BioMetals **15:** 225–235, 2002.

Structural and catalytic chemistry of magnesium-dependent enzymes

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Introduction

Magnesium dependent enzymes are ubiquitous in both general metabolic pathways and in nucleic acid biochemistry (Cowan 1995; Wilcox 1996), and their enzymatic reactions fall into one of two general classes (Figure 1). First, an enzyme may bind the magnesium-substrate complex. In this case the enzyme interacts principally with the substrate and shows little or at best weak interaction with Mg^{2+} (for example Mg-isocitrate in isocitrate lyase shown in Figure 2). Alternatively, Mg^{2+} may bind directly to an enzyme and alter its structure and/or serve a catalytic role. Complex formation with the protein or enzyme may arise either through direct coordination to protein sidechains (inner-sphere binding) or by indirect interactions through metal bound waters (outer-sphere binding), Although other divalent metal ions may also activate these enzymes this is frequently accompanied by a reduction of enzyme efficiency and/or substrate specificity.

Magnesium binds weakly to proteins and enzymes ($K_a \leq 10^5\ M^{-1}$), and magnesium-activated enzymes are not necessarily isolated in the metal-bound form. Magnesium must be added to the enzyme solution for *in vitro* reactions, but the intracellular free Mg^{2+} concentration is approximately 0.5 mM in the cytosol of most cells, thus providing sufficient Mg^{2+} to activate Mg^{2+}-dependent enzymes. Typically the magnesium cofactor functions as a Lewis acid toward a bound substrate, serving to activate a bound nucleophile by promoting ionization to a more reactive anionic form (for example, water to hydroxide) or stabilization of an intermediate (Figure 1). The protein-bound metal ion also provides a template that brings the reactant species into close proximity. The use of metal cations as Lewis acids allows a large number of hydrolysis and condensation reactions to proceed under physiological conditions that would otherwise require extremes of pH.

Characteristics of magnesium-promoted activity in general metabolic biochemistry

Many metabolic cycles in higher organisms (e.g., citric acid cycle, glycolytic cycle, etc.) are mediated by magnesium-dependent enzymes and follow a general mechanistic theme for involvement of magnesium ion which is presented in Figure 1 (Cowan 1995). In spite of the apparent complexity of the structural and kinetic models that have been developed, several common themes do emerge. First, most enzymes on these pathways require at least two metal binding sites: an allosteric regulatory site modulating either structure or binding, while a second ion typically serves a catalytic role (Figure 3). In these cases the substrate binds to the metal-activated enzyme, and the principal substrate molecules (xylose, pyruvate, etc.) do not themselves have a high affinity for Mg^{2+}. Enzymes using metals other than Mg^{2+} tend to show isolated high-affinity metal binding sites where the metal cofactor serves to stabilize an important reaction intermediate during the catalytic pathway. Crystallographic evidence and supporting mutagenesis studies suggest that substrate binding occurs at only one of the metal sites. In some cases the catalytic metal is delivered as a chelate complex of the substrate (for example Mg^{2+}-isocitrate with isocitrate lyase as shown in Figure 2). However, in others the enzyme makes a genuine complex with the Mg^{2+} cofactor, and in these examples the coordinating ligands are typically carboxylates. Occasionally an amide carbonyl from the backbone or a side-chain are used as ligands (Black *et al.* 1994), but the residual ligands that complete the octahedral coordination set are most often water molecules, some of which may be displaced by substrate binding. Illustrative examples

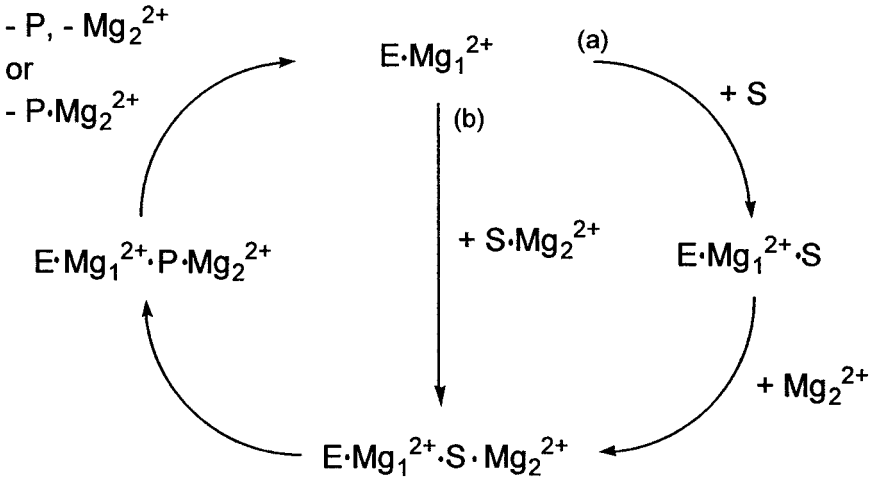

Fig. 1. General mechanistic features for magnesium-catalyzed transformations of enzymes on the glycolytic pathway. Pathways (a) and (b) correspond to cases where the substrate does not, or does chelate the second Mg^{2+} cofactor Mg_2^{2+}, respectively.

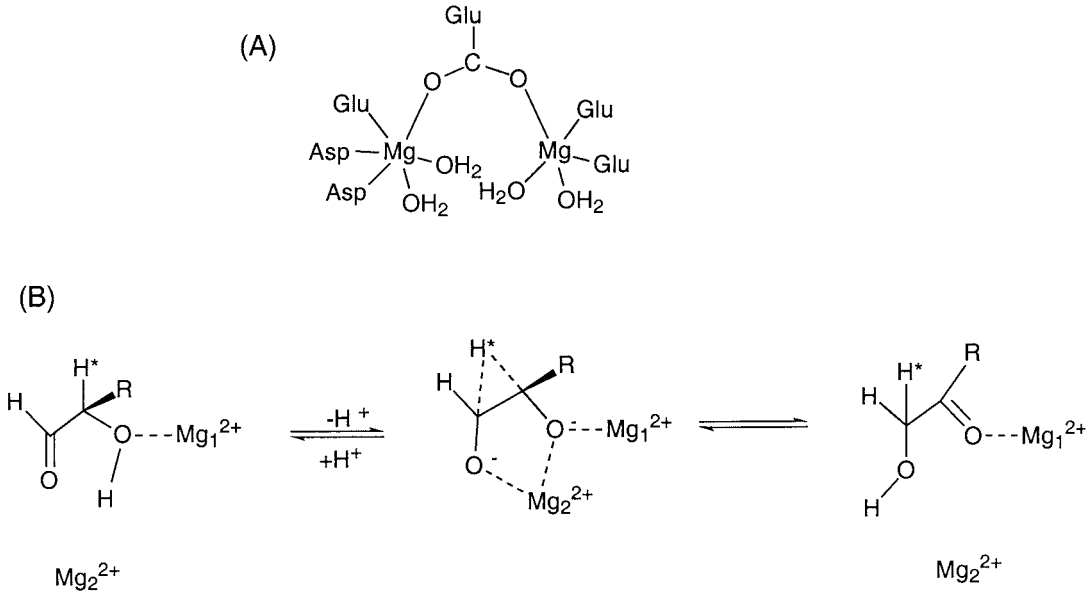

Fig. 2. A proposed mechanism for the metal-catalyzed 1,2-hydride shift in *Arthrobacter* xylose isomerase. The labile hydrogen is starred. Divalent magnesium ions maintain octahedral coordination with extensive coordination to carboxylate residues. Note that $Mg(2)^{2+}$ is thought to be the catalytic ion, while the other $Mg(1)^{2+}$ most likely plays a structural role.

of the function of magnesium-dependent enzymes are presented below.

Kinetic data for Mg^{2+}-activated *xylose isomerase* (van Bastelaere *et al.* 1995) suggests that the first step in catalysis (Figure 3) is the binding of Mg^{2+} to site 1 (having the higher binding affinity). This facilitates substrate binding and proper orientation and by binding to ligand atoms on the substrate stabilizes reaction intermediates. Subsequently, the second cation binds and turnover is initiated. A Mg^{2+}-bound water molecule is likely to assist in proton transfer between the hydroxyl and carbonyl oxygens. In the case of xylose isomerase, this second ion is essential for the isomerization reaction (that is, for H^+ transfer from C1 to C2, and H^+ transfer from O2 to O1), and most probably both stabilizes the anionic substrate and polarizes the C-O bonds (Figure 3).

Isocitrate lyase catalyzes the reversible cleavage of isocitrate to glyoxylate and succinate (Figure 2) and shows an absolute requirement for divalent mag-

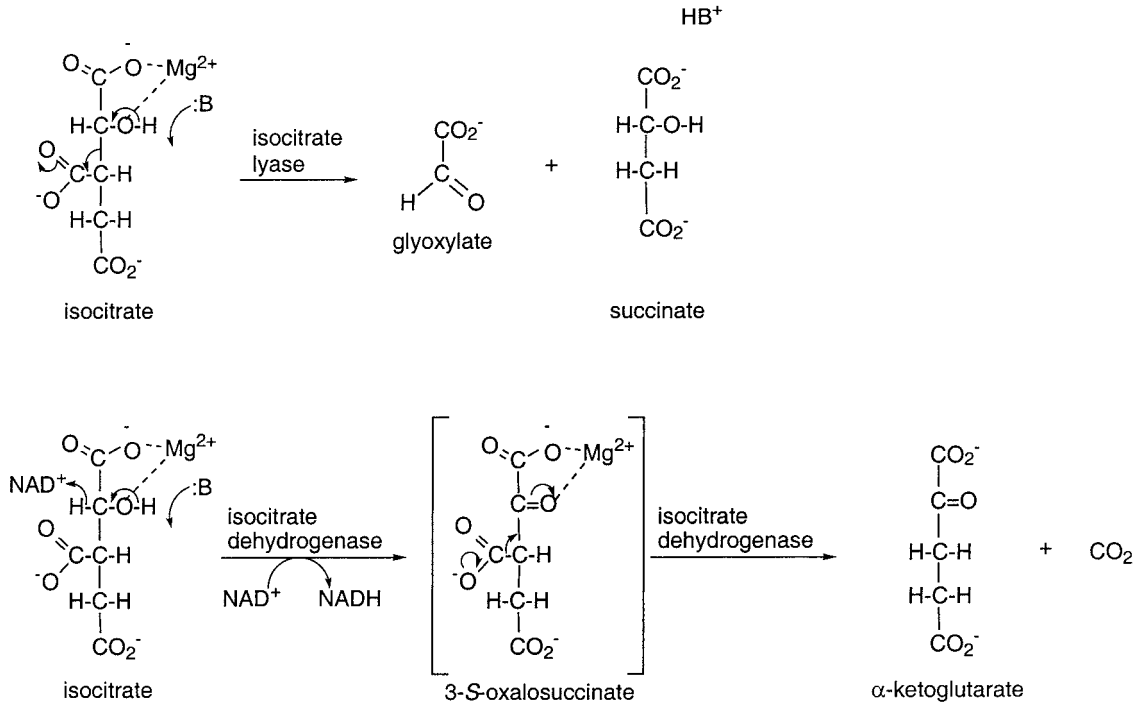

Fig. 3. A proposed mechanism for isocitrate lyase illustrating the role of the catalytic magnesium cofactors.

nesium to promote enzyme activity. The enzyme is tetrameric, with four identical subunits, each of which possesses an active site (Britton *et al.* 2000). The substrate is the Mg^{2+}-isocitrate complex, although a second, non-catalytic high affinity Mg^{2+} binding site ($K_D \sim 200\ \mu M$) has been identified that activates the enzyme by inducing a conformational change. This structural shift presumably optimizes the alignment of key catalytic residues in the active site. The Mg^{2+}-isocitrate complex binds tightly to the active site ($K_D \sim 40\ \mu M$) although free Mg^{2+} can also bind weakly ($K_D \sim 6$ mM) and presumably accounts for the inhibition observed at high [Mg^{2+}]. Enzyme inhibition at elevated [Mg^{2+}] typically only arises in those cases where excess free Mg^{2+} can compete with a Mg^{2+}-substrate complex. So, for example, inhibition is also observed for xylose isomerase, but not in the case of L-aspartase.

The chemistry of *glutamine synthetase*, a dodecamer that catalyzes the formation of glutamine from glutamate with accompanying hydrolysis of ATP (Liaw & Eisenberg 1994), is summarized in Figure 4. Crystallographic and kinetic studies have identified two metal binding sites (of high and low affinity) with an internuclear distance of 5.8 Å (Almassey *et al.* 1986; Yamashita *et al.* 1989). The high affinity site corresponds to the catalytic cofactor, while the weakly bound ion has been associated with binding of a MgATP chelate. Again, the catalytic magnesium provides a template for the reaction, electrostatic stabilization during the transition state, and Lewis acid catalysis.

L-Aspartase catalyzes the reversible deamination of L-aspartic acid to form fumaric acid and ammonia (Saribas *et al.* 1994; Schindler & Viola 1994). Unlike glutamine synthetase there is no need for a MgATP co-substrate, and so only one metal cofactor is required. At low pH the strict cation requirement disappears and residual activity occurs without the metal cofactor. The metal cofactor stabilizes the active site conformation of the enzyme, but does not coordinate the substrate.

Characteristics of magnesium-promoted activity in nucleic acid biochemistry

Another class of magnesium-dependent enzyme uses either free divalent magnesium as a cofactor, or a Mg-NTP complex as substrate (where NTP = nucleotide triphosphate). First, it is necessary to consider pertinent facts relating to the suitability of Mg^{2+} for its

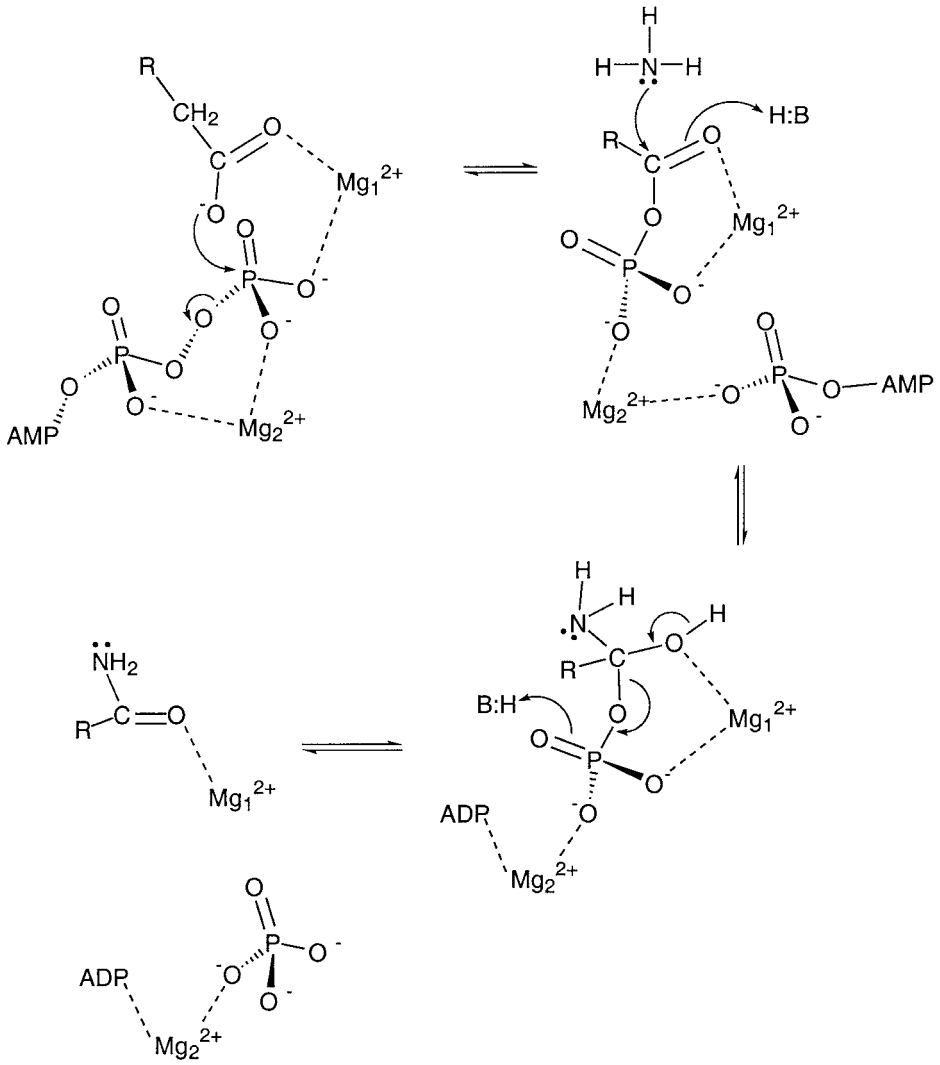

Fig. 4. Structural details and a proposed mechanism for *E. coli* glutamine synthetase amination. Two basic residues are thought to be involved in the reaction, supplying protons to the carboxyl and the phosphoryl groups. Divalent magnesium ions maintain octahedral coordination with extensive coordination to carboxylate residues.

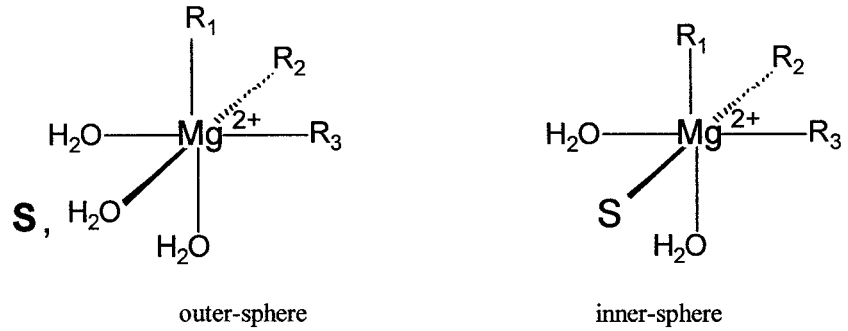

Fig. 5. Comparison of inner- and outer-sphere modes of activation. A substrate **S** interacts with a catalytic Mg^{2+} ion that is tethered to an enzyme through ligation to residues R_1, R_2, and R_3, for example. (Note that the number of inner sphere contacts to protein ligands varies considerably, depending on the enzyme. From zero to five contacts have been observed). Both activation modes are observed for magnesium-promoted reactions with the latter more typical of nuclease activity.

role as an activator of enzymes that act on nucleic acid substrates, the required metal ion stoichiometry, and mechanistic details of function. This will provide an essential background for a subsequent review of specific enzyme families.

Relative to other alkaline earth and transition metal ions, the small ionic radius and resulting high charge density of divalent magnesium results in a tendency to bind water molecules rather than bulkier ligands in the inner coordination shell, leading to appropriate hydration states and slow solvent exchange rates (Cowan 1998). These facts are also reflected in the hydration numbers for crystalline salts of magnesium relative to other alkaline earth metal ions (Cowan 1998b) and illustrate the difficulty of coordinating large counterions or ligands to the rather small magnesium cation. Outer-sphere mediated hydrolytic pathways are followed by a large group of magnesium-dependent enzymes that carry out reactions on nucleic acid substrates (Figure 5) (Cowan 1998). Such pathways are favored by the hydrogen bonding network that can form between hydrated Mg^{2+} and nucleic acid substrate (Figure 6). 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 (Black *et al.* 1996; Cowan 1998). 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 (Cowan 1998). For example, Figure 7 illustrates a selection of magnesium-binding modes to proteins with varying extents of solvation. Nevertheless, the K_a's are comparable and are tuned to physiological availability.

A mechanistic model that has been put forward for metal-mediated hydrolysis of the phosphodiester backbone of nucleic acids involves two metal ions working in concert (Steitz & Steitz 1993). This two-metal-ion model is perhaps best defined as the requirement for two metal ions located in close proximity (< 4 Å)and bridged by a common substrate. This allows a clear distinction from other enzymes that may bind two spatially separated and non-bridged metal cofactors that do not function as a coherent catalytic

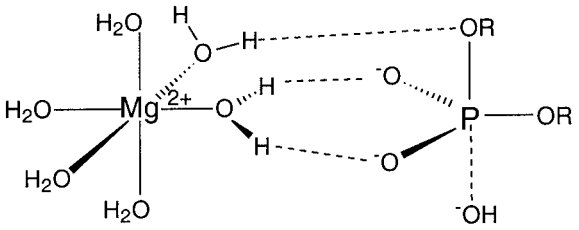

Fig. 6. Illustration of transition-state stabilization by outer-sphere complex formation to hydrated magnesium ion. Both hydrogen bonding and electrostatics contribute to the stabilization of the increased negative charge in the transition state.

unit, and presumably follow a distinct reaction pathway. This latter group includes examples such as T4 RNase H, where two Mg^{2+} sites are separated by 7 Å (Mueser *et al.* 1996). Crystallographic analysis of T5 5'-exonuclease (Ceska *et al.* 1996; Mueser *et al.* 1996) also shows two Mn^{2+} sites separated by 8.1 Å (10 Å in the case of Taq 5'-exonuclease). The key idea behind the first stated two-metal-ion model is that concerted action by two bridged divalent ions can provide more extensive Lewis acid catalysis as a result of the increased positive charge, while also combining a template effect (drawing together substrate and nucleophile), with activation of bound H_2O (see for example the proposed model for Klenow activity summarized in Figure 8A). The higher charge density of Mg^{2+} however provides a strong electrostatic barrier to such a model, while the higher pK_a of Mg^{2+}-bound H_2O makes metal activation of H_2O less favorable in the case of divalent magnesium (Cowan 1997). An insightful theoretical analysis of the Klenow fragment from DNA polymerase I identifies electrostatic stabilization of a free hydroxide nucleophile as the principal role of the metal cofactor(s) (Aaqvist 1990; Cowan 1998) rather than having a magnesium-bound hydroxide as the active nucleophile (Figure 8B). That is, 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 an acidic side chain. Some of the problems of interpretation in this area arise from inconsistencies in data from crystallographic and solution studies. In part this arises from the use of transition metal analogues in place of divalent magnesium (Cowan 1998). Illustrative examples of metalloenzymes in nucleic acid biochemistry are described next.

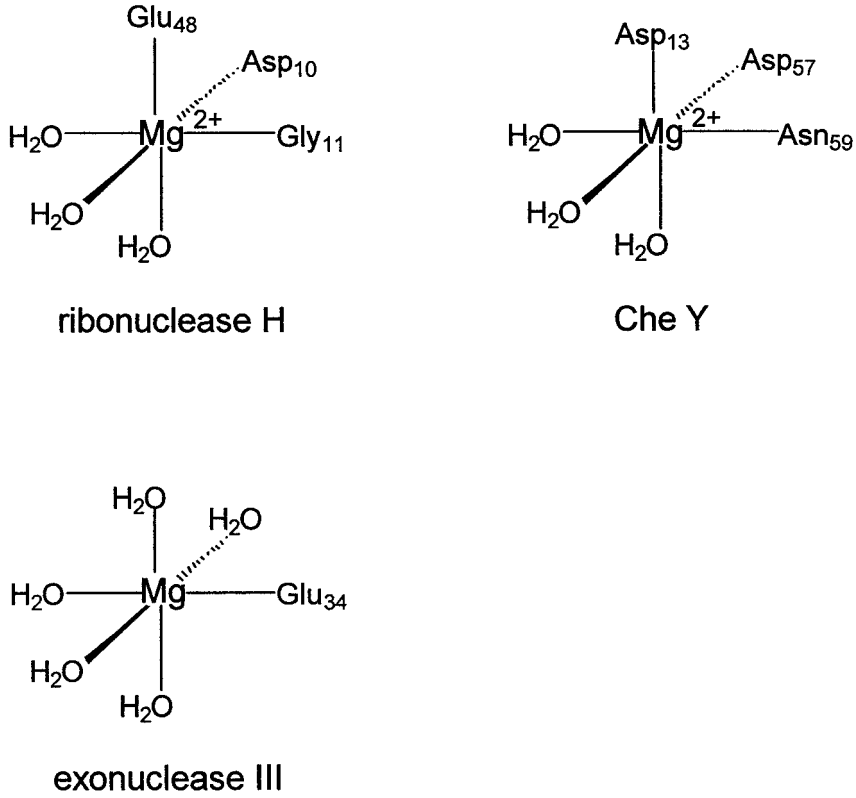

Fig. 7. Comparison of magnesium coordination spheres for selected magnesium proteins (the metal binding domains of *E. coli* ribonuclease H, the chemotaxal protein CheY, and the DNA repair enzyme exonuclease III).

Restriction endonucleases

Few magnesium-dependent nuclease enzymes have been studied in detail with regard to the role of the essential metal cofactor. Noteworthy exceptions are the restriction enzymes *Eco*RI, and especially *Eco*RV (Jeltsch *et al.* 1992, 1993; Baldwin *et al.* 1995; Vipond *et al.* 1995; Pingoud & Jeltsch 1997). A recent review summarizes what is known of the recognition and cleavage chemistry of type-II endonucleases (Pingoud & Jeltsch 1997), the family to which both of the aforementioned enzymes belong. *Eco*RI is also a type-II endonuclease and shows a high degree of structural homology with the active site of *Eco*RV (Figure 9), although there is no general sequence homology. A mechanistic model has been suggested for both *Eco*RI and *Eco*RV (Figure 9). Some uncertainty remains over the metal cofactor stoichiometry; however, an inner sphere pathway appears likely since probes of outer sphere paths do not promote activity. This possibly reflects stricter substrate recognition requirements of such enzymes.

Exonuclease

Exonuclease activity can be defined as the removal of nucleotide fragments from either the 3'-end or 5'-end of a strand of nucleic acid (usually one strand of a double-stranded substrate) by hydrolysis of the terminal phosphodiester linkage. The activity is usually associated with enzyme domains showing multifunctional behavior (for example, the Klenow fragment of DNA polymerase, and the DNA repair enzyme exonuclease III). Both single-metal and two-metal-ion mechanisms have been proposed and a detailed analysis of the metal promoted activity of Klenow, supporting a one-metal-ion mechanism has been reported (Black & Cowan 1998). Crystallographic evidence suggests a two-metal-ion mechanism, however, 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. X-ray structures of proteins with labile Mg^{2+} cofactors are usually obtained by doping pre-existing crystals of enzyme with very high concentrations of metal cofactor (relative to phys-

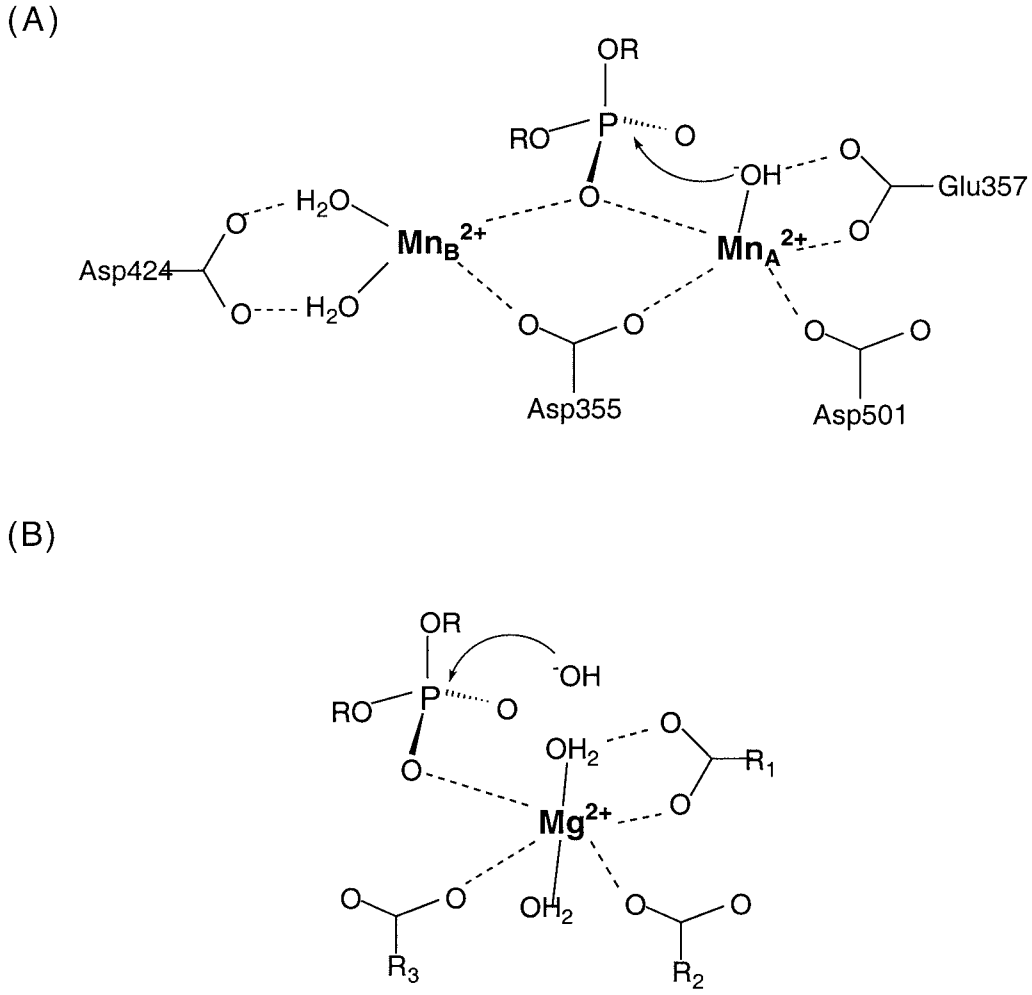

Fig. 8. (A) Schematic illustration of the metal binding sites identified from crystallographic studies of the Mn^{2+} derivative of the DNA polymerase I Klenow fragment. Site B is weakly populated in the presence of substrate or a substrate analog. Site A is the principal site of both metal coordination and catalytic chemistry. (B) A minimalist scheme for Klenow activity showing one essential metal cofactor and an activated nucleophilic solvent water (Cowan 1998).

iological concentrations). Several caveats that must be borne in mind include the use of high metal ion concentration in doping experiments, and so weak and physiologically irrelevant sites may be occupied. With this in mind, the high affinity 'structural site' populated by Zn^{2+} or Mn^{2+} in Klenow, showing 4- or 5-coordination depending on whether substrate is bound, would not be populated by Mg^{2+} at normal physiological concentrations. Moreover, neither Zn^{2+} nor Mn^{2+} bind at this site at normal physiological concentrations. Similar investigations of exonuclease III (Black & Cowan 1997) also support a one-metal-ion model for Mg^{2+} cofactor, but provide evidence for additional metal binding in the case of Mn^{2+} (Casareno 1996). The catalytic relevance of the second Mn^{2+} in the manganese promoted reaction has not, however, been addressed.

Polymerases

A large number of enzymes utilize magnesium as a chelate with nucleotidyl di- or tri-phosphates (especially ATP and ADP), where the metal cofactor serves as a mediator of phosphoryl or nucleotidyl transfer chemistry. In such enzymes the role of the metal is fairly well understood. Association of the metal to the enzyme is a consequence of nucleotide binding. The function of the metal ion was considered in the previous section. Nucleotidyl transferases are the subject of

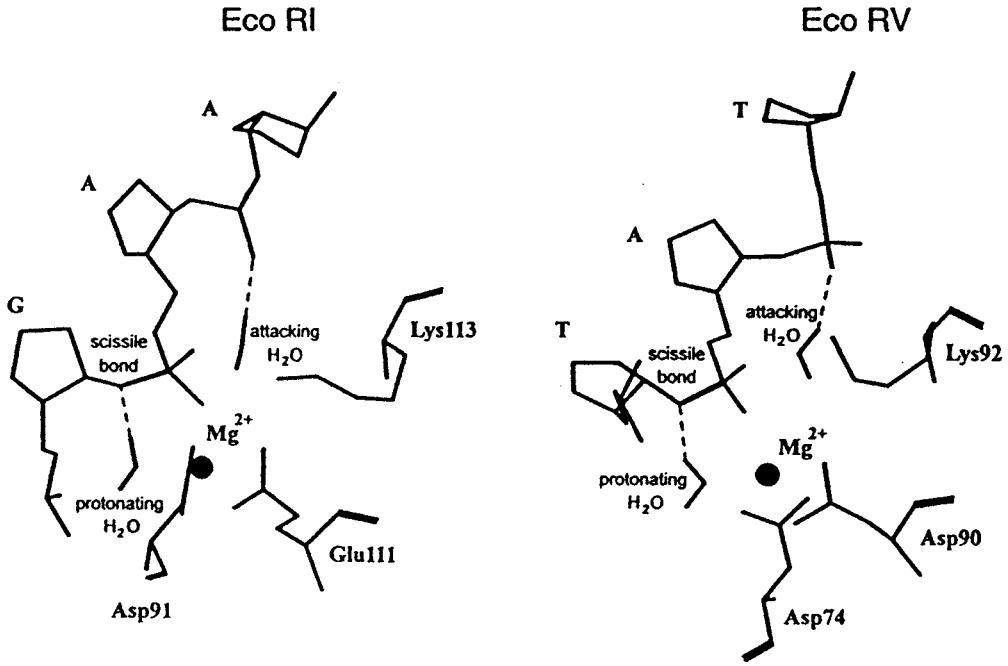

Fig. 9. A comparison of the metal cofactor binding domains, and other features of the catalytic pockets of *Eco*RI and *Eco*RV, illustrating the high degree of local homology for these two restriction endonucleases and proposed mechanistic details. Adapted from (Jeltsch *et al.* 1992).

greater focus here since this activity is relevant to the chemistry of RNA and DNA 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 (Cowan 1998). Some of these enzymes are multifunctional and contain other exonuclease or ribonuclease H activities required for their overall operation. These different catalytic activities are carried out at distinct sites on the enzyme. A common feature of the polymerase active site is possession of a labile metal binding site in addition to the Mg^{2+} that is carried in as a chelate with the NTP substrate (Figure 10). Acidic active site residues can interact with this metal-phosphate center and contribute to active site chemistry.

Divalent magnesium binds to ATP^{4-} as a β,γ-chelate. This serves to promote nucleophilic attack at the γ-phosphate during phosphoryl transfer reactions (discussed below) (Cowan 1991). Similarly, the chelate serves to stabilize the pyrophosphate leaving group following nucleophilic attack at the □-phosphate during nucleotidyl transfer reactions of polymerases (Figure 10). The presence of the Mg^{2+} ion also tends to direct any H^+ delivered during catalysis to the β-phosphate (with release of Mg^{2+} to the more negatively-charged terminal phosphate), which further enhances pyrophosphate as a leaving group.

Phosphoryl transfer

Enzymes that catalyze phosphorylation of substrates (usually Ser, Thr, or Tyr residues on protein targets) typically use magnesium chelates of ATP as a co-substrate. As detailed earlier, the bound Mg^{2+} serves to facilitate nucleophilic attack at the γ-phosphate of the highly negative ATP substrate (Figure 10). Such enzymatic reactions are therefore mediated by Mg^{2+}. However, we should note that the hydrolysis of simple phosphate esters is often catalyzed by enzymes containing transition metal cofactors. For example, alkaline phosphatase and purple-acid phosphatase contain binuclear zinc and iron centers, respectively (Wilcox 1996). The terms alkaline and acid phosphatases indicate the pH optima for activity and reflect the relative pK_a's for bound water to the zinc and iron cofactors, respectively. Presumably these substrates are less reactive and require more potent Lewis acid catalysts. 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 (Cowan 1997).

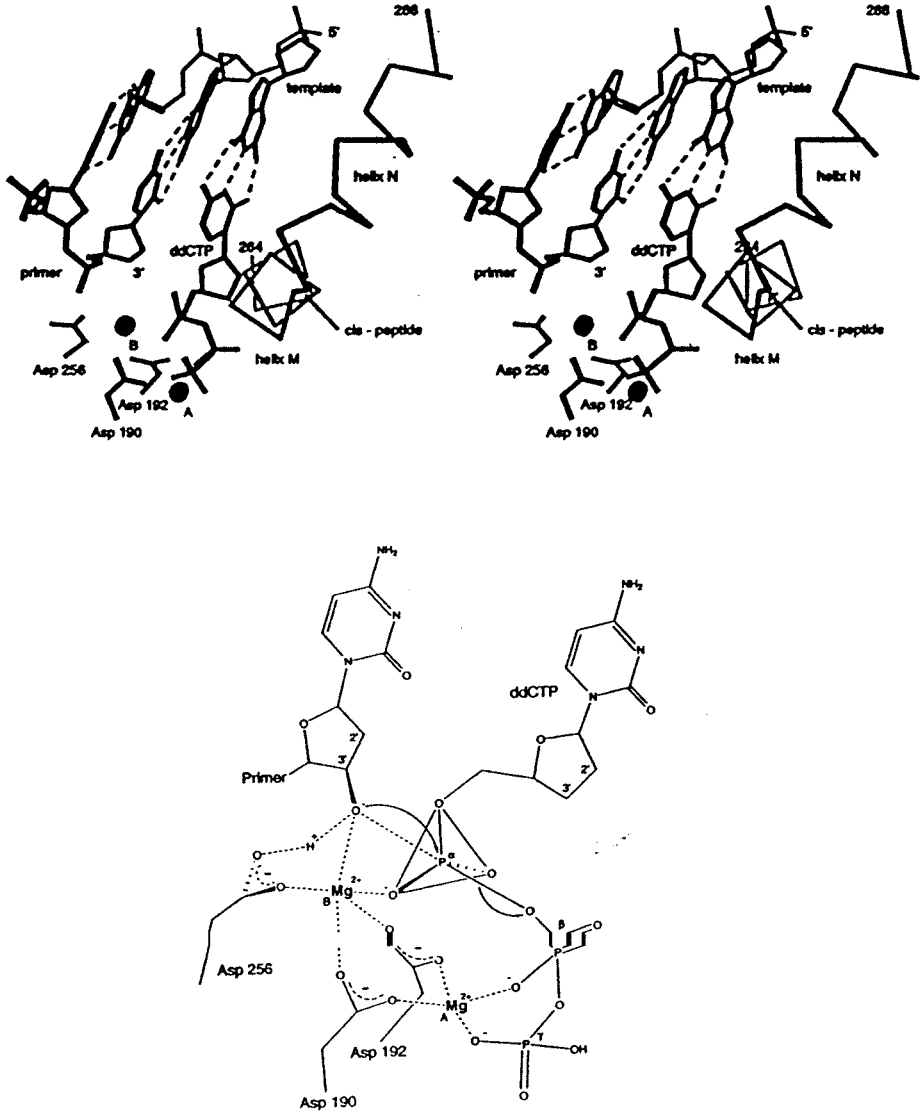

Fig. 10. Stereoview of the polymerase active site of rat DNA polymerase and a schematic model of the reaction chemistry. Adapted from (Pelletier *et al.* 1994).

Phosphorylation and dephosphorylation

Phosphate and phosphoryl transfer reactions are ubiquitous in cellular biochemistry, and require magnesium ion as an essential cofactor (Cowan 1995, 1998). Distinctions between enzymes in this class can be related to the function of magnesium. Generally divalent magnesium will either make its principal bonding contacts with the phosphate moiety of a nucleotide di- or tri-phosphate or with side-chains in the enzyme. The catalytic role for Mg^{2+} in promoting NTP hydrolysis is well established, with activation of the β-phosphate toward protonation and formation of a 'pyrophosphate' leaving group and/or relief of electrostatic repulsion between an incoming nucleophile and the terminal phosphate (Cowan 1991; Sigel 1998).

In the case of regulatory proteins, hydrolysis chemistry must be coupled to structural change (Cowan 1995). For example, the family of Ras genes code for regulatory proteins that bind guanine nucleotides (G-proteins). Ha-Ras p21 binds a divalent magnesium-GTP complex and has been examined by time-resolved crystallographic characterization (Schlichting *et al.* 1990). A change in metal ion coordination triggers substantial changes in structure during hy-

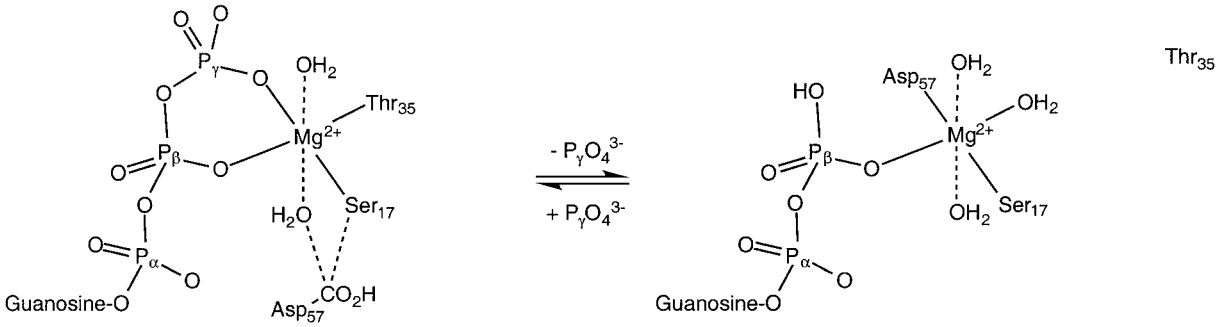

Fig. 11. The magnesium binding site in Ha-Ras p21 before and after γ-phosphate transfer (Schlichting *et al.* 1990). Note the retention of two inner sphere water molecules. Also, Asp-57 replaces the γ-phosphate after transfer. Thr-35, which is part of the effector loop, moves to ~ 4 Å distance from Mg^{2+} after transfer. The uptake and release of protein residues results in structural change of the protein.

drolysis. Although the details are still unclear, it is now known that upon hydrolysis of the γ-phosphate, Mg^{2+} coordinates to the carbonyl group of Asp-57, leaving only one phosphate ligand (Figure 11). In addition to an added aspartate contact, the Thr-35 contact is lost. These differences in the coordination sphere around magnesium lead to large conformational changes in an 'effector loop' of approximately 7 residues in length. This loop is believed to be responsible for the binding of GAP (GTPase activating protein), which activates Ha-Ras p21 for catalysis. From this structural data, and previous mechanistic evidence, either a protein-bound water situated directly opposite the leaving group, or the β-phosphate oxygen may be the attacking nucleophile. After loss of the nucleophilic water molecule, a 5-coordinate magnesium-bound phosphate intermediate is formed. It is likely that magnesium increases the electrophilicity of the γ-phosphate center through coordination (Figure 11) and stabilizes the product after transfer.

Concluding remarks

The importance of divalent magnesium as an activator of cellular enzymes on metabolic pathways and in nucleic acid biochemistry is clear. In spite of the apparent complexity of the structural and kinetic models described above, several common themes do emerge. Notably, the enzymes in the glycolytic or glyoxylate pathways each require at least two metal binding sites: an allosteric regulatory site modulating either structure or binding, while the second ion typically serves a catalytic role. Also, in these cases the substrate binds to the metal-activated enzyme and the principal substrate molecules (xylose, pyruvate, etc.) do not themselves have a high affinity for Mg^{2+}. Other enzymes tend to show isolated high-affinity metal binding sites where the metal cofactor serves to stabilize an important reaction intermediate during the catalytic pathway. In nucleic acid biochemistry, key points include the role of water in mediating enzymatic phosphate ester hydrolysis and the design of the catalytic site that includes formation of coordination pockets for essential magnesium cofactors. These emphasize the importance of the solvation state of the metal in catalysis, and suggest a model that defines the coordination state of the magnesium cofactor in terms of the requirement for bound solvent water as a mediator of catalysis. This varied structural and catalytic chemistry reflects the adaptability of magnesium to a variety of protein structural contexts. Many questions remain to be answered concerning the detailed roles of magnesium in catalysis, cooperativity, cofactor stoichiometry, and factors underpinning the selection of inner and outer-sphere modes of activation, it is hoped that this article will highlight some experimental systems where these questions can be fruitfully addressed and encourage other workers to join this exciting field of research.

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