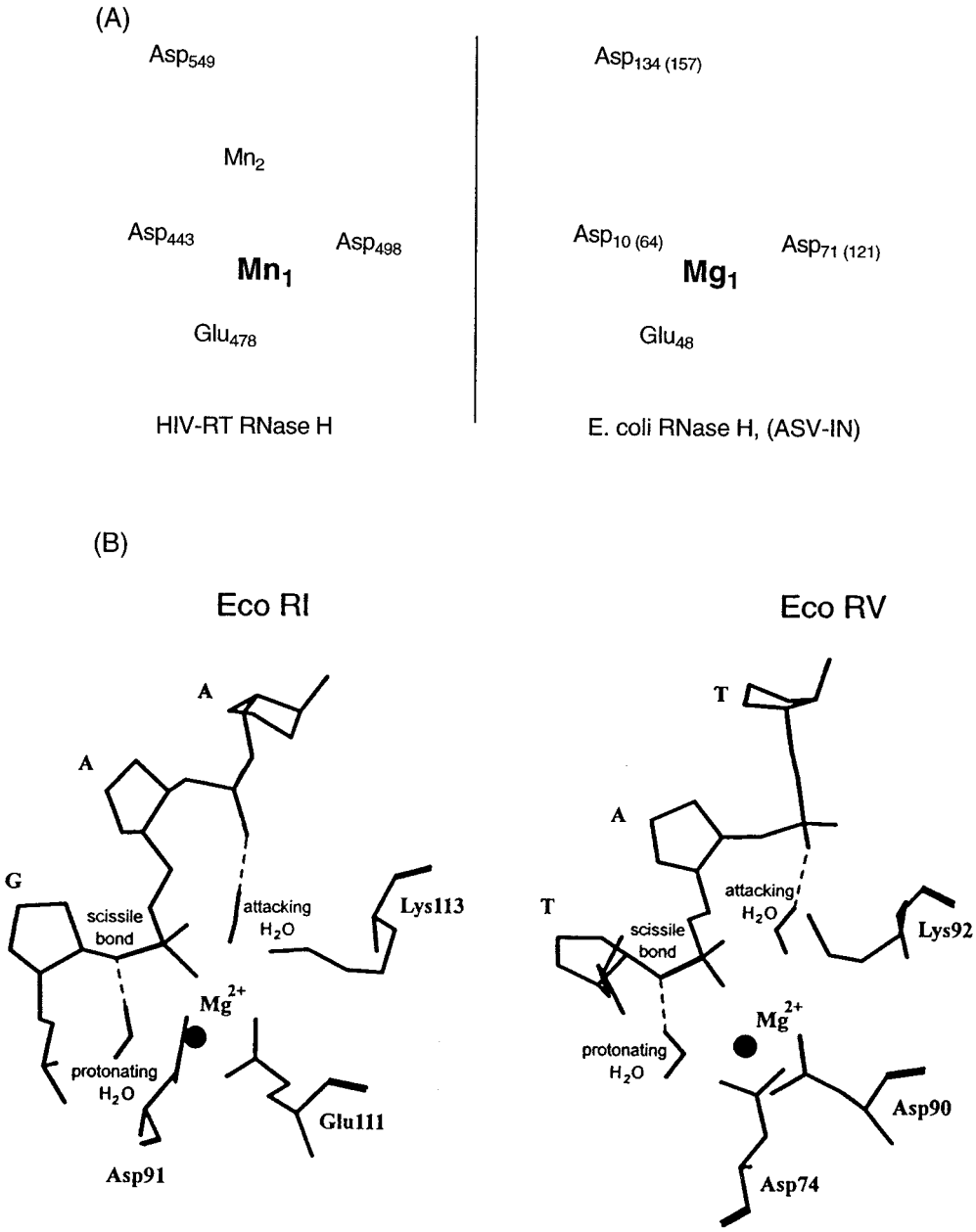

Figure 5. Homology in the tertiary structural arrangement of the active-site metal binding residues in some magnesium-binding proteins. (A) (Left) Schematic illustration of the two Mn^{2+} ions identified in the active site of the HIV-RT RNase H domain after doping the cofactor-free crystal with 45 mM Mn^{2+}. The positions of key carboxylate residues are shown. (Right) The structurally homologous sites in *E. coli* RNase H and avian sarcoma viral integrase, showing the positions of conserved carboxylate residues (the numbering for ASV-IN is shown in parentheses). Only one metal-binding site (Mg^{2+} or Mn^{2+}) has been identified.[49,97] The metal ions in bold are the major homolgous sites common to the enzymes shown in A and B. (B) A comparison of the metal cofactor binding domains, and other features of the catalytic pockets of *Eco*RI and *Eco*RV, illustrating the high degree of local homology for these two restriction endonucleases.[38]

of nuclease activity. This issue has been most thoroughly addressed by the laboratories of Pingoud,[38,41] Halford,[39,42] and Cowan.[17,34] A general observation in almost all plots of enzyme activity as a function of metal cofactor concentration is an initial increase in activity with increasing [Mg^{2+}], followed by a gradual decrease in activity (Figure 6). While direct enzyme inhibition is a possibility, no direct evidence has been advanced for such, and the author of this review is inclined to see this effect as a general example of substrate inhibition, where the metal ions bind to the nucleic acid substrate, perturbing the charge density of the substrate and its interaction with the enzyme. This may also be thought of as a form of competitive binding between the metal ions and enzyme, to the substrate. Such a viewpoint is supported by the similarity of this response over a diverse group of enzymes,[34−36,43] and the similarity of apparent binding constants for metal−substrate complexation determined from these kinetic and thermodynamic measurements. The effect is clear in the case of *E. coli* RNase H, where there is no evidence for secondary magnesium binding sites on the enzyme,[34] while metal−hybrid and metal−enzyme binding constants ($K_I \approx 14$ mM, and $K_{MET} \approx 0.2$ mM, respectively), obtained by kinetic measurements for *E. coli* RNase H[34] and by independent NMR and calorimetric titration methods, are found to be

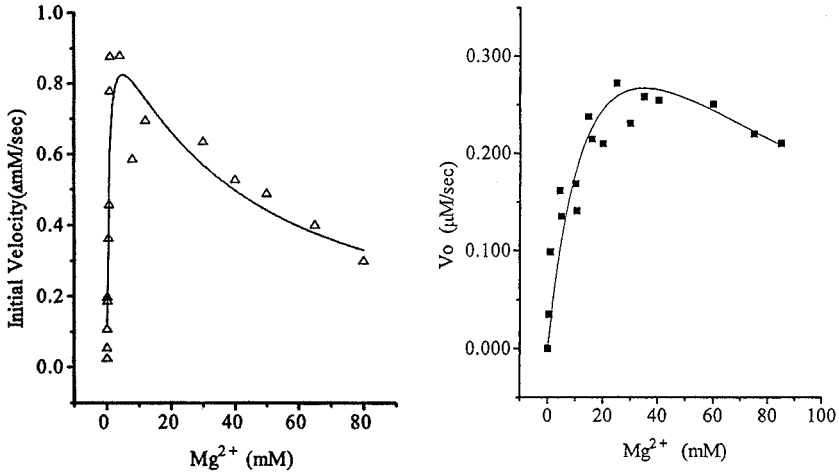

Figure 6. Variation of initial velocity (v_o) with magnesium concentration for *E. coli* RNase H (left) and the exonuclease activity of Klenow (right). The data clearly shows the inhibitory influence of high concentrations of Mg^{2+}, and was fit to a model that assumes metal-mediated substrate inhibition.[34,62]

similar.[44,47,62] This also provides good evidence for catalytic activation by the same metal ion that binds to the enzyme alone.

An important observation is that this kind of inhibition effect is diminished by the presence of polyamines, which competitively displace Mg^{2+} ions, but have an overall reduced charge density relative to a number of divalent magnesium ions bearing an equivalent charge.[36] Such an observation may be taken as a diagnostic test for this kind of metal-mediated inhibition. The generality of a metal-mediated inhibitory influence on substrate interactions with enzyme is consistent with the observation that polyamines (spermine, spermidine, and putrescine) modulate DNA-dependent DNA polymerase activity.[45] These species shift the optimal Mg^{2+} concentration of polymerase from 10 mM to a more physiological level. For example, polyamines were found to increase (by 2−6-fold) the activity of malarial α-like DNA polymerase at suboptimal concentrations of divalent magnesium.[46] This general phenomenon can be attributed to the requisite neutralization of charges on the nucleic acid substrate, but without the inhibitory influence observed with higher concentrations of Mg^{2+}. The higher binding affinities of polyamines also renders these cations more effective than monovalent alkali metal ions.

The observation of sigmoidal behavior, or a lag phase, in some kinetic profiles of activity versus metal cofactor concentration may be taken as evidence for the requirement of additional weakly bound metal cofactors that may either be required for catalytic activity or to stimulate substrate binding.

VI. Mechanistic Issues

In sections II and III, I have presented evidence that favor outer-sphere-mediated hydrolytic pathways for a large section of magnesium-dependent enzymes that carry out reactions on nucleic acid substrates. The activity of substitutionally inert complexes indicates that metal-bound H_2O is not necessarily required as a nucleophile, but rather a free solvent molecule will serve as the hydrolytic agent, perhaps with base catalysis from a neighboring side chain. While H_2O bound to Zn^{2+} and other

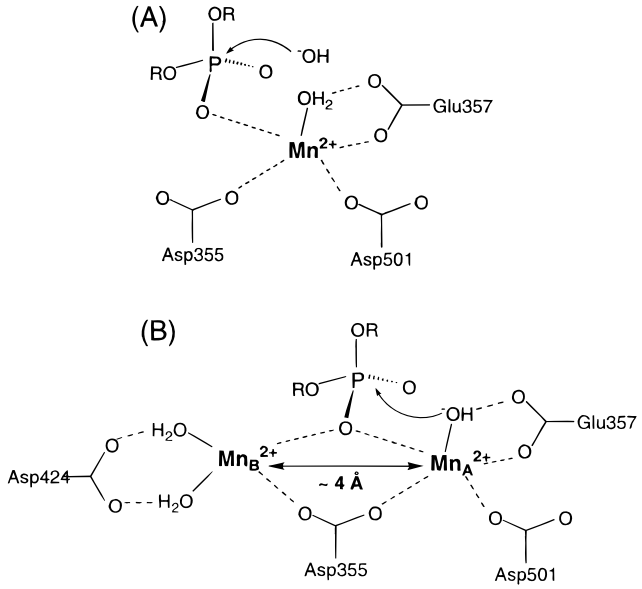

Figure 7. (A) An alternative minimalist scheme for Klenow activity, based on the theoretical analysis of Aaqvist and Warshel,[58] showing one essential metal cofactor and an activated nucleophilic solvent water. (B) Schematic illustration of the metal binding sites identified from crystallographic studies of the Mn^{2+} derivative of the DNA polymerase I Klenow fragment.[48,100] Site B is weakly populated in the presence of substrate or a substrate analogue. Site A is the principal site of both metal coordination and catalytic chemistry.

transition metals is often viewed as the nucleophile, the higher pK_a of Mg^{2+}-bound H_2O makes metal activation of H_2O less favorable in the case of divalent magnesium. Warshel and co-workers have published an insightful theoretical analysis of the Klenow fragment from DNA polymerase I, which is more generally applicable and identifies electrostatic stabilization as the principal role of the metal cofactor(s).[64] These workers argued against a magnesium-bound hydroxide as the active nucleophile, but favored an external hydroxide that is stabilized electrostatically by the metal cofactor. Stabilization of this hydroxide could be accounted for in terms of the electrostatic influence of the high affinity metal ion alone (Figure 7A). The role of the putative low-

affinity metal ion (site B) was discussed in terms of electrostatic stabilization of the negative charge that is transferred from hydroxide to phosphate. Such a model was also favored for one metal ion enzymes (such as Staphylococcal nuclease) where the single cation carries out both roles.[58] In this, and other cases where single metal ion cofactors have been identified, the metal is typically divalent calcium. Inasmuch as all Ca^{2+}-dependent nucleases act by one metal ion catalytic pathways, it seems reasonable to infer that magnesium, with a charge density that is significantly higher than that of Ca^{2+},[67] would also show a requirement for one metal cofactor. This is indeed generally substantiated by available evidence.

A. Stoichiometry

The stoichiometry of metal cofactor that is required to mediate phosphate ester hydrolysis is an important variable for mechanistic considerations. This problem has been the subject of debate as a result of contradictions between solution and crystallographic studies[47−50] and has fueled recent efforts by chemists to construct synthetic models to address this issue.[51−53] Several binuclear metallohydrolases that contain a pair of bridged transition metal ions at the active site have been characterized.[2,54] However, while there are a few examples of metabolic enzymes that appear to utilize two magnesium centers in close proximity, such as xylose (glucose) isomerase,[55] and glutamine synthase,[56] the evidence for binuclear magnesium in nucleic acid hydrolysis is not so clear-cut and is supported by a rather small body of crystallographic data with relatively few confirmatory results from solution experiments. The two metal ion mechanism has been proposed for numerous metal hydrolase,[2] nuclease,[39,57,84] polymerase,[58] and ribozyme reactions;[59,60] however, solution thermodynamic and kinetic measurements on substrate-free enzyme typically indicate a single metal cofactor (Table 3). An important caveat to note is that simple binding experiments do not directly indicate the number of metal ions required for catalytic turnover; for example, it is possible that an additional metal binding site is found in the enzyme−substrate complex, as suggested for the Klenow fragment of DNA pol I.[48,61]

Table 3. Summary of Metal Binding Constants and Stoichiometries Evaluated from Solution Measurements and Crystallographic Analysis[10]

	crystallography		solution	
enzyme	no. of M^{2+}	$[M^{2+}]$ used (mM)	no. of M^{2+}	K_D (mM)
S. nuclease	1	0 (Ca^{2+})[a]	1	0.005
Klenow exonuclease	2	12 to 50 (Mn^{2+})	1	0.40
Exo III	1	20 (Mn^{+})	1	0.11
*Eco*RV	1 or 2	10 to 30 (Mg^{2+})	1	ND[b]
*Eco*RI	1		1	ND
HIV-RNase H	2	45 (Mn^{2+})	ND	
E. coli RNase H	1	50 to 100 (Mg^{2+})	1	0.5
Che Y	1	50 (Mn^{2+})	1	0.2
ASV-IN	1	200 (Mg^{2+})	ND	

[a] The Ca^{2+} cofactor binds sufficiently tightly that it can be isolated in the metal-bound state and requires no additional solution Ca^{2+}(aq) to maintain saturation of this site. [b] ND, not determined.

However, this question can be addressed by kinetics methods. One strategy that has been used successfully involves determination of metal binding parameters from kinetics experiments, and comparison of the fitted binding parameters with results from independent calorimetry experiments carried out in the presence of substrate analogues.[34,62] The results are analyzed with a view to identifying specific mechanistic models that yield consistent results from each independent approach and, importantly, that are consistent with the physiological availability of the divalent metal ions. Nature imposes well-defined limits on metal binding stoichiometry. This is controlled by both the magnitude of the dissociation constant and the bioavailability of metal cofactor in vivo. For example, a 40 mM binding affinity is of little significance if the available concentration of the cofactor is 0.5 mM. In effect, the dissociation constant for a biologically relevant divalent magnesium cofactor should be ~0.5 mM, since this is the concentration of free magnesium in a cellular environment.[14] This is a powerful constraint in assessing metal ion stoichiometry and distinguishing kinetic models.

Evaluations of metal cofactor stoichiometry by crystallographic and solution studies have been made for several enzymes (summarized in Table 3). There is now extensive data available for *E. coli* Klenow fragment, RNase H, Exo III, and *Eco*RV. Some of these results will be presented in greater detail in the sections dealing with specific enzymes that follow; however, some general comments can be made that shed considerable light on this dichotomy. X-ray structures of proteins with labile Mg^{2+} cofactors are usually obtained by doping preexisting crystals of "apo" enzyme with very high concentrations of metal cofactor to permit population of metal binding sites in a kinetically reasonable time frame. Often the metal used is not the natural magnesium cofactor, which has a low electron density and is difficult to distinguish from oxygen in water, but rather a transition metal ion carrying a higher electron density which is more easily observed. Increasingly, crystallographic studies are using the natural Mg^{2+} cofactor and making use of the strong pattern of octahedral coordination by metal-bound H_2O to distinguish Mg^{2+} from general solvent water. Although much useful information has come from studies with magnesium analogues, a few simple caveats must be kept in mind. (1) Coordination sites in a preexisting structure of an apoenzyme may not exist in the structure of the active metalloenzyme. (2) Coordination preferences for Mg^{2+} and analogues are often different. (3) Under conditions of high metal concentration used in doping experiments, weak and physiologically irrelevant sites may be occupied.

The paradigm of the two metal ion model is perhaps best defined as the requirement for two metal ions, located in close proximity (<4 Å), that are bridged by a common substrate. This allows a clear distinction from other enzymes that may bind two metal cofactors in the same catalytic domain, but do not function as a coherent catalytic unit. For

example, crystallographic analysis of T5 5′-exonuclease shows two Mn^{2+} sites for data collected in the presence of 25 mm Mn^{2+}.[63] These sites are, however, separated by 8.1 Å (10 Å in the case of Taq 5′-exonuclease), which is significantly greater than the <4 Å separation observed in the putative two-metal nucleases. Two Mg^{2+} sites are separated by 7 Å in T4 RNase H (see Figure 16, and the discussion in section VII.D). Accordingly, one would expect a difference in mechanism from the two-metal variety for these, and related enzymes.

The stoichiometry of catalytic metal ions is better characterized for calcium-dependent nucleases. These have been more extensively studied, for reasons that include: (1) calcium-binding proteins typically bind the cofactor with higher affinity than magnesium, and so the site is more readily populated; (2) calcium is a more electron-rich cation, and is therefore easier to identify; and (3) there are significantly more examples of well-defined structures of calcium proteins available for analysis. Overall, there is extensive experimental data from solution and structural studies on a wide variety of enzymes to support a one metal ion model as the general mechanistic scheme in considerations of magnesium- or calcium-mediated hydrolysis reactions in nucleic acid biochemistry.

B. Inner-Sphere/Outer-Sphere Pathways

With the background provided by sections II and III, it is clear that protein-bound Mg^{2+} shows extensive hydration. It might be expected, then, that these metal-bound water molecules would be utilized in enzyme-mediated chemistry. While direct binding of a substrate molecule to a metal cofactor is common for transition metals, the assumption that magnesium cofactors generally serve as simple Lewis acids is inconsistent with the observation that substitutionally inert transition metal complexes, incapable of direct binding to substrate, can promote the reactions of a number of important magnesium-dependent enzymes.[16,35,37] In magnesium biochemistry, outer-sphere pathways that require a solvated metal cofactor are important, and are perhaps the major mechanistic class displayed during metal-mediated phosphate ester hydrolysis. As an aside, it is intriguing to note that crystallographic data collected on zinc carboxypeptidase suggests that the peptide carbonyl oxygen never forms a direct coordinate bond to the zinc ion.[65] The metal cofactor seems to electrostatically stabilize the buildup of negative charge on the carbonyl O in the transition state, and also facilitates deprotonation of a bound nucleophilic H_2O.

This idea of outer-sphere activation is, therefore, an important one that is likely to be of wide relevance. Figure 8 illustrates outer-sphere catalysis by stabilization of the developing negative charge in the transition state of the reaction. According to this mechanism the metal cofactor serves principally to stabilize the transition state, either electrostatically, and/or through hydrogen bonding from the metal-bound waters. The latter appears to be the larger contribution in the few cases that have been examined.[16,35] Experimental data clearly show that transition-state stabilization is effected by hydrogen bonding with essentially no contribution from electrostatic stabilization through the charge on the cofactor.[16,35,66] The solvation state of the metal cofactor is of critical importance for the proper functioning of these enzymes and is defined by the number of protein ligand contacts. The metal binding pockets of metal-dependent nucleases have evolved to allow a large variation in the number of protein ligands (and thereby solvent water), while optimizing the binding affinity to physiological requirements. The distinct coordination properties of Mg^{2+} (six coordinate), versus the larger Ca^{2+} (six to eight coordinate), most likely favors inner-sphere over outer-sphere chemistry for the latter, as described in section II.

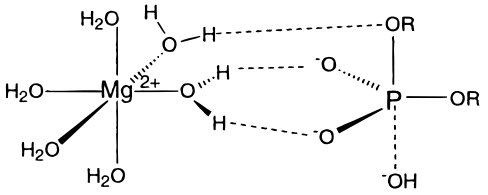

Figure 8. Schematic illustration of transition-state stabilization by outer-sphere complex formation to hydrated magnesium ion. Both hydrogen bonding and electrostatics contribute to the stabilization of the increased negative charge in the transition state; however, for divalent magnesium, the hydrogen-bonding contribution is dominant.[16,35]

A major difficulty in distinguishing inner- and outer-sphere pathways for magnesium-promoted reactions stems from the kinetic lability of this metal ion. One approach that has been successfully applied is the use of cobalt and chromium complexes that are substitutionally inert within the time frame of the enzyme-catalyzed reactions.[9,37,68,69] If such complexes promote a magnesium-dependent reaction, this is evidence in favor of an outer-sphere pathway. Of course the absence of activity promoted by such complexes does not necessarily preclude such a pathway.

Catalytic activation of *E. coli* ribonuclease H by a series of inert chromium complexes $[Cr(NH_3)_{6-x}(H_2O)_x]^{3+}$ ($x = 0-6$) that bear water and ammonia ligands in well-defined geometries in the inner coordination shell has been examined.[16,35] This approach has been applied also to *E. coli* exonuclease III,[35] topoisomerase I,[70] and other enzymes and ribozymes[9,71] to establish outer-sphere pathways. Such complexes are observed to function by transition-state stabilization, and the approach permits one to distinguish hydrogen bonding from electrostatic contributions inasmuch as NH_3 bound to Cr(III) or Co(III) centers has negligible H-bonding propensity.[66] Moreover, since complexes of the general form $[Cr(NH_3)_{6-x}(H_2O)_x]^{3+}$, with well-defined, but variable, coordination geometry are synthetically available, these also afford a probe of the preferred structural arrangement for hydrogen-bonding interactions. For example, it has been found that only those complexes with a facial array of bound water molecules promote catalysis of *E. coli* ribonuclease H; as expected from comparison with the ligation of the enzyme-bound Mg^{2+} cofactor. Figure 9 illustrates the crystallo-

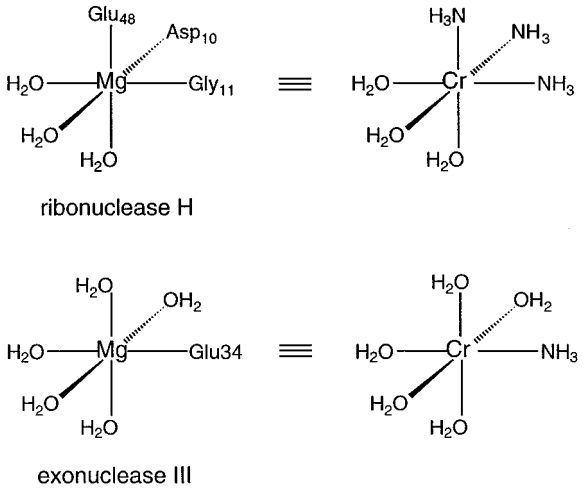

Figure 9. The crystallographic coordination modes for magnesium binding to *E. coli* ribonuclease H and exonuclease III (compare Figure 3), illustrating the distinct solvation states of each ion. These coordination states are compared to inert chromium complexes that show the minimal hydration requirements for efficient promotion of enzyme-catalyzed phosphate ester hydrolysis for each enzyme. For ribonuclease H, the *mer*-$[Cr(NH_3)_3(H_2O)_3]^{3+}$ complex is inactive, while for exo III, those complexes with one less water of hydration (namely *cis*- or *trans*-$[Cr(NH_3)_2(H_2O)_4]^{3+}$]) show significantly reduced activity.[16,35]

graphically defined hydration state of the natural magnesium cofactor and also shows the minimal hydration states required by the inert metal probe complex to efficiently mediate catalytic activity.[16,35] There is an apparent relationship between the requirements for metal-bound solvent interactions with the substrate, and the number of coordinating protein ligands, which ultimately controls the hydration state of the bound metal. Relative to ribonuclease H, exonuclease III requires more extensive solvation of the metal cofactor for optimal activity, and this is reflected in the smaller number of protein ligands (Figure 3).

C. Engineering the Active Site To Remove the Metal Dependency

The preceding section has described a mechanistic model for enzyme-catalyzed metal-mediated hydrolysis of a nucleotide backbone,[16,35] through stabilization of a transient intermediate by formation of a hydrogen-bonded outer-sphere complex with the positively charged hydrated metal cofactor. Consideration of this model led to the hypothesis that mutation of active-site carboxylate residues to positively charged Lys or Arg might provide sufficient positive charge density and hydrogen-bonding propensity in the active-site domain to mimic the role of hydrated divalent magnesium. This idea has been successfully demonstrated by engineering novel metal-independent active mutants of *E. coli* RNase H (Figure 10).[72]

Of the three principal active-site carboxylate residues, crystallographic evidence suggested that both Glu48 and Asp10 are bound to Mg^{2+}, while Asp70 has been proposed to serve as a catalytic base that is required for deprotonation of water prior to hydrolysis of the backbone (Figures 2 and 10).[73] Mutation of the essential catalytic base (Asp70Asn),

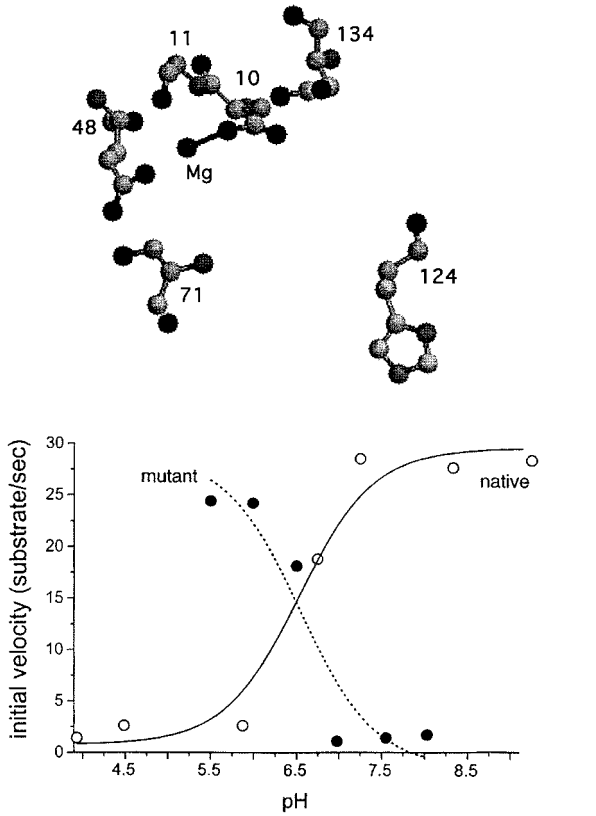

Figure 10. Active site of native *E. coli* ribonuclease H. A double mutant of active-sites residues (Asp10Arg/Glu48Arg) yields a functional enzyme with a distinct pH profile, relative to native.[72]

Table 4. Activities of Mutants Relative to Native *E. coli* RNase H[72]

enzyme	k_{cat} (s^{-1})
native	28 ± 8
native (-Mg^{2+})	*a*
Glu48Asp	1.1 ± 0.3
Glu48Gln	*a*
Asp70Asn	*a*
Asp10Glu	14 ± 4
Asp10Asn	*a*
Asp10Ser	*a*
Asp10Arg	*a*
Asp10Gly	*a*
Asp10Arg/Asp70Lys	*a*
Asp10Arg/Glu48Arg (-Mg^{2+})	27 ± 7^b

[a] Activity is below detection levels. [b] For the Asp10Arg/Glu48Arg mutant the activity obtained at pH $\approx$ 5.5 is reported. Other data was obtained at pH 7.3.

or single-point mutations of the magnesium-binding residues, Asp10 and Glu48, yield inactive enzyme, either with or without added Mg^{2+} (Table 4), with the expected exception of the Asp10Glu and Glu48Asp mutants which retained their ability to bind metal cofactor and exhibited 50% and 4% activity, respectively. Introduction of a single positive charge (for example, Asp10Arg) was found to be insufficient for activation, while also serving to inhibit binding of magnesium cofactors. In contrast, at low pH the double mutant Asp10Arg/Glu48Arg demonstrated almost native levels of initial activity, even in the absence of added Mg^{2+}, although significant product inhibition was observed, presumably as a result of the increase in positive charge density in the catalytic

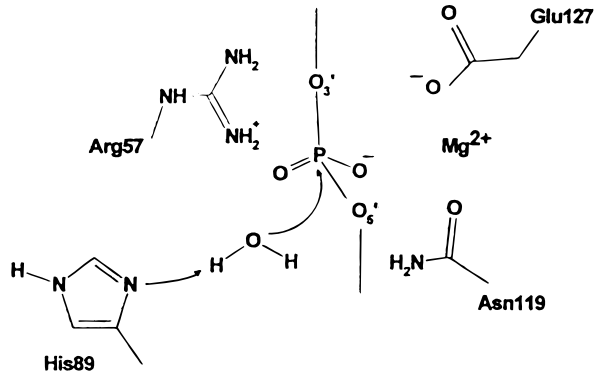

Figure 11. Active site of Serratia nuclease, showing the metal cofactor and Arg residues that likely contribute to transition state stabilization.[36]

pocket. Both an increase in product inhibition and a requirement for a lower pH to achieve reasonable levels of activity are reasons for the use of metal cofactors rather than positively charged side chains in enzyme design. These observations are consistent with a model of substrate activation by native enzyme that is dominated by hydrogen bonding from waters of solvation. In the double mutant, the role of the hydrogen bond donor is most likely accommodated by the guanidinium centers of the mutant side chains. The use of guanidinium centers in natural enzymes to further enhance transition-state stabilization is well-documented for *Serratia* nuclease and homologues (Figure 11),[36] RNase T1,[74] and nuclease P1.[75]

VII. Nucleases (Restriction Enzymes)

Few magnesium-dependent nuclease enzymes have been studied in detail insofar as the role of the essential metal cofactor is concerned. Noteworthy exceptions are the restriction enzymes *Eco*RI, and especially *Eco*RV, which has been the subject of lively competition between the laboratories of Halford and Pingoud.[38,39,65] There is now an extensive body of structural and mechanistic information concerning the role of the metal cofactors in the function of this enzyme, and Pingoud and Jeltsch have recently published a thorough review of the recognition and cleavage chemistry of type II endonucleases, the family to which both of the aforementioned enzymes belong.[76]

A. *Eco*RV and *Eco*RI

Figure 12 shows that *Eco*RV binds and cleaves double-strand DNA in a blunt-end fashion with a high degree of specificity at sites defined by a unique sequence of six contiguous bases (5′-GATATC-3′).[38]

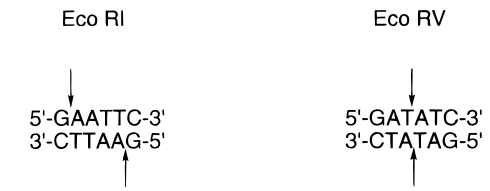

Figure 12. Schematic illustration of the recognition sequences for *Eco*RI and *Eco*RV. The arrows indicate the cleavage sites.

*Eco*RI is also a type II endonuclease and shows a high degree of structural homology with the active site of *Eco*RV (Figure 5B),[38,41,82] although there is no general sequence homology. Divalent magnesium is an essential cofactor for type II endonucleases. A mechanistic model has been suggested for *Eco*RI and *Eco*RV where the attacking water is activated by the phosphate placed 3′ to the scissile phosphodiester and the leaving group is protonated by a water molecule associated with the Mg^{2+} cofactor.[38,41] This effect has been described as substrate-assisted catalysis and has been supported by experiments that remove the phosphate group (substituting with an H-phosphonate) and by phosphorothioate substitution. In general, activity is lost or severely diminished following these substitutions, and in the latter case, activity is only observed if the negatively charged sulfur is in the R_P configuration. Nevertheless, there would appear to be a requirement for participation by a carboxylate residue to provide base catalysis, since the pK_a of a phosphodiester is too low (pK_a < 2) for this role.

Unlike *Eco*RV, *Eco*RI produces sticky end products, since the homologous active site is offset relative to the DNA recognition sequence (5′-GAATTC-3′; Figure 12). In the presence of Mg^{2+}, association of *Eco*RI with palindromic tridecadeoxynucleotides containing the recognition site occurs at an almost diffusion-controlled rate.[83] Figure 5B illustrates how both enzymes show a similar structural arrangement in the vicinity of the scissile phosphate, and form a motif that is composed of four active-site residues (Pro90, Asp91, Glu111, and Lys113 for *Eco*RI; and Pro73, Asp74, Asp90, and Lys92 for *Eco*RV) that appear to form a binding pocket for the essential divalent metal cofactor.[38,41] For *Eco*RI, both kinetic and structural studies are consistent with one essential metal cofactor in the active site.[38,41] The role of the metal cofactor has been most clearly elucidated for *Eco*RV and this is now taken up, although the mechanistic details of metal-promoted hydrolysis are most likely similar for each enzyme.

Unlike other restriction enzymes in this class, which demonstrate specific binding in the absence of Mg^{2+}, *Eco*RV binds nonspecifically in the absence of the divalent cofactor.[77] In 1993, on the basis of kinetic evidence, Pingoud and co-workers proposed a requirement for one metal ion to effect *Eco*RV catalysis,[38,41] but also pointed out that binding of an additional high-affinity metal ion could not be excluded since an additional residue (Glu45) was available for binding and coordination of heavy metals (such as Pb^{2+}) had been recognized in crystallographic experiments. The 2-Å resolution structures of dimeric *Eco*RV cocomplexed with both an undecamer substrate and product (Figure 13),[50] published by Kostreiva and Winkler, showed one Mg^{2+} bound to the scissile phosphodiester group and two carboxylate oxygens from Asp 74 and Asp 90 in the substrate complex, while the product complex showed two Mg^{2+} bound to each 5′-phosphate ester oxygen (Figure 13C) (Mg_3^{2+} and Mg_4^{2+}). On the basis of this structure, and other kinetic data, Halford and Winkler and co-workers subsequently proposed a two

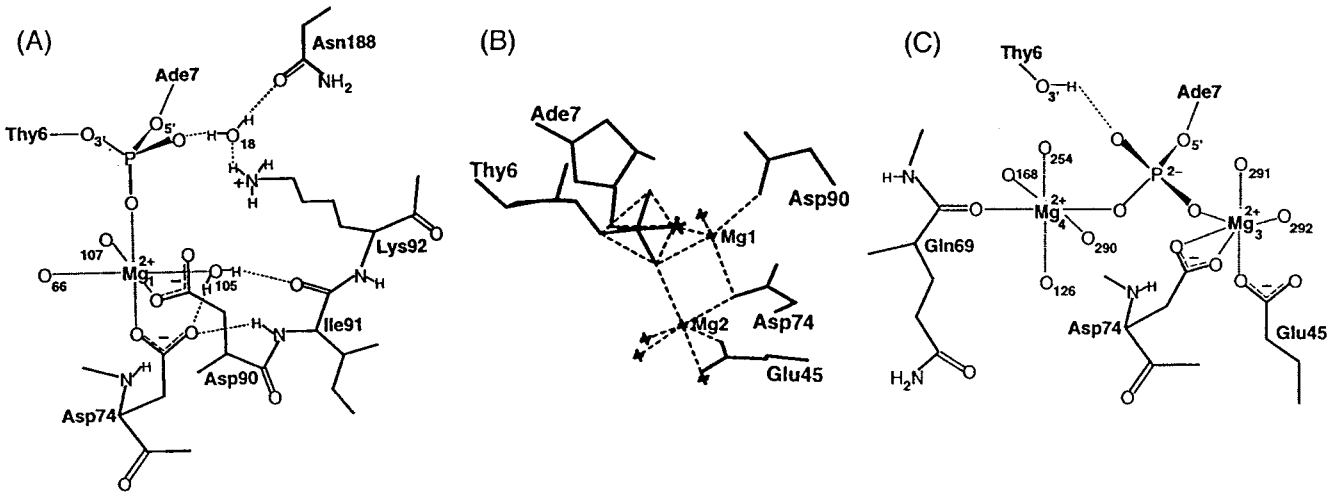

Figure 13. (A) Schematic illustration of the catalytic pocket for the *Eco*RV−substrate complex. Note that only one metal cofactor is identified. (B) Proposed model for the transition state, showing recruitment of a second metal cofactor. However, in other work, the critical residue Glu45 has been mutated to residues that do not bind magnesium and the mutants were found to be active. (C) Schematic illustration of the catalytic pocket for the *Eco*RV−product complex. Note that now two metal ions are observed, although metal ion concentrations in the range of 10 to 30 mM were used. Adapted from ref 50.

metal ion transition-state model. Later, Halford and co-workers also examined the metal requirement for *Eco*RI and *Eco*RV and correctly pointed out that the Pingoud analysis incorrectly assumed total cooperativity.[39] They suggested a catalytic requirement of one metal cofactor for the former, and a two metal ion mechanism for the latter. It was observed that Ca^{2+} inhibits Mg^{2+} activation of *Eco*RV, but stimulates Mn^{2+} activation of the same enzyme, suggesting that Ca^{2+} can displace a metal from one of the two sites. Such a model was supported by stopped-flow fluorescence experiments that examined the influence of metal ions on DNA binding to *Eco*RV.[42] Two Mg^{2+}-dependent transitions were observed and interpreted in terms of a model where one Mg^{2+} binds to the active site before phosphodiester hydrolysis, with a second binding subsequently to a preformed enzyme−DNA complex.

These facts notwithstanding, several problems nevertheless remained with this interpretation. First, the crystal complex with the substrate lacked activity, even in the presence of saturating magnesium, and the active site bound only one divalent magnesium ion (Figure 13A). Second, a Mg^{2+} concentration of $\geq$10 mM was required to populate both sites; however, the key issue is whether these sites can be populated under physiological conditions (<1 mM). Third, and perhaps most importantly, the critical residues that define the binding site of the second metal ion (Mg_2^{2+} in Figure 13B), especially Glu45, have been mutated to nonbinding residues (such as Ala) and the mutants were found to be active.[78] It has therefore been suggested that a second, but spatially distinct and noncatalytic site might exist,[77] since the DNA binding specificity is increased by Mg^{2+} binding to a site that is distinct from that of the catalytic center (bound by Glu 45, Asp 74, Asp 90). Also, the Ala triple mutant which cannot bind catalytic metal, was found to bind specifically to DNA in the presence of Mg^{2+}. Phosphorothioate derivatives provided further evidence for a Mg^{2+} site distinct from the catalytic center. Only one of these ions is required for catalysis; the other appears to promote binding of the substrate molecule. This most likely corresponds to the additional metal cofactor suggested by the stopped-flow kinetic studies of Halford and co-workers.[39,42]

Specific recognition of DNA by *Eco*RV can be promoted by Ca^{2+} ion. By analyzing gel shifts with a permuted set of DNA fragments, the degree of DNA bending was shown to be similar to that seen in the crystal structure of the cognate DNA−protein complex in the presence of Mg^{2+}.[57] Calcium ion, therefore, mimics the ability of Mg^{2+} to generate a specific protein−metal−DNA complex, but is incapable of inducing the cleavage reaction. This may also reflect the tendency of Ca^{2+} to engage in inner-sphere rather than outer-sphere interactions, if the latter are required for catalysis. Outer-sphere activation is also consistent with the absence of any significant influence on the relative cleavage rates for Mg^{2+}- versus Mn^{2+}-induced activity with phosphorothioate substrates, since direct coordination to the metal cofactor should discriminate between a hard Mg^{2+} ion and softer Mn^{2+} ion.[79]

Recent results for an Ile91Leu mutant show a greater than 1000-fold decrease in activity.[80] A change in metal ion dependency was observed, with a preference for Mn^{2+} rather than Mg^{2+} as for the native enzyme. The mutant shows evidence of nonspecific DNA binding in gel-shift experiments, as does the native. At noncognate sites that differ from *Eco*RV by one base pair, Mn^{2+} gives higher cleavage rates than Mg^{2+}, but the effect is reversed for the Ile91 Leu mutant.[81] Since each mutant requires the same carboxylate residues for binding, compared to native, it is likely that the switch in metal preference arises from a structural perturbation of the metal binding pocket, as described in section IV.

B. Other Restriction Nucleases

There is a paucity of structural and mechanistic information concerning other restriction enzymes,

especially with regard to the chemistry of the metal cofactor. Nevertheless, the constellations of active-site metal-binding residues in other structurally characterized endonucleases (*Bam*HI,[84] and *Pvu*II[85]) is similar to that of *Eco*RI and *Eco*RV, summarized in Figure 5B,[86] suggesting that each of these enzymes proceed by similar one metal catalyzed mechanisms, and most likely by outer-sphere pathways (although such enzymes are not activated by substitutionally inert complexes[9]). Two points are particularly noteworthy from these structural studies. First, the similarity in tertiary structure in the magnesium binding site is not reflected in the primary sequence; and second, there is extensive structural similarity in the active sites of these restriction enzymes relative to the 3′-5′ exonuclease domain of the Klenow fragment. The restriction nucleases are likely to proceed by a one metal ion pathway, while the stoichiometry for Klenow will be addressed in section VIII.B later in the review. The key issues of blunt or sticky end cutting, and sequence recognition for the restriction endonucleases, are most likely dictated by the positioning of other residues at the enzyme−substrate interface, with little involvement (except for a few cases such as *Eco*RV) by divalent metal cofactors. As a side note, it has been observed that introduction of phosphoramide bonds at the cleavage position of one strand blocks cleavage of that strand for the endonucleases *Eco*RII and *Sso*II, and in the case of *Eco*RII reduces the rate of cleavage of the other natural strand.[87] In the presence of the Mg^{2+} cofactor, association rates are reduced 3-fold and dissociation rates are increased 1.5-fold, and so recognition and specific binding is strongly influenced by the metal cofactor. This is consistent with the breakdown in recognition specificity of *Eco*RI and other endonucleases (such as *Apo*I, *Ase*I, *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Pst*I, *Pvu*II, and *Taq*I, among others)[31] with magnesium analogues, as previously noted in section IV.

C. Exonuclease III

Escherichia coli exonuclease III is a monomeric low molecular weight ($M_r \approx 28$ kDa) apurinic/apyrimidinic endonuclease that serves an integral role in DNA repair.[88] The crystal structure of the enzyme has been elucidated in the metal-bound state (for Mn^{2+} and Sm^{2+} derivatives) and shows one metal cofactor at the active site (Figure 3).[89] This is a particularly valuable example for illustration of the difference in bound metal stoichiometry when evaluated for different metal ions. The binding of Mg^{2+}, Ca^{2+}, and Mn^{2+} to the *E. coli* ribonuclease H and exonuclease III enzymes has been compared in solution, and clearly demonstrates 1:1 stoichiometry for metal binding to ribonuclease H, but not for exonuclease III (Figure 14).[47] For both Mg^{2+} and Ca^{2+} ions, one binding site was determined. In contrast, the binding profile for Mn^{2+} in exo III is distinct, with clear evidence for (at least) two classes of metal binding site, one showing exothermic binding and the other showing endothermic binding. The X-ray structure of exo III obtained by Tainer and co-workers

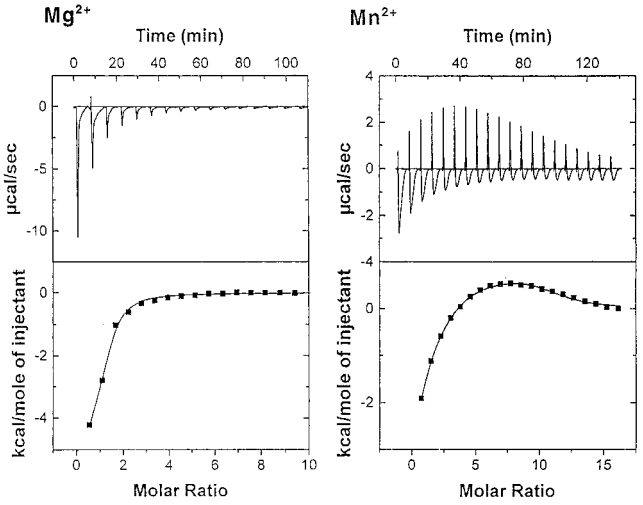

Figure 14. (Left) Calorimetric titration of *E. coli* exonuclease III with Mg^{2+}. The raw data is shown above, and the integrated heats and fitted data are below. (Right) A similar experiment with Mn^{2+} as titrant.[10,47]

showed only one bound Mn^{2+};[89] and so one class of site appears not to be populated, and is possibly sterically inaccessible for the crystals of native enzyme with the doping strategy employed (compare the case of Klenow, vide supra). This result is particularly pertinent to the issue of *one metal* versus *two metal* ion mechanisms in phosphate ester hydrolysis. Studies with substitutionally inert complexes support an outer-sphere mechanism of hydrolysis,[35] and inhibition at higher metal cofactor concentrations is again observed.[35,90]

D. Ribonuclease H

One of the best characterized examples is the *Escherichia coli* endoribonuclease H, a low molecular weight enzyme ($M_r \approx 17\,580$) that hydrolytically cleaves the ribonucleotide backbone of RNA·DNA hybrids, producing 5′-phosphate and 3′-hydroxyl oligonucleotides. Both native[73,91] and mutant[92−94] *E. coli* RNase H enzymes have been structurally characterized, as well as other members of this general family. Other relevant proteins include CheY, a chemotaxal protein that shows significant structural homology with *E. coli* RNase H in the metal-binding active site.[95] *E. coli* ribonuclease H is structurally homologous to the RNase H domain of HIV reverse transcriptase (RT) (Figure 15) and shows conservation of key active-site residues (Figure 5A),[91,96,97] nevertheless, no consensus has been reached on the stoichiometric requirement for metal cofactors in these two enzymes. The *E. coli* enzyme has been extensively characterized by NMR methods (both structural and functional studies),[98] including metal binding.[99]

A crystallographic analysis of the Mn^{2+}-doped crystals of the RNase H domain of HIV-RT revealed two bound Mn^{2+} ions (Figure 5A),[97] located among four acidic residues (Asp443, Glu478, Asp498, and Asp549) in a Mn^{2+}-doped crystal.[97] These acidic residues are four of the seven conserved residues found in all bacterial and retroviral RNase H domains, including the *E. coli* enzyme (Figure 5A).

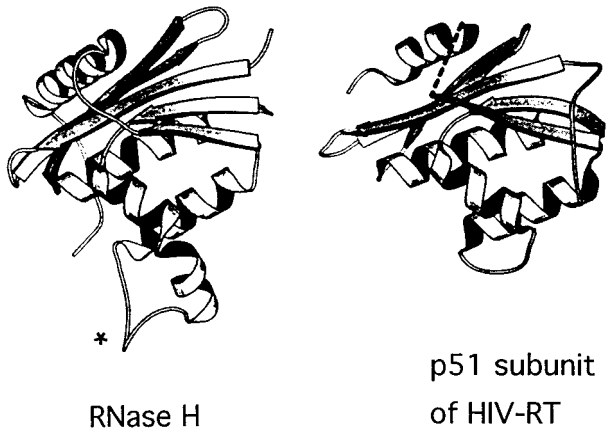

Figure 15. The substrate binding loop (∗) of *E. coli* RNase H substrate binding is missing in the p51 subunit of HIV-RT, and is replaced by the p66 subunit (not shown) in the case of HIV-RT. Adapted from ref 101.

Either the HIV-RT RNase H domain does indeed bind two divalent metal ions, or this result again reflects the specific use of Mn^{2+} and/or the high concentrations of Mn^{2+} used (~45 mM) in the doping technique. The discussion that follows for reverse transcriptase (section VIII.C) and retroviral integrase (section X) provides further insight on this point. On the basis of crystallographic evidence from the HIV-RT RNase H domain, it was proposed that the enzyme most likely acts in a manner analogous to the exonuclease domain of DNA polymerase I (Figure 7B).[100] By analogy with the two metal ion model proposed for the Klenow fragment, a similar metal cofactor arrangement was also proposed in preliminary structural studies of the *E. coli* ribonuclease H.[96] However, more detailed crystallographic work, with magnesium-bound enzyme,[73,91] has suggested one metal binding site in an analogous fashion to the magnesium binding pocket of the CheY chemotaxal protein (Figure 3).[95] A one metal ion model is also strongly indicated by solution kinetic, NMR, and calorimetric studies,[34,44] for both Mg^{2+} and Mn^{2+} binding.

Although the *E. coli* RNase H active-site domain is structurally similar to that of the HIV RNase H domain, one distinct feature is the presence of a basic loop in the former, that is absent in the HIV RNase H domain where it is replaced by a piece of the polymerase domain from the other subunit (Figure 15). Marqusee and co-workers have demonstrated that the basic loop is not essential for catalytic activity.[101] A significantly reduced activity with a strict Mn^{2+} requirement was found, while the Mn^{2+} affinity also decreased by 2 orders of magnitude.

Structural studies have provided valuable insight on metal cofactor chemistry for this family of enzymes. The structure of bacteriophage T4 RNase H has been determined and shows two bound Mg^{2+} ions (Figure 16).[102] One is surrounded by six H_2O and is therefore held in an outer-sphere mode, while the other is ligated by five H_2O and the carboxylate of Asp132. The Ba^{2+} derivative also showed two bound ions at distinct sites from the Mg^{2+} sites, with intermetal distances of 5.0 and 7.0 Å, respectively. In both cases, this is greater than the putative two

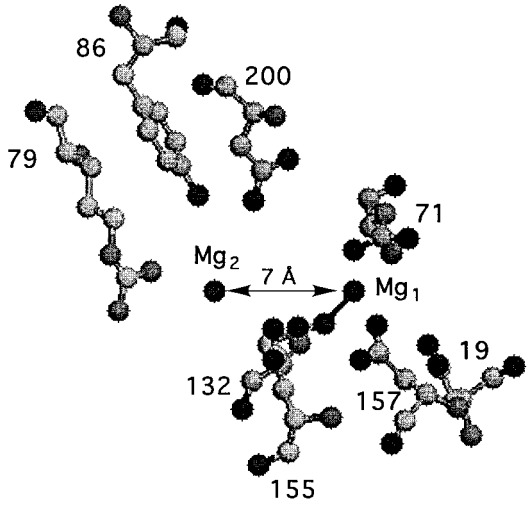

Figure 16. The active site of bacteriophage T4 RNase H showing the two Mg^{2+}-binding sites. The intermetal distance is 7 Å.

metal ion model proposed for certain nucleases. The Ba^{2+} ions were ligated by Asp19, Asp132, Asp155, and Asp132, Asp155, Asp157, respectively. With Mn^{2+}, only one bound ion was observed.

The metal cofactor regulates many facets of enzyme chemistry. Crouch and co-workers have examined the kinetics of hybrid binding to *E. coli* RNase H using surface plasmon resonance.[103] The presence of Mg^{2+} appears to decrease k_{on} by almost 30-fold, and k_{off} by 5-fold, with an effective increase in binding affinity of 6-fold.

E. General Nucleases

There exist many families of endo- and exonuclease enzymes that digest target DNA molecules in a relatively nonspecific fashion. Many of these require Mg^{2+} and/or Ca^{2+} cofactors. Pingoud and co-workers have identified a cluster of conserved residues in a family of six nonspecific nucleases related to Serratia nuclease, which has been crystallographically characterized (Figure 11).[36] Five of these are very important for catalytic activity (Arg 57, Arg 87, His 89, Asn 119, and Glu 127). Some influence k_{cat} and others K_m, and metal ion and pH dependence studies led to a tentative assignment of function. As described earlier in section VI.C, the Arg residue most likely contributes to enhance transition-state stabilization.

There are many nucleases, especially localized in the nucleus of eukaryotic organisms, which require both Mg^{2+} and Ca^{2+} for optimal activity. Typically, Ca^{2+} serves a structural role and Mg^{2+} is catalytic. Both Mg^{2+} and Mg^{2+}/Ca^{2+} endonucleases have been isolated from rat liver nuclei,[104] while several nuclear nucleases have been isolated from rat thymocytes.[105] These nuclear Ca^{2+}/Mg^{2+}-dependent endonucleases have been implicated in the extensive internucleosomal DNA fragmentation arising during programmed cell death (apoptosis) and are potential targets for drug design.[106−110] Physiological concentrations of

Zn^{2+} known to inhibit apoptosis also inhibit the activity of these Ca^{2+}/Mg^{2+} nucleases, which are found extensively in lymphocyte tissue. Other than inhibition by zinc, the activity of these nucleases may be regulated at the transcriptional level. For example, an 18 kD Ca^{2+}/Mg^{2+}-dependent nuclease (putatively a cyclophilin) identified in lymphoid cells undergoing apoptosis, is regulated by the glucocorticoid receptor.[111,112]

For many nucleases the metal cofactors appear to mediate not only direct catalytic activity, but also structural changes that regulate substrate recognition and binding specificity. In the absence of Mg, the periplasmic nuclease from *Streptomyces antibioticus* does not form tight specific complexes with cognate (dG) sequences ($n \geq 4$).[113] Changes in site specificity of single strand specific endonucleases, such as mung bean nuclease, have been observed with a change in Mg^{2+} concentration, and also by varying temperature and ionic strength.[114] Conformational changes of supercoiled DNA were proposed. As previously noted for nuclear nucleases, many fungal multisubunit nucleases require two types of metal for optimal activity. Studies with metal-selective chelating agents suggest a Ca^{2+} requirement to stabilize quaternary structure and a Mg^{2+} requirement to promote activity.[115] The fungal nuclease from *Aspergillus* may be related to *Serratia* nuclease, and again Ca^{2+} has been found to increase the thermostability of this enzyme.[36,116] The acid-soluble nuclease γ has been purified from *U. maydis* cell extracts.[117] Either Mg^{2+} or Ca^{2+} promotes nicking of one strand of the substrate DNA duplex, while Mn^{2+}, Co^{2+}, or Zn^{2+} promotes double-strand cleavage.

Many nucleases serve as general hydrolytic agents to break down nucleic acids. One of the best-studied examples is DNase, although this class of enzyme uses Ca^{2+} as the principal catalytic site, with Mg^{2+} and other Ca^{2+} ions serving as structural cofactors. Early work on pancreatic DNase A demonstrated distinct Mg^{2+} and Ca^{2+} sites, with the number of sites varying with solution pH.[118] Aaqvist and Warshel have examined the effect of metal ion substitution on the activity of staphylococcal nuclease by free energy perturbation calculations.[58] Their results suggest a protein architecture that is designed to optimize the activity of Ca^{2+}. More electrophilic cations with large hydration free energies increase the activation barrier for hydrolysis as a result of overstabilization of the putative HO^- nucleophile. Optimization of metal cofactors is related to the dual requirement of stabilizing both the entering nucleophile, and also the transition state for the reaction. One obvious problem in over-generalizing these issues stems from the discrete coordination chemistries and pK_as for metal-bound H_2O that must be considered when comparing results from transition metals versus alkaline earths.

VIII. Polymerases

Polymerases are enzymes that catalyze the replication and synthesis of strands of DNA or RNA from a single-strand or double-strand template polynucleotide. Some of these enzymes contain other exonuclease or ribonuclease functions that are required for their overall operation. A large number of polymerases have now been structurally characterized and can be divided into three major categories; namely RNA and DNA polymerases, and reverse transcriptases. Divalent magnesium is an essential cofactor for these three classes of polymerase enzyme, and serves a variety of roles.

RNA polymerases are commonly used in transcription, where the information encoded in a genomic DNA (or in some retroviruses, a genomic RNA) molecule is transcribed into a single strand of messenger RNA. DNA polymerases replicate double-strand DNA, and usually possess an exonuclease activity as a proof-reading function. Finally, reverse-transcriptases are commonly associated with retroviruses. This enzyme catalyzes three reactions: first the conversion of single-strand genomic RNA to a double-strand RNA−DNA hybrid (DNA polymerase function); second, the digestion of the RNA strand of the hybrid (ribonuclease H function); and third, a second DNA polymerase reaction that converts the single-strand DNA to double-strand DNA prior to integration into the host cell's genomic DNA.

A. RNA Polymerases

In addition to its well-known catalytic role, Mg^{2+} induces structural changes in the *E. coli* RNA polymerase open complex with the λPR promoter that have been examined by chemical and enzymatic footprinting.[119−121] Sigman and co-workers[122] have demonstrated a Mg^{2+} requirement for *E. coli* RNA polymerase to form single-stranded DNA structures that resemble open transcription complexes. The influence of Mg^{2+} on formation of the transcription bubble upon binding of *E. coli* RNA polymerase to the T7A1 promoter has also been examined by use of chemical probes, including hydroxyl radical footprinting.[19,120] These data suggest that formation of the transcription active complex requires two steps: one Mg^{2+}-dependent, and the other Mg^{2+}-independent. The data also suggests that one or two Mg^{2+} ions activate polymerase activity, while other ions are responsible for enlarging the transcription bubble.

Magnesium binding motifs have been identified in primase, a ssDNA-dependent RNA polymerase (Table 2 and Figure 4).[123] Also, the Mg^{2+} binding site of *E. coli* RNA polymerase has been investigated by use of hydroxyl radical footprinting,[19] and mutagenesis studies have implicated a -NADFDGD- motif in Mg^{2+} binding (Table 2 and Figure 4).

The structure of bacteriophage T7 RNA polymerase has been reported at 3.3 Å resolution.[124] Both Asp537 and Asp812 have been proposed as metal-binding residues; however, the Asp537Asn and Asp812Asn mutants demonstrate only slightly lower K_ds for Mn^{2+} relative to native enzyme, although the activity was greatly reduced.[125] Scatchard analysis of EPR Mn^{2+} binding data has suggested two bound ions;[125] however, such an approach is not a reliable method for the estimation of metal ion stoichiometry. It is proposed that the Asp537 and Asp812 residues bridge the metal cofactors and that subtle changes in the geometry of these residues might have a strong influence on catalytic activity.

Table 5. Thermodynamic Binding Parameters from Calorimetric Studies of Mg^{2+} Binding to Klenow[62]

parameters	Mg^{2+}	Mg^{2+} (+ TMP^{2-})	Mn^{2+}	Mn^{2+} (+ TMP^{2-})
	Polymerase			
no. of bound cations	1	1	1	1
K_1 (mM)	0.16	0.08	0.02	0.03
	3′-5′ Exonuclease			
no. of bound cations	1	1	1	1
K_2 (mM)	0.40	0.20	0.60	0.07

Mutation of the aspartic acid residues of the -GDD- acid sequence motif of poliovirus RNA-dependent RNA polymerase results in enzymes with different metal ion requirements for optimal activity.[21] The repeated carboxylate motif is a common feature of polymerase domains (Table 2 and Figure 4). Both aspartate residues are required for Mg^{2+} binding; however, Asn mutations are found to be active with transition metal replacements. Mutation of either Asp to Asn knocks out activity with Mg^{2+} as cofactor, however, the -GDN- mutation is active with Mn^{2+}.[21] Inasmuch as an amide linkage may serve as a ligand, especially to Mn^{2+}, it is possible that the activity of this mutant reflects retention of the metal cofactor.

B. DNA Polymerases

The Klenow fragment is a proteolysis product of DNA polymerase I, an important enzyme in DNA replication. This fragment contains both the 3′-5′ exonuclease and polymerase domains[126] and has become a paradigm for a class of metalloenzymes that follow a two metal ion mechanism of phosphate ester hydrolysis.[48,61] This is based extensively on the crystallographic work of Steitz and co-workers.[48,61] Early studies of Klenow demonstrated two metal ions (illustrated in Figure 7B) bound in the exonuclease active site in the presence of the substrate mimic thymidine monophosphate (TMP^{2-}).[61] In the absence of TMP^{2-} only one metal ion (A in Figure 7B) was observed to bind. Multiple bound ions have also been proposed on the basis of solution studies of Klenow by kinetic, optical, and EPR methods with Co^{2+} and Mn^{2+} cofactors.[127,128] After these studies, other enzymes have been crystallographically characterized with evidence of two active-site metal ions.[97] A common feature of each of these studies is the use of Mn^{2+}, and occasionally Zn^{2+} and Co^{2+}, as analogues for the presumed natural Mg^{2+} cofactor, since the heavier manganous ion facilitates discrimination of the metal ion from solvent water.[49] Subsequent characterization of bound Mg^{2+} is often carried out by difference methods, by subtracting electron density maps of an enzyme from those obtained with a manganese or heavy metal complex of an enzyme;[48] however, the difficulties associated with the analysis of metal cofactor stoichiometry from crystallography have been alluded to earlier.

The binding stoichiometries to Klenow for both Mg^{2+} and Mn^{2+} have been examined by titration calorimetry (Table 5), both in the presence and absence of the substrate analogue thymidine monophosphate, TMP^{2-}.[62] Considering their physiological availablity, divalent magnesium is the physiological cofactor rather than Mn^{2+} or any other transition metal ion; however, studies with Mn^{2+} allow a direct comparison with crystallographic data. In the presence of TMP^{2-}, one Mg^{2+} binds at each of the active sites (one at the 3′-5′ exonuclease site, and one at the polymerase site) and the binding constants are slightly augmented relative to binding in the absence of TMP^{2-}. A similar result is obtained with Mn^{2+} and is supported for both cofactors by a detailed kinetic analysis.[62]

Warshel and co-workers have carried out a theoretical analysis of the role of metal ions in phosphate ester hydrolysis catalyzed by the Klenow fragment of *E. coli* DNA polymerase I[64] and argued against a metal-bound hydroxide as the active nucleophile, but favored an external hydroxide that is stabilized electrostatically by the metal cofactor. Stabilization of this hydroxide could be accounted for in terms of the electrostatic influence of the high-affinity metal ion alone (Figure 7A).

The conserved amino acids in region I (-YGDTDG-) of human DNA polymerase α are most likely involved in formation of a metal binding domain (Table 2 and Figure 4). Both Asp1002Asn and Thr1003Ser mutants were found to utilize Mn^{2+} more effectively than Mg^{2+};[22,23] however, these mutants show enhanced misinsertion fidelity as a result of a 850- and 62-fold higher K_D. Mutation of Asp1004 resulted in a loss of activity for either metal cofactor, presumably arising from loss of metal binding. The results are consistent with metal binding to each Asp residue; more critically for Asp1004. In the case of Mn^{2+} activation, a significant increase in misincorporation of nucleotide bases was observed, but not for Mg^{2+}, even for the active Asp/Asn and Thr/Ser mutants described earlier. The effect for Mn^{2+} was manifest by the influence of this cation on K_Ms, and the results suggest that subtle changes in Mn^{2+} coordination chemistry resulting from these mutations gives rise to structural changes in the active-site pocket that diminish its ability to recognize specific nucleotide bases. The consequences of these structural perturbations for increased mutagenicity were discussed in section IV.

The DNA polymerase φ29 shares many of the characteristics of α-like DNA polymerases, including two conserved regions of amino acid similarity (D-NSLYP and K-NS(L/V)YG) that have been proposed to form part of the active site.[129,130] Tyr to Phe mutations of the Tyr residues in these two domains were found to influence the polymerization activity of Mg-NTP substrates, but not Mn-NTP substrates. In the first domain, an Asp to Glu mutation greatly influenced the polymerase activity, but not the exonuclease function. Polymerase activity could be partially recovered by use of Mn-NTPs. These results are consistent with involvement of these two amino acid regions in the polymerase, rather than the exonuclease domain. Also, the recovery of partial activity for the Asp to Glu mutation with Mn^{2+} is consistent with direct interaction with the NTP-

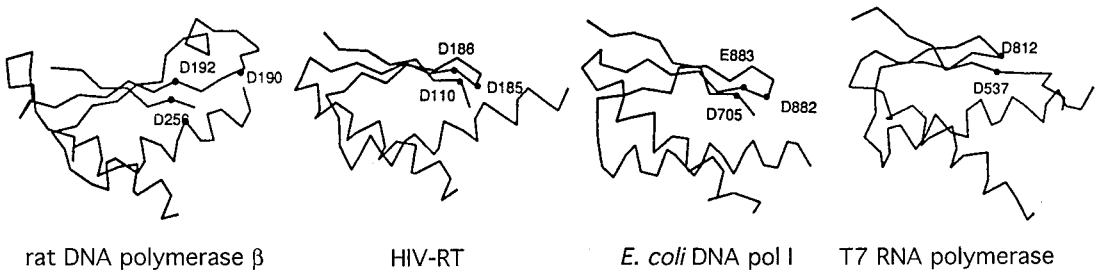

Figure 17. α-Carbon trace structures of rat DNA polymerase *β*, HIV-RT, *E. coli* DNA pol I, and T7 RNA polymerase, showing the positioning of common aspartate residues. Adapted from ref 132.

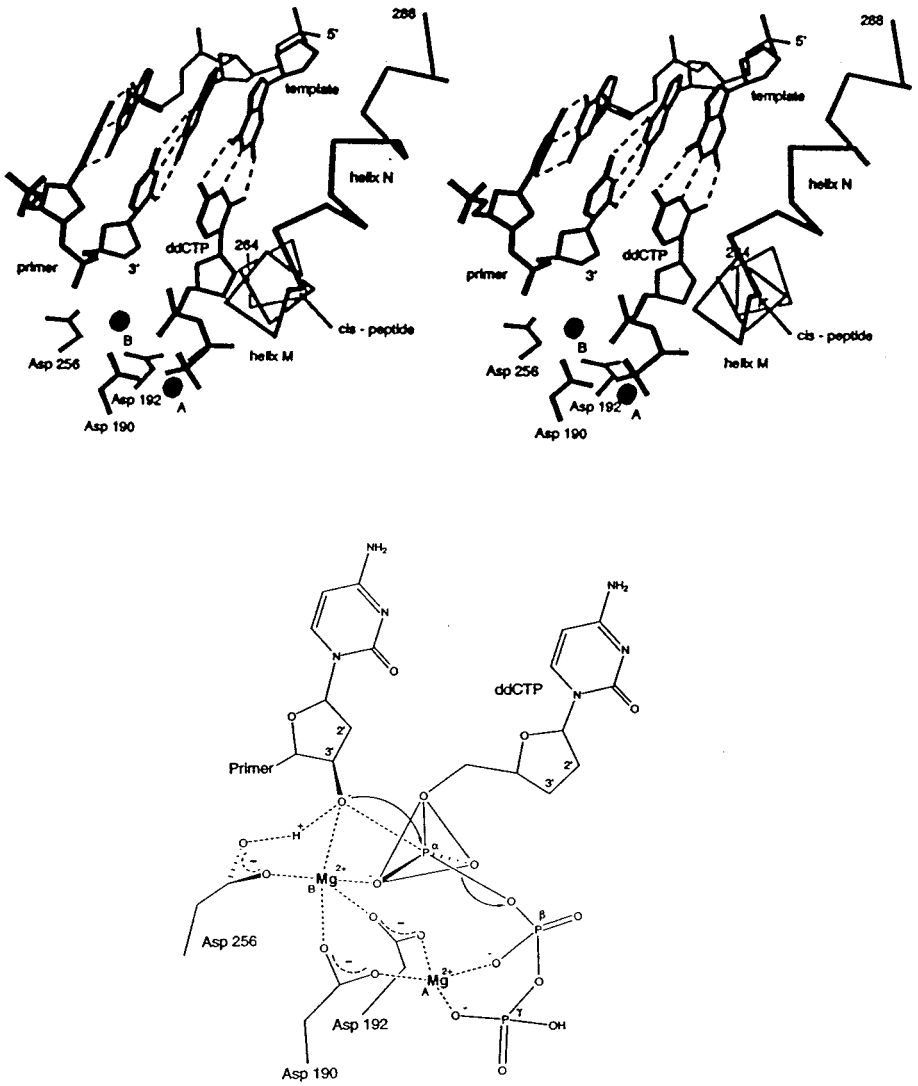

Figure 18. Stereoview of the polymerase active site of rat DNA polymerase *β*, and a schematic model of the reaction chemistry. Adapted from ref 131.

bound metal cofactor: a more favorable interaction arising in the case of Mn^{2+}, relative to Mg^{2+}.

In addition to DNA polymerases α, the polymerases *β* have also been the subject of intense structural investigation. Ternary complexes of rat DNA polymerase *β* complexed with a DNA template primer and dideoxycytidine (ddCTP) have been determined at 2.9 and 3.6 Å and yield significant mechanistic insight.[131,132] Three conserved aspartates are located at the active polymerase site of all DNA polymerases thus far characterized, while RNA polymerases show two of these (Figure 17). For nucleotidyl transferase reactions, one divalent cofactor is carried into the active site as a tight complex with dNTPs (shown with ddCTP in Figure 18).[131] There is an additional magnesium binding site that is most likely stabilized by the high charge density resulting from the multiple carboxylates and the triphosphate. The Mg^{2+} bound to the nucleotide triphosphate is well-known to facilitate nucleophilic attack,[133−136] although the 3′-OH of the attacking sugar need not necessarily be directly coordinated to the magnesium center as shown schematically in Figure 18, but may be stabilized electrostatically as an outer-sphere com-

plex.[16,64] Also, the use of the metal-bound carboxylate as a catalytic base is not appealing and has been discussed in the literature.[137,138] Previously it has been shown that a DNA polymerase can be converted to an RNA polymerase by changing the metal cofactor from Mg^{2+} to Mn^{2+}.[139] Figure 18 shows that all three phosphates on ddCTP bind to one or other of the two metal cofactors, and so it is likely that the metal ions help to position the nucleotide. It is argued that a slight change in orientation of ddCTP would be sufficient to reduce steric hindrance at the C2′ ribose of ddCTP and allow RNA to become as good a substrate as DNA.[131,139] This could be brought about by subtle changes in the coordination chemistry of Mg^{2+} relative to Mn^{2+}, as suggested earlier in section IV on the origins of mutagenicity.

In a later study of rat DNA pol β, using Mn^{2+} as metal cofactor, two metal centers were observed even in the absence of a nucleotide triphosphate.[140] These two cations were held to the enzyme by a single bridging aspartate: a mode of binding that seems unreasonable for Mg^{2+}. It is likely that these sites reflect specific binding preferences for Mn^{2+} under the high metal concentrations used. Other mechanistic aspects of polymerase activity, relating to orientation of the primer template, have been debated; however, this issue is not relevant to the focus of this review.[137,138]

C. Reverse Transcriptase

Blain and Goff have examined the RNase H activity of native and mutant Moloney murine leukemia virus reverse transcriptase.[141] Two of these mutants, designated Δ5 and ΔC, contain deletions in the RNase H domain. These were found to be active with in vitro assays using Mn^{2+}, but were found to be inactive in vivo, where the physiological cofactor is Mg^{2+}. These results are consistent with perturbation of the metal cofactor binding site in the RNase H domain, but apparently the deletion mutations do not completely remove the Mn^{2+} binding propensity. Three point mutants (R657S, Y598V, and S526A) were found to show full activity with Mn^{2+}, and partial activity in the presence of Mg^{2+}. Again, mutation of these residues are likely to perturb the metal-binding site (Table 2 and Figure 4). Two other point mutants (Y586F and D524N) showed no activity for Mg^{2+} activation, and only minimal levels (5%) for Mn^{2+} in the Y586F mutant. In these cases, the metal-binding capability has clearly been more seriously damaged, suggestive of direct coordination by Asp524 and Tyr586. Variations in DNA polymerase and processivity were also observed with the use of Mn^{2+} in these RNase H-deficient mutants, consistent with the mutagenic effects of this cation described earlier in section IV.

The stimulatory effect of Mg^{2+} compared to Mn^{2+} has been examined for HIV-RT.[142] Wild-type enzyme shows normal functional properties for both cations, although RNase H* activity (hydrolysis of double-strand RNA) was observed with Mn^{2+}. An E478Q mutant in the p66 domain, which participates in metal binding, was found to be inactive with Mg^{2+}, although Mn^{2+} restored both its endoribonuclease activity and the ability to catalyze specific removal of the tRNA replication primer. These observations point to obvious structural and/or coordination changes for Mg^{2+}, relative to Mn^{2+} activation, and may even be indicative of distinct coordination sites.

Conservative mutation Q151N in the polymerase domain of HIV-1 RT demonstrated similar activity to that of WT,[143] although the Q151A mutant demonstrated reduced activity. The preference for divalent cation changed from Mg^{2+} to Mn^{2+}. The influence of each of the p66 and p51 subunits on substrate and inhibitor binding, and metal ion preference, has been tested by subunit-selective mutagenesis studies on Glu 89. Evaluation of Michaelis and inhibition constants, and metal preference was evaluated for E89G mutants of HIV-1 RT (for both the p66 and p51 domains).[144] Only those mutants containing a p66 WT domain demonstrated a preference for Mg^{2+} (i.e., p51 mutants had no effect), while substrate and inhibitor binding were also unchanged. However, absolute activity was influenced by mutation of the p66 Glu89 residue. Clearly, the p66 domain contributes the most important side-chain functionality to metal-mediated chemistry.

The inhibitory influence of the antiviral drugs AZT (azidothymidylate) and *N*-ethylmaleimide (NEM) on the RNase H activity of HIV-1 RT is distinct when using either Mg^{2+} or Mn^{2+} as cofactors.[145] AZT monophosphate is a competitive inhibitor in the presence of Mg^{2+}, but is noncompetitive with a Mn^{2+} cofactor. This observation is also consistent with distinct metal-binding sites for Mg^{2+} and Mn^{2+} in the RNase H active site. Furthermore, the mode of substrate cleavage is distinct, with endonucleolytic hydrolysis of a poly(rA)/poly(dT) substrate in the presence of Mg^{2+} and both endo- and exonucleolytic hydrolysis in the presence of Mn^{2+}.[145,146] With poly(rG)/poly(dC) both exo- and endonucleolytic cleavage are observed for Mg^{2+} and Mn^{2+}. The authors suggest that the RNase H domain of HIV-1 RT undergoes conformational changes upon binding hybrid substrates and that these changes depend on the divalent cofactor.

All RT enzymes possess a common stretch of seven amino acids termed the -YXDD- box. Amino acid substitution of X results in changes in divalent metal ion preference and enzyme activity, and so the -YXDD- box appears to play a role at the active center of RTs by binding the metal cofactor.[147]

The metal cofactor carries out functions in addition to catalysis. Mg^{2+} stabilizes HIV-1 RT binding to DNA−DNA substrates, with a 20−60-fold decrease in k_{off}, and an increase in productive interaction with DNA.[148−150] Other charged species may enhance or diminish the influence of magnesium. For example, Ca^{2+} competitively inhibits Mg^{2+}-promoted functions.[151] Spermine enhances, but cannot replace Mg^{2+} in reactions catalyzed by avian myeloblastosis virus reverse transcriptase,[152] in a manner consistent with the function of polyamines as described in section V.

Finally, a reverse transcriptase motif has been identified in the catalytic subunit of telomerase, the ribonucleoprotein required for replication of chromo-

some ends, isolated from *Euplotes aediculatus* and yeast (*S. cerevisiae*).[166] Many metal binding residues are conserved, and mutations in these motifs results in loss of activity and telomere shortening. Interestingly the -YXDD- motif described earlier for RT enzymes, appears as -LXDD- in telomerase.

IX. Phosphatases

Crystallographic studies of *E. coli* inorganic pyrophosphatase, a hexameric soluble enzyme, provide evidence for 1.5 Mg^{2+} cofactors per monomer (Figure 19).[153] One ion resides in a "tight" binding site and is coordinated by three aspartates (Asp65, Asp70, and Asp102). The other Mg^{2+} is shared by two monomers, and appears to stabilize the interfacial contact region, and is coordinated by outer-sphere H-bond contacts to Asp26 and Asp24 on each monomer. At higher metal concentrations the phosphatase is observed to bind up to 4.5 Mg^{2+} per monomer, however, it is not clear that any site other than the "tight" site is physiologically relevant. The structure of *Saccharomyces cerevisiae* yeast inorganic pyrophosphatase has also been obtained at 2.4 Å resolution (Figure 19) using Mn^{2+} as an analogue for Mg^{2+}.[154] This structure showed four bound Mn^{2+} ions and two bound phosphates that putatively are derived from pyrophosphate. The same group had previously structurally characterized the *E. coli* enzyme and identified one active-site Mn^{2+} ion,[164] as found earlier by Kankare et al.[153] It remains uncertain whether these two enzymes do in fact follow distinct mechanistic pathways, or at least differ in their use of metal

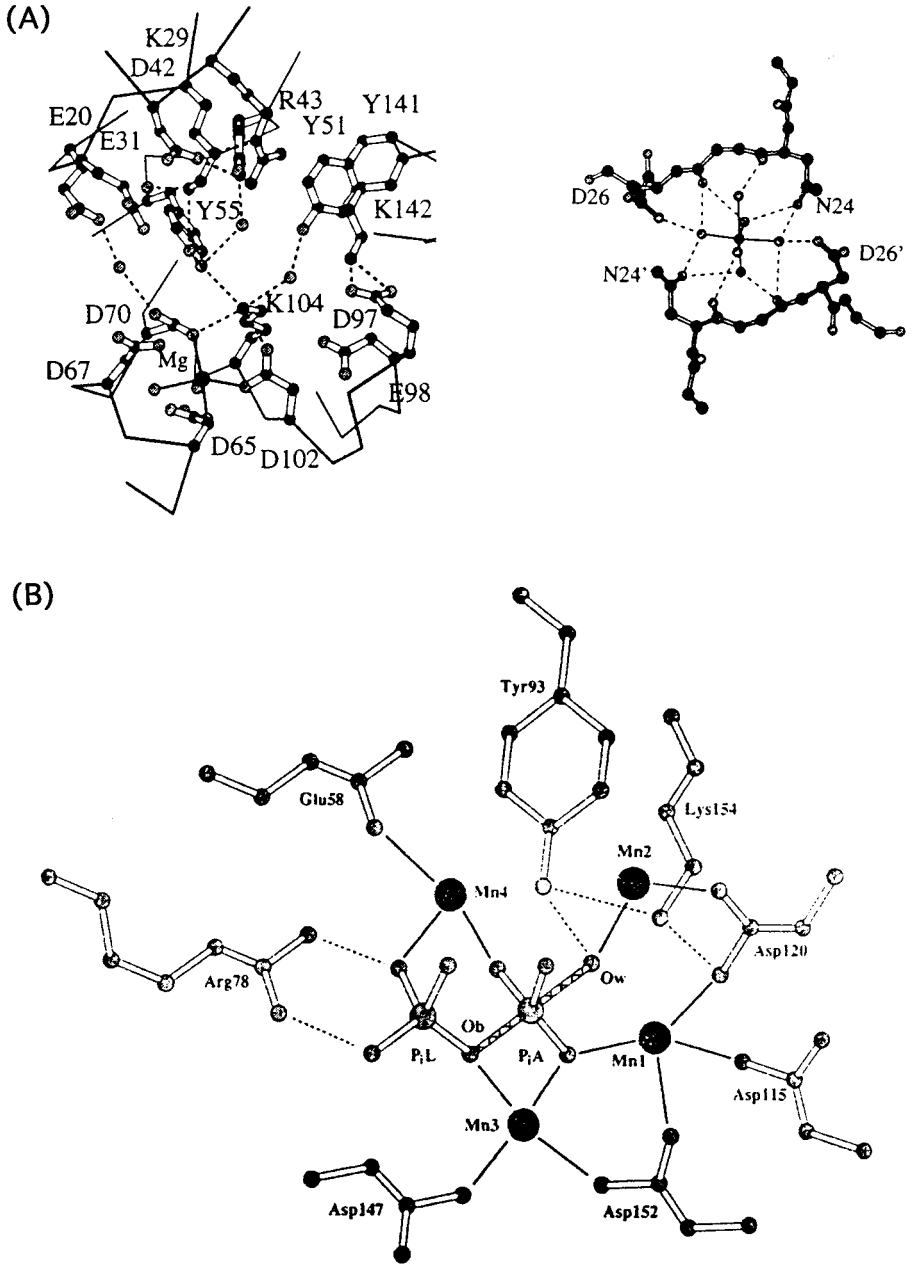

Figure 19. (Top) The catalytic (left) and interfacial (right) Mg^{2+} sites of inorganic pyrophosphatase. Adapted from ref 153. (Bottom) Schematic illustration of the proposed transition state for the pyrophosphatase activity of the *Saccharomyces cerevisiae* enzyme. The four Mn^{2+} ions and two phosphates, identified crystallographically, are indicated. Adapted from ref 154.

cofactors, or if the additional metal cofactors for the yeast enzyme arise from the use of Mn^{2+} ion in the structural study. In passing, it can be noted that a structural role is played by the Mg^{2+} ion cofactor for the dinuclear zinc *E. coli* enzyme, alkaline phosphatase.[155]

X. Integrases

Retroviral integrase (IN) enzymes catalyze insertion of virally encoded DNA into the genomic DNA of a host[156,157] and belong to a structurally related superfamily that includes RNase H, RuvC resolvase, and MuA transposase.[49,158] Detailed structural information is not available on the latter two, but recent reports describe the structures of HIV and avian sarcoma virus (ASV) integrase enzymes.[49,97] Only for ASV-IN has the position of the divalent metal cofactor (Mg^{2+} or Mn^{2+}) been defined. A single metal ion was resolved and the placement of aspartate side chains and metal ions was found to be similar to *E. coli* RNase H. Figure 5A summarizes the metal binding sites for the two RNase H structures (*E. coli* and HIV-RT) and ASV-IN. There appears to be a common metal binding site, and only the additional Mn^{2+} site illustrated in the HIV-RT RNase H domain in Figure 5A is unusual. A unique metal binding site has also been tentatively identified in crystallographic studies of the RuvC enzyme.[159] On the basis of epitope-mapping studies with antibodies, binding of Mg^{2+} is suggested to regulate the conformation of the monomeric subunits of HIV-IN.[160] The accessibility of epitopes (surface peptide fragments recognized by antibodies) to the C-terminal domain and catalytic core is reduced in the presence of the magnesium cofactor, supportive of a conformational change. Such structural changes may contribute to the aggregation mechanism of the monomers to form an active multimeric complex.

Clearly, a unique structural and catalytic metal ion is the common theme that emerges from structural studies of this superfamily of enzymes. This provides strong support for the argument presented earlier that the second Mn^{2+} site identified crystallographically for the HIV-RT RNase H is likely to be physiologically irrelevant, is populated as a consequence of the experimental conditions and that only the common metal binding site is of functional relevance.

XI. Closing Remarks

Magnesium is a truly versatile metal cofactor. It mediates a large range of enzymatic reactions, by both outer- and inner-sphere pathways and demonstrates remarkable variability in coordination chemistry. Much of the biochemistry of magnesium ion is dictated by a few key properties (namely, a high charge density and extensive hydration state) that discriminate it from transition metal analogues. Accordingly, it is not surprising that coordination changes for the latter should give rise to mutagenic effects, loss of binding and recognition specificity, and typically an increase in activity. For nucleic acids, outer-sphere interactions through magnesium-bound water molecules are extremely important for deciphering the chemistry of this essential metal cofactor. This fact contributes to the design of magnesium binding pockets in proteins and enzymes. The importance of reading the relevance of metal binding chemistry within the context of natural abundance has been repeatedly emphasized.

XII. Acknowledgments

This work was supported by grants from the Petroleum Research Fund (administered by the American Chemical Society), and the National Science Foundation (CHE-9706904). J.A.C. is a Camille Dreyfus Teacher−Scholar (1994−1999) and a National Science Foundation Young Investigator (1992−1997).

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CR960436Q