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The Role of Magnesium in Hydrolytic Nuclease Enzymes. The Use of Inert Chromium and Cobalt Probes in Understanding Magnesium Activation.

Dissertation

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Graduate School of The Ohio State University by Christopher B. Black, B.Sc.

******

The Ohio State University

1996

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Adviser
Department of Chemistry
To Beth, Tess, my Family and Friends, for their support,

friendship and love
ACKNOWLEDGEMENTS

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VITA

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• Development of UV kinetic assay which monitors enzyme steady-state kinetics utilizing hypochromicity of nucleic acids.

• Measurement of steady-state rate constants for ribonuclease H and exonuclease III using an optical stopped-flow apparatus.

• Calorimetric studies on ribonuclease H-nucleic acid interactions with direct measurement of n binding sites, K binding constant and ΔH of binding.

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PROFESSIONAL ORGANIZATIONS

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1992 Jan.- Member of American Association for the Advancement of Science
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>ds</td>
<td>Double-stranded</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiolthreitol</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>Exo III</td>
<td>Exonuclease III</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl Thiogalactosidase</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>Pol I</td>
<td>DNA Polymerase I</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RNase H</td>
<td>Ribonuclease H</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium-dodecyl Sulfate</td>
</tr>
<tr>
<td>ss</td>
<td>Single-stranded</td>
</tr>
<tr>
<td>TE</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>TMP</td>
<td>Thymine Monophosphate</td>
</tr>
</tbody>
</table>
CHAPTER I
OVERVIEW

1.1 Introduction

Magnesium ion exists as the fourth most abundant cation in cellular organisms, playing an intimate role in every aspect of cellular life. As an example, Figure 1 illustrates the divalent cation's essential role in the mitochondrial glycolysis pathway. In addition to its ubiquitous role in the cell, magnesium is the sixth most abundant element in the lithosphere with an extensive mineral history. The name derives from the Magnesia district in Thessally, Greece where the magnesia stone (talc; [Mg₃Si₄O₁₀(OH)₂]) was first obtained. Many of magnesium’s mineral forms (dolomite, epsomite, gypsum) derive from the cation's ability to form insoluble carbonates, sulfates and silicates. The cation is an essential element (Table 1) constituting 0.06% by weight of a typical human. In fact an average sized human can store up to ca. 25 g in mineral
Figure 1. The importance of magnesium in the glycolytic pathway. All enzymes and cofactors which intimately use magnesium are in bold and underlined.
**Table 1.** Distribution of Magnesium in the Cellular Environment. (Adapted from Cowan, 1995). Table 1A shows cation distribution in the hydrosphere and in living cells. Table 1B shows approximate elemental composition of a typical 70 kg human.

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<tr>
<th>Ion</th>
<th>Blood Plasma (mM)</th>
<th>Seawater (mM)</th>
</tr>
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<tr>
<td>Na⁺</td>
<td>138</td>
<td>470</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>K⁺</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>100</td>
<td>55</td>
</tr>
<tr>
<td>HPO₄⁻</td>
<td>1</td>
<td>1 x 10⁻³</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>1</td>
<td>28</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>2 x 10⁻²</td>
<td>1 x 10⁻⁴</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>2 x 10⁻²</td>
<td>1 x 10⁻⁴</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>1.5 x 10⁻²</td>
<td>1 x 10⁻³</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>2 x 10⁻³</td>
<td>3.1 x 10⁻⁶</td>
</tr>
</tbody>
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Table 1 (con.)

B.

<table>
<thead>
<tr>
<th>Element</th>
<th>Quantity</th>
<th>Element</th>
<th>Quantity</th>
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<tr>
<td>Oxygen</td>
<td>44 kg</td>
<td>Potassium</td>
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<tr>
<td>Carbon</td>
<td>12.6 kg</td>
<td>Chlorine</td>
<td>115 g</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>6.6 kg</td>
<td>Sulfur</td>
<td>100 g</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>1.8 kg</td>
<td>Sodium</td>
<td>70 g</td>
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<tr>
<td>Calcium</td>
<td>1.7 kg</td>
<td>Magnesium</td>
<td>42 g</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>680 g</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
form (~ 65% in bones) and in interaction with nucleic acids and proteins (~ 32%) [Marcus, 1985]. Also, magnesium is known to be necessary for the metabolic cycles of plants. This fact is manifested in chlorophyll, the light harvesting pigment that is responsible for funneling the energy of visible light into photosynthesis.

The biological chemistry of magnesium is not restricted to questions of abundance and mineral forms insofar as the solution properties finally dictate its role in cellular organisms. Much of the current thinking with respect to magnesium solution chemistry has evolved from work on the Mg-ATP complex. ATP, the energy currency of the cell, chelates magnesium to ensure its stability in the cytosol until it can be used as an energy source (e.g. glycolysis) or as an enzyme cofactor (e.g. pyruvate kinase). It has been found that Mg$^{2+}$ directly coordinates ATP through the $\beta$ and $\gamma$ phosphate oxygens (Figure 2A). In so doing the cation affords ATP protection from hydrolytic breakdown. Note that coordination occurs through magnesium’s inner coordination sphere. This is in contrast to outer sphere binding which necessarily occurs through metal bound waters (Figure 2B). The importance of inner sphere versus outer sphere binding and catalysis will be discussed below and is the focus of the body of this work.
Figure 2. (A) Mg$^{2+}$-ATP Complex. Note the direct coordination of the $\beta$- and $\gamma$-phosphate oxygens with Mg$^{2+}$. Divalent magnesium is necessary to prevent hydrolysis of the $\gamma$-phosphate in aqueous solution. (B) Representation of hydrated magnesium interacting with a nucleic acid backbone through its outer sphere coordination shell. Specifically in this case the waters form a hydrogen bonding network with the phosphate backbone.
Figure 2 (con.)

B.
Table 2  Physicochemical Properties of Selected Hydrated Cations

<table>
<thead>
<tr>
<th>Ion</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Cs⁺</th>
<th>Mg²⁺</th>
<th>Ca²⁺</th>
<th>Ba²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radius (Å)</td>
<td>1.16</td>
<td>1.52</td>
<td>1.88</td>
<td>0.86</td>
<td>1.14</td>
<td>1.56</td>
</tr>
<tr>
<td>Charge Density (q²/r)</td>
<td>0.86</td>
<td>0.66</td>
<td>0.53</td>
<td>4.65</td>
<td>3.51</td>
<td>2.56</td>
</tr>
<tr>
<td>ΔH°₉₅ (kJ mole⁻¹)</td>
<td>-405</td>
<td>-321</td>
<td>-263</td>
<td>-1922</td>
<td>-1592</td>
<td>-1304</td>
</tr>
<tr>
<td>kₑₓ (H₂O) (s⁻¹)</td>
<td>8x10⁸</td>
<td>10⁹</td>
<td>4x10⁹</td>
<td>10⁵</td>
<td>3x10⁸</td>
<td>10⁹</td>
</tr>
<tr>
<td>pKₐ</td>
<td>14.7</td>
<td>14.5</td>
<td>&gt;15</td>
<td>11.4</td>
<td>12.6</td>
<td>13.2</td>
</tr>
</tbody>
</table>
1.2 Physicochemical Properties of Magnesium

The biological choice of an appropriate metal ion (transition metal, alkali or alkaline earth) is determined by its physicochemical properties and abundance. Some of these relevant properties are listed in Table 2. For example, redox active enzymes generally employ transition metal ions due to the relative ease with which electron transfer can occur. It is readily seen that both the alkali and alkaline earths would be inadequate for such a role. Frequently, it is easy to describe many of the properties listed in Table 2 in terms of a simplified concept. One such concept is the “hard-soft” theory which refers to the preference of various cations toward anions. Pearson coined the term in the early 60’s [Pearson, 1963] as a result of the Irving-Williams series of stability [Irving, 1953]. This generalized rule can be stated as: hard cations prefer to bind to hard anions and soft cations prefer to bind soft anions. This empirical rule explains preferences such as Na⁺ and K⁺ (hard cations) with hard donors (phospholipids, cryptands). Magnesium clearly demonstrates this behavior by the abundance of oxygen ligands, and its ligand preference as a hard cation is clearly illustrated in Table 3 and in a recent review [Black, et al., 1994a]. A related property concerns the extremely high charge-radius ratio or charge density of magnesium. This has several implications for solution behavior: first, magnesium remains heavily solvated, and second, a large preference exists for electrostatic interactions (i.e. for hard donors). These issues are
Table 3. Ligands coordinating magnesium from select proteins based on crystal structures [Black, et al., 1994a]. Each of these proteins and enzymes belong to distinct families and have an intimate dependence on magnesium. Note the abundance of oxygen donors and bound waters.

<table>
<thead>
<tr>
<th>Enzyme/Protein</th>
<th>Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>alkaline phosphatase</td>
<td>$3\times\text{H}_2\text{O}$, Glu, Thr, Asp</td>
</tr>
<tr>
<td>fructose-1,6-bisphosphatase</td>
<td>$2\times\text{H}_2\text{O}$, $2\times$Glu, $2\times$Asp</td>
</tr>
<tr>
<td>ribulose-1,5-bisphosphate</td>
<td>$2\times\text{H}_2\text{O}$, Glu, Asp, Asn, carbamate</td>
</tr>
<tr>
<td>Che-Y</td>
<td>$\text{H}_2\text{O}$, $3\times$Asp, Asn, $\text{SO}_4^{2-}$</td>
</tr>
</tbody>
</table>
central in describing the interactions of Mg$^{2+}$ with polynucleotides and enzyme systems.

1.3 The Biochemistry of Magnesium

The biochemistry of magnesium is more varied than what was imagined even a few years ago pervading almost all biochemical cycles and cellular functions. Due to its relative spectroscopic silence this fact has eluded investigators for many years, however, as attention has increasingly focused on this divalent cation, newer methods to avoid these problems have proliferated. In terms of cytosolic concentrations, the cation is ca. 1 mM in most types of cells; however, specific cells, such as brain cells, can maintain up to 20 mM since divalent cations (Ca$^{2+}$ and Mg$^{2+}$) have been found to act as secondary messengers. In general, approximately 90% of all intracellular magnesium is bound to ribosomes or polynucleotides.

1.3.1 Structural Role

One distinct cellular function magnesium maintains is in lending structural stability to substrate and enzymatic systems. Illustrative enzymes in this category are pyruvate kinase, where a separate site utilizes the cation for stability and allosteric control [Muirhead, H., 1987]; and ribonucleotide reductase, where the magnesium binds at the subunit interface and stabilizes the tertiary structure [Nordlund, et al., 1990]. Structural roles are not abundant in large part due to the lability of the cation (Table 2). Under this
category of structural stability falls its interaction with ATP and polynucleotides. As mentioned previously, ATP$^{4+}$, when complexed to Mg$^{2+}$, is hydrolytically protected in the cytosol. In addition, all of the bulk electrolytes: Ca$^{2+}$, Na$^+$ K$^+$ and Mg$^{2+}$ act on polynucleotides by binding to the negatively charged backbone and electrostatically neutralizing the phosphate oxygens. This serves several purposes. Magnesium, with a higher charge density, populates sites and increases structural integrity by minimizing repulsion. In doing so, it also acts to increase base stacking and further stabilize the base pairing between strands, (as reflected by an increase in melting temperatures). At sufficiently high concentrations, magnesium can effect changes in polynucleotide conformation, for example, changing from a B- to A- or Z-comformation [Davies, et al., 1981]. Equally significant is the effect on catalytic RNA molecules or ribozymes. Magnesium has been determined to control the tertiary structural integrity of the enzyme and effect catalysis [Labuda, et al., 1982; Teeter, et al., 1980]. Another structural function magnesium plays, with analogy to polynucleotides, is in stabilizing cell membranes. These phospholipid aggregates maintain a negatively-charged outer and inner envelope. The binding of divalent cations, especially the abundant magnesium, lends stability and integrity to the membrane such that the influx/efflux of substrates and cations can be done in a controlled manner, either through the various ion channels or by direct interaction with the phospholipid layer [Beveridge, et al., 1976].
1.3.2 Catalytic Role

A second well known function of magnesium relates to enzyme activation. A survey of several unrelated enzymes reveals that the function of magnesium is extremely diverse (Table 4). In general, magnesium is found to interact with enzymes in two contexts. First, the divalent cation may largely interact with the enzyme forming intimate contacts. This is typically where bonds are formed to specific enzyme residues (ribonuclease H, CheY) causing structural changes or catalysis to occur [Black, C.B., et al., 1994a]. Second, the enzyme may interact with a Mg$^{2+}$-substrate complex (ATPase, isocitrate dehydrogenase), where magnesium is present to stabilize substrate interactions with the enzyme [Black, C.B., et al., 1994a]. A combination of these possibilities is most often the case, however, one type of interaction usually predominates.

A few words must be said concerning magnesium's interaction with substrate since these interactions are the principal concern of this work. That interaction can be characterized, in the broadest sense, as either being outer sphere or inner sphere in nature. Work done initially with Mg$^{2+}$-ATP complexes has lead to a widespread belief that Mg$^{2+}$ binding consists of contacts through the cation's inner sphere (Figure 2). However, there are ample lines of evidence based on x-ray crystallography, NMR and kinetic data to demonstrate that divalent magnesium interacts in many systems through its outer sphere waters [Black, et al., 1994a]. Inner sphere interactions would largely be characterized by lewis acid binding, typically polarizing a scissile bond or activating the
Table 4.  Selected magnesium binding proteins and functional roles.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enzyme Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eco R1</td>
<td>Restriction nuclease, DNA cleavage at GAATTC sequences</td>
</tr>
<tr>
<td>DNA Polymerase I</td>
<td>Polymerizes a complementary strand of DNA to a single stranded template</td>
</tr>
<tr>
<td>Topoisomerase I</td>
<td>Relaxation of supercoiled DNA by cleavage and ligation</td>
</tr>
<tr>
<td>Che-Y</td>
<td>Phosphatase, where signal transduction occurs through phosphate transfer</td>
</tr>
</tbody>
</table>
Figure 3. Mg$^{2+}$ acting as a lewis acid. The divalent cation is bound to a nucleic acid processing enzyme (not shown) and polarizes a scissile phosphate oxygen bond, resulting in a phosphate center that is activated toward nucleophilic attack. This is an example of ground state catalysis where the binding of Mg$^{2+}$ affects the energy of the enzyme-substrate complex.
substrate toward nucleophilic attack during activation. This can be seen in Figure 3 in relation to nucleic acid hydrolysis. A reaction mechanism based on this scheme, involving polarization, consists of ground state catalysis, or destabilization of the enzyme-substrate complex to lower the overall transition barrier (Figure 4A). Outer sphere interactions tend to lower the transition barrier by forming bonds with the substrate. This is illustrated in Figure 2B by way of formation of a hydrogen bonding network to the phosphate backbone through metal-bound waters. Mechanistically, the ground state remains unchanged and stabilization occurs when the transition state is formed. During bond breaking and bond formation steps during catalysis, the metal ion can stabilize this energy state and effectively reduce the transition state barrier (Figure 4B).

Although magnesium can act as a cofactor in many systems and in a variety of ways, there are surprisingly few mechanistic modes of action. As eluded to in the above the dominant mechanistic pathways involve inner sphere interactions (ground state destabilization) or outer sphere interactions (transition state stabilization). These are by no means the only viable mechanisms for magnesium. For example, the alkaline earths maintain a $pK_{a1} \approx 12$ for a metal bound water. As such, it is conceivable that a metal bound hydroxide could be a potent nucleophile. The evidence for this is sparse at best with such a pathway proposed for ribozymes [Haydock, et al., 1985; Guerrier-Takada, et al., 1986] and in a more indirect sense the 3'-5' exonuclease activity of DNA polymerase
I [Steitz, et al., 1993]. In terms of indirect catalysis though, magnesium can electrostatically neutralize negatively-charged active site residues or coordinate an active center orienting the substrate for attack. It is important to keep in mind however, that the majority of cases involve mechanisms that are a combination of the possibilities outlined above. Specifically, any such pathway can be thought of in terms of its smaller contributions. For example, in *E. coli* ribonuclease H, magnesium interacts with the substrate through metal-bound waters. This indicates the mechanistic pathway occurs through transition state stabilization by hydrogen bonding interaction with the substrate. A lesser contribution is electrostatic, probably necessary in minimizing the negative charge at the active site (see Chapter 4). Other complications can be seen with systems such as DNA polymerase I which require multiple cations for full functionality, where each of these cations has a distinct function and distinct interactions.

It is the aim of this body of work to investigate in a detailed and quantitative manner the interactions and mechanistic role magnesium plays in activating three nucleic acid processing enzymes: namely, *E. coli* ribonuclease H, *E. coli* exonuclease III and *E. coli* DNA polymerase I. Each of these enzymes requires or utilizes magnesium for catalytic function, has been crystallized, and has previously been the subject of extensive investigation. However, the role of magnesium, especially in the cases of RNase H and Exo III, has remained unclear. Based on this work it is concluded that for RNase H and Exo III, Mg$^{2+}$ acts through its bound waters to stabilize the developing transition state via
a hydrogen bonding network. The numbers of waters, geometry of the waters, and electrostatic contributions are evaluated. In DNA Pol I a different picture has emerged, where one magnesium functions at the 3'-5' exonuclease site, most likely effecting catalysis through inner sphere chemistry.
Figure 4. (A) Destabilization of the enzyme-substrate complex. Graphical representation of metal ion mediated catalysis through ground state destabilization, reducing the overall reaction barrier. (B) Stabilization of the transition state. Representation of metal-mediated transition state stabilization. The formation of hydrogen bonds or electrostatic contacts can lower the activation barrier and result in faster turnover.
B.

Figure 4 (con.)

Free Energy
(G)

\[ E + S \]

\[ \Delta G^\ddagger_{ES} \]

\[ E.S \]

\[ E.S^{*} \]

\[ E.S.Mn^{+*} \]
1.4 Research Objective

The goal of this research study was to investigate the functional role of magnesium in the nucleolytic enzymes *E. coli* ribonuclease H, *E. coli* exonuclease III, and the Klenow fragment of *E. coli* DNA polymerase I. The field of inorganic biochemistry has traditionally involved the study of the role of metal ions in biology. Whereas metal ions were once thought to be principally important as background electrolytes, more recently have been found to facilitate electron transfer, impart structural integrity, and hydrolytically activate many protein systems. Understanding the detailed mechanisms of these processes is currently the focus of this field. Traditionally, magnesium has been viewed with little interest, in part, due to its spectroscopic silence, despite the fact that many of the biochemical and molecular biology protocols require magnesium. In fact, very little is known regarding mechanistic details of this elusive cation. To broaden the breadth of mechanistic knowledge concerning magnesium, a convenient vehicle was necessary to begin studies on this divalent cation. In particular, *E. coli* ribonuclease H, which is thought to be involved with primer removal, was a prime candidate since it has been studied extensively by crystallography, solution NMR and mutagenesis. However, the function of magnesium in ribonuclease H has remained unclear. Extending the mechanistic detail for ribonuclease H has led to analysis of the function of magnesium in exonuclease III and the Klenow fragment.
Since magnesium is relatively spectroscopically silent, it was initially important to develop a methodology to study this cation. Previous work from our laboratory utilized $^{25}\text{Mg NMR}$ as a way of monitoring binding properties to nucleic acids and ATP$^4^+$ [Black, et al., 1994b; Cowan, 1991]. However, technical problems, such as line broadening due to magnesium’s quadrapolar nucleus, limit this technique from enzyme turnover experiments. One possibility, employed recently, utilizes the hypochromicity inherent in nucleic acids [Waters, et al., 1992]. With this method, quantitation of enzymatic turnover under steady-state conditions can be made for any nucleic-acid processing enzyme. Due to the spectroscopic silence of magnesium, direct observation of the cation during enzymatic turnover is impossible; spectroscopic techniques allow direct understanding of the active species. Therefore, in addition to developing a suitable assay, we have developed the use of probes to study the interactions of magnesium in protein systems. These probes, substitution inert cobalt and chromium complexes ($\text{[Cr(H}_2\text{O)}_{6-x}(\text{NH}_3)_x]^{3+}$ where $x = 0 - 6$), allow systematic variation of the inner sphere complement of the ammine to water ratio. The characterization and use of such probes was carried out and yielded previously unknown information on metal ion-mediated outer sphere catalysis in enzyme systems.
CHAPTER II
MATERIALS AND METHODS

2.1 General Materials

Unless otherwise indicated, all chemicals were purchased from Sigma or Aldrich (molecular biology grade) and synthesized oligonucleotides from IDT (Integrated DNA Technologies). DNase I, DTT and streptomycin sulfate were purchased from Boehringer Mannheim. Measurements of solution pH were made with an Accumet model 910 pH meter equipped with a Corning semi-micro combination pH-electrode. Sephadex G-100 gel filtration material (used for desalting dT$_{20}$) was obtained from Sigma, and DE-52 and P-11 ion-exchange resins from Whatman.

2.2 Bulk Growth of Escherichia coli

Specific conditions for cell growth are described below. Both ribonuclease H and exonuclease III plasmids were initially expressed behind the temperature dependent λ $P_L$ promoter, but were eventually cloned into a pET system which uses IPTG induction.
promoter, but were eventually cloned into a pET system which uses IPTG induction (specific plasmids and related details are outlined in each section below). The protocol described below for RNase H follows the original procedure with induction arising through a temperature shift. This material was used for the work described in Chapter 3, while the work described in Chapter 4 used RNase H which had been cloned into the pET system. For all strains described below, frozen culture stocks were prepared by mixing 850 µL of the relevant cell strain (O.D. ~ 1) with 150 µL of sterile glycerol. This mixture was flash frozen in a carbon dioxide slush bath (solid CO₂ and 95% ethanol). Using the relevant frozen stock, a starter culture (1 mL thawed stock into 9 mL growth media) was grown for 4-6 hours in an autoclaved 75 mL flask. A second starter culture was initiated by adding (under sterile conditions) the 10 mL (from the first inoculum) to 100 mL sterile growth media in a 250 mL flask. The 100 mL starter is added to two flasks (50 mL to each flask), each containing 1 L autoclaved LB media. On this scale the flasks grew for 3-4 hours and were then induced. Cells were harvested 2-3 hours after induction.

2.3 Enzyme Purification

2.3.1 Purification of E. coli RNase H using pSK58

An overexpressing vector (pSK58), containing a synthetic gene for RNase H and the temperature sensitive λ P₁-promoter was kindly provided by Dr. R.C. Crouch (NIH). E. coli (N4830), containing this plasmid, was grown at The Ohio State University
Fermentation Center with help from Don Odaz at 32°C in LB medium containing ampicillin (100 µg/mL). When the optical density of the cell culture reached 1.0, the temperature was shifted to 42°C to stimulate expression of RNase H. After an additional 5 hr incubation, the cells were harvested. The enzyme was isolated and purified using a protocol in the literature [(a) Yang et al., 1990]. All procedures were carried out at 4°C. Buffers A, B and C were prepared as follows: A (50 mM Tris, pH 7.5; 5 mM MgCl₂; 200 mM KCl; 0.1 mM EDTA; 1 mM 2-mercaptoethanol), B (50 mM Tris, pH 7.5; 0.1 mM EDTA; 1 mM 2-mercaptoethanol), C (50 mM Tris, pH 7.5; 50 mM KCl; 0.1 mM EDTA; 1 mM 2-mercaptoethanol). A 150 g batch of frozen cells was thawed, 50 ml of buffer A added, and cell lysis carried out using a sonic dismembrator (Fisher, model 300) at 90% maximum power (10 x 2 min). DNase I (20 µg/mL) was added and incubated at 0°C for 30 min to reduce the viscosity. After centrifuging at 10,000 rpm for 30 min (Sorval/du Pont, Model RC5B) the solution was ultracentrifuged for 16 hr at 4°C and 30,000 rpm (Beckman, model L8-M). The upper part of the solution was taken (a dark oily brown layer remained at the bottom of the tube with the pellet), diluted 3-fold with buffer B and applied to a DE-52 column (5 x 15 cm) equilibrated in buffer C. The column was eluted with buffer C and the absorbance monitored at 280 nm. The first broad band (0-600 ml) contains RNase H. After concentrating to a volume of ca. 100 mL by Amicon ultrafiltration, (NH₄)₂SO₄ was added to a final concentration of 0.6 mg/mL. The solution
was stirred for 1 hr and the precipitate collected by centrifugation, redissolved in a minimum of buffer C, and dialyzed against buffer C. The solution was applied to a phosphocellulose P-11 column (5 x 20 cm) and eluted with a gradient from 0 to 1 M NaCl in buffer C (2L). Ribonuclease H eluted as the third band at [NaCl] ~ 0.3 M. Approximately 80 mg of enzyme was obtained from 150 g of cell paste.

2.3.2 Purification of *E. coli* RNase H using pET 21b(+)

Recombinant native ribonuclease H was overexpressed in *E. coli* BL21 (DE3) harboring a pET 21b(+) expression vector (Novagen) following IPTG induction. The 465 base pair gene for native ribonuclease H was cloned behind the T7 *lac* promoter of the pET 21b(+) vector in the restriction sites Nde I and Hind III. Purification was performed essentially as above with the noted modifications. *E. coli* BL21 (DE3) was grown at 37°C until the optical density reached 0.4-0.6 at which point 0.24 mg of IPTG per liter of media was added. The cells were allowed to grow for another 2-3 hours and then harvested. After ultracentrifugation the supernatant was applied to a DE-52 column equilibrated in buffer C, and subsequently eluted with the same buffer. The large peak obtained as above was applied directly to a P-11 column equilibrated with buffer C and a gradient from 0 to 1 M NaCl was applied (2 L) (Figure 5). The resulting band collected from the P-11 column showed a single band on a polyacrylamide gel (20% homogeneous PAGE) run using the same conditions and materials outlined in Figure 14. An
Figure 5. Profile for the purification of *E. coli* ribonuclease H from a phosphocellulose column (P-11). The enzyme peak (middle large peak) elutes slightly above 0.5 M NaCl.
Figure 6. Absorbance spectrum for 3μM *E. coli* ribonuclease H after final purification. Note the characteristic absorbance at 280 nm. Aside from extinction coefficient differences, *E. coli* exonuclease III and *E. coli* DNA polymerase I are qualitatively similar.
absorbance spectrum after purification is shown in Figure 6.

2.3.3 Purification of E. coli Exonuclease III

The exonuclease III (xth) gene (provided by Dr. B. Weiss) was overexpressed from an E. coli strain harboring an expression vector, and isolated and purified following published procedures (Kuo et al., 1993). Modification of this procedure consisted of ultracentrifugation of the cytosolic remnants (after the initial sonication) for 12 hr at 4°C and 30,000 rpm (Beckman, model L8-M). The supernatant was applied to a DE-52 cellulose column (5 x 20 cm) equilibrated with buffer C (20 mM NaH₂PO₄ / Na₂HPO₄, pH 7.4; 1 mM 2-mercaptoethanol) at 4°C. Exo III eluted as the third band (1.2 L) from a phosphate gradient (0.1 M to 0.3 M mM NaH₂PO₄ / Na₂HPO₄, pH 7.4) at 4°C. Figure 7 shows a typical elution profile with exonuclease III eluted as the large band. Each tube collected approximately 10 mL of elute. Figure 8 shows the fluorescence spectrum where excitation occurred at 280 nm. To ensure large structural changes did not occur upon metal ion binding, several spectra were obtained with different concentrations of metal ions present. No significant change occurred (Figure 8).

2.3.4 Purification of the Klenow Fragment from CJ379

The Klenow fragment has been cloned into the overproducing strain of E. coli CJ379 which contains two plasmids: pCJ136 (chloramphenicol resistance) and pCJ122
Figure 7. Profile for the purification of *E. coli* exonuclease III from a DE-52 (Whatman) cellulose column (anion exchange). The third peak (centered around 800 mL) contained exonuclease III.
Figure 8. Fluorescence spectrum for 2.5 μM *E. coli* exonuclease III. The bottom spectrum is buffer only. The overlapping spectra are: exo III only, exo III and 12 mM Mg$^{2+}$, and exo III and 35 mM Mg$^{2+}$ with slightly increasing intensities (ca. 580 nm) with increasing Mg$^{2+}$. Excitation occurred at 280 nm. No change upon addition of Mg$^{2+}$.
Figure 9. Absorbance spectrum for 25 μM *E. coli* exonuclease III in 20 mM Tris-base, pH 7.5, 50 mM KCl, 0.1 mM DTT.
(ampicillin resistance) (gift from Prof. C. Joyce). The Klenow fragment gene, located in pCJ122, was transcribed from the $\lambda$ P$_L$ promoter in the vector pAS1. A 20 mL overnight culture was grown at 30°C in sterile LB media containing 20 $\mu$L ampicillin (50 mg/mL) and 27.2 $\mu$L chloramphenicol (34 mg/mL). The starter culture was added to 2 L of sterile LB media containing 4 mL (50 mg/mL) ampicillin and grown at 30°C until OD(550) ~ 0.4-0.6. The culture was heat shocked by incubating at 42°C for 2 minutes and grown for a further 2-3 hours. The cells were harvested through centrifugation at 6000 rpm for 20 mins at 4°C.

Approximately 8 mL of buffer A (50 mM Tris, pH 7.5, 1 mM DTT) per gram of cell paste was added and the resulting solution sonicated at 90% power until a homogenous solution was obtained. The crude extract was obtained by centrifuging at 10,000 rpm for 10 mins. To the extract supernatant ammonium sulfate was added to 50% saturation (0.291 g/mL). The solution was centrifuged (12,000 rpm for 20 mins) and the supernatant retained; additional ammonium sulfate was added to give an 85% saturated solution (an additional 0.23 g/mL). The slurry was stable and can be stored at 4°C for many months (Prof. Joyce).

To further purify, the slurry was centrifuged (10,000 rpm for 20 mins), suspended in buffer A (7.5 mL/g original cell paste). The solution is dialyzed (2 x) against 1 liter of buffer A at 4°C for 2 hours. The dialyzed solution was filtered through a 0.22 $\mu$m filter and applied to a Mono Q column equilibrated with buffer A. A gradient (0-25%) was applied
using buffer B (buffer A + 2 M NaCl). The Klenow fragment eluted at ca. 10% buffer B. To obtain a working stock, the solution was concentrated by use of amicon filtration with a 30,000 Da M.W. cut-off membrane and finally treated several times with Chelex-100 to remove adventiously bound metal ions. Treatment with Chelex-100 involved addition of 0.5 g of the resin added to ca. 15 mg of Klenow (approximately 10 mL) and stirring on ice for 10 to 12 hours. To check for complete removal of metal ions, the activity was measured using the hypochromicity assay described in section 2.6.3. Figure 10 shows an illustrative FPLC trace, where the collected peak was typically not easily separable from the contaminant peaks. To circumvent this, only the very center of the peak was collected to ensure the highest purity. Figure 11 shows a typical absorbance spectrum of the Klenow fragment.

During the course of kinetic work with the Klenow fragment, it was necessary to check the intrinsic fluorescence of the protein as a possible means of monitoring binding or reaction turnover. To that end, the protein was mixed with 5 mM Mg$^{2+}$ and the change monitored (Figure 12). Both the excitation spectrum and emission spectrum were scanned for optimal intensity, where $\lambda_{ex} = 345$ nm was the optimal excitation wavelength. This reflects the intrinsic fluorescence of the tryptophan and tyrosine residues and not the characteristic absorbance wavelength (Figure 11). The scanning speed was 240 nm/min, with the excitation slit width of 5 mm and an emission slit width of 10 mm. All measurements were performed on a Perkin-Elmer fluorimeter at room temperature. From
Figure 10. Fast Protein Liquid Chromatogram (FPLC) for the purification *E. coli* DNA polymerase I. See text for purification details.
Figure 11. Absorbance spectrum for 8 µM *E. coli* DNA polymerase I in 20 mM Tris-base, pH 7.5, 50 mM KCl, 1 mM DTT.
Figure 12. Fluorescence spectrum for 1 μM *E. coli* DNA polymerase I in 20 mM Tris-base, pH 7.5, 50 mM KCl, 1 mM DTT with the addition of 5 mM Mg$^{2+}$. The lowest intensity spectrum is at $t = 1$ min (which is identical to the Klenow-only spectrum), the middle is at $t = 8$ min and the highest at $t = 15$ min. The excitation was at $\lambda_{ex} = 345$ nm and emission was scanned from 200-600 nm.
Figure 13. Circular dichroism spectrum of *E. coli* DNA polymerase I Klenow fragment.

Conditions: \([\text{Klenow}] = 10 \, \mu\text{M}, \, [\text{Mg}^{2+}] = 5 \, \text{mM}, \) and 25°C. The scanning range was 200 - 500 nm with a 0.2 nm resolution (only 200-300 nm shown here). The y-axis was +20.00 to -80.00 mdeg. The scanning rate was 100 nm/min with 4 scans averaged. The spectrum was identical with and without addition of Mg$^{2+}$. 
Figure 12, it is clear that very little change occurs with addition of Mg\(^{2+}\), indicating that if such a change does occur with binding it is either fast, or the fluorescent residues are not in close proximity to reflect a change in binding. To further check any possible structural changes upon binding of Mg\(^{2+}\), a circular dichroism spectrum was taken. The scanning range was 200 - 500 nm with a 0.2 nm resolution. The scanning rate was 100 nm/min were only 4 scans were averaged. This spectrum (Figure 13) showed however, over several hours, that little change occurs in the secondary structure upon Mg\(^{2+}\) binding.

### 2.4 Synthesis of (A·dT)\(_{20}\) Hybrid Substrate

Substrate RNA · DNA hybrid was synthesized from dT\(_{20}\) (4 mg) and poly(rA) (4 mg) (Pharmacia LKB Biotechnology Catalog). The components were dissolved in buffer (50 mM Tris, pH 7.5; 100 mM KCl) and annealed (42-44°C) for 15 min. To concentrate the annealed oligonucleotide, ethanol precipitation was carried out. For this, two volumes of -20°C absolute ethanol was added and mixed briefly. This mixture was stored at -20°C for 15 mins. The solution was then centrifuged (4°C) at 12,000 rpm for 15-20 mins and the supernatent discarded. To the pellet, ca. 1 mL -20°C 70% ethanol was added and mixed to remove any ions. This mixture was centrifuged (4°C) at 12,000 rpm for 10 mins and the supernatent removed. The pellet was dried under vacuum. After precipitation, the resulting pellet was incubated with 500 U mung bean nuclease (30 min,
To purify the hybrid from remaining nuclease a phenol extraction was performed. Phenol, which is equilibrated at pH ~ 7 using TE buffer, is first mixed with chloroform and isoamylalcohol 25:24:1 (equilibrated phenol:chloroform:isoamylalcohol). This mixture is added in equal volume to the hybrid sample and vortexed vigorously for 10 sec. Following mixing, the solution is spun down for 20 sec. at 12,000 rpm (4°C) in order to create an organic-aqueous layer. The phenol-denatured protein lies at the interface. The top phase is removed containing the nucleic acids. An equal volume of chloroform is added with the mixing and centrifuging steps repeated to remove any remaining phenol. The resulting chloroform containing hybrid is finally treated with ethanol as described above to re-precipitate the pure hybrid. The purity of the 20-mer hybrid was verified by comparison with appropriate molecular weight markers on 20% homogeneous PAGE (Figure 14). A single band was observed. The hybrid was stored at -20°C. Hybrid concentration is defined by phosphate equivalents which was determined from a weighed mass of substrate.

To further assess the stability and behavior of the synthetically prepared (A·dT)_{20} substrate, a series of melting temperature curves were carried out (Figure 15). To determine the melting temperature, the absorbance was monitored at 260 nm while an incremental increase in temperature was applied. Taking advantage of the hypochromic effect, a large increase in absorbance intensity corresponds to the dissociation of the two strands. For background check, the hybrid mixture before treatment with Mung Bean
Figure 14. Polyacrylamide gel of (A·dT)_{20} from the Phast-gel electrophoresis system (Pharmacia). The gel, purchased from Pharmacia, is a 20% polyacrylamide homogeneous gel with SDS buffer strips. The first lane is before treatment with Mung Bean nuclease, where the smear is a large strand of poly(A) with dT_{20}-mer units annealed. The lane in the middle shows hybrid purity. The gel was stained comassie blue.
Figure 15. Melting temperature curves for hybrid under different electrolyte conditions.

All curves were obtained in 50 mM Tris-base, pH 7.5, 100 mM KCl where: (■) denotes (dT)$_{20}$ and poly(A), (●) denotes (dT)$_{20}$ and poly(A) with 1 mM MgCl$_2$, (▼) denotes (dT)$_{20}$ and poly(A) with 1 mM Co(NH$_3$)$_6$Cl$_3$, (▲) denotes (A·dT)$_{20}$. All T$_m$ ~ 45°C except for the Co(NH$_3$)$_6^{3+}$ treated mixture which is shifted slightly (T$_m$ ~ 50°C).
Nuclease was checked with and without 1 mM Mg\textsuperscript{2+}. Also the final product, [(A·dT)\textsubscript{20}], is shown for comparison (specific conditions listed in Figure 15). The large signal increase for the mixtures of dT\textsubscript{20} and poly(A) are due to the larger amounts of poly(A) present. The melting behavior is roughly the same for all cases, yielding a T\textsubscript{m} \sim 45°C, except for the Co(NH\textsubscript{3})\textsubscript{6}\textsuperscript{3+} treated poly(A) dT\textsubscript{20}, where T\textsubscript{m} \sim 50°C due to the trivalent charge on the complex.

### 2.5 Preparation of Chromium Complexes

Chromium complexes were prepared using standard literature procedures:

\[ \text{[Cr(H}_2\text{O)}\textsubscript{6}]\text{Cl}_3: \text{ Brauer, et al.}, 1962; \text{[Cr(H}_2\text{O)}\textsubscript{5}(NH\textsubscript{3})]\textsubscript{2}[(SO}_4]\textsubscript{3}]: \text{ Ardon, et al.}, 1962; \]

\[ \text{trans-[Cr(H}_2\text{O)}\textsubscript{4}(NH\textsubscript{3})\textsubscript{2}]\text{Cl}_3: \text{ Bjerrum, et al.}, 1958; \text{cis-[Cr(H}_2\text{O)}\textsubscript{4}(NH\textsubscript{3})\textsubscript{2}](ClO}_4)_3: \]

\[ \text{Andersen, et al.}, 1984(a); \text{mer-[Cr(H}_2\text{O)}\textsubscript{3}(NH\textsubscript{3})\textsubscript{3}]Cl}_3: \text{ Riesenfeld, et al.}, 1909; \text{fac-}\]

\[ \text{[Cr(H}_2\text{O)}\textsubscript{3}(NH\textsubscript{3})\textsubscript{3}]Cl}_3: \text{ Andersen, et al.}, 1984(b) and Andersen, et al., 1990; \text{cis-}\]

\[ \text{[Cr(H}_2\text{O)}\textsubscript{2}(NH\textsubscript{3})\textsubscript{4}](NO}_3)_3: \text{ Springborg, et al.}, 1962; \text{trans-[Cr(H}_2\text{O)}\textsubscript{2}(NH\textsubscript{3})\textsubscript{4}](NO}_3)_3: \]

\[ \text{Hoppenjans, et al.}, 1969; \text{[Cr(H}_2\text{O})(NH\textsubscript{3})\textsubscript{5}](NO}_3)_3: \text{ Schlessinger, 1962; } \]

\[ \text{[Cr(NH\textsubscript{3})\textsubscript{6}](NO}_3)_3: \text{ Schlessinger, 1962. } \]

Product complexes were purified by ion-exchange chromatography and/or recrystallization and characterized by electronic absorption spectroscopy. All complexes were stored as solids in the dark under desiccating conditions. Solutions were freshly prepared immediately before use. The specific synthetic procedures are described below.
trans-\([\text{Cr(NCS}_4](\text{NH}_3)_2] \text{NH}_4\cdot5\text{H}_2\text{O (Reincke's Salt)}\). This precursor was prepared by addition of 100 g of NH$_4$SCN in a 600 mL beaker and slowly heated to 150°C on a hot plate until the salt completely fused. While stirring, 17 g of powdered NH$_4$Cr$_2$O$_7$ was added in small amounts. This fusion mixture is stirred well after each addition waiting until the frothing stops to add the next portion. The temperature remained at 170°C (the reaction is exothermic). This purple mixture was allowed to cool and solidify under desiccating conditions and was subsequently broken up with a spatula and transferred to a beaker containing 60 g of ice (frozen from distilled water). Stir until only small pieces of ice remain and the solution was filtered off with a fritted filter and drained without washing. This solid was added to 250 mL of H$_2$O at 70°C and stirred continuously with the temperature of 65°C. The purple-red solution was filtered and rapidly cooled on ice. The salt was filtered off and air-dried obtaining the red salt trans-[
Cr(NCS)$_4$(NH$_3$)$_2]$NH$_4\cdot5$H$_2$O.

\([\text{Cr(H}_2\text{O})_6]\text{Cl}_3\). In a 100 mL round bottom flask 2.5 g KCr(SO$_4$)$_2$ was added to a 12.5 mL cooled solution of HCL (10 mL conc. HCl, and 2.5 mL H$_2$O). This mixture was stirred for one hour and the dark blue solution filtered. This mixture was saturated with HCl$_{(g)}$ on ice. After ca. 15 mins. blue crystals had formed and were filtered using a fritted glass filter. The crystals were washed with acetone first, then redissolved in 1.75 mL
H$_2$O and reprecipitated with HCl$_g$/acetone again. The blue-violet crystals were collected, dried and stored under desiccating conditions in the dark.

$[Cr(H_2O)_5(NH_3)_2][(SO_4)_3]$. 3.5 mL H$_2$O was degassed in a 100 mL round bottom flask with a stirrer. 1 g CrCl$_3$ and 1 g Zn (powder) were added and stirred until the solution was dark green. Separately, 0.122 g NaN$_3$ was added to 20 mL 1 M HCl in a 100 mL round bottom flask. Using a double-tipped needle, the Cr(III) solution was added to the hydrazoic acid solution under anaerobic conditions. With an N$_2$ atmosphere, the pressure forced the Cr(III) solution (green) through the needle into the hydrazoic acid where Cr(II) forms (dark blue solution). After 10 min. of stirring, the mixture was diluted to 400 mL with H$_2$O (in air). A Dowex 50W-X8 column was prepared by first treating the resin with 2 M H$_2$SO$_4$, stirring for ca. 1 hour. The resin was washed with H$_2$O until neutral pH and packed on a 25 cm column. The chromium mixture (400 mL) was applied to the column and first washed with 0.2 M H$_2$SO$_4$. A green band and a violet band formed near the top of the column. The green band eluted with ~ 0.7 - 1 M H$_2$SO$_4$, whereas the violet band eluted in 2 M H$_2$SO$_4$. To crystallize, ethanol was added (2:1) to a final volume ~ 900 mL and placed at -20°C for 1 week. In addition, the solution was rotoevaporated and the resulting solution resuspended in ethanol and recrystallized.
trans-\(\{\text{Cr}(\text{H}_2\text{O})_4\text{NH}_3\}_2\text{Cl}_3\). trans-[Cr(NCS)_4(NH_3)_2]NH_4·5H_2O (Reincke’s Salt) was oxidized with H_2O_2: the hydrogen peroxide (30%) was added dropwise on ice to Reincke's salt which was slightly moistened with conc. HCl (very vigorous reaction). The resulting dark red solution was frequently decanted and all fractions were pooled and saturated with HCl(g) (10 mins) on ice. The solution was left at room temperature for one day and the precipitate used (blue-green). Filtered this mixture on a fritted glass filter, washed with acetone and dried. After drying, the solid was dissolved in 0.1 M HCl:acetone (1:4) mixture. The solution was again filtered and saturated at 0°C with HCl(g) for 5 mins. and stored overnight at 4°C. The green crystals were filtered and washed with acetone (trans-[Cr(H_2O)_2(NH_3)_2(Cl)_2]Cl). This mixed chloride salt (~ 0.3 g) was stirred into 5 mL 0.6 M HCl at 50°C for 3 hours. After 3 hours, the solution was pink and placed at 4°C overnight. A Dowex 50W-X8 column was prepared as above for the [Cr(H_2O)_5(NH_3)_2][(SO_4)_3], except HCl was used (3 M HCl to regenerate then H_2O). The pink diammine solution was diluted to ~ 300 mL and loaded on the column. The product red band was eluted with ~ 300 mL H_2O. The solution was recrystallized with acetone.

cis-[Cr(H_2O)_4(NH_3)_2](Cl)_3. The initial precursor was obtained generously from Prof. Andersen, cis-[Cr(NH_3)_3(H_2O)(Cl)_2]Cl with synthetic steps shown previously for the perchlorate salt [Andersen, et al., 1984]. To completely hydrolyze this compound, a
silver oxide compound was first prepared. In a 50 mL flask, 2 g AgNO₃ were dissolved in 10 mL distilled H₂O. In a separate 25 mL flask, 0.5 g 99.5% NaOH in a minimum amount of H₂O was dissolved. The NaOH solution was pipetted into the AgNO₃, and immediately a brown precipitate formed, the liquid was decanted and washed 3 times of H₂O (10 mL) draining the water layer. To the moist Ag₂O, 4.5 mL H₂O and 0.5 g cis-[Cr(NH₃)₃(H₂O)(Cl₂)]Cl were added and stirred. The solution was filtered and 3 mL more HNO₃ was added. The solution was cooled (reddish-orange) and 10 mL 95% ethanol was added while the mixture was still cold (on ice). The pink crystals were filtered and washed with 95% ethanol followed by acetone. Finally the crystals were air dried in the dark yielding ~0.2 g

fac-[Cr(H₂O)₃(NH₃)₃]Cl₃. The initial precursor was obtained generously from Prof. Andersen, fac-[Cr(Cl)₃(NH₃)₃]Cl₃ with synthetic steps shown previously [Andersen, et al., 1990]. To hydrolyze this compound, a silver oxide compound was first prepared. In a 50 mL flask, 2 g AgNO₃ were dissolved in 10 mL distilled H₂O. In a separate 25 mL flask, 0.5 g 99.5% NaOH in a minimum amount of H₂O was dissolved. The NaOH solution was pipetted into the AgNO₃, and immediately a brown precipitate formed, the liquid was decanted and washed 3 times of H₂O (10 mL) draining the water layer. To the moist Ag₂O, 4.5 mL H₂O and 0.5 g fac-[Cr(Cl)₃(NH₃)₃]Cl₃ were added and stirred. The solution was filtered and 3 mL more HNO₃ was added. The solution was cooled
(reddish-orange) and 10 mL 95% ethanol was added while the mixture was still cold (on ice). The red crystals were filtered and washed with 95% ethanol followed by acetone. Finally the crystals were air dried in the dark yielding ~ 0.3 g.

mer-\([\text{Cr(H}_2\text{O)}_3(N\text{H}_3)_2]\text{Cl}_3\). Dissolved 25 g CrO₃ in 50 mL H₂O and added, with stirring, 10% ammonia solution (85 mL NH₄OH and 163 mL H₂O). Placed the brown solution on ice and slowly added 50 mL 30% H₂O₂. The brownish bubbling solution was kept on ice for ca. 1 hour. The mixture was warmed up to 50°C until evolution of O₂ ceased with stirring, and the solution was filtered with a buchner funnel. The resulting solution was transferred to a 500 mL flask for cooling at -20°C. Filtered brown crystals and washed with cold ethanol and anhydrous ether and place filtrate at -20°C. In a 50 mL round bottom flask, ~ 0.1 g of the crystals were slowly mixed with dilute HCl (1:4 H₂O), where the brownish color turned dark red. The solution, on ice, was left stirring for 1.5 hours. After cooling, the crystals were precipitated with HCl₉ on the ice bath for 0.5 hours. The crystals were filtered on a fritted filter and finally washed with absolute ethanol followed by ether. The crystals appeared fine and red.

trans-\([\text{Cr(H}_2\text{O)}_2(N\text{H}_3)_4]\). First the precursor rhodo chloride must be prepared, [Cr(NH₃)₅(OH)Cr(NH₃)₃](Cl)₃. Once this compound is prepared, the hydroxoerythro perchlorate is obtained, [Cr(NH₃)₅(OH)Cr(NH₃)₄(OH)](ClO₄)₄. Following cleavage and
hydrolysis, the trans-[Cr(NH₃)₄(OH)₂(NO₃)]₃ can be obtained. For the Rhodo chloride, 110 g CrCl₃·6H₂O were dissolved with stirring in 150 mL H₂O and reduced by addition of 110 g Zn when the solution was degassed. Finally, 150 mL conc. HCl was added when the solution was blue and kept cold on ice under N₂. In a separate 100 mL flask, 150 g NH₄Cl and 750 mL NH₄OH were mixed and degassed on ice. The Cr(II) solution (blue) was transferred to the NH₃ mixture using a double-tipped needle under inert atmosphere. Under the inert atmosphere (using a side-arm flask), the mixture was stirred for 1-2 hours upon which crystals had formed. The solution was decanted from the undissolved NH₄Cl. These crystals were filtered and washed with 10% HCl, ethanol and finally ether.

To prepare the erythro chloride compound, 100 g of the rhodo chloride were dissolved in 800 mL 2 N NH₃ and filtered with a frit filter. After stirring the solution on ice, the color changed from a blue to red. Then, with stirring, 1.2 L conc. HCl were slowly added dropwise (over ca. 1 hour). The subsequent crystals were washed with dilute HCl, then 100% ethanol and finally ether. The erythro can by recrystallized by dissolving 10 g of erythro crystals in 60 mL H₂O at room temperature in air, and filtering into an ice cold flask. After sitting for ca. 0.5 hours, 200 mL 10% HCl dropwise was added while on ice.

For the trans final product, 40 g of the erythro chloride were ground, and 125 mL of 70% HClO₄ were slowly added with stirring. When the evolution of gas ceased (HCl), the mixture was diluted to 700 mL with H₂O (in a separate flask). This mixture was
heated to 60°C with constant stirring (~ 0.5 hours). The solution was filtered while still hot through a fritted filter, and then immediately cooled on ice. Then 400 mL 70% conc. HCl was added and the precipitate filtered off through a fritted filter. The brownish crystals were washed with ethanol first, followed by ether. The formed salt was recrystallized by dissolving in a minimum of 0.01 M HCl and adding an equal volume of conc. HCl on ice dropwise with stirring (trans-[Cr(NH$_3$)$_4$(OH$_2$)Cl](Cl)$_2$). To hydrolyze this compound, 2 g AgNO$_3$ were dissolved in 10 mL distilled H$_2$O. In a separate 25 mL flask, 0.5 g 99.5% NaOH in a minimum amount of H$_2$O was dissolved. The NaOH solution was pipetted into the AgNO$_3$, and immediately a brown precipitate formed, the liquid was decanted and washed 3 times of H$_2$O (10 mL) draining the water layer. To the moist Ag$_2$O, 4.5 mL H$_2$O and 0.5 g trans-[Cr(NH$_3$)$_4$(OH$_2$)Cl](Cl)$_2$ were mixed and 3 mL more HNO$_3$ was added. The solution was cooled (reddish-orange) and 10 mL 95% ethanol was added while the mixture was still cold (on ice). The red crystals were filtered and washed with 95% ethanol followed by acetone. Finally the crystals, trans-[Cr(NH$_3$)$_4$(OH$_2$)$_2$](NO$_3$)$_3$, were air dried in the dark yielding ~ 0.3 g.

cis-[Cr(H$_2$O)$_2$(NH$_3$)$_4$](NO$_3$)$_3$. In a 100 mL round bottom flask, 6.4 g NH$_4$Cl and 30 mL of 25% NH$_3$(aq) were stirred and heated to 25°C. After mixing, 14.4 g Cr$_2$(SO$_4$)$_3$ was added and stirred. The solution became blue from colorless and the temperature raised to 50°C. After 0.5 hours at 50°C, the solution turned dark-red or purple and was poured into
40 mL of cold conc. HCl very slowly. After the solution was homogenously cooled, it was filtered using a fritted filter. There were a large number of purple crystals. The filtrate was mixed with 70 mL conc. HCl and covered with foil. After sitting overnight, red crystals had formed and were filtered with a fritted filter. The crystals were washed with 4 M HCl, followed by a wash of 100% ethanol and finally ether. The red crystals were dissolved in 12 mL ice cold HNO₃ and stirred. Roughly 1 g (NH₄)SO₄ was added, shaken until dissolved and allowed to sit on a ice bath. The resulting crystals were washed as previously done.

\[ \text{[Cr(H₂O)(NH₃)₅(NO₃)₂]} \]

In a 50 mL round bottom, 3 g CrCl₃, 3 g powdered Zn and 5 mL H₂O were stirred and degassed with N₂. Conc. HCl (3 mL) was added to reduce the mixture and stirred. In a separate 100 mL flask, 15 g NH₄Cl and 30 mL NH₄OH were mixed and degassed on ice. The Cr(II) solution (blue) was transferred to the NH₃ mixture using a double-tipped needle under inert atmosphere. The mixture immediately became dark purple. While stirring, air was allowed into the mixture through a pipette and the solution gradually became red. This red solution was transferred to 100 mL conc. HCl in 250 mL H₂O and heated gently for ca. 25 min. Upon settling, red crystals formed with a green aqueous layer. The solution was filtered with a fritted filter and washed with 10 mL ice cold 6 M HCl, then 20 mL 50% ethanol and finally dried with acetone. The remaining red-pink fine crystals were \[ \text{[Cr(NH₃)₅Cl]}(\text{Cl})₂ \].
In a 50 mL flask, 2 g AgNO₃ were dissolved in 10 mL distilled H₂O. In a separate 25 mL flask, 0.5 g 99.5% NaOH in a minimum amount of H₂O was dissolved. The NaOH solution was pipetted into the AgNO₃, and immediately a brown precipitate formed, the liquid was decanted and washed 3 times of H₂O (10 mL) draining the water layer. To the moist Ag₂O, 4.5 mL H₂O and 0.5 g [Cr(NH₃)₅Cl](Cl)₂ were stirred into the solution. The solution was stirred and filtered and 3 mL more HNO₃ was added. The solution was cooled (orange) and 10 mL 95% ethanol was added while the mixture was still cold (on ice). The orange crystals were filtered and washed with 95% ethanol followed by acetone. Finally the crystals were air dried in the dark yielding ~ 0.3 g.

\[Cr(NH₃)₆(NO₃)₃\]. In a degassed (with Ar) 2-neck 250 mL flask with a stirbar, 50 mg Fe(NH₃)₂(SO₄)₂ was added. After 1 hour, 0.1 g Na (dipped in petroleum ether, cut) was added to the flask. On a dry ice/acetone bath, anhydrous NH₃ was slowly added. With the solution color still blue, but after addition of sufficient NH₃, small portions of CrCl₃ (2.5 g which was dried in a vacuum oven) were added and the solution stirred under Ar. A yellow-orange solid formed. After most of the CrCl₃ had reacted, the mixture was opened to air and the remaining NH₃ allowed to evaporate. In a 2-neck flask, added HCl (14 mL H₂O and 1 mL conc. HCl) dissolving the solid (solution slightly heated to dissolve). Filtered with fritted filter and finally precipitated [Cr(NH₃)₆(NO₃)₃ with 6 mL cold conc. HNO₃. After drying in a desiccator overnight, the solid was recrystallized.
from warm H₂O followed by cold conc. HNO₃. The green solid was stored under desiccating conditions and in the dark.

2.6 Estimation of Extinction Coefficients and Turnover Rates

Reaction velocities were determined from the initial slope of absorbance versus time plots. To estimate reaction velocities in concentration units, an extinction coefficient was required for the final product mixture. The final products of any of the enzyme reactions (ribonuclease H, exonuclease III, Klenow) are not known; the formation of various oligonucleotides is possible due to the fact these are not restriction enzymes. It is not necessary to know the mixture of product oligonucleotides since the number of bases involved is the same before and after enzyme reaction. The only quantity that changes during enzymatic reaction is the extinction coefficient of the substrate mixture. To that end, measurement of the reaction at several substrate concentrations (different amounts of DNA or hybrid) yielded the same change in absorbance. Simply, the final absorbance from the absorbance versus time trace was used to determine the extinction coefficient of the product solution and consequently initial reaction velocity (v₀). The conversion was handled by use of Equations (2-1) and (2-2), where subscript “p” refers to chromophoric products formed during the reaction, and subscript “s” refers to chromophoric reactants. Combining Equations (2-1) and (2-2) yields Equation (2-3) and, by rearrangement,
\[ A_{\text{obs}} = \varepsilon_p \cdot b \cdot c_p + \varepsilon_s \cdot b \cdot c_s \]  
\hspace{1cm} (2-1)

\[ c_{\text{tot}} = c_p + c_s \]  
\hspace{1cm} (2-2)

Equation (2-4). Equation (2-4) was used routinely to convert from initial velocities in units of absorbance to concentration units.

\[ \Delta A_{\text{obs}} = (\varepsilon_p - \varepsilon_s) \cdot b \cdot \Delta c_p \]  
\hspace{1cm} (2-3)

\[ \Delta c_p = \Delta A_{\text{obs}} / (\varepsilon_p - \varepsilon_s) \cdot b \]  
\hspace{1cm} (2-4)

2.6.1 *E. coli* Ribonuclease H

The extinction coefficient for ribonuclease H is 39,500 [Kanaya, et al., 1990]. To obtain the initial velocity in units of concentration, rather than absorbance, it proved necessary to evaluate the extinction coefficient for the mixture of reaction products, and to account for the absorbance of both reactant and product species during turnover. To ensure that the extinction coefficient reflected the products formed from ribonuclease H hydrolysis of \([A.dT]_{20}\), several time courses were run until the absorbance did not change (reaction completion). Since the number of bases did not change the increase in
absorbance reflects the only a change from reactant to product. Therefore, the change in absorbance from reactants to products reflects the change from polymer nucleotides to free oligonucleotides. The change in absorbance was identical for reactions with different amounts of hybrid, meaning substrate inhibition or the exact composition of product oligonucleotides is inconsequential. Defining concentration in bases allows the same value to be used for reactants and products. The resulting change is only due to a change in molar absorptivity and therefore the difference between reactant and product is only the extinction coefficient. Once the change in absorbance and the reactant extinction coefficient is known then the product extinction can be determined. The product is a combination of smaller oligonucleotides since ribonuclease H is nonspecific. The extinction coefficient, $\varepsilon_{260} = 6,550 \text{ M}^{-1} \text{ cm}^{-1}$, used for poly(A)$\cdot$poly(dT) was determined by annealing known amounts of poly(A) and poly(dT) (Pharmacia).

2.6.2 *E. coli* Exonuclease III

The extinction coefficient of exonuclease III at 280 nm was evaluated from a solution containing a known mass of enzyme. The latter was determined by quantitative amino acid analysis and an extinction coefficient $\varepsilon_{280} \sim 55,800 \text{ M}^{-1} \text{ cm}^{-1}$ was calculated. Poly(A)$\cdot$poly(dT) was purchased from Pharmacia and used after dissolving in the standard reaction buffer described earlier. To estimate the extinction coefficient, a known amount of poly(A) was incubated with an equimolar amount of poly(dT) at 63 °C for 0.5
h and allowed to cool for several hours to 20 °C. From the absorbance of the resulting solution, an extinction coefficient $\varepsilon_{260} \approx 6,550 \text{ M}^{-1} \text{ cm}^{-1}$ was calculated, assuming a concentration in base pair equivalents. Calf thymus DNA was purchased from Sigma Chemical Co with a reported extinction coefficient $\varepsilon_{260} \approx 6,600 \text{ M}^{-1} \text{ cm}^{-1}$.

### 2.6.3 *E. coli* DNA Polymerase I Klenow Fragment

The extinction coefficient used in all studies was 63,200 M$^{-1}$ cm$^{-1}$ for the Klenow fragment (Mr $\approx 68,000$ Da) [Mullen, et al., 1990]. The substrate was single stranded oligomers (IDT) synthesized between 16 and 22 bases long with various sequence combinations using A, T, G, and C bases (c.f. Figure 17 for structures). Using the same methodology in Section 2.6.1, $(\varepsilon_p - \varepsilon_s)$ was found to be 2396 M$^{-1}$ cm$^{-1}$. For the DNA substrate, a value of $\varepsilon_s = 8245 \text{ M}^{-1} \text{ cm}^{-1}$ was used (Pharmacia).

### 2.7 Steady-State Kinetic Studies on RNase H, Exo III and Klenow

#### 2.7.1 Development of a Kinetic Model: Measurement of $K_m$, $k_{cat}$, $K_{MET}$ and $K_i$

The metal ion dependence of enzyme activity ($v_o$) was analyzed according to the saturation model in Scheme 1. Inasmuch as the enzyme is pre-saturated with Mg$^{2+}$,
The binding of substrate (S) to metal-free enzyme (E) can be ignored. Substrate (S) can, however, bind excess \( \text{Mg}^{2+} \) which may become inhibitory at high concentrations. The inhibitory effect of metal ion binding to substrate is considered by viewing the substrate as a polyanion with multiple binding sites. The binding constant \( (K_i) \) is an apparent binding constant for \( \text{Mg}^{2+} \). This is a standard type of analysis commonly used in studies of metal ion binding to polyelectrolytes [Black, et al., 1994a; McGhee, et al., 1974; Han, et al., 1991; Record, et al., 1978]. Only the metal-enzyme complex (E-Mg) is active which we relate to the total enzyme concentration \( [E^o] \) through the mass balance Equations (2-5) and (2-6). At high metal concentration \( \text{Mg}^{2+} \) serves as a substrate.

\[
[E^o] = [E] + [E - \text{Mg}] \quad (2-5)
\]

\[
[E - \text{Mg}] = [E^o](K_{\text{MET}}[\text{Mg}^o])/(1 + K_{\text{MET}}[\text{Mg}^o]) \quad (2-6)
\]
inhibitor (Scheme 1), forming an inhibition complex \( S(Mg^{2+}) \). Note that

\( S(Mg^{2+}) \) is used to represent a substrate molecule with many bound metal ions (see \([S-Mg_n]\) in Scheme 1). Typically one considers an apparent binding affinity per \( Mg^{2+} \) ion, in this case represented by \( K_i \). Again, by mass balance and assuming uncompetitive inhibition (where \( K_I = [S][Mg]/[S - Mg] \)), Equation (2-7) is obtained. This equation can

\[
[S] = \frac{K_i[S^o]}{([Mg^o] + K_i)} \tag{2-7}
\]

also be derived from a serial expansion of the expression from Segel’s text [Segel, 1975]

\[
[S] = \frac{(([M^o] - [S^o] + K_i)^2 + 4K_i[S^o])^{1/2} - ([M^o] - [S^o] + K_i)}{2}, \text{ assuming } [M^o] > [S^o].
\]

Equations derived assuming competitive or non-competitive inhibition do not give acceptable fits to the data. Substituting Equations (2-6) and (2-7) into the standard

\[
V_0 = \frac{k_{cat}[E - Mg][S]/([S] + K_m)} \tag{2-8}
\]

\[
V_0 = \frac{(k_{cat}[E^o]K_{Mg}[Mg^o]K_i[S^o])/(1 + K_{Mg}[Mg^o])(K_i[S^o] + ([Mg^o] + K_i)K_m)} \tag{2-9}
\]

Equation (2-8) yields (2-9), which was routinely used to analyze the variation of reaction
velocity with metal concentration. Equation (2-9) properly addresses the kinetic role of
the cofactor since the requirement for binding at the active site of the enzyme, and the
inhibitory influence of binding excess metal ion to the substrate are directly accounted
for.

2.7.2 Stopped-Flow Instrumentation and Methods

Standard reaction conditions included use of Trizma buffered at pH 7.5, 50-100
mM KCl, metal cofactor, enzyme and substrate. Depending on the experiment and
enzyme, metal cofactor concentrations varied up to 80 mM and substrate concentrations
up to mM levels. A schematic is shown in Figure 18 where detection occurred linearly to
the incident light. The timescale in all of the enzymatic reactions described never
exceeded the minutes timescale and was faster than 10 seconds. This means that the full
capabilities of a stopped-flow apparatus are not used in these measurements. For
exonuclease III the stopped-flow measurements were carried out using an OLIS (On-Line
Instrument Systems, Inc.) stopped-flow apparatus. A broad band 75 watt xenon arc lamp
source (Ischio) powered by an OLIS XL150 power supply was filtered through a
monochromator (model H10 by Instruments SA) with a resolving power of 8 nm/mm. A
photomultiplier tube (Homatsu) was mounted linearly from the source to detect the
change in absorbance of the hybrid substrate at 260 nm. The cell pathlength is 20 mm.
Experiments described in Chapters 4 and 5 used an OLIS RSM (rotating scanning
monochromator) stopped-flow setup which was functionally equivalent to the one above in all but two respects. First, the source was a water-cooled broad band 150 watt xenon arc lamp (Ischio) and second, the monochromator was a scanning disk designed by OLIS to acquire 1000 spectral scans per second.

The observed signal in all stopped-flow experiments is the change in the absorbance of DNA or hybrid during cleavage arising from the change in hypochroism. Hypochroism results from the arrangement of dipoles in chromophoric-polymeric type molecules such as nucleic acids. Using a more classical analogy (versus quantum mechanical) the effect can be explained by summing the dipoles in an electric field. In the presence of an electric field polarizable molecules maintain a nonzero transition dipole moment such that the induced dipole moment can be described as in equation (2-10):

\[ \mu_{\text{ind}} = \alpha(\nu) \times \mathbf{E}(\nu) \]  

(2-10)

where \( \mu_{\text{ind}} \) is the light induced dipole, \( \alpha(\nu) \) is the electric polarizability tensor of frequency \( \nu \), and \( \mathbf{E} \) is the electric field vector of the radiation. The induced dipoles do not dissipate this absorbed energy, rather they re-radiate. To illustrate with the quantum mechanical analog, the electric polarizability is a function of the transition dipole moments \( \langle \Psi_0 | \mu | \Psi_i \rangle \) of all the excited states. The light induced dipole moment of the
chromophore can interact with the transition dipole moments of the neighboring chromophores. The nature of this interaction gives rise to hypochromicity. The dipole (and thus intensity) of the chromophore can either be enhanced or inhibited by the neighboring chromophores; this effect specifically relates to geometry. If the dipoles in a molecule are stacked on top of each other, then all of the dipoles are mutually repelling in terms of the electric field (Figure 16A). If the chromophores have dipoles which are aligned one in front of another, then they are mutually attracting (Figure 16B). In the latter case, exciting a single chromophore with light causes the induced dipole to be “attracted” to the surrounding dipoles, or hyperchroism; the opposite results in hypochroism. Nucleic acids, which undergo hypochroism due to the stacking of chromophoric bases, can yield as much as a 30% reduction in the transition dipole for a single base (Table 5). This effect is used to take advantage in the analysis of DNA cleavage. When the nucleotide bases are released into solution, specifically their dipoles only interact with the surrounding water molecules, and so during a nucleolytic reaction, the absorbance at 260 nm (the characteristic absorbance maxima) increases as a function of enzymatic turnover due to the breakdown of the polymer. The sensitivity of this methodology obviates the inaccuracies common to the use of radiolabeled substrates to measure reaction rates [Dahlberg, et al., 1991].

The use of chromium probes under the conditions of the stopped-flow experiment yields a further potential source of experimental difficulty. Specifically, since light is
used in the detection of hypochromicity there remains the possibility of photolabilization of cobalt and chromium complexes. However, under the conditions described above there is no evidence for significant photochemistry. No change is observed in the absorbance in the absence of enzyme, while absorbance spectra of solutions of the metal complexes taken before and after going through the stopped-flow are effectively the same. This is consistent with the power output of the lamp, which directs approximately 4 \times 10^{-10} \text{ moles photons/sec} through the stopped flow cell, using an area estimate for the stopped flow and assuming 180\degree incident light. The quantum yield for the photoaquation of monoammine to hexaaquo product at 366 nm is 10^{-4} \text{ mol per einstein}. We would therefore expect only \sim 2 \times 10^{-14} \text{ mol of complex} to be converted in 5 seconds, which is insignificant.
Figure 16. (A) Schematic of dipole moments stacked as bases in a polynucleotide. Incident radiation excites one dipole (in bold) where the induced dipole is lessened due to interaction with nearby bases. (B) Schematic representation of dipoles oriented end-to-end. Incident light is absorbed by one dipole (bold) where the induced dipole is augmented due to interaction with nearby dipoles. The dipoles refer to the polarizability of each of the nucleic acid base units in a polymeric environment (see also Figure 17).
Figure 17. Structural units of nucleic acids. The four most common purine and pyrimidine bases (B) are shown, Adenine (A), Guanine (G), Cytosine (C), and Thymine (T). Also a ribonucleotide unit (R) is shown where B stands for a glycosidic linkage to a base unit. The combination of a base and ribonucleotide yield the basic unit for RNA. Removal of a 2'-OH from the ribonucleotide and addition of a base gives the unit for DNA.
Table 5. Absorbance maxima, fluorescence and extinction coefficients of nucleic acid bases. All measurements are made in aqueous solutions at pH 7 [Cantor, et al., 1980]

<table>
<thead>
<tr>
<th>Bases</th>
<th>$\lambda_{\text{abs}}_{\text{max}}$ (nm)</th>
<th>$\varepsilon_{\text{max}}$ (M$^{-1}$ cm$^{-1}$)</th>
<th>$\lambda_{\text{em}}_{\text{max}}$ (nm)</th>
<th>$\phi_F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>260</td>
<td>13,400</td>
<td>321</td>
<td>2.6x10$^{-4}$</td>
</tr>
<tr>
<td>Guanine</td>
<td>275</td>
<td>8,100</td>
<td>329</td>
<td>3.0x10$^{-4}$</td>
</tr>
<tr>
<td>Cytosine</td>
<td>267</td>
<td>6,100</td>
<td>313</td>
<td>0.8x10$^{-4}$</td>
</tr>
<tr>
<td>Uracil</td>
<td>260</td>
<td>9,500</td>
<td>308</td>
<td>0.4x10$^{-4}$</td>
</tr>
</tbody>
</table>
**Table 6.** Absorbance maxima and extinction coefficients of *E. coli* ribonuclease H, *E. coli* exonuclease III and *E. coli* DNA polymerase I Klenow fragment. All measurements are made in 20 mM Tris, pH 7.5, 50 mM KCl and 0.1 mM DTT.

<table>
<thead>
<tr>
<th></th>
<th>$\lambda_{\text{abs}}\text{max}$</th>
<th>$\varepsilon_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ribonuclease H</td>
<td>280</td>
<td>39,500</td>
</tr>
<tr>
<td>exonuclease III</td>
<td>280</td>
<td>55,800</td>
</tr>
<tr>
<td>DNA polymerase I Klenow</td>
<td>280</td>
<td>63,200</td>
</tr>
<tr>
<td>$(A,dT)_{20}$</td>
<td>260</td>
<td>6800</td>
</tr>
<tr>
<td>poly(A):poly(dT)</td>
<td>260</td>
<td>6,550</td>
</tr>
</tbody>
</table>
**Figure 18.** Schematic Diagram of the Stopped-Flow Apparatus. Enzyme with metal ion in one syringe with substrate and metal ion in the other syringe (reproduced from Burgess, 1988).
2.7.3 Titration Calorimetric Methods

A titration calorimeter allows measurement of the heat change resulting from addition of a controlled amount of ligand. Titration calorimetry is the only technique which allows definition of $\Delta G^\circ$, $\Delta H^\circ$, $\Delta S^\circ$, $K$ and $n$ in a single binding experiment. This instrument, different from a scanning differential calorimeter, is ideal in the thermodynamic characterization of binding events. Addition of a ligand or metal ion to the enzyme solution will generate heat based on occupancy. The heat of binding is directly related to concentration (or occupancy) and thus it is desirable to maintain the highest concentration to generate the largest measurable signal. Defining the binding constant (Equation 2-11) and concentration of ligand (Equation 2-12) the heat content after any injection can be found (Equation 2-13):

$$K = \frac{\Theta}{(1 - \Theta)[X]} \quad (2-11)$$

$$X_t = [X] + n\Theta M_t \quad (2-12)$$

where $K$ is the binding constant, $X_t$ is the total amount of ligand $X$, $[X]$ is the free concentration of ligand $X$, $\Theta$ is the fraction of sites occupied by ligand $X$, $n$ is the number of sites, and $M_t$ is the free concentration of macromolecule (enzyme). Combining (2-11)
and (2-12), where the total heat refers to unligated macromolecule at fractional saturation, equation (2-13) is obtained.

\[ Q = M^c V_o n \Theta \Delta H \]  

(2-13)

In Equation (2-13), \( \Delta H \) is the molar heat of ligand binding and \( V_o \) is the active cell volume. One drawback in working with enzymes however is the viscosity and possibility of precipitation which occurs at high concentrations. Given these problems (low signal, concentration) measurements were made on an Omega titration calorimeter (Figure 19). The Omega calorimeter employs a thermoelectric device between the reference cell and the sample cell. The use of a such a device allows sensitive adjustment in temperature between the reaction cell (where binding occurs) and the reference cell (background buffer). This is the principal way sensitive measurements can be obtained with small concentrations of enzyme. When heat is generated (or absorbed) from a binding event the system produces a voltage proportional to that difference such that \( \Delta T \) goes to zero. The time integral of the power deflection (raw data) is a measure of the heat generated. In an attempt to increase sensitivity, especially with low concentrations, the temperature of the adiabatic shield is regulated in the same manner. Another temperature difference is measured between the shield and the cells, which is also allowed to tend toward zero.
A typical experiment involves using 5-10 mM metal ion (Mg$^{2+}$, Mn$^{2+}$) and ca. 0.2-0.3 mM enzyme (Klenow). The metal ion solution is injected into the reaction cell with continuous stirring at 150 rpm. Under isothermal conditions, the resultant heat is monitored over the time of the injection. Initially large heats are generated due to excess enzyme metal ion binding sites, until saturation of all binding sites occurs. At this point further injections only result in background or dilution effects where a small amount of heat is generated (< 0.1 kcal) and can be subtracted using the control (no enzyme).

2.7.4 Steady-State Kinetic Measurements on the Klenow Fragment

The hypochromic effect for the 3'-5' exonucleaseolytic reaction of the Klenow fragment on oligonucleotide ssDNA was monitored using a Hewlett-Packard 8425A spectrophotometer (run by software from On-Line Instrument Systems) using a 1 cm path length quartz cuvette. A control was taken against background buffer at 260 nm, and a Fisher circulating temperature bath maintained the temperature at 37°C. The sample was incubated for 5-10 minutes without the presence of Klenow (reaction buffer, ssDNA and metal ion). After addition of Klenow (1 μM) the instrument collected data at 260 nm for 10-20 minutes. The initial velocity was calculated (5-10% of total reaction) and the resulting plots were fitted using Equation 2-9 described above.
Figure 19. Schematic drawing of the Omega titration calorimeter (reproduced from the Omega titration calorimeter manual).
CHAPTER III

ACTIVATION OF RIBONUCLEASE H. EVIDENCE FOR ONE CATALYTIC METAL ION

3.1 Introduction

*Escherichia coli* ribonuclease H is a crystallographically characterized low molecular weight endonuclease (M_r ~ 17,580) that hydrolytically cleaves the ribonucleotide backbone of RNA:DNA hybrids, producing 5'-phosphate and 3'-hydroxyl oligonucleotides [Yang, et al., 1990b; Katayanagi, et al., 1990; Davies, et al., 1991; Kanaya, et al., 1989; Nakamura, et al., 1991; Jou, et al., 1991]. Ribonuclease H activity has been implicated in bacterial plasmid replication and forms an essential catalytic domain on retroviral reverse transcriptase [Yang, et al., 1990b; Katayanagi, et al., 1990; Davies, et al., 1991]. The *E. coli* enzyme is structurally homologous to the RNase H domain of HIV reverse transcriptase and shows retention of key active site residues (Figure 20) [Davies, et al., 1991; Kanaya, et al., 1989; Nakamura, et al., 1991].
Figure 20. Ribbon diagram of *E. coli* ribonuclease H showing the active site residues and the position of the magnesium ion [Kanaya, et al., 1990].
Metal ions are essential for the activity of a multitude of enzymes and ribozymes in nucleic acid biochemistry, [Black, et al., 1994a; Cowan, 1992; Steitz, et al., 1993; Piccirilli, et al., 1993; Perreault, et al., 1991] however, a molecular understanding of their functional role is lacking. Both the molecular mechanism of ribonuclease H activation, and the stoichiometric requirement for Mg$^{2+}$ during turnover are uncertain. A recent structural analysis of the Mn$^{2+}$- doped crystals of the RNase H domain of HIV-reverse transcriptase revealed two bound Mn$^{2+}$ ions in close proximity to the catalytic site [Davies, et al., 1991]. On this basis it was proposed that the enzyme most likely acts in a manner analogous to the exonuclease domain of DNA polymerase I [Steitz, et al., 1993; Beese, et al., 1991]. The involvement of one ion during catalytic turnover has also been proposed [Yang, et al., 1990b; Jou, et al., 1991]; however, in neither case has evidence been advanced to support metal stoichiometry during active turnover.

In this chapter experiments evaluate the stoichiometric requirement for Mg$^{2+}$ and Co(NH$_3$)$_6$$^{3+}$ during turnover and distinguish between these two possible reaction pathways. In addition, the use of the change in absorbance of nucleotide substrate to monitor rates of reaction is used. Kinetic equations are derived that rationally account for experimental rate data. Comparison of thermodynamic data and kinetic data obtained with ribonuclease H suggests that there is one essential catalytic cofactor, and that activation does not proceed by a two-metal-ion mechanism.
3.2 Results

Divalent magnesium ion is an essential cofactor for many enzymes in nucleic acid biochemistry. Although putative models for catalytic mechanisms have been published [Davies, et al., 1991; Kanaya, et al., 1989; Nakamura, et al., 1991], the specific details of metal activation have not been addressed. Contrary to previous speculation [Davies, et al., 1991; Steitz, et al., 1993], herein we provide experimental evidence that RNase H activity is promoted by a single metal cofactor rather than a binuclear metal site. This result is in accord with a recent crystallographic analysis of the magnesium-bound enzyme [Katayanagi, et al., 1993].

3.2.1 Initial Velocity Dependence on Metal Ion

Using Equation (2-9), characterization of the metal ion dependence of ribonuclease H activity by stopped-flow kinetic methods was performed. Using photomultiplier detection to monitor the hypochromic effect described in 2.7.2, time traces were generated by degradation of an (A·dT)_{20} hybrid substrate (Figure 21). One syringe contained RNase H while the other contained saturating amounts of substrate. Each of the two syringes were loaded with the same concentration of varying metal cofactor (0.5-80 mM) in order to minimize the effects of secondary binding chemistry. In addition, ionic strength was kept constant between both syringes. In the absence of enzyme, no
Figure 21. Stopped-flow trace showing the change in absorbance of the digested RNA-DNA hybrid. Reaction conditions are [Trizma] = 50 mM, pH 7.5, [K\(^+\)] = 100 mM, [Mg\(^{2+}\)] = 10 mM, [RNase H] = 90 nM, [(A·dT\(_{20}\)] = 0.35 mM phosphate equivalents. The cell path length is 20 mm.
Figure 22. Variation of initial velocity ($v_0$) with hybrid concentration ($[\text{Mg}^{2+}] = 10 \text{ mM}, [\text{Na}^+] = 100 \text{ mM}$). The fit (dotted line) was obtained using equation (2-9), giving a $\chi^2$ error of 0.012.
Figure 23. Variation of initial velocity (v₀) with magnesium concentration. In the upper curve [Na⁺] = 0 mM, while in the lower [Na⁺] = 100 mM. The optimal turnover rates obtained for each of these curves are actually similar [kcat(opt) in Table 7]. The fits (dotted lines) were obtained using equation (2-9), giving a $\chi^2$ error of 0.008.
Table 7. Kinetic Parameters from Ribonuclease H Digestion of (A-dT)$_{20}$ Substrate$^a$

<table>
<thead>
<tr>
<th>Metal Cofactor</th>
<th>$K_m$</th>
<th>$10^{-3} k_{cat}^b$</th>
<th>$10^{-3} k_{cat (opt)}^c$</th>
<th>$10^{-3} k_{cat}/K_m$</th>
<th>$K_{MET}$</th>
<th>$K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mM)</td>
<td>(s$^{-1}$)</td>
<td>(s$^{-1}$)</td>
<td>(mM$^{-1}$ s$^{-1}$)</td>
<td>(mM)</td>
<td>(mM)</td>
</tr>
<tr>
<td>Mg$^{2+}$ (0 mM Na$^+$)</td>
<td>0.8(±0.2)</td>
<td>5(±1)</td>
<td>6(±1)</td>
<td>7</td>
<td>0.5(±0.2)</td>
<td>14(±3)</td>
</tr>
<tr>
<td>Mg$^{2+}$ (10 mM Na$^+$)</td>
<td>1.0(±0.2)</td>
<td>4(±1)</td>
<td>9(±1)</td>
<td>4</td>
<td>9(±2)</td>
<td>6(±3)</td>
</tr>
<tr>
<td>Mg$^{2+}$ (100 mM Na$^+$)</td>
<td>0.4(±0.1)</td>
<td>2(±0.4)</td>
<td>4(±1)</td>
<td>5</td>
<td>31(±5)</td>
<td>5(±3)</td>
</tr>
<tr>
<td>Mg$^{2+}$ (100 mM K$^+$)</td>
<td>3.8(±1.1)</td>
<td>1(±0.3)</td>
<td>5(±1)</td>
<td>0.4</td>
<td>21(±5)</td>
<td>17(±3)</td>
</tr>
<tr>
<td>Co(NH$_3$)$_6^{3+}$</td>
<td>0.8(±0.2)</td>
<td>0.02(±0.006)</td>
<td>1(±0.3)</td>
<td>0.03</td>
<td>4(±1)</td>
<td>2(±0.6)</td>
</tr>
</tbody>
</table>
a Standard reaction conditions: 50 mM Trizma, pH 7.5, [Mg$^{2+}$] = 10 mM, [RNase H] = 170 nM ($\varepsilon = 3.9 \times 10^4$ M$^{-1}$ cm$^{-1}$) ([b] Kanaya, et al., 1990] and 37°C. For metal titrations (to determine $K_{MET}$ and $K_i$, as in Figure 23), [(A·dT)$_{20}$] = 0.8 mM phosphate equivalents and [Mg$^{2+}$] varied up to 80 mM. Unit conversion for initial velocity from absorbance units to ΔmM/s was carried out using an extinction coefficient $\varepsilon = 6800$ M$^{-1}$ cm$^{-1}$ with 40 phosphate equivalents per substrate molecule.

b $k_{cat}$ values reflect the production of product in units of phosphate equivalents.

c The value of $k_{cat(\text{opt})}$, determined from a fit to equation (2-9), reflects maximal rates obtained in the presence of optimal concentrations of Mg$^{2+}$ rather than the fixed cofactor concentrations noted in footnote a.

d Value in parentheses is the affinity for thermodynamic binding of Mg$^{2+}$ to the hybrid [Black, et al., 1994b].
change in absorbance was observed. Reactants were preequilibrated at 37°C prior to mixing. At least four data sets were averaged for each \( v_0 \). Figure 22 illustrates the substrate dependence of RNase H which yields the standard Michaelis-Menten kinetic parameters, \( K_m \) and \( k_{cat} \). Figure 23 represents the metal ion dependence of RNase H with the fitting results for both types of plots tabulated in Table 7.

Table 7 lists steady-state parameters, metal binding (\( K_{MET} \)) and inhibition constants (\( K_i \)) determined from these studies. Inasmuch as the magnesium binding constant suggests lower affinity in solutions of higher salt concentration, the data demonstrates that monovalent ions (\( Na^+ \) and \( K^+ \)) compete for the enzyme metal-binding site and result in at most a 5-fold decrease in \( k_{cat} \) at fixed concentrations of activating metal cofactor. Maximal rates [\( k_{cat}(opt) \) noted in Table 7], obtained by varying the concentration of metal cofactor as defined by equation (2-9) and fitting as in Figure 23, are found to be relatively similar and independent of \( K^+ \) or \( Na^+ \) levels. That is, the monovalent ions simply compete for the carboxylate ligands in the active site. Otherwise these ions have only a moderate influence on substrate binding and activation (Table 7). The effect of cofactor charge is illustrated by analysis of the \( v_0 \) dependence on \( Co(NH_3)_6^{3+} \) as tabulated in Table 7. Intrinsic binding \( Co(NH_3)_6^{3+} \) to the enzyme is similar to the \( Mg^{2+} \) case (\( K_{MET} \)), while a substantial drop in \( k_{cat}(opt) \) occurs. In addition, the sharper inhibition dependence exhibited by \( Co(NH_3)_6^{3+} \) reflects the increased net charge on the metal complex.
Figure 24. Arrhenius plots: (Δ) denotes the Mg$^{2+}$ activated reaction while (■) for Co(NH$_3$)$_6^{3+}$ activation. Mg$^{2+}$-RNH: [RNH] = 80 nM, [(A·dT)$_{20}$] = 20 μM, [Mg$^{2+}$] = 10 mM. Co(NH$_3$)$_6^{3+}$-RNH: [RNH] = 170 nM, [(A·dT)$_{20}$] = 50 μM, [Mg$^{2+}$] = 10 mM. In each case the reaction buffer contained 50 mM Trizma, 100 mM KCl, pH 7.5.
3.2.2 Initial Velocity Dependence on Temperature

To further characterize the differences between Mg$^{2+}$ and Co(NH$_3$)$_6$$^{3+}$ activation of ribonuclease H, the temperature dependence was carried out. The fits were obtained using the Arrhenius equation relating the rate to temperature (Equation 3-1). A plot of $k_{\text{cat}}$ against the inverse temperature gives the activation energy for both metal cofactors. The resulting plots are shown in Figure 24 and is evident that a slight difference in $k_{\text{cat}}$ exists between the two cofactors. However, the slopes are similar [$E_a$(Mg$^{2+}$) ~ 20 kcal mole$^{-1}$ and $E_a$(Co(NH$_3$)$_6$$^{3+}$) ~ 16 kcal mole$^{-1}$] suggesting a similar mode of action. This view is supported by Table 7.

$$k_{\text{cat}} = A \cdot \exp[-E_a/RT] \quad (3-1)$$

3.2.3 Competitive Inhibition Using Poly(A)

In an effort to identify what effect non-active substrate nucleic acids had on the activity of ribonuclease H, an inhibition study was undertaken. Poly(A) was chosen due to its structural rigidity and also due to its A-conformation in solution which is thought to be similar to hybrid. The activity was monitored using a 20-mer hybrid substrate while different concentrations of poly(A) were added (Figure 25). Competitive inhibition (Scheme 2) was observed where the $K_m$ value did not change but the initial velocity did decrease. Fitting the data to an equation
Figure 25. Inhibition of poly(A) in ribonuclease H hydrolysis of (A\cdotdT)_{20}. [RNH] = 270 nM, [(A\cdotdT)_{20}] = 30 \mu M bases, [Mg^{2+}] = 10 mM, reaction buffer, 37°C.
Figure 26. Illustration of poly(A) inhibition of ribonuclease H hydrolysis of (A·dT)$_{20}$.

The top trace has no poly(A) added while the bottom plot has 44 μM poly(A) present.

[RNH] = 270 nM, [Mg$^{2+}$] = 10 mM, reaction buffer, 37°C.
describing competitive inhibition (Equation 3-2) yielded an inhibition constant \(K_i\) for poly(A) of 0.4 mM. Upon inspection of Table 7, it is also apparent that this inhibition constant correlates with the observed \(K_m\) for \((A\cdot dT)_{20}\) and is indicative of similar binding modes for the hybrid and poly(A).

\[
\begin{align*}
E\cdot Mg + S &\rightleftharpoons E\cdot Mg\cdot S \rightarrow E\cdot Mg + P \\
\uparrow I \\
E\cdot Mg\cdot I 
\end{align*}
\

Scheme 2

\[
V_o = \frac{([E^0][S]k_{cat})}{([S] + K_m(1 + [I]/K_i))}
\]

(3-2)

The notation used in equation (3-2) is the same as that used in equations (2-6) - (2-9) except that \([I]\) and \(K_i\) refer to \([\text{poly}(A)]\) and the poly(A) inhibition constant for binding to RNase H.
3.3 Discussion

Previously it has been demonstrated that one tight binding site exists on RNase H for Mg$^{2+}$ [Huang, et al., 1994]. Inasmuch as data on the isolated enzyme alone does not directly indicate the number of critical metal ions required for catalytic turnover we chose to address this issue by kinetic methods. The optimal magnesium concentration for kinetic activation [$K_{\text{met}} \sim 0.5 \text{ mM}$] is similar to the thermodynamic binding affinity of the free enzyme [$K_{\text{met}} \sim 0.1 \text{ mM}$]. [Huang, et al., 1994] This provides good evidence for catalytic activation by the same metal ion that binds to the enzyme alone. Association constants for additional metals that form binuclear metal sites to effect enzymatic turnover in the enzyme-substrate complex are typically small [Beese, et al., 1991; Han, et al., 1991; Record, et al., 1978] and would be reflected by a larger value for the binding constant ($K_{\text{met}}$) that is estimated from the kinetic measurements. Furthermore, since RNase H lacks significant secondary binding sites for metal ions, [Huang, et al., 1994] the inhibitory effect of increasing [Mg$^{2+}$] most likely arises from binding to the negatively-charged polynucleotide. Michaelis-Menten $k_{\text{cat}}$ values are very different between the two cofactors possibly indicating alternate mechanisms; however, examination of $k_{\text{cat}}$(opt) values show similarity and thus further emphasize these cofactors bind to the same pocket and are acting in a similar manner [Huang, et al., 1994; Jou, et al., 1991]. At sufficiently high concentrations of metal cofactor the excess charge can impair binding to the relatively nonpolar enzyme surface that surrounds the catalytic site.
[Yang, et al., 1990b; Katayanagi, et al., 1990; Davies, et al., 1991] The inhibition constants ($K_i$) listed in Table 7 ($K_i \sim 4.4-18$ mM) correlate well with previous estimates of metal ion binding constants to an A-conformer nucleotide ($K_d \sim 4.5$ mM) [Black, et al., 1994b; Cowan, 1991]. These values are consistent with results obtained from fits of data for metal binding to nucleic acids using a simple binding model such as that assumed in Scheme 1. When analyzed in terms of the more elaborate McGhee-von Hippel model, the absolute binding constants change [Black, et al., 1994b], but this level of analysis is inappropriate for simple comparison.

Use of the substitutionally inert complex Co(NH$_3$)$_6^{3+}$ has already demonstrated an outer sphere pathway for the metal cofactor in ribonuclease H [Jou, et al., 1991]. This means the cofactor interacts through its inner sphere ligands, bound H$_2$O, forming contacts with the substrate; however, the the substitution of Co(NH$_3$)$_6^{3+}$ does not distinguish between metal-assisted organization of active site residues or interaction with substrate via stabilization of the transition state. The data in Table 7 acts to further distinguish these two possibilities. If interaction with the enzyme pocket residues is a dominant pathway in hydrolytic activation, increasing the charge on the cofactor should increase binding ($K_{MET}$). This is not the case; in fact the term describing substrate interaction ($K_i$) is the only term changing significantly along with $k_{cat}$. This indicates that the dominant interaction of the metal cofactor in activating ribonuclease H toward
hydrolysis is its interaction with the substrate. This is further characterized by the use of inert chromium probes (Chapter 4).

It is expected that inhibition by nucleic acids takes place through a competitive inhibitive mechanism; where the polymer binds at the same active site where hybrid is hydrolyzed. To test this, poly(A) was used in conjunction with hybrid substrate. Figure 26 clearly shows different k_{cat} values with different concentrations of poly(A), while K_m values stay the same; it appears that poly(A) inhibits competitively in the manner of Scheme 2. Based on this evidence it is relevant to point out that the 2'-OH does not appear to be an essential requirement for ribonuclease H activity (both the hybrid and poly(A) contain 2'-OH). Rather, it indicates that certain structural features are important for hybrid catalysis, possibly hybrid conformational flexibility in solution.

In reference to fitting results, it should be noted that a priori our data can also be fit by assuming any number of equivalent tight binding sites on the enzyme. There is the intrinsic assumption that a second metal ion will bind with lower affinity to the active site than the existing ion. This is rather easy to justify from literature precedent. First, a binding constant on the order of 10^{-4} M is the upper limit thus far observed for Mg^{2+} binding to proteins and enzymes. Second, if we assume a model where two or equivalent high affinity sites are required to be populated, then the apparent binding constant (K_{MET}) determined from the fit to our data actually falls (binding is tighter) and approaches unreasonable levels (K_{MET} \leq 10^{-5} M for two sites). Literature precedent demonstrates that
for contiguous metal ions (particularly alkaline earths), binding of the second metal results in a marked lowering of the observed binding affinity, presumably as a result of electrostatic interactions. For example, the Mg$^{2+}$ site on DNA polymerase I is weak [Mullen, et al., 1990], while recent studies of calcium channels demonstrate that while one Ca$^{2+}$ ion binds at μM levels, a second contiguous ion results in a lowering of the affinity of both to mM levels [Yang, et al., 1993]. Submillimolar binding constants are also incompatible with binding to double-strand nucleic acids, under these solution conditions [Black, et al., 1994b; Han, et al., 1991; Record, et al., 1978]. The consistency of this body of kinetic and thermodynamic data lends support to one catalytically relevant metal cofactor during turnover. This is also in accord with crystallographic analyses of RNase H. [Katayanagi, et al., 1993], and other proteins and enzymes that show patterns of carboxylate residues at the metal binding site that are similar to those of RNase H [Black, et al., 1994a; Huang, et al., 1994]. The two bound metal ions crystallographically identified in the RNase H domain of Mn$^{2+}$-doped HIV-reverse transcriptase [Davies, et al., 1991] may reflect either the distinct coordination chemistry of Mn$^{2+}$ versus Mg$^{2+}$, or the constraints of the doping procedure as a vehicle for introducing metal cofactors to enzyme active sites.
CHAPTER IV

MECHANISM OF METAL-PROMOTED CATALYSIS OF NUCLEIC ACID HYDROLYSIS BY RIBONUCLEASE H. USE OF INERT CHROMIUM COMPLEXES TO EVALUATE HYDROGEN BONDING AND ELECTROSTATIC STABILIZATION OF THE TRANSITION STATE

4.1 Introduction

In the previous chapter, the focus remained on the stoichiometry of the metal cofactor and the question of an inner sphere or outer sphere mechanism. The questions were answered by the use of Co(NH$_3$)$_6^{3+}$ as a probe of outer sphere chemistry and quantitating the kinetic behavior of ribonuclease H. Although these were fundamental questions concerning RNase H activation, the previous studies do not address the role of the cofactor during turnover. The focus of this Chapter is to elucidate the mechanistic role of the magnesium cofactor.
Following the precedent of Cleland and coworkers, [Rawlings, et al., 1993a; Rawlings, et al., 1993b; Speckhard, et al., 1991; Haromy, et al., 1990; Smith, et al., 1981] we have developed a strategy for studying the metalllobiochemistry of metal-dependent nucleases by use of transition metal complexes of defined coordination chemistry [Jou, et al., 1991; Kim, et al., 1992; Black, et al., 1994a]. Previously our lab has found that the substitutionally inert complex, Co(NH$_3$)$_6^{3+}$, promotes significant levels of activity, suggesting an outer sphere pathway [Jou, et al., 1991]. This evidence alone does not, however, exclude the possibility of a structural role for the cofactor involving reorientation of active site residues in a manner similar to the structural perturbations identified in RNase H mutants [Katayanagi, et al., 1993a; Katayanagi, et al., 1992]. Also, this experiment does not address the critical issue of the relative contribution of charge (electrostatics) and hydration sphere (hydrogen bonding) to catalytic activation. In this chapter we quantitatively demonstrate that the essential metal ion catalyzes hydrolysis by transition state stabilization through outer-sphere complex formation with a metal cofactor, and that this stabilization, in the case of the natural alkaline earth cofactor, results predominantly from hydrogen bonding contacts to waters of solvation. These experiments appear to exclude a structural role for the metal cofactor.
4.1.1 Consideration of the Inert Chromium Complexes as Probes

The chromium probes used as metal cofactors in activation of RNase H are shown in Figure 27 (the Cr(NH$_3$)$_6$$^{3+}$ and Co(NH$_3$)$_6$$^{3+}$ are not shown for brevity). Chromium complexes carrying a water ligand show an ionization ($pK_{a1}$) between pH 4 and 5 [Basolo, et al., 1967]. Under the experimental pH conditions (pH ~ 7.5) the [Cr(NH$_3$)$_6$-x(H$_2$O)$_x$]$^{3+}$ complexes will exist predominantly in an ionized form, bearing an hydroxide ligand and yielding a net (+2) charge [Basolo, et al., 1967]. That is, the solution state of such complexes will correspond to [Cr(NH$_3$)$_6$-x(H$_2$O)$_x$-1(HO$^-$)]$^{2+}$, as represented in Table 8, and the apparent charge is therefore similar to divalent magnesium. A second ionization ($pK_{a2}$) can occur between pH 6 and 7 for Cr(H$_2$O)$_6$$^{3+}$, however, even at the pH ~ 7.5 used in our experiments, there is a sufficient concentration of the singly ionized species present in solution to saturate the binding site. In fact at pH ~ 6.5 the rate increases less than two-fold. For other complexes that carry ammine ligands, the $pK_{a2}$ is significantly larger [Basolo, et al., 1967] and need not be considered here.

4.1.2 Evaluation of Catalytic Parameter

Michaelis-Menten parameters were characterized in the normal fashion by monitoring the variation in initial velocity (Figure 22) with substrate concentration. For systematic studies, the catalytic rate constant $k_{cat}$ was obtained from the initial reaction
Figure 27. Representation of the chromium probes. Note that each maintains an ionized water at the pH of the experiment.

Also, the Cr(NH₃)₆³⁻ and Co(NH₃)₆³⁺ are not shown for simplicity.
velocity under conditions of saturating substrate (30 μM). Michaelis K_m constants (Table 8) were evaluated from standard fits to plots of initial velocity versus substrate concentration (Figures 28-35). Parameters v_max and k_cat were determined in the usual fashion [Palmer, 1985]. Kinetic parameters determined by these procedures are generally comparable to results obtained from alternative assays that employ radiolabeled substrates (allowing for variation in the definition of substrate concentration). Specific reaction conditions are summarized in the legend to Table 8.

We assume that the limiting rates measured herein are defined by strand scission, rather than melting of the annealed product oligonucleotides. Our reasons for this include: (1) The pattern of reactivity observed for modified substrates and the reactivity of mutant enzymes is consistent with strand cleavage as rate limiting [Nakamura, et al., 1991; Kanaya, et al., 1989; Hogrefe, et al., 1990; Kanaya, et al., 1995]. (2) The pattern of reactivity for the various metal complexes used in our studies is not consistent with monitoring a melting rate. (3) We find that the measured rates using either (A·dT)_20 or poly(A)-poly(dT) substrates are comparable within the range of error [Black, et al., 1994b], a fact that also supports a more processive mode of action for RNase H.
Figure 28. Eadie-Hofstee plot for RNase H and Mg\textsuperscript{2+} (K\textsubscript{m} = 9.6 \mu M). [poly(A)-poly(dT)] = 30 \mu M base-pairs, [Mg\textsuperscript{2+}] = 10 mM, [RNase H] = 540 nM, and 25 \pm 1 \degree C.
Figure 29. Eadie-Hofstee plot for RNase H and Cr(H₂O)₆³⁺ (Kₘ = 8.2 μM). Conditions:
[poly(A)•poly(dT)] = 30 μM base-pairs, [Cr(H₂O)₆³⁺] = 10 mM, [RNase H] = 540 nM,
and 25 ± 1 °C.
Figure 30. Eadie-Hofstee plot for RNase H and Cr(H₂O)₅(NH₃)³⁺ (Kₘ = 14.2 μM).

Conditions: [poly(A)-poly(dT)] = 30 μM base-pairs, [Cr(H₂O)₅(NH₃)³⁺] = 10 mM,
[RNase H] = 540 nM, and 25 ± 1 °C.
Figure 31. Eadie-Hofstee plot for RNase H and cis-Cr(H$_2$O)$_4$(NH$_3$)$_2$$^{3+}$ ($K_m = 6.92$ $\mu$M).

Conditions: [poly(A)·poly(dT)] = 30 $\mu$M base-pairs, [cis-Cr(H$_2$O)$_4$(NH$_3$)$_2$$^{3+}$] = 10 mM, [RNase H] = 540 nM, and 25 ± 1 $^\circ$C.
Figure 32. Eadie-Hofstee plot for RNase H and fac-Cr(H$_2$O)$_3$(NH$_3$)$_3$$^3^+$ ($K_m = 6.92 \mu M$).

Conditions: [poly(A)poly(dT )] = 30 μM base-pairs, [fac-Cr(H$_2$O)$_3$(NH$_3$)$_3$$^3^+$] = 10 mM, [RNase H] = 540 nM, and 25 ± 1 °C.
Figure 33. Eadie-Hofstee plot for RNase H and Cr(NH$_3$)$_6^{3+}$ ($K_m = 14.6 \mu$M). Conditions:

[poly(A)-poly(dT)] = 30 \mu M base-pairs, [Cr(NH$_3$)$_6^{3+}$] = 10 mM, [RNase H] = 540 nM,
and 25 ± 1 °C.
Figure 34. Eadie-Hofstee plot for RNase H and Co(NH$_3$)$_6^{3+}$ ($K_m = 10.6$ $\mu$M).

Conditions: [poly(A)-poly(dT)] = 30 $\mu$M base-pairs, [Co(NH$_3$)$_6^{3+}$] = 10 mM, [RNase H] = 722 nM, and 25 ± 1 °C.
Figure 35. Eadie-Hofstee plot for RNase H and trans-Cr(H$_2$O)$_4$(NH$_3$)$_2^{3+}$ ($K_m$ = 22.6 μM).

Conditions: [poly(A)-poly(dT)] = 30 μM base-pairs, [trans-Cr(H$_2$O)$_4$(NH$_3$)$_2^{3+}$] = 10 mM, [RNase H] = 540 nM, and 25 ± 1 °C.
4.2 Results and Discussion

Throughout the body of this work, we have developed a program of experiments to evaluate the molecular mechanisms employed by magnesium-dependent nucleases. Such reactions are of fundamental importance in nucleic acid biochemistry, but the role of the essential metal cofactor has not usually been delineated in a satisfactory manner. Recent evidence suggests a variety of mechanistic roles for the divalent magnesium center [Jou, et al., 1991; Black, et al., 1994a; Black, et al., 1994c; Cowan, J.A., 1992; Cowan, ed., 1995], however, the lability and spectroscopic silence of this alkaline earth ion has precluded detailed elucidation of its chemistry during active turnover. Here we describe an approach based on the use of substitutionally inert transition metal complexes that bear ammine and aquo ligands in well-defined geometries.

4.2.1 Classification of Metal Complexes by Reactivity

To fully delineate the relative contributions of electrostatic stabilization and hydrogen bonding from metal-bound water molecules to catalytic activation, one would ideally like to systematically vary the number of water molecules in the inner coordination sphere of the activating metal cofactor. Unfortunately, cobalt(III) complexes carrying significant numbers of H$_2$O are unstable, undergoing autoreduction to cobalt(II) species [Basolo, et al., 1986]. To avoid this problem, and also provide a more systematic probe of the mechanistic issues summarized above, we have synthesized a series of inert
chromium complexes \([\text{Cr(NH}_3\text{)}_{6-x}(\text{H}_2\text{O})_x]^{3+} \) \((x = 0 \text{ to } 6)\) that carry water and ammine ligands in defined geometries in the inner coordination shell \([\text{trans-}[\text{Cr(NH}_3\text{)}_4(\text{H}_2\text{O})_2]\text{Cl}_3\): Bjerrum, et al., 1958; \([\text{Cr(NH}_3\text{)}(\text{H}_2\text{O})_5]\text{Cl}_3\): Ardon, et al., 1962; mer-
\([\text{Cr(NH}_3\text{)}_3(\text{H}_2\text{O})_2]\text{Cl}_3\): Riesenfeld, et al., 1909; cis-\([\text{Cr(NH}_3\text{)}_4(\text{H}_2\text{O})_2]\text{Cl}_3\) Springborg, et al., 19; \([\text{Cr(NH}_3\text{)}_5(\text{H}_2\text{O})](\text{NO}_3)_3\): Schlessinger, 1962; trans-\([\text{Cr(NH}_3\text{)}_4(\text{H}_2\text{O})_2](\text{NO}_3)_3\): Hoppenjans, et al., 1969; \([\text{Cr(NH}_3\text{)}_6](\text{NO}_3)_3\): Schlessinger, 1962; cis-
\([\text{Cr(NH}_3\text{)}_2(\text{H}_2\text{O})_4]\text{Cl}_3\): Andersen, et al., 1984a; fac-\([\text{Cr(NH}_3\text{)}_3(\text{H}_2\text{O})_3]\text{Cl}_3\): Andersen, et al., 1984b, and Andersen, et al., 1990). Comparison of the catalytic parameters of these complexes [Black, et al., 1994c], allows us to quantitatively evaluate the influence of electrostatics and hydrogen bonding to substrate binding and catalytic activation [Black, et al., 1994b; Black, et al., 1994c].

Table 8 lists kinetic rate constants for metal-complex promoted RNase H hydrolysis of a poly(A)-poly(dT) hybrid substrate. Within experimental error, the relative activities of these metal complexes fall into three major categories: (A) promote significant levels of activity; (B) promote either no or low levels of activity; and (C) promote moderate levels of activity. Figure 36 illustrates the difference in replacing an inner sphere water with an ammine. The data in Table 8 show a clear pattern of reactivity. We will argue below that this reflects the interaction between the metal cofactor and the nucleotide substrate. Other explanations will be considered; however,
### Table 8. Kinetics parameters for metal-promoted ribonuclease H digestion of hybrid substrate.\(^a\)

<table>
<thead>
<tr>
<th>Category</th>
<th>Cofactor</th>
<th>(K_m) (μM)</th>
<th>(k_{cat}) (substr. s(^{-1}))</th>
<th>(\Delta G^*) (kcal mole(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mg(H(_2)O(_6))(^{2+})</td>
<td>10 ± 3</td>
<td>28 ± 8</td>
<td>15.5</td>
</tr>
<tr>
<td></td>
<td>[Cr(H(_2)O)(_5)(H(_2)O(^-))](^{2+})</td>
<td>9 ± 3</td>
<td>16 ± 5</td>
<td>15.8</td>
</tr>
<tr>
<td>A</td>
<td>[Cr(NH(_3))(H(_2)O(_4))(H(_2)O(^-))](^{2+})</td>
<td>13 ± 3</td>
<td>14 ± 4</td>
<td>15.9</td>
</tr>
<tr>
<td></td>
<td>cis-[Cr(NH(_3))(H(_2)O)(_3)(H(_2)O(^-))](^{2+})</td>
<td>9 ± 3</td>
<td>11 ± 3</td>
<td>16.1</td>
</tr>
<tr>
<td></td>
<td>fac-[Cr(NH(_3))(H(_2)O)(_2)(H(_2)O(^-))](^{2+})</td>
<td>13 ± 3</td>
<td>9 ± 3</td>
<td>16.0</td>
</tr>
<tr>
<td>B</td>
<td>trans-[Cr(NH(_3))(H(_2)O)(_3)(H(_2)O(^-))](^{2+})</td>
<td>18 ± 7</td>
<td>0.24 ± 0.07</td>
<td>18.3</td>
</tr>
<tr>
<td></td>
<td>mer-[Cr(NH(_3))(H(_2)O)(_2)(H(_2)O(^-))](^{2+})</td>
<td>-</td>
<td>&lt;0.05</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>trans-[Cr(NH(_3))(H(_2)O)(H(_2)O(^-))](^{2+})</td>
<td>-</td>
<td>&lt;0.05</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>cis-[Cr(NH(_3))(H(_2)O)(H(_2)O(^-))](^{2+})</td>
<td>-</td>
<td>&lt;0.05</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>[Cr(NH(_3))(H(_2)O(^-))](^{2+})</td>
<td>-</td>
<td>&lt;0.05</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>Cr(NH(_3))(_6)(^{3+})</td>
<td>13 ± 4</td>
<td>3 ± 0.9</td>
<td>16.8</td>
</tr>
<tr>
<td></td>
<td>Co(NH(_3))(_6)(^{3+})</td>
<td>10 ± 3</td>
<td>2 ± 0.6(^d)</td>
<td>17.1</td>
</tr>
</tbody>
</table>
a An $\varepsilon_{260} = 6.550$ M$^{-1}$ cm$^{-1}$ (per base pair) was used for poly(A)-poly(dT) substrate, and an $\varepsilon_{280} = 35,500$ M$^{-1}$ cm$^{-1}$ was used for RNase H. All reactions were carried out in 20 mM Tris base, pH 7.5, 50 mM KCl, 0.1 M DTT, 30 µM base-pairs poly(A)-poly(dT), 10 mM $M^{n+}$, 540 nM RNase H, and 25 ± 1 °C. Experimental errors arose mostly from estimates of protein and substrate concentration. Parameters $k_{cat}$ and $K_m$ were determined both from fits to standard hyperbolic plots of initial velocity versus substrate concentration and from Eadie-Hofstee plots (Figures 28-35). The activation free energy $\Delta G^*$ was calculated from the standard equation, $k_{cat} = (kT/h) \exp(-\Delta G^*/RT)$, where symbols have their usual meanings.

b To assess any effects due to species with two ionized water ligands, complete activity versus pH profiles were determined for reactions promoted by the chromium complexes. Although pH-dependent activity behavior was observed, the difference in observed maximal activity relative to that obtained for the conditions noted in Table 8 was less than a factor of two, and does not influence any of the conclusions detailed in the text. A complete discussion of results from these, and related studies, will be reported elsewhere.

c With Co(NH$_3$)$_6^{3+}$ as the cofactor, the RNase H concentration was 722 nM. Previously we had reported that the catalytic activities of Co(NH$_3$)$_6^{3+}$ and Mg$^{2+}$(aq) were similar [Jou, R. et al., 1991]. The moderately lower activity reported here for Co(NH$_3$)$_6^{3+}$
reflects in part the greater precision of the absorbance method for evaluating reaction velocity, but also our previous results did not account for the phenomenon of metal-dependent inhibition and the ionic strength dependence of this interaction, which has been detailed in this work.
we aim to show that these are inconsistent with the body of experimental data described herein.

4.2.2 Evaluation of Electrostatic and Hydrogen Bonding Contributions

Relatively high levels of activity are observed for category (A) complexes carrying a significant hydration shell: such as \([\text{Mg}(\text{H}_2\text{O})_6]^{2+}\), \([\text{Cr}(\text{H}_2\text{O})_6(\text{HO}^-)]^{3+}\), \([\text{Cr}(\text{NH}_3)(\text{H}_2\text{O})_4(\text{HO}^-)]^{2+}\), cis-\([\text{Cr}(\text{NH}_3)_{2}(\text{H}_2\text{O})_3(\text{HO}^-)]^{2+}\), and fac-\([\text{Cr}(\text{NH}_3)_{3}(\text{H}_2\text{O})_2(\text{HO}^-)]^{2+}\). Michaelis constants \((K_m)\) are found to be similar for turnover by each cofactor \((\sim 10 \text{ mM})\) [this is equivalent to \(\sim 0.5 \text{ mM}\) using the method of calculation in Chapter 3 and is consistent with the data reported therein] suggesting that the metal cofactor serves no significant role in the binding of hybrid substrate. Also, the binding affinity of the enzyme for the complexes are similar. For the limiting cases of \([\text{Mg}(\text{H}_2\text{O})_6]^{2+}\), \([\text{Cr}(\text{H}_2\text{O})_6]^{3+}\), and \([\text{Co}(\text{NH}_3)_6]^{3+}\) (a category C complex), the binding constant to the enzyme was \(\sim 0.2 \text{ mM}, 0.3 \text{ mM}, \text{ and } 1 \text{ mM}\), respectively, by use of NMR titration experiments, kinetic studies, or calorimetry using the protocols defined previously [(c) Black, et al., 1994c; (a) Speckhard, et al., 1993a; (b) Speckhard, et al., 1993b; Speckhard, et al., 1991; Haromy, et al., 1990; Smith, et al., 1981]. Clearly the difference in the \text{NH}_3 or \text{H}_2\text{O} ligand environments do not significantly perturb the enzyme-complex binding, and so at the metal cofactor concentrations employed, we can assume that the enzyme cofactor binding site is saturated and partial occupation does not account for the trends in activity seen in Table 8.
Figure 36. Time traces illustrating the observed change in absorbance over the course of the reaction. The top trace was obtained from [Cr(NH$_3$)(H$_2$O)$_4$(HO$^-$)]$^{2+}$ activated RNase H, while the lower trace was obtained from trans-[Cr(NH$_3$)$_2$(H$_2$O)$_3$(HO$^-$)]$^{2+}$ activated RNase H.

Note the marked dependence of reaction rate on the number of water molecules in the hydration sphere. Experimental details are summarized in the legend to Table 8.
The complexes \([\text{Cr(NH}_3\text{)}_{6-x}\text{(H}_2\text{O})_{x-1}(\text{HO}^\text{-})]^{2+}\) (where \(x \leq 4\)) fall into category (B) and do not promote RNase H activity, except in the case of the fac-isomer, a class (A) complex, where a facial arrangement of the waters allows for maximal hydrogen bonding with the substrate. Even though the trans-tetraaquo complex coordinates four waters, the reduced activity can be attributed to the absence of facially arranged waters; illustrating the importance of maximal hydrogen bonding between cofactor and substrate [Black, et al., 1994b].

The hexaammine complexes fall into category (C). In the absence of a critical number of hydrogen bond donors, activity can be promoted by a trivalent complex such as \(\text{Cr(NH}_3\text{)}_6^{3+}\) or \(\text{Co(NH}_3\text{)}_6^{3+}\). This trivalent charge offsets the poorer hydrogen bonding abilities of the ammine ligands. We have previously discussed the evidence for the poor hydrogen bonding abilities of \(\text{NH}_3\) coordinated to trivalent cobalt (or chromium) substrate [Black, et al., 1994b].

The absence of significant activation by mer-[\(\text{Cr(NH}_3\text{)}_3\text{(H}_2\text{O})_2(\text{HO}^\text{-})]\)^{2+}, trans-[\(\text{Cr(NH}_3\text{)}_4\text{(H}_2\text{O})(\text{HO}^\text{-})]\)^{2+}, cis-[\(\text{Cr(NH}_3\text{)}_4\text{(H}_2\text{O})(\text{HO}^\text{-})]\)^{2+}, and [\(\text{Cr(NH}_3\text{)}_5(\text{HO}^\text{-})]\)^{2+} complexes, which possess the same formal charge as \(\text{Mg}^{2+}\text{(aq)}\), suggests that stabilization of the transition state arises predominantly through hydrogen-bonding. Moreover, since all of these complexes are formally “chromium-hydroxide” species, the absence of activity for the category (B) complexes argues against the hydroxide as the reactive center that promotes activity. The activity of class (A) complexes indicates that there is a minimum number, and a preferred geometry for bound waters of hydration to effect
catalysis. Compare, for example, the relative activities of mer-[Cr(NH\textsubscript{3})\textsubscript{3}(H\textsubscript{2}O)\textsubscript{3}(HO\textsuperscript{-})]\textsuperscript{2+} \textit{versus} fac-[Cr(NH\textsubscript{3})\textsubscript{3}(H\textsubscript{2}O)\textsubscript{2}(HO\textsuperscript{-})]\textsuperscript{2+}, and the low level of activity from trans-[Cr(NH\textsubscript{3})\textsubscript{2}(H\textsubscript{2}O)\textsubscript{3}(HO\textsuperscript{-})]\textsuperscript{2+}. The moderate activities of class (C) complexes demonstrates that if the charge is large enough, and the metal-ligand bonds are unable to autoionize, as for Co(NH\textsubscript{3})\textsubscript{6}\textsuperscript{3+} and Co(NH\textsubscript{3})\textsubscript{6}\textsuperscript{3+}, then electrostatics alone can promote catalysis. The differences in $k_{\text{cat}}$ must therefore reflect transition state stabilization: the increased negative charge on phosphate in the transition state is offset by hydrogen bonding and/or electrostatic interaction with the neighboring metal cofactor. In this regard, our thoughts are in line with recently revised proposals for zinc activation of carboxypeptidase A [Christianson, et al., 1989]. The pattern of enzyme activity previously observed with substrate analogues and derivatives supports the hydrolysis step as rate-limiting [Hogrefe, et al., 1990; Inoue, et al., 1987]. It is difficult to relate the relative activities of the various cofactors described in Table 8 to a mechanism that involves an alternative limiting step, such as product release.

4.2.3 Geometric Considerations

The availability of [Cr(NH\textsubscript{3})\textsubscript{6-\ensuremath{x}}(H\textsubscript{2}O)\textsubscript{k-\ensuremath{y}}(HO\textsuperscript{-})]\textsuperscript{3+} complexes of varying coordination geometry provides control over the disposition of H-bond interactions and affords a direct probe of the geometric preference for metal-bound water molecules. The
crystallographically characterized coordination geometry of ligands to enzyme-bound Mg$^{2+}$ is illustrated below (Scheme 3) [Katayanagi, et al., 1993]. Although the substitutionally inert complexes are unable to coordinate directly to the Asp, Glu, and Gly ligand atoms, we have previously demonstrated that complexes such as

![Scheme 3](image)

these bind at this location and promote enzyme catalysis [Huang, et al., 1994]. Clearly, direct coordination to the enzyme is not a prerequisite for enzyme activity, and is essentially irrelevant for a discussion of catalysis. Also, the ionic radius of the trivalent ions is smaller than for divalent magnesium [Cotton, et al., 1983], offsetting any spacial disparity arising from the distinct binding modes to the enzyme. The nature of the interaction between the metal cofactor and the nucleotide substrate is the critical issue.
The metal binding protein residues simply serve to tether a metal cofactor to the catalytic pocket on the enzyme surface. In addition, the difference between metal cofactors must be pointed out. The probes used above in Table 8 are substitution inert meaning exchange of inner sphere ligands does not occur. This means that interaction with the enzyme residues consists principally of electrostatic and possibly hydrogen bonding contacts (Scheme 4). The data in Table 8 is consistent with the metal cofactor modulating reactivity by largely interacting with the substrate and not the enzyme pocket.

Scheme 4

We propose that the data summarized in Table 8 indicates a requirement for a facial array of water molecules that can be presented to the substrate in a fashion analogous to that implied for the enzyme-bound magnesium in Scheme 3. Alternatively, it may be argued that the variation in activities demonstrated by these complexes reflects
specific structural perturbations of active site residues that either favor or disfavor catalysis. We believe that this explanation is inconsistent with the body of experimental data described herein. Comparison of $k_{\text{cat}}$ values for each isomer demonstrates a clear correlation with the orientation of $\text{NH}_3$ and $\text{H}_2\text{O}$ ligands to the hybrid substrate and enzyme pocket. Those complexes that possess a facial array of water molecules that can be presented to the substrate are able to promote catalysis. Those that do not possess such an array of water ligands demonstrate either minimal or no activity. In each case the net charge on the complex is the same (+2). The effective charge on category (B) complexes is equivalent to those in category (A), while category (C) complexes, which lack a hydration shell, maintain a trivalent charge. It is difficult to rationalize these trends in terms of specific side-chain perturbations for the series of $[\text{Cr(H}_2\text{O)}_{6-x}(\text{NH}_3)_x]^{3+}$ complexes, while a fundamental requirement for reorientation of active site residues is inconsistent with the significant levels of activity exhibited by the $[\text{Cr(\text{NH}_3})_6]^{3+}$ and $[\text{Co(\text{NH}_3})_6]^{3+}$ complexes. Accordingly, we suggest that these experiments reasonably exclude the possibility of a structural role for the cofactor, involving reorientation of active site residues. Only the electrostatic/H-bonding arguments developed in this chapter, and the mechanism of transition state stabilization through outer sphere complex formation with the metal cofactor is fully consistent with the results presented in Table 8 (Figure 37). Explanations based on preferential binding of the metal complexes to the enzyme or substrate are inconsistent with the binding parameters reported earlier, and
with the observed pattern of activity. We believe that the experimental approach
described here will be widely applicable to mechanistic studies of metallonucleases in
nucleic acid biochemistry.
Figure 37. Proposed function of the metal ion in ribonuclease H. The Mg$^{2+}$ is tethered in a cleft or pocket by three enzyme contacts (Asp-10, Glu-48, Gly-11) and interacts the hybrid through hydrogen bonds with its inner sphere waters. Only one strand of the hybrid is shown here for simplicity and Ad refers to an adenosine base.
CHAPTER V

INERT CHROMIUM AND COBALT COMPLEXES AS PROBES OF MAGNESIUM DEPENDENT ENZYMES. EVALUATION OF THE STOICHIOMETRY AND MECHANISTIC ROLE OF THE ESSENTIAL METAL COFACTOR IN *E. coli* EXONUCLEASE III

5.1 Introduction

*Escherichia coli* exonuclease III is a monomeric low molecular weight (Mr \( \sim 28 \) kDa) apurinic/apyrimidinic endonuclease that serves an integral role in DNA repair [Weiss, 1981; Weiss, 1976; Demple, et al. 1986; Loeb, et al., 1980]. It targets DNA strands where the base has been lost or damaged and excises the damaged fragment. In addition to this endonucleolytic activity, the enzyme also hydrolytically removes nucleotide fragments from the 3'-end of ds-DNA by cleavage of the terminal phosphodiester linkage, and carries out a similar 3’-5’ exoribonuclease H (RNase H) reaction by step-wise removal of ribonucleotides from an RNA-DNA hybrid [Weiss,
1981; Demple, et al., 1986; Loeb, et al., 1980; Richardson, et al., 1964; Kow, et al., 1989; Henner, et al., 1983]. It is known that each of these functions is promoted by an essential divalent magnesium cofactor, and it has been proposed that the same catalytic site is used for each of these various reactions [Weiss, 1981; Kow, et al., 1989; Henner, et al., 1983; Mol, et al., 1995], although the hydrolysis mechanism remains to be elucidated [Kow, et al., 1989; Henner, et al., 1983; Kim, et al., 1992; Black, et al., 1996].

Recently the crystal structure of *E. coli* exonuclease III has been solved at 1.7 Å resolution and structural similarities have been identified with the active sites of bovine DNase I and *E. coli* ribonuclease H [Mol, et al., 1995] (Figure 38). On the basis of the published structure of a ternary complex with bound Mn$^{2+}$ and dCMP, a reaction mechanism involving a carboxylate-His relay has been proposed, although a specific role for the divalent metal cofactor has not yet been ascribed. Prior dogma that the essential alkaline earth cofactors for such enzymes generally serve as simple Lewis acids has been shown to lack general applicability inasmuch as substitutionally inert transition metal complexes, incapable of direct binding to substrate, can promote the reactions of a number of important magnesium-dependent enzymes [Kim, et al., 1992; Jou, et al., 1991; Black, et al., 1996; Black, et al., 1994b,c]. These, and related studies have demonstrated a more complex chemistry for divalent magnesium with biological macromolecules, and emphasize the need for further studies to delineate the mechanisms of metal-mediated
Figure 38. Ribbon structure of *E. coli* exonuclease III shown complexed to a strand of dsDNA. Note that the surface contacting the DNA is \( \sim 55 \, \text{Å} \) long. [Mol, et al., 1995].

The ribbon structure is taken from the x-ray coordinates, while the DNA is an idealized model.
phosphate ester hydrolysis. Both the molecular mechanism of exonuclease III activation, and the stoichiometric requirement for Mg\(^{2+}\) during turnover are uncertain. Although one metal ion has been identified in crystallographic studies, this contrasts with the case of the exonucleolytic domain of the Klenow fragment where two metal ions are proposed [Steitz, et al., 1993]. Only recently, however, have solution kinetic studies been reported that seek to address the issue of metal cofactor stoichiometry during bona fide turnover [Black, et al., 1996a]. Such experiments are essential for the reliable estimation of metal cofactor requirements, since crystallographic data alone cannot exclude the possible recruitment of an additional weakly bound cofactor in the presence of bound substrate.

In this chapter we address these issues for the 3'-5' exonuclease and RNase H activities of *E. coli* exonuclease III. In particular, we report the results of (1) solution studies that quantitate metal cofactor stoichiometry during turnover, (2) an evaluation of the mode of interaction (inner or outer sphere) of the metal cofactor with substrate, (3) the roles of hydrogen bonding and electrostatics and hydrogen bonding in catalytic activation, (4) a comparison of the reaction profiles for exonuclease and RNase H activities to provide experimental support for a common reaction site, (5) studies with inert transition metal complexes of defined coordination sphere and geometry that have proven helpful in evaluating the issues just summarized, and (6) a rationalization of the coordination state of the metal cofactor and its functional role. This work complements previous studies in our laboratory on the *E. coli* ribonuclease H enzyme, of which the metallobiochemistry
has been extensively studied by $^1$H and $^{25}$Mg NMR, isothermal titration calorimetry, and solution kinetics [Jou, et al., 1991; Black, et al., 1996a; Casareno, et al., 1995; Huang, et al., 1994]. In spite of the significant differences in protein ligation displayed by each enzyme for the magnesium cofactor (three and one protein-derived ligand, respectively, for ribonuclease H and exonuclease III), magnesium binding affinities are comparable (~$10^4 \, \text{M}^{-1}$). A comparison of the active site chemistry of the exo III and ribonuclease H enzymes is made, and insight is obtained on the relationship of the ligation/solvation states of the metal cofactor and the requirements for catalytic activation.

5.2 Results

5.2.1 Consideration of the Effective Charge and Form of the Inert Chromium Complexes

While the chemistry of magnesium-dependent nuclease is of fundamental importance in nucleic acid biochemistry, the role of the essential metal cofactor has not usually been delineated in a satisfactory manner due to the lability and spectroscopic silence of this alkaline earth ion. To overcome this problem we have developed an approach based on the use of substitutionally inert transition metal complexes that bear ammine and aquo ligands in well defined coordination geometries [Jou, et al., 1991; Black, et al., 1996a]. Previously we have demonstrated the use of this strategy to delineate the relative contributions of electrostatic stabilization and hydrogen bonding
from metal-bound water molecules to catalytic activation [Black, et al., 1996a; Black, et al., 1994b]. The series of inert chromium complexes (Figure 27) \([\text{Cr(NH}_3\text{)}_{6-x}(\text{H}_2\text{O})_x]^{3+}\) (\(x = 0\) to 6) carry water and ammine ligands in the inner coordination shell and comparison of the catalytic parameters of these complexes allows quantitative evaluation of the contribution of electrostatics and hydrogen bonding to substrate binding and catalytic activation. A discussion of the effective charge on the chromium complexes used in these studies has been presented in Chapter 4. The salient points of that discussion are described here in brief. Under the pH conditions reported in the experimental section the \([\text{Cr(NH}_3\text{)}_{6-x}(\text{H}_2\text{O})_x\cdot(\text{HO}^-)]^{3+}\) complexes will exist predominantly in an ionized form (\(\text{pK}_{a1} \sim 4\)) [Jorgensen, et al., 1958; Schaffer, 1964; Bjerrum, 1910], bearing an hydroxide ligand and yielding a net (+2) charge. The solution state of such complexes is best represented as \([\text{Cr(NH}_3\text{)}_{6-x}(\text{H}_2\text{O})_x\cdot(\text{HO}^-)]^{2+}\), as shown in Tables 8 in Chapter 4, 12 and 13. A second ionization (\(\text{pK}_{a2}\)) can arise between pH 6 and 7 for \(\text{Cr(H}_2\text{O})_6^{3+}\), however, even at the pH values used in our experiments, there is a sufficient concentration of the singly ionized species present in solution to saturate the binding site. For other complexes that carry ammine ligands, the \(\text{pK}_{a2}\) is significantly larger and need not be considered here. For convenience, the ionized state of the complex will usually not be shown, however, it should be realized that any chromium(III) complex carrying a water ligand will exist in an ionized state around neutral pH.
A further source of potential problems for studies with the hexaaquo-chromium complex stems from the known propensity of these species to form aggregates in solution. However, from published data obtained from solution studies [Monsted, et al., 1973; DePamphilis, et al., 1973; Thompson, et al., 1981; Rotzinger, et al., 1986], it is clear that the formation of dimeric, trimeric or polynuclear clusters is not significant under the solution conditions, concentrations, and timescales reported herein. This is further supported by the absence of significant increases in absorption at wavelengths attributed to these aggregates [Monsted, et al., 1973; DePamphilis, et al., 1973; Thompson, et al., 1981; Rotzinger, et al., 1986], either in the presence or absence of enzyme. The distinct pH-profiles obtained for the various enzymatic activities is also consistent with the view that the observed data does not correlate with the formation of any specific cluster species.

Inasmuch as the available crystallographic data clearly shows one glutamate binding in an inner sphere fashion to magnesium, it is appropriate to consider the fact that these inert complexes will not bind in such a fashion. To address this issue requires us to distinguish the binding modes of the metal cofactor to the enzyme and the interaction with the substrate. Surface magnesium binding sites have been previously shown, from work in our lab and others [Casareno, et al., 1996; Needham, et al., 1993], to exhibit only a minor dependence of the binding affinity on the size of the metal ion (or complex), consistent with a surface site that displays extensive flexibility. The affinities for metal
binding to the enzyme ($K_{M_{\text{ET}}}$) have been determined by kinetic methods (Table 10) and clearly show efficient binding for each metal cofactor. The binding site is perhaps best considered as a hollow on the surface of the enzyme in which ions or complexes of varying sizes can fit. For this reason, the binding of the metal cofactor to the enzyme is not a dominant factor for the understanding of catalysis. The interaction of the $\text{Mg}^{2+}$ and inert complexes with the substrate is a different issue. The data to be reported later in this work will support an outer-sphere interaction mode for the catalytic metal. There is extensive crystallographic evidence to support the propensity of magnesium to interact with oligonucleotides in an outer sphere fashion [Black, et al., 1994a].

5.2.2 Metal Ion Activation of E. coli Exonuclease III Catalyzed Hydrolysis of dsDNA and RNA-DNA Hybrid

The 3'-5' exonucleolytic activity of exonuclease III results in the nonprocessive hydrolysis of one strand of DNA from the 3'-terminae, releasing 5'-monophosphate base units [Thomas, et al., 1978]. In addition, the enzyme exhibits RNase H activity and can hydrolyze the RNA strand of RNA-DNA hybrids, leaving single-stranded DNA and 5'-monophosphate base units. Exonuclease III does not catalyze hydrolysis of single-stranded DNA [Weiss, 1981]. Table 9 summarizes Michaelis-Menten kinetic constants
Figure 39. Substrate dependence of *E. coli* exonuclease III 3'-5' exonuclease activity on dsDNA with Cr(H$_2$O)$_6$$^{3+}$. See Table 9 for reaction conditions.
Figure 40. Substrate dependence of *E. coli* exonuclease III RNase H activity on poly(A)-poly(dT) with Mg$^{2+}$ (aq). See Table 9 for reaction conditions.
Table 9. Steady-state parameters for exonuclease III activities by (A) Mg$^{2+}$ and (B) Cr(H$_2$O)$_6^{3+}$.

<table>
<thead>
<tr>
<th>(A) Mg$^{2+}$ Activation</th>
<th>3'-5' exonuclease</th>
<th>RNase H</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ ($\mu$M)</td>
<td>12.1</td>
<td>4.3</td>
</tr>
<tr>
<td>$k_{cat}$ (sec$^{-1}$)</td>
<td>0.11</td>
<td>0.043</td>
</tr>
<tr>
<td>$k_{cat}/K_m$ (sec$^{-1}$mM$^{-1}$)</td>
<td>9.1</td>
<td>10.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(B) Cr(H$_2$O)$_6^{3+}$ Activation</th>
<th>3'-5' exonuclease</th>
<th>RNase H</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ ($\mu$M)</td>
<td>79.1</td>
<td>15.5</td>
</tr>
<tr>
<td>$k_{cat}$ (sec$^{-1}$)</td>
<td>38.6</td>
<td>4.1</td>
</tr>
<tr>
<td>$k_{cat}/K_m$ (sec$^{-1}$mM$^{-1}$)</td>
<td>0.5</td>
<td>0.3</td>
</tr>
</tbody>
</table>

*Experimental solution conditions for evaluation of Michaelis-Menten parameters were as follows: 0.7 $\mu$M *E. coli* exonuclease III; 10 mM Mg$^{2+}$ or 8 mM Cr(H$_2$O)$_6^{3+}$; 20 mM Tris-base pH 7.4; 50 mM KCl; 0.1 mM Na$_2$EDTA; 0.1 mM DTT; and a temperature of 25 ± 1 °C. All concentrations for nucleic acids are in units of base pairs. All errors are ± 50%.
determined for each of these enzyme activities when promoted by the Mg\(^{2+}\) (aq) and Cr(H\(_2\)O)\(_6^{3+}\) cofactors. The specificity constants (k\(_{cat}/K_m\)) are similar for the 3'→5' exonuclease and RNase H activities since the approximately four-fold lower K\(_m\) for RNase H activity is offset by the almost three-fold higher k\(_{cat}\) for the 3'-5' exonuclease activity. Certain substitutionally-inert chromium(III) complexes were also found to promote enzyme activity. These will be the subject of detailed discussion in a later section.

The influence of metal cofactor concentration on initial velocity was also evaluated. Metal ion inhibition was observed for both 3'-5' exonuclease and RNase H activities using either Mg\(^{2+}\) or Cr(H\(_2\)O)\(_6^{3+}\) cofactors (Table 10 and Figures 41-44). This form of substrate inhibition has previously been observed and rationalized for E. coli ribonuclease H [Black, et al., 1994c], but was not explained in the case of E. coli exonuclease III [Kow, 1989]. Plots of initial velocity versus metal cofactor concentration were analyzed by use of equation (2-9), which accounts for the inhibitory effect of metal ion binding to substrate, and the results are summarized in Table 10. The binding affinity of each metal cofactor for the enzyme (K\(_{MET}\)) is similar for each of the 3'-5' exonuclease and RNase H activities. The binding affinity for the substrate shows greater variation for dsDNA versus hybrid ligands [Black, et al., 1994b]. The value of k\(_{cat}(\text{opt})\) listed in Table 10, and determined from a fit to equation (2-9), reflects the maximal rates that might be
Figure 41. Metal cofactor dependence of *E. coli* exonuclease III 3'-5' exonuclease activity on Mg$^{2+}$ (aq) concentration. See Table 10 for reaction conditions.
Figure 42. Metal cofactor dependence of *E. coli* exonuclease III RNase H activity on Mg\(^{2+}\) (aq) concentration. See Table 10 for reaction conditions.
Figure 43. Metal cofactor dependence of *E. coli* exonuclease III 3′-5′ exonuclease activity on Cr(H$_2$O)$_6^{3+}$ concentration. See Table 10 for reaction conditions.
Figure 44. Metal cofactor dependence of *E. coli* exonuclease III RNase H activity on Cr(H$_2$O)$_6^{3+}$ concentration. See Table 10 for reaction conditions.
**Table 10.** Kinetic parameters evaluated from the metal dependence of the two enzymatic activities of *E. coli* exonuclease III.\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>3'-5' exonuclease</th>
<th>RNase H</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\text{Mg(H}_2\text{O)}_6^{2+})</td>
<td>(\text{Cr(H}_2\text{O)}_6^{3+})</td>
</tr>
<tr>
<td>(K_m) ((\mu\text{M}))</td>
<td>12.1</td>
<td>79</td>
</tr>
<tr>
<td>(k_{\text{cat (opt)}}) ((\text{sec}^{-1}))</td>
<td>1.0</td>
<td>340</td>
</tr>
<tr>
<td>(K_{\text{MET}}) (mM)</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>(K_i) (mM)</td>
<td>0.6</td>
<td>5.3</td>
</tr>
</tbody>
</table>

\(^a\)Experimental solution conditions for evaluation of Michaelis-Menten parameters were as follows: 0.7 \(\mu\text{M}\) exonuclease III; saturating substrate; 20 mM Tris-base pH 7.4; 50 mM KCl; 0.1 mM Na\(_2\)EDTA; 0.1 mM DTT; and a temperature of 25 ± 1 °C. Substrates for the 3'-5' exonuclease and RNase H reactions were calf thymus DNA and poly(A)-poly(dT) hybrid, respectively. All concentrations for nucleic acids are in units of base pairs. All errors are ± 20%. For the purpose of data fitting, the \(K_m\) values in Table 9 were used as constants. All other parameters were floating. \(K_{\text{MET}}\) is the metal binding affinity for the enzyme, while \(K_i\) is the binding affinity of the metal cofactor for the
substrate in the presence of a fixed enzyme concentration (0.5 μM). These parameters were obtained by fitting to Equation (2-9).
obtained in the presence of optimal concentrations of Mg\(^{2+}\) with no inhibitory effects.

Comparison of the results summarized in Table 10 for activation of 3'-5' exonuclease and RNase H catalytic functions by Mg\(^{2+}\) versus Cr(H\(_2\)O)\(_6\)\(^{3+}\) shows a clear similarity in the kinetic and binding parameters for each metal cofactor, irrespective of the activity, which lends considerable support to arguments for a common reaction site.

5.2.3 Dependence of Activity on pH

The systematic pH dependence of the exonuclease activity was also measured using both Mg\(^{2+}\) and Cr(H\(_2\)O)\(_6\)\(^{3+}\) as cofactors. The pH dependence of the 3'-5' exonuclease and RNase H activities of \textit{E. coli} exonuclease III are similar. In this section we focus on the comparison with the pH profile obtained with \textit{E. coli} ribonuclease H.

The plots in Figures 45-48 illustrate that strikingly different pH profiles were obtained for each metal cofactor, and each enzyme (\textit{E. coli} exonuclease III relative to \textit{E. coli} ribonuclease H) over the pH range from 3 to 8. In all instances, chromium (III) complexes were observed to precipitate at pH values above 7.5, and so the comparison could not be extended to higher pH values. The pH dependence of metal-promoted enzyme activities was measured using both Mg\(^{2+}\) and Cr(H\(_2\)O)\(_6\)\(^{3+}\) as metal cofactors. The data were analyzed according to either equations (5-1) or (5-2) below [Findlay, et al.,
\[ v_{\text{max}} = \frac{v_{\text{opt}}}{(1 + 10^{pH/pK_a})} \] (5-1)

\[ v_{\text{max}} = \frac{v_{\text{opt}}}{(1 + 10^{pH/pK_a^1} + 10^{pK_a^2/pH})} \] (5-2)

1961], where \( v_{\text{max}} \) represents the maximal activity under saturating conditions, \( v_{\text{opt}} \) represents the optimum initial velocity with respect to pH, and pKa represents the fitted ionization constants. Equation (5-1) is used when only one ionization is evident in the data, while equation (5-2) accommodates two ionization constants. For \( E. \ coli \) exonuclease III, the \( \text{Mg}^{2+} \) activated enzyme shows a maximal activity at approximately pH 7.5 (Figure 45) with lower activity on either side, while the \( \text{Cr(H}_2\text{O)}_6^{3+} \) exhibits fairly uniform activity over the entire pH range (Figure 46). The possibility of direct nucleophilic attack on the phosphate ester by a \( \text{Cr-OH} \) species, yielding a chromium-bound product, was eliminated by examining the optical spectra of the acid soluble products after reaction. No change was observed in the absorbance bands associated with the hexaaquo complex relative to a control solution of the substrate and chromium complex and no enzyme.
Figure 45. Dependence of the 3'-5' exonuclease activity of *E. coli* exonuclease III with pH for the Mg$^{2+}$ promoted reaction. See Table 11 for reaction conditions.
Figure 46. Dependence of the 3’-5’ exonuclease activity of E. coli exonuclease III with pH for the Cr(H₂O)₆³⁺ promoted reaction. See Table 11 for reaction conditions.
Figure 47. Dependence of *E. coli* ribonuclease H with pH for the Mg$^{2+}$ promoted reaction. See Table 11 for reaction conditions.
Figure 48. Dependence of *E. coli* ribonuclease H with pH for the Cr(H₂O)₆³⁺ promoted reaction. See Table 11 for reaction conditions.
Table 11. Ionization constants of *E. coli* exonuclease III 3'-5' exonuclease activity and *E. coli* ribonuclease H with Mg\(^{2+}\) and Cr(H\(_2\)O)\(_6\)\(^{3+}\) metal cofactors.\(^a\)

<table>
<thead>
<tr>
<th>Cofactor</th>
<th><em>E. coli</em> Ribonuclease H</th>
<th><em>E. coli</em> Exonuclease III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pK(_{a1})</td>
<td>pK(_{a2})</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>6.5</td>
<td>-</td>
</tr>
<tr>
<td>Cr(H(_2)O)(_6)(^{3+})</td>
<td>3.2</td>
<td>7.2(^d)</td>
</tr>
</tbody>
</table>

\(^a\) The plots are shown in Figures 45 to 48. Initial velocity was measured under conditions of saturating substrate (to provide k\(_{cat}\)) as a function of pH for both *E. coli* enzymes. Experimental conditions are given in the Figure legends, and equations used for analysis are described in the experimental section. Substrates for the 3'-5' exonuclease and RNase H reactions were calf thymus DNA and poly(A)-poly(dT) hybrid, respectively.

\(^b\) Only one ionization was observed in the pH profile.

\(^c\) An apparent pK\(_{a2}\) is observed at ~ 7.8, however, the decrease in activity observed at higher pH corresponds to irreversible inactivation of the enzyme rather than ionization of either the cofactor or a protein residue.
Since it was not practical to vary the pH to values where $v_o \sim 0$, the pK_a's were estimated from the pH values where the activity $\sim v_o/2$.

Constant activity was observed over a pH range of 2.7 to 7.5 without any observable ionization constants.
To provide a point of reference and comparison for understanding the reactivity of the *E. coli* exonuclease III enzyme, we also examined the pH dependence for the metal-promoted hydrolysis of hybrid substrate by *E. coli* ribonuclease H using Mg$^{2+}$, Cr(H$_2$O)$_6^{3+}$ and fac-[Cr(NH$_3$)$_3$(H$_2$O)$_3$]$^{3+}$ as cofactors. Titration profiles with the magnesium-activated enzyme exhibited typical titration behavior (Figure 47), indicating the ionization of a catalytically important residue with a pK$_a$ ~ 6.5 (Table 11). One likely candidate is Asp-10, which has been implicated as a binding ligand for Mg$^{2+}$ by crystallography and NMR studies [Kanaya, et al., 1990; Oda, et al., 1994]. The relatively high pK$_a$ value reflects electrostatic interactions among the cluster of carboxylates in the catalytic core [Oda, et al., 1994]. In contrast, enzyme-catalyzed reactions using Cr(H$_2$O)$_6^{3+}$ as the cofactor were found to display biphasic behavior (Figure 48) with two ionization constants (pK$_a$'s) of 3.2 and 7.2 (Table 11). Since the fully protonated metal complex should promote activity, we attribute the former to the apparent ionization constant of Asp-10, which is shifted to a significantly lower pH as a result of the binding of fully protonated Cr(H$_2$O)$_6^{3+}$, and is consistent with the influence of the divalent metal ion cofactor on the pK$_a$ of Asp-10 ($\Delta\delta$ ~ 2 ppm) [Oda, et al., 1994]. The second ionization with pK$_a$ ~ 7.2 corresponds to ionization of a second inner sphere water molecule on chromium(III), resulting in the loss of binding affinity by the essential metal cofactor. The facial isomer showed behavior similar to Mg$^{2+}$ with a rise in activity until pH 7.5,
above which precipitation was observed. The pKₐ can be extrapolated and estimated to be ~ 4.5 (data not shown), and again is consistent with a decrease in the pKₐ of active site carboxylates as a result of binding of the trivalent complex (the second ionization for the triammine chromium complex lies at a higher pH and is outside of the accessible pH range).

5.2.4 Inert Transition Metal Complexes as Probes of Enzyme Activity

For *E. coli* ribonuclease H we have previously reported used of substitutionally inert cobalt(III) and chromium(III) complexes to investigate the role of the metal ion cofactor in catalysis (Chapter 4). We have employed a similar strategy in our studies of *E. coli* exonuclease III. In contrast to *E. coli* ribonuclease H, however, we have found that neither Co(NH₃)₆³⁺ nor Cr(NH₃)₆³⁺ complexes were capable of activating *E. coli* exonuclease III. Apparently the metal hydration shell is an absolute requirement for the 3'-5' exonucleolytic and RNase H activities of the latter enzyme. To quantitatively evaluate both the number and geometric preference for the critical waters of solvation required for catalysis, an array of geometric isomers of the substitutionally inert Cr(NH₃)₆₋₃(H₂O)ₓ³⁻ complexes were used to activate the enzyme, allowing systematic variation of the number of inner sphere water molecules. The data obtained are summarized in Tables 12 and 13 for the 3'-5' exonucleolytic and RNase H activities,
respectively. We have previously used this approach in studies of *E. coli* ribonuclease H and demonstrated the requirement for three water molecules to be arranged in a facial geometry for optimal activity (Chapter 4). In this study the critical number of waters for the 3'-5' exonuclease and RNase H activities is 4. Increased activity relative to Mg$^{2+}$ was measured using Cr(H$_2$O)$_6$$^{3+}$, decreasing with fewer numbers of waters. However, even with all of the water ligands replaced with ammonia, the trivalent charge was not sufficient to allow activation. This indicates an absolute requirement for inner sphere water ligands. The similarity in activity profiles for the exonuclease and RNase H functions catalyzed by the various complexes provides an additional indication that the reaction site and catalytic hydrolysis mechanisms are common for both activities.
Table 12. Activation parameters for metal-promoted 3'-5' exonuclease digestion of double-stranded calf-thymus DNA for *E. coli* exonuclease III.\(^a,b\)

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>(k_{\text{cat}}) (substrate s(^{-1}))</th>
<th>(\Delta G^*) (kcal mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{Mg(H}_2\text{O)}_6^{2+})</td>
<td>1.4 ± 0.4</td>
<td>17.2</td>
</tr>
<tr>
<td>([\text{Cr(H}_2\text{O)}_5(\text{HO}^-)]^{2+})</td>
<td>340 ± 70</td>
<td>14.2</td>
</tr>
<tr>
<td>([\text{Cr(NH}_3)(\text{H}_2\text{O)}_4(\text{HO}^-)]^{2+})</td>
<td>52 ± 10(^d)</td>
<td>15.1</td>
</tr>
<tr>
<td>cis-([\text{Cr(NH}_3)_2(\text{H}_2\text{O})_3(\text{HO}^-)]^{2+})</td>
<td>14 ± 0.5</td>
<td>15.9</td>
</tr>
<tr>
<td>trans-([\text{Cr(NH}_3)_2(\text{H}_2\text{O})_3(\text{HO}^-)]^{2+})</td>
<td>10 ± 2</td>
<td>16.0</td>
</tr>
<tr>
<td>fac-([\text{Cr(NH}_3)_3(\text{H}_2\text{O})_2(\text{HO}^-)]^{2+})</td>
<td>&lt; 0.05</td>
<td>-</td>
</tr>
<tr>
<td>mer-([\text{Cr(NH}_3)_3(\text{H}_2\text{O})_2(\text{HO}^-)]^{2+})</td>
<td>&lt; 0.05</td>
<td>-</td>
</tr>
<tr>
<td>trans-([\text{Cr(NH}_3)_4(\text{H}_2\text{O})(\text{HO}^-)]^{2+})</td>
<td>&lt; 0.05</td>
<td>-</td>
</tr>
<tr>
<td>cis-([\text{Cr(NH}_3)_4(\text{H}_2\text{O})(\text{HO}^-)]^{2+})</td>
<td>&lt; 0.05</td>
<td>-</td>
</tr>
<tr>
<td>([\text{Cr(NH}_3)_5(\text{HO}^-)]^{2+})</td>
<td>&lt; 0.05</td>
<td>-</td>
</tr>
<tr>
<td>([\text{Cr(NH}_3)_6^{3+})</td>
<td>&lt; 0.05</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^d\) Assay conditions: ***E. coli*** exonuclease III (EccIII), double-stranded calf-thymus DNA (150 ng), MgSO\(_4\) (20 μM), 5 μM MAMP, 50 μM Tris-HCl (pH 7.5), 100 μM spermidine, 10 mM NaCl, 100 μM spermine, 100 μM MnCl\(_2\), and 0.5 units/ml of EccIII in a total volume of 50 μl. Assay time: 30 min. 0.1 M concentration in all cases.
An $\varepsilon_{260} \sim 6,600 \text{ M}^{-1} \text{ cm}^{-1}$ was used for dsDNA and an $\varepsilon_{280} = 55,800 \text{ M}^{-1} \text{ cm}^{-1}$ was used for exonuclease III. Reactions were carried out in 20 mM Tris-base, pH 7.5, 50 mM KCl, 0.1 mM DTT, 8 mM Mn$^{3+}$, 500 nM enzyme, with saturating substrate at 25 ± 1°C. The activation energy was calculated from the standard equation, $k_{\text{cat}} = (kT/h)\exp(-\Delta G^*/RT)$, where symbols have their usual meanings. In the lower part of the table the $k_{\text{cat}}$ limit of < 0.05 corresponds to the detection limit of the experiment and effectively denotes no reaction.

Although pH-dependent activity behavior was observed for the related 3'-5' exonuclease reaction, the difference in observed maximal activity (Figures 45 and 46) relative to that obtained for the conditions noted in Table 12 was less than a factor of two, and does not influence any of the conclusions detailed in the text.

The chromium complexes are shown explicitly in the singly ionized state.

Enzyme concentration was 0.7 μM.
Table 13. Activation parameters for metal-promoted RNase H digestion of poly(A)-poly(dT) for E. coli exonuclease III.a,b

<table>
<thead>
<tr>
<th>Cofactor c</th>
<th>( k_{\text{cat}} ) (substrate s(^{-1}))</th>
<th>( \Delta G^* ) (kcal mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg(H₂O)₆²⁺</td>
<td>0.6 ± 0.2</td>
<td>17.7</td>
</tr>
<tr>
<td>[Cr(H₂O)₆(HO⁻)]²⁺</td>
<td>15.5 ± 4</td>
<td>15.8</td>
</tr>
<tr>
<td>[Cr(NH₃)(H₂O)₄(HO⁻)]²⁺</td>
<td>9.3 ± 2(^d)</td>
<td>16.1</td>
</tr>
<tr>
<td>cis-[Cr(NH₃)₂(H₂O)₃(HO⁻)]²⁺</td>
<td>8.1 ± 2</td>
<td>16.2</td>
</tr>
<tr>
<td>trans-[Cr(NH₃)₂(H₂O)₃(HO⁻)]²⁺</td>
<td>5.8 ± 2</td>
<td>16.4</td>
</tr>
<tr>
<td>fac-[Cr(NH₃)₃(H₂O)₂(HO⁻)]²⁺</td>
<td>&lt; 0.05</td>
<td>-</td>
</tr>
<tr>
<td>mer-[Cr(NH₃)₃(H₂O)₂(HO⁻)]²⁺</td>
<td>&lt; 0.05</td>
<td>-</td>
</tr>
<tr>
<td>trans-[Cr(NH₃)₄(H₂O)(HO⁻)]²⁺</td>
<td>&lt; 0.05</td>
<td>-</td>
</tr>
<tr>
<td>cis-[Cr(NH₃)₄(H₂O)(HO⁻)]²⁺</td>
<td>&lt; 0.05</td>
<td>-</td>
</tr>
<tr>
<td>[Cr(NH₃)₅(HO⁻)]²⁺</td>
<td>&lt; 0.05</td>
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<tr>
<td>Cr(NH₃)₆³⁺</td>
<td>&lt; 0.05</td>
<td>-</td>
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<tr>
<td>Co(NH₃)₆³⁺</td>
<td>&lt; 0.05</td>
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</table>
a An $\varepsilon_{260} = 6,550 \text{ M}^{-1} \text{ cm}^{-1}$ was used for poly(A)-poly(dT). Other reaction conditions are described in the legend to Table 12. No reaction was observed with the other complexes listed in Table 12. In the lower part of the table the $k_{\text{cat}}$ limit of < 0.05 corresponds to the detection limit of the experiment and effectively denotes no reaction.

b Although pH-dependent activity behavior was observed, the difference in observed maximal activity (Figures 45 and 46) relative to that obtained for the conditions noted in Table 13 was less than a factor of two, and does not influence any of the conclusions detailed in the text.

c The chromium complexes are shown explicitly in the singly ionized state.

d Enzyme concentration was 0.7 μM.
5.3 Discussion

5.3.1 Metal Ion Stoichiometry During Turnover and Comparison of 3'-5' Exonuclease and RNase H Activities

By comparison of kinetic parameters obtained from the dependence of initial velocity on the concentrations of substrate and metal cofactor (Tables 9 and 10, respectively), two conclusions may be drawn. First, the general similarity in Michaelis-Menten constants for dsDNA and hybrid substrates, and especially the similarity in metal-enzyme binding constants \(K_{M_{ET}}\) derived from equation (2-9), and summarized in Table 10, strongly suggest that both activities are supported at the same reaction site on the enzyme. This conclusion is consistent with published structural data from recent crystallographic studies [Mol, et al., 1995].

Second, and in line with our recent findings for \(E.\ coli\) ribonuclease H, there appears to be a requirement for only one metal ion in the catalytically competent state of the enzyme during turnover. If more than one metal ion is assumed necessary for activation, it is found that fits to the data yield binding constants that approach unreasonable limits for Mg\(^{2+}\) binding proteins [Black, et al., 1994c; Huang, et al., 1994; Jenkins, et al., 1992; Kim, et al., 1991]. Further support is provided by calorimetric studies of magnesium binding to exonuclease III, which revealed one binding site for Mg\(^{2+}\) [Casareno, et al., 1996]. The stoichiometry of metal cofactor required to effect enzyme-promoted hydrolysis has been the subject of much speculation. Much of the
current thinking on this subject has been inferred from analysis of a rather small body of crystallographic data (in particular the exonuclease domain of the Klenow fragment) [Steitz, et al., 1993] with relatively few results from solution experiments. We have previously demonstrated one-metal-ion activation in the case of *E. coli* ribonuclease H [Black, et al., 1994c]. Herein we show that this is also the case for exonuclease III. This conclusion is again supported from the recent crystal structure, which shows that under the solution conditions employed only one metal binding site can be populated by a variety of added cofactors; including Mn$^{2+}$, Zn$^{2+}$ and Sm$^{2+}$ cations, although results with the natural Mg$^{2+}$ cofactor were not reported [Mol, et al., 1995]. It was also found that the electron density around the metal ion increased in the presence of bound nucleotide, and so it has been proposed that the cocrystallized nucleotide and one enzyme residue contribute the binding ligands to the metal cofactor.

Comparison of the apparent binding constants of substrates with different metal cofactors indicates a slight decrease in binding affinity with Cr(H$_2$O)$_6$$^{3+}$ (Table 10). This can be explained by the fact that a chromium(III)-bound water is ionized at pH 7.4 ($pK_a$ ~ 4), and so the bound hydroxide partially inhibits binding of the nucleic acid backbone. For both activities the metal inhibition constants ($K_i$) are similar to estimated constants for metal ion binding to nucleic acids [Black, et al., 1994b,c]. One very notable and surprising feature is the hyperactivity of Cr(H$_2$O)$_6$$^{3+}$ over Mg$^{2+}$. This will be discussed in
reference to the pH data below, but, in brief, we believe that the ionized water on the metal cofactor is taking an active role in base catalysis (the ionized water facilitates deprotonation of a nucleophilic H$_2$O) such that an increase in activity is observed relative to the normal enzyme-Mg$^{2+}$ mediated activity. This point is discussed in greater detail in the mechanistic section that immediately follows.

5.3.2 The Role of Ionizable Sites on the Enzyme and Metal Cofactor

As a further probe of catalytic activity, a comparative study was undertaken of the pH dependence of the initial velocity of reactions catalyzed by *E. coli* exonuclease III, relative to those for *E. coli* ribonuclease H. The pH dependence of initial velocity for *E. coli* ribonuclease H catalyzed hydrolysis of poly(A)-poly(dT) hybrid is illustrated in Figures 47 and 48. Figure 47 shows the titration of a residue which significantly affects Mg$^{2+}$-promoted turnover (Table 11). This single ionization constant appears to corresponds to the apparent pK$_a$ for Asp-10, since the pK$_a$ ~ 6.5 determined here correlates very well with the constant observed independently through $^{25}$Mg and $^1$H NMR studies [Huang, et al., 1994; Oda, et al., 1994]. This residue is also known to strongly influence Mg$^{2+}$ binding to the enzyme [Katayanagi, et al., 1993], and so the plot reflects titration of a metal binding residue. Similar pH titration studies using Cr(H$_2$O)$_6^{3+}$ as the metal cofactor gave rise to a distinct pH profile. The first ionization constant (pK$_{a1}$ ~ 3.2)
again corresponds to Asp-10, while the second (pKα2 ~ 7.2) correlates with the ionization of a second inner sphere water that results in a doubly ionized complex, \([\text{Cr(H}_2\text{O)}_4(\text{HO}^-)_2]^+\). The affinity of the enzyme for the monovalent complex is markedly reduced, and so activity falls off.

In contrast to these observations, *E. coli* exonuclease III behaves in a very distinct manner, insofar as \(\text{Cr(H}_2\text{O})_6^{3+}\) apparently activates the enzyme over the entire pH range (Figure 46), while the \(\text{Mg}^{2+}\) profile shows two apparent ionization constants of 7.4 and 7.8 (Figure 45 and Table 11). Based on the available crystal structure, several ionizable sites have been implicated in a hydrolytic reaction pathway. These include Asp 151, Asp 229, His 259, a bulk \(\text{H}_2\text{O}\), and \(\text{Mg}^{2+}(aq)\) [Mol, et al., 1995]. We ascribe the first ionization constant (pKα1 ~ 7.4) for the magnesium promoted reaction (Figure 45) to protonation of His 259 [Findlay, et al., 1961]. In contrast with results obtained with *E. coli* ribonuclease H, we do not appear to be influencing activity by perturbing the metal binding site; that is, by protonation of a metal binding residue that results in loss of both the metal cofactor and enzyme activity. The second ionization is less readily explained. Histidine is the only reasonable candidate for the ionizable site that results in the second ionization with pKα2 ~ 7.8, since Asp-229, \(\text{Mg}^{2+}(aq)\), and Glu-34 (bound to \(\text{Mg}^{2+}\)) have ionization constants well below and above the two observed constants. A distinct possibility was considered; namely, that the enzyme is irreversibly inactivated at higher
pH. To test this hypothesis, a solution of enzyme was equilibrated at a range of pH values between 9 and 12 for several minutes before lowering the pH to 7. It was found that the resulting activity of the enzyme (at pH 7) tracked the pH profile shown in Figure 45, and so it appears that the loss of activity results from irreversible inactivation of exonuclease III at high pH.

The inert complex Cr(H$_2$O)$_6^{3+}$ shows drastically different behavior for activation of \textit{E. coli} exonuclease III relative to the previously discussed data for \textit{E. coli} ribonuclease H, and is also distinct from the Mg$^{2+}$ activation profiles obtained for either enzyme. Moderate activity over the pH range 3.5 to 7.5 suggests that the inner sphere hydroxide can function as the H$^+$ acceptor (general base). Specifically, by having available an inner sphere hydroxide, significant activity can be observed over the entire pH range of interest, i.e. there is no dependence on His-259 acting as the general base. At higher pH values we might anticipate double ionization of the chromium complex, which would no longer bind to the enzyme. Unfortunately, the instability of the chromium complexes at higher pH precludes the rigorous testing of this idea. These data appear to suggest that for exonuclease III, unlike the situation for \textit{E. coli} ribonuclease H, the magnesium and chromium cofactors may act in a different manner. However, the details differ only with regard to the nature of the catalytic acids and bases. The essential mechanism of transition state stabilization through hydrogen bonding from solvent waters is maintained.
5.3.3 Evaluation of the Catalytic Mechanism for Metal-Promoted Hydrolysis Reactions

In contrast to previous observations with *E. coli* ribonuclease H [Black, et al., 1994c; Jou, et al., 1991; Black, et al., 1996a], neither of the inert Co(NH$_3$)$_6^{3+}$ or Cr(NH$_3$)$_6^{3+}$ complexes yielded measurable activity with *E. coli* exonuclease III. It is evident with exonuclease III that there is an absolute requirement for inner sphere water ligands, with $x \geq 4$ for Cr(NH$_3$)$_{6-x}$(H$_2$O)$_x^{3+}$. In contrast to *E. coli* ribonuclease H [Jou, et al., 1991; Black, et al., 1996a], the trivalent charge of the hexaammine complexes is insufficient for activity. Since the chromium(III) complexes with at least one water ligand have a bound HO' ($pK_a \sim 4$) [Rotzinger, et al., 1986], the net charge is identical to Mg$^{2+}$; and since no activity was observed for $x<4$, it can be concluded that the net charge on the complex is not the only issue here. Certainly a net divalent charge is required to promote binding to the enzyme, but this does not contribute to catalytic activation.

While the inactivity of the inert hexaammine complexes may appear to suggest an inner sphere model for catalysis, where the metal ion is bound directly to a phosphate oxygen on the substrate, this conclusion is not supported by other data. In particular, the inert chromium complexes [Cr(NH$_3$)$_{6-x}$(H$_2$O)$_x$.1(HO')]$^{3+}$ (where $x \geq 4$) do significantly promote enzyme activity (Tables 10, 12 and 13), but cannot exchange inner sphere
waters, and so catalysis must necessarily be occurring via outer sphere chemistry. This data reinforces the importance of the bound water molecules for activity. In addition to offering information about the number of water molecules required for activation \(x \geq 4\), the chromium(III) complexes provide further insight on metal-promoted exonuclease III activation. For \(\text{Cr}(\text{H}_2\text{O})_6^{3+}\) at least one inner sphere water remains ionized \((\text{pK}_a \sim 4)\).

Although the effective net charge is the same for both \(\text{Mg}^{2+}\) and \([\text{Cr}(\text{H}_2\text{O})_5(\text{HO}^-)]^{2+}\), the latter maintains a bound \(\text{OH}^-\). Since the observed turnover is on the order of 100 fold higher for \([\text{Cr}(\text{H}_2\text{O})_5(\text{HO}^-)]^{2+}\) over \(\text{Mg}^{2+}\), we propose that the inner sphere \(\text{OH}^-\) must be taking part in catalysis. This in itself does not suggest that \(\text{Mg}^{2+}\) acts in this manner (that is, through its inner sphere hydroxide) since the \(\text{pK}_a\) for a water bound to \(\text{Mg}^{2+}\) is 11.4.

The equilibrium concentration of \(\text{Mg-OH}^-\) species is low, and so the difference in rates is not readily explicable by the relative concentrations of \(\text{Mg-OH}^-\) and \(\text{Cr-OH}^-\) species in the active site pocket. Rather, this indicates that hydrogen bonding is critical for activity. In the case of \([\text{Cr}(\text{H}_2\text{O})_5(\text{HO}^-)]^{2+}\), an inner sphere hydroxide can increase catalysis over the wild type, possibly by circumventing the role of His 259 as a catalytic base (Figure 59).

An alternative is to consider nucleophilic attack by this bound hydroxide. This should result in products with bound chromium, however, no evidence for such product species were found.
5.3.4 Evaluation of Electrostatic and Hydrogen Bonding Contributions

Relatively high levels of activity are observed for both the exonuclease and RNase H activities for complexes carrying a significant hydration shell: such as $[\text{Mg}(\text{H}_2\text{O})_6]^{2+}$, $[\text{Cr}(\text{H}_2\text{O})_6(\text{HO}^-)]^{2+}$, $[\text{Cr}(\text{NH}_3)(\text{H}_2\text{O})_4(\text{HO}^-)]^{2+}$, cis- and trans-$[\text{Cr}(\text{NH}_3)_2(\text{H}_2\text{O})_3(\text{HO}^-)]^{2+}$. Michaelis constants ($K_m$) are found to be similar for turnover by each cofactor (79 mM, 72 mM, 71 mM, and 72 mM respectively, for the exonuclease activity; and 16 mM, 20 mM, 18 mM, and 18 mM respectively, for the RNase H activity). The other complexes $[\text{Cr}(\text{NH}_3)_{6-x}(\text{H}_2\text{O})_{x+1}(\text{HO}^-)]^{2+}$ (where $x < 4$) do not promote activity. Since the effective charge on these complexes is similar to those that activate the enzyme, these data clearly illustrate that stabilization of the transition state arises predominantly through hydrogen-bonding between the waters of hydration of the metal cofactor and the substrate (Figure 59). Moreover, since all of these complexes are formally “chromium-hydroxide” species, the absence of activity for the remaining complexes argues against the hydroxide as the reactive center that promotes activity. This is further indicated by the absence of enzyme activity with the trivalent complexes $\text{Cr}(\text{NH}_3)_6^{3+}$ or $\text{Co}(\text{NH}_3)_6^{3+}$. This contrasts with the results for $E. coli$ ribonuclease H where significant activation from these trivalents was observed since the electrostatic stabilization from the increased charge proved to be sufficient.
Figure 49. Lineweaver-Burk plot for the 3'-5' exonuclease activity for 500 nM *E. coli* exonuclease III with 10 mM Mg$^{2+}$ at $T = 25^\circ$C. The $K_m$ was $\sim 79 \mu$M.
Figure 50. Lineweaver-Burk plot for the 3'-5' exonuclease activity for 500 nM *E. coli* exonuclease III with 8 mM Cr(H$_2$O)$_5$(OH)$^{3+}$ at $T = 25^\circ$C. The $K_m$ was $\sim 72$ $\mu$M.
Figure 51. Lineweaver-Burk plot for the 3'-5' exonuclease activity for 500 nM *E. coli* exonuclease III with 8 mM Cr(H₂O)₆(NH₃)(OH')₃⁺ at T = 25°C. The $K_m$ was ~ 71 μM.
Figure 52. Lineweaver-Burk plot for the 3'−5' exonuclease activity for 500 nM *E. coli* exonuclease III with 8 mM cis-Cr(H$_2$O)$_3$(NH$_3$)$_2$(OH)$^{3+}$ at T = 25°C. The $K_m$ was ~ 71 μM.
Figure 53. Lineweaver-Burk plot for the 3'-5' exonuclease activity for 500 nM *E. coli* exonuclease III with 8 mM trans-Cr(H$_2$O)$_3$(NH$_3$)$_2$(OH)$^{3+}$ at $T = 25^\circ$C. The $K_m$ was $\sim 72$ $\mu$M.
Figure 54. Lineweaver-Burk plot for the RNase H activity for 500 nM *E. coli* exonuclease III with 10 mM Mg$^{2+}$ at T = 25°C. The $K_m$ was ~16 μM.
Figure 55. Lineweaver-Burk plot for the RNase H activity for 500 nM *E. coli* exonuclease III with 8 mM Cr(H$_2$O)$_5$(OH)$^{3+}$ at T = 25°C. The $K_m$ was ~ 16 μM.
Figure 56. Lineweaver-Burk plot for the RNase H activity for 500 nM *E. coli* exonuclease III with 8 mM Cr(H₂O)₄(NH₃)(OH⁻)³⁺ at T = 25°C. The Kₘ was ~ 20 μM.
Figure 57. Lineweaver-Burk plot for the RNase H activity for 500 nM *E. coli* exonuclease III with 8 mM cis-Cr(H$_2$O)$_3$(NH$_3$)$_2$(OH)$^{3+}$ at T = 25°C. The $K_m$ was ~ 18 $\mu$M.
Figure 58. Lineweaver-Burk plot for the RNase H activity for 500 nM *E. coli* exonuclease III with 8 mM trans-Cr(H₂O)₃(NH₃)₂(OH)₃⁺ at T = 25°C. The Kᵌ was ~ 18 μM.
5.3.5 Geometric Considerations

The availability of \([\text{Cr(NH}_3\text{)}_{6-x}(\text{H}_2\text{O})_x]^{3+}\) complexes of varying coordination geometry provides control over the disposition of H-bond interactions and affords a direct probe of the geometric preference for metal-bound water molecules. The crystallographically characterized coordination geometry of ligands to enzyme-bound \(\text{Mg}^{2+}\) is illustrated in Figure 59 [Mol, et al., 1995]. Although the substitutionally inert complexes are unable to coordinate directly to Glu34, we earlier argued that the metal binding pockets of surface magnesium sites typically show poor selectivity and can accommodate a large variation in metal size. The critical issue is the mode of interaction with the substrate. The activity of the inert cofactors clearly shows that direct coordination to the enzyme is not a prerequisite for enzyme activity, and is essentially irrelevant for a discussion of catalysis. The nature of the interaction between the metal cofactor and the nucleotide substrate is the critical issue. The metal binding protein residues simply serve to tether a metal cofactor to the catalytic pocket on the enzyme surface.

The data summarized in Tables 12 and 13 indicates a need for extensive metal solvation. This is consistent with the formation of an extensive hydrogen bonding network between the cofactor and substrate [Black, et al., 1994a,b] and is the expected mode of interaction on the basis of crystallography, NMR and calorimetric studies. In earlier studies of \(E.\ coli\) ribonuclease H we demonstrated the requirement for a facial
array of three water molecules to promote activity [Black, et al., 1996a]. This correlated well with the fact that three coordination sites on the natural magnesium cofactor were taken up by protein ligands, leaving a face of water molecules to interact with the substrate (Figure 59). Now we see a requirement for more extensive solvation of the metal cofactor, which correlates well with the single protein ligand to the natural cofactor, leaving a heavily solvated magnesium to interact with the substrate. There appears to be an interesting relationship between the requirements for metal-bound solvent interactions with the substrate and the coordination number with protein ligands, which ultimately controls the hydration state of the bound metal. This is addressed in the following section.

5.3.6 Comparison of Metal Binding Domains for E. coli Exonuclease III and Ribonuclease H. Insight on the Design of Cofactor Coordination Number

Our research efforts in this area have demonstrated the use of physicochemical methods to monitor magnesium binding chemistry (titration calorimetry, $^{25}\text{Mg NMR, } ^{1}\text{H NMR}$) [Black, et al., 1994b; Huang, et al., 1994], and have developed the use of inert inorganic complexes of defined coordination state to address mechanistic questions relating to magnesium biochemistry [Kim, et al., 1992; Black, et al., 1994b,c; Jou, et al., 1991]. Detailed studies on ribonuclease H have revealed the factors underlying metal-promoted catalysis, and allowed the rational design of new metal independent catalytic
pockets [Casareno, et al., 1995]. We believe that similar results can be obtained from other enzymes, assuming a fundamental understanding of metal-enzyme and metal-substrate interaction chemistry. The metal binding pockets for the magnesium-dependent enzymes ribonuclease H, exonuclease III (both summarized in Figure 59) [Mol, et al., 1995; Kanaya, et al., 1990], and the chemotaxal protein CheY differ markedly [Needham, et al., 1993]. A key issue in resolving the structure/function relations of these enzymes is raised by the following fact and questions. Both the coordination environment and charge density in the metal binding pockets are distinct (the number of carboxylate ligands varies as 3 x Asp for CheY, 1 x Asp, 1 x Glu and 1 x Gly carbonyl for ribonuclease H, and 1 x Glu for exonuclease III); nevertheless, the binding affinity for Mg$^{2+}$ is similar ($\sim 10^4 \text{ M}^{-1}$) for each of these magnesium binding proteins. For ribonuclease H and exonuclease III the $K_{MET}$ data in Table 10 indicates that the similarity in binding constants for these two active sites extends also to the inert chromium complexes. How does this arise, and why should it be so? One possible explanation for these observations is as follows. It appears that the solvation state of the metal cofactor is of critical importance for the proper functioning of these enzymes. The solvation state in turn is defined by the coordination state. Since the metal binding affinities of metal-dependent nucleases are tuned to the normal physiological concentration, Nature has evolved a structural mechanism that permits permutation of the ligand environment of the essential metal cofactor, while optimizing the binding affinity to physiological requirements. We have shown that the
electrostatic contribution to catalytic activation is smaller for exonuclease III than for ribonuclease H, inasmuch as trivalent hexaammine complexes activate the latter but not the former, and so it is tempting to speculate that this is offset by increasing the solvation state of the metal cofactor for exonuclease III to promote hydrogen bonding from the metal-bound water to the substrate. The experimental approach described herein affords a direct means of investigating these mechanisms in these and related enzymes.

5.4 Summary

It is clear that the waters of hydration for the essential metal cofactor are essential for activity, and that charge is relatively unimportant, serving only to electrostatically stabilize the metal-enzyme complex. Inner sphere ammines, having a reduced ability to hydrogen bond relative to water [Black, et al., 1994c], do not activate *E. coli* exonuclease III. Chromium complexes, Cr(NH₃)₆₋ₓ(H₂O)ₓ³⁺, where x<4, do not promote catalysis. Also, a higher charge on the metal cofactor does not compensate for the absence of hydrogen bonding propensity; neither Co(NH₃)₆³⁺ nor Cr(NH₃)₆³⁺ promote enzyme activity. Apparently hydrogen bonding is an *absolute requirement* for both the 3'-5' exonuclease and RNase H activities of exonuclease III. When an inner sphere hydroxide is available, then increased catalytic activity may result; however, as a result of the low equilibrium concentration of Mg²⁺-OH⁻, this hyperactivity is not observed for magnesium
promoted reactions. While the possibility of a structural role for the metal cofactor, involving reorientation of active site residues cannot be excluded, the mechanism of transition state stabilization illustrated in Figure 59, through outer sphere complex formation with the metal cofactor is most likely its dominant role. Finally, this work again demonstrates the utility of inert transition metal complexes for mechanistic studies of metallonucleases in nucleic acid biochemistry.

The major conclusions from this Chapter may be summarized as follows: (1) The metal cofactor appears to activate substrate by outer-sphere stabilization of the transition state; (2) The difference between the ligation states for bound Mg\textsuperscript{2+}, comparing the *E. coli* exonuclease III and ribonuclease H enzymes, stems from the requirement for a more extensively solvated metal cofactor for the former enzyme, which can only be achieved by limiting the number of protein ligands to the metal; (3) We have reasonably excluded the possibility of a two-metal-ion mechanism for exonuclease III by steady-state kinetic studies in solution. Crystallographic data alone cannot exclude the possible recruitment of a second ion in the presence of bound substrate; (4) The 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; (5) The phenomenon of metal-promoted substrate inhibition has been kinetically characterized; (6) 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.
Figure 59. Summary of the crystallographic coordination modes for magnesium binding to *E. coli* exonuclease III [Mol, et al., 1995] and ribonuclease H [Katayanagi, et al.,]
1993] illustrating the distinct solvation states of each ion. A proposed reaction scheme for exonuclease III shows the importance of outer-sphere hydrogen bonding interactions with the substrate. His-259 is the putative catalytic base [path (a)], although a metal-bound hydroxide [path (b)] is a possibility for the aquo-chromium complexes. Asp-151 is a putative proton donor to the product phosphate
CHAPTER VI
MAGNESIUM ACTIVATION OF THE 3'-5' EXONUCLEASE DOMAIN OF THE DNA POLYMERASE I KLENOW FRAGMENT. EVALUATION OF METAL ION STOICHIOMETRY IN SOLUTION BY CALORIMETRIC AND KINETIC EXPERIMENTS. EVIDENCE FOR ACTIVATION BY ONE METAL ION DURING TURNOVER

6.1 Introduction

The Klenow fragment of Escherichia coli DNA polymerase I maintains two distinct catalytic activities that are essential for the faithful replication of DNA [Ollis, et al., 1985]. The DNA polymerase and 3'-5' exonuclease functions work in synchrony to achieve polymerase fidelity of approximately one mismatch error in $10^{10}$ polymerized bases, even though they are spacially separated by 25-30 Å [Ollis, et al., 1985; Englisch, et al., 1985]. This is vastly superior to the basal level of ca. $10^2$ mismatch errors that is expected on the basis of the energy difference between correct and incorrect complementation of the bases [Loeb, et al., 1982]. The largest domain, which can be proteolytically cleaved, is known as the Klenow fragment. This fragment functions as a
Figure 60. Crystal structure of the Klenow fragment. The cleft at the top and left side are the 3'-5' exonuclease and polymerase active sites respectively. The fragment was cocrystallized with TMP (not shown) [Ollis, et al., 1985].
68,000 Da monomer and has been crystallographically characterized [Ollis, et al., 1985]. In an effort to evaluate the functional role of active site residues, several mutants of Klenow have been prepared and the structures solved [Derbyshire, et al., 1988]. Through this mutational analysis (D242A, D355A and E357A) it was determined that the active site binds two metal ions, and it has been suggested that one of these metal cofactors serves to aid binding of the substrate while the other plays a direct role in catalysis of the exonuclease reaction [Derbyshire, et al., 1988]. This result has found support from kinetic studies of native and mutant enzymes [Derbyshire, et al., 1988; Polesky, et al., 1990; Derbyshire, et al., 1991]. The published structural studies have delineated the coordination state of the bound metal cofactors, showing that one metal ion (metal A) remains slightly solvated with one metal bound water, while the other (metal B) is more extensively solvated maintaining 3 bound waters [Steitz, et al., 1993; Beese, et al., 1991; Freemont, et al., 1988]. The two metal ions are located at the exonuclease active site sharing Asp-355 as a bridging ligand [Derbyshire, et al., 1988; Steitz, et al., 1993].

Each of the three enzymatic activities of DNA polymerase I requires divalent cations for optimal function. In vivo this requirement is most likely met by divalent magnesium; the most abundant intracellular divalent cation and a potent activator of polymerase activity. However, all of the reported structural work has been carried out with Mn\(^{2+}\), while many of the published kinetic studies have utilized Mn\(^{2+}\) or Co\(^{2+}\). The x-ray crystal structure and site directed mutagenesis have shown two adjacent Mn\(^{2+}\) (in 50
mM MnSO₄) at the 3'-5' exonuclease site on the Klenow fragment [Ollis, et al., 1985; Derbyshire, et al., 1988]. Based on binding studies to mutant enzyme, it was determined that metal ion A (Mn²⁺) was bound more tightly to the Klenow fragment with a \( K_D \approx 2.5 \) mM [Mullen, et al., 1990]. Upon addition of substrate analogs (TMP, dGTP), a combined apparent \( K_D \approx 10 \) µM was measured for all of the bound metal ions [Mullen, et al., 1990]. This suggests that both metal cofactors interact with the substrate and that the weaker binding site (metal ion B) is created only upon substrate binding. On the basis of such data the 3'-5' exonuclease reaction has been proposed to proceed through a two-metal ion mechanism [Steitz, et al., 1993; Beese, et al., 1991]. Metal ion A is proposed to maintain the nucleophilic water which attacks the phosphate center, while metal ion B is believed to uphold the structural integrity of the enzyme-substrate complex and facilitate the 3' leaving group [Beese, et al., 1991]. It is thought that this two metal ion mechanism is likely to be ubiquitous in nucleic acid hydrolysis [Beese, et al., 1991], although there is an increasing volume of evidence to indicate that this notion is incorrect.

In recent years our laboratory has explored the biological chemistry of magnesium ion, and has developed strategies to investigate the structural and catalytic chemistry of this cation. In this chapter we have employed these methods to rigorously characterize the chemistry of the Klenow fragment with the natural divalent magnesium cofactor,
including calorimetric and kinetic studies of binding and turnover. A critical comparison is made with results obtained with the divalent analogues, Mn²⁺.

6.2 Results

Initial velocity plots were fit to equations that described activation of the Klenow fragment by either one or multiple metal ions. Equation (2-9) was used for all one metal ion fits. In Equation (2-9), \( k_{\text{cat}} \) is the maximal catalytic rate constant that would be obtained in the absence of the inhibitory influence of excess metal ion, \( K_{\text{MET}} \) is the observed binding affinity of the metal cofactor for the enzyme, \( K_m \) is the Michaelis constant for the substrate, \( K_i \) is the binding affinity of the metal cofactor for the substrate in the presence of a fixed enzyme concentration, and \([\text{Mg}^\circ], [E^\circ], \text{and } [S^\circ] \) are the total concentrations of magnesium, enzyme, and substrate present in the reaction solution. Inasmuch as the enzyme is pre-saturated with Mg²⁺, binding of substrate (S) to metal-free enzyme (E) can be ignored. The derivation of Equation (2-9) has been previously described in Chapter 2. To properly account for multiple metal ion activation, the scheme described in Chapter 2 must be modified. The modification is described in Scheme 5 where the fully-activated Klenow fragment binds \( n \) metal ions. This scheme is a generalized model of Equation (2-9) (where \( n=1 \)). Alternative models are considered and discussed at the end of this chapter.
Modification of Equation 2-9 with Scheme 5 accounts for multiple metal ion activation, with the possibility of distinct binding contacts, and yields Equation 6-1:

\[ v_o = \frac{v_{\text{max}} \cdot K_{\text{MET}} \cdot K_i \cdot S^0 \cdot (M^o)^n}{[1 + K_{\text{MET}} \cdot (M^o)^n][S^0 \cdot K_i + K_m \cdot (M^o + K_i)]} \]  

(6-1)

where the symbols are the same as that in Equation (2-9), except \( n \), which refers to the optimal number of metal ions necessary for catalytic turnover. Both Equations (2-9) and
(6-1) were used in the fitting of plots of initial velocity versus metal cofactor concentration.

6.2.1 Activity Studies with Inert Probe Complexes

In an effort to better characterize the metal dependence of the Klenow fragment, a combination of kinetic and thermodynamic data has been collected. Previously our laboratory has developed a general methodology for characterization of the active site chemistry of magnesium during enzymatic turnover of nucleic acids. This has yielded insight previously unavailable by use of chromium and cobalt exchange inert probes. It has been proposed for the Klenow fragment that a two metal ion mechanism occurs for the 3'-5' exonuclease reaction where both metals are bound through inner sphere contacts with the enzyme [Steitz, et al., 1993]. It was also seen from the crystal structure that the cations maintained partial hydration, although the mechanism involves direct attack by an activated water. The catalytic metal ion is thought to activate the nucleophilic water and also orient the phosphate center by ligation of the phosphate oxygen. The second metal ion is thought to stabilize the pentacoordinate intermediate and facilitate the 3' leaving group through ligation of the substrate (c.f. Figure 79). These proposed roles involve inner sphere contacts with the enzyme and also ground state catalysis. Assuming this mechanism to be correct, then exchange inert probes should be ineffective at hydrolysis due to their inability to directly ligate the substrate. This was born out insofar as
Cr(H$_2$O)$_6^{3+}$, Cr(H$_2$O)$_5$(NH$_3$)$_3^{3+}$, fac-Cr(H$_2$O)$_3$(NH$_3$)$_3^{3+}$, Cr(H$_2$O)(NH$_3$)$_5^{3+}$, Co(NH$_3$)$_6^{3+}$ and Cr(NH$_3$)$_6^{3+}$ did not mediate the 3’-5’ exonuclease reaction when used as metal cofactors. Only Mg$^{2+}$ and Mn$^{2+}$ cofactors showed catalytic turnover. Since the chromium and cobalt complexes are exchange inert, meaning no inner sphere contacts are possible, it clearly demonstrates that either inner sphere binding to the enzyme, or inner sphere catalysis (or a combination) is the dominant mode of action.

6.2.2 Kinetic Measurements

Table 14 summarizes the available data for Mn$^{2+}$ binding to DNA polymerase 1, the Klenow fragment, and Klenow mutants obtained by EPR measurements [Mullen, et al., 1990]. These experiments revealed one tight site in the 3’-5’ exonuclease active site pocket, and a weaker secondary binding site that is populated upon addition of TMP. Also, one divalent cation was found to bind at the polymerase active site through binding experiments to the exonuclease-deficient Klenow fragment (Table 14) [Mullen, et al., 1990]. Additional weaker sites were deduced from the binding data (not listed in Table 14) for the enzyme alone, which most likely correspond to the additional metal ions that are more clearly defined, with higher affinity, after addition of TMP. Binding of dGTP, which binds at the polymerase site, generates another metal binding site to yield a total of
Table 14. Metal binding parameters from previous work. All metal binding is to the “static” enzyme (not during turnover) and uses Mn\(^{2+}\). This table is reproduced from Mullen, et al., 1990. Symbols for the metal ions are: A for 3'-5' exonuclease metal ion A; B for 3'-5' exonuclease metal ion B; P for the metal ion at the polymerase site.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>n</th>
<th>(K_{\text{obs}}) ((\mu\text{M}))</th>
<th>Metal</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Polymerase I</td>
<td>-</td>
<td>~1</td>
<td>A</td>
</tr>
<tr>
<td>Klenow Fragment</td>
<td>-</td>
<td>~1</td>
<td>A</td>
</tr>
<tr>
<td>Klenow Fragment(^b)</td>
<td>TMP</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Klenow Fragment(^c)</td>
<td>TMP,dGTP</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Klenow Fragment (D424A)(^d)</td>
<td>-</td>
<td>1</td>
<td>67</td>
</tr>
<tr>
<td>Klenow Fragment (D424A)</td>
<td>TMP</td>
<td>~1</td>
<td>72</td>
</tr>
<tr>
<td>Klenow Fragment (D355A, E357A)(^e)</td>
<td>-</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Klenow Fragment (D355A, E357A)</td>
<td>dGTP</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

\(^a\) All conclusions refer to data obtained using Mn\(^{2+}\) EPR and fitted by Scatchard analysis. This table for Mn\(^{2+}\) binding is simply a summary of the most recent binding data for comparison [Mullen, et al., 1990].
b  TMP binds to the 3'-5' exonuclease site and is increases binding at that site [Derbyshire, et al., 1988].

c  dGTP binds to the polymerase site [Mullen, et al., 1989].

d  D424 binds only metal ion B.

e  D355 binds both metal ion A and B while E357 binds metal ion A only.
three metal ions on the Klenow fragment. Experiments with mutant proteins supported these conclusions (Table 14).

Previous to the work undertaken in this chapter, there has only been one study pertaining to the kinetic behavior of metal ions at the 3'-5' exonuclease active site of the Klenow fragment [Han, et al., 1991]. The substitution of Co$^{2+}$ and Mn$^{2+}$ employed in an attempt to understand coordination environment at the active site. In addition to these measurements, Hill coefficients, $K_{0.5}$ (concentration of cation yielding half-maximal rate), and turnover numbers for Co$^{2+}$, Mn$^{2+}$, and Mg$^{2+}$ were measured. These constants are comparable to those reported in Table 15, obtained from the rate dependence on substrate concentration. The analysis carried out in Tables 16 (and subsequent Tables) accounts for optimal metal ion concentration, substrate concentration, inhibition and stoichiometry during turnover. The measured $K_{0.5}$ (Mg$^{2+}$) = 0.34 mM and $k_{cat}$(Mg$^{2+}$) = 0.05 sec$^{-1}$ [Han, et al., 1991]. The half-saturation constant ($K_{0.5}$) is seen to be very similar to the value of $K_{MET}$ determined from our kinetic studies (Table 15). The turnover number determined by Han, et al. is a factor of two less than $k_{cat}$(opt) observed in Table 15. A complete discussion is made at the end of this chapter comparing the present work with the previous studies.

We have evaluated the metal ion stoichiometry during turnover by use of a kinetic approach that has been previously described in Chapter 2. The dependence of the initial velocity on substrate was examined and is summarized in Table 15 (Figures 61 and 62).
Kinetic activation parameters are summarized in Tables 16 and 17 for Mg$^{2+}$ and Mn$^{2+}$ cofactors. These parameters have not previously been reported in the literature, and so a direct comparison cannot be made. It is seen that Mn$^{2+}$ is slightly more effective than Mg$^{2+}$ in promoting hydrolysis of the substrate oligonucleotide. This is consistent with the observation made with other Mg$^{2+}$-dependent enzymes where Mn$^{2+}$ is used as an in vitro analogue cofactor. Typically, the observed turnover rate tends to increase for Mn$^{2+}$ relative to Mg$^{2+}$-promoted enzymatic hydrolysis [Taylor, et al., 1989], although this increase is often accompanied by a loss of specificity. Table 15, which gives the usual $k_{\text{cat}}$ determined from the substrate dependence of initial velocity, varies by a factor of 10 between the two cofactors, while the optimal rate constant is approximately the same within error, and so the difference in rate for the two cofactors appears to arise from differences in the inhibition reaction. Tables 16 and 17 summarizes values for $k_{\text{cat(opt)}}$ which is the catalytic rate constant that would be obtained under optimal metal and substrate concentrations in the absence of substrate inhibition.
Figure 61. Initial velocity dependence on the DNA oligonucleotide substrate concentration for the Klenow fragment. Conditions: [Klenow] = 800 nM, [Mg\(^{2+}\)] = 5 mM and, T = 37°C.
Figure 62. Initial velocity dependence on the DNA oligonucleotide substrate concentration for the Klenow fragment. Conditions: [Klenow] = 800 nM, [Mn$^{2+}$] = 5 mM and, T = 37°C.
Table 15. Michaelis-Menten kinetic parameters for the 3'-5' exonuclease activity of the Klenow fragment and metal cofactor varying DNA oligonucleotide.\(^8\)

<table>
<thead>
<tr>
<th></th>
<th>Mg(^{2+})</th>
<th>Mn(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\chi^2)</td>
<td>4.8x10(^{-5})</td>
<td>3.7x10(^{-3})</td>
</tr>
</tbody>
</table>

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(K_m) (µM)</td>
<td>60±18</td>
<td>25±7</td>
</tr>
<tr>
<td>(k_{cat}) (sec(^{-1}))</td>
<td>0.20±0.06</td>
<td>0.90±0.30</td>
</tr>
<tr>
<td>(k_{cat}/K_m) (sec(^{-1}) mM(^{-1}))</td>
<td>3.3</td>
<td>36</td>
</tr>
</tbody>
</table>

\(^a\) The concentration of Mg\(^{2+}\) and Mn\(^{2+}\) was 5 mM. The concentration of Klenow fragment in both cases was 800 nM. All solutions were incubated 5-10 mins at 37 °C prior to data collection.
Figure 63. Initial velocity dependence on Mg$^{2+}$ for the Klenow fragment. Conditions:

\[ [\text{Klenow}] = 800 \text{ nM}, \ [\text{oligonucleotide}] = 180 \mu\text{M}, \text{ and } T = 37^\circ\text{C}. \] Fits are to Equation (6-1) using \( n=1 \).
Figure 64. Initial velocity dependence on Mg\(^{2+}\) for the Klenow fragment. Conditions:

\([\text{Klenow}] = 800\ \text{nM}, [\text{oligonucleotide}] = 180\ \mu\text{M}, \text{ and } T = 37^\circ\text{C}\). Fits are to Equation (6-1) using \(n=2\).
Figure 65. Initial velocity dependence on Mn$^{2+}$ for the Klenow fragment. Conditions: [Klenow] = 800 nM, [oligonucleotide] = 160 μM, and T = 37°C. Fits are to Equation (6-1) using n=1.
Figure 66. Initial velocity dependence on Mn$^{2+}$ for the Klenow fragment. Conditions: [Klenow] = 800 nM, [oligonucleotide] = 160 μM, and T = 37°C. Fits are to Equation (6-1) using n=2.
Table 16. Steady-state kinetic parameters for Mg\textsuperscript{2+} dependence of the 3',5'-exonuclease activity of the Klenow fragment.a

<table>
<thead>
<tr>
<th></th>
<th>Mg\textsuperscript{2+} (n\textsubscript{tot}=1)</th>
<th>Mg\textsuperscript{2+} (n\textsubscript{tot}=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\chi^2$</td>
<td>2.8 x 10\textsuperscript{-9}</td>
<td>3.0 x 10\textsuperscript{-9}</td>
</tr>
<tr>
<td>n (fixed)\textsuperscript{b}</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>$K_m$ (μM) (fixed)\textsuperscript{c}</td>
<td>80±24</td>
<td>80±24</td>
</tr>
<tr>
<td>$k_{cat}$ (opt) (sec\textsuperscript{-1})</td>
<td>1.1±0.3</td>
<td>1.1±0.3</td>
</tr>
<tr>
<td>$K_{MET}$ (mM)</td>
<td>0.48±0.14</td>
<td>0.086±0.026</td>
</tr>
<tr>
<td>$K_i$ (mM)</td>
<td>41±12</td>
<td>42±12</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The concentration of single stranded DNA oligonucleotide (prepared by IDT) for Mg\textsuperscript{2+} activation was 180 μM. The concentration of Klenow fragment in both cases was 430 nM. All solutions were incubated 5-10 mins at 37 °C prior to data collection. All fitting results are from simulation using Equation (6-1). The bold column represents the fitting results which most accurately characterize the metal cofactor interactions.
For fitting, the \( n \) parameter was fixed at values of 1 or 2 to represent the number of activating metal ions.

For the purposes of fitting, \( K_m \), the Michaelis-Menten constant, was fixed. In addition, \( K_1 \) was determined first by fitting to the one metal ion equation where this term does not change upon successive metals binding to the enzyme. The two metal ion equation was used for the above parameters for which only \( k_{cat}(opt) \), \( K_{MET1} \) and \( K_{MET2} \) were allowed to vary.
Table 17. Steady-state kinetic parameters for Mn$^{2+}$ dependence of the 3'-5' exonuclease activity of the Klenow fragment.$^a$

<table>
<thead>
<tr>
<th></th>
<th>Mn$^{2+}$ (n$_{tot}$=1)</th>
<th>Mn$^{2+}$ (n$_{tot}$=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\chi^2$</td>
<td>$3.2 \times 10^{-9}$</td>
<td>$3.9 \times 10^{-9}$</td>
</tr>
<tr>
<td>$n$ (fixed)$^b$</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>$K_m$ (μM) (fixed)$^c$</td>
<td>$25\pm7$</td>
<td>$25\pm7$</td>
</tr>
<tr>
<td>$k_{cat(opt)}$ (sec$^{-1}$)</td>
<td>$3.6\pm1.1$</td>
<td>$0.70\pm0.21$</td>
</tr>
<tr>
<td>$K_{MET}$ (mM)</td>
<td>$0.84\pm0.25$</td>
<td>$0.74\pm0.22$</td>
</tr>
<tr>
<td>$K_i$ (mM)</td>
<td>$0.90\pm0.27$</td>
<td>$8.2\pm2.5$</td>
</tr>
</tbody>
</table>

$^a$ The concentration of single stranded DNA oligonucleotide (prepared by IDT) for Mn$^{2+}$ activation was 160 μM bases. The concentration of Klenow fragment in both cases was 430 nM. All solutions were incubated 5-10 mins at 37 °C prior to data collection. All fitting results are from simulation using Equation (6-1). The bold column represents the fitting results which most accurately characterize the metal cofactor interactions.
b For fitting, the n parameter was fixed at values of 1 or 2 to represent the number of activating metal ions.

c For the purposes of fitting, $K_m$, the Michaelis-Menten constant, was fixed. In addition, $K_i$ was determined first by fitting to the one metal ion equation where this term does not change upon successive metals binding to the enzyme. The two metal ion equation was used for the above parameters for which only $k_{cat(\text{opt})}$, $K_{\text{MET1}}$, and $K_{\text{MET2}}$ were allowed to vary.
Fitting Results

The kinetic data shown in Tables 16 and 17 represents activation by both Mg$^{2+}$ and Mn$^{2+}$ cofactors for the 3'-5' exonuclease activity of DNA polymerase I. After generating the rate dependence on substrate concentration (Table 15), the fitted $K_m$ was used as a fixed parameter for the metal dependence fits (Tables 16 and 17). For both cofactors, the plots were fit to Equation (6-1) fixing n to 1 or 2. For Mg$^{2+}$ activation, it is clear that there is only one metal ion hydrolytically important for turnover. The Mg$^{2+}$ dependent kinetic data for one metal ion activation are reasonable as compared to magnesium binding proteins as well as previously determined inhibition constants ($K_i$) from our lab [Black, et al., 1994a; Black, et al., 1994b; Black, et al., 1994c]. Both one and two metal ion activation was considered through use of Equation (6-1). If the assumption that two metal ions are necessary for complete activation, then the observed binding constant reflects two identical sites where the true binding constant is the square of the observed binding constant. From Table 16, this translates into $K_{\text{MET}}=(K_{\text{obs}})^2$, or $K_{\text{MET}}=(86 \mu M)^{\frac{1}{2}}$, a fitted dissociation constant of $\sim 9$ mM for each metal ion (Table 16). Such a low affinity binding constant is unreasonable insofar as dissociation constants rarely exceed 4 mM for magnesium-binding proteins [Black, et al., 1994a]. Meaning, both metal ions bind 4-5 Å apart in the same active site pocket and must maintain little or no electrostatic repulsion due to identical dissociation constants. This mechanistic scheme where both cations maintain the same binding constant is highly speculative. In
fact, it was previously observed binding Mg$^{2+}$ to xylose isomerase, that a 60-fold decrease between $K_1$ and $K_2$ is measured (separate metal binding constants) where both metal ions constitute a binuclear active site [Van Bastelacre, et al., 1992; Lambeir, et al., 1992]. Binding of both metal ions at one active site (e.g. 3’-5’ exonuclease site) should result in successive decreased binding due to electrostatic repulsion unlike what was observed for the two metal ion fit (Table 16). For the assumption that n=1, Equation (6-1) yields a fitted binding constant reflective of the catalytically significant divalent cation. If, in fact for n=1, two metal ions are necessary for activation, then only the weaker metal ion would be observed. Only when this lower affinity metal ion was bound would catalytic turnover occur. Therefore, the dissociation constant for the one metal ion fit, $K_{MET} = 0.48$ mM, reflects binding of the catalytically important magnesium cation.

The fitting results for Mn$^{2+}$ activation are shown in Table 17, where both one and two metal ion activation were considered. Unlike Mg$^{2+}$, there is not a clear difference between the two sets of parameters. While the binding constants are similar for both sets of numbers, there is a difference between the inhibition ($K_i$) and $k_{cat(opt)}$ parameters. The $k_{cat(opt)}$ for Mn$^{2+}$ would be expected to be similar to Mg$^{2+}$ in accordance with previous substitutions using both cofactors Mn$^{2+}$ [Mullen, et al., 1990]. Only in this respect is assuming one metal ion activation more reasonable than the two metal ion case. On the basis of the crystal structure doped with MnSO$_4$, it has been proposed that a binuclear site carries out catalysis. We find no reason to exclude this possibility for
Mn$^{2+}$, however this is certainly not the case with Mg$^{2+}$. This most probably this reflects the propensity of Mn$^{2+}$ in forming multinuclear centers.

Tables 16 and 17 summarizes the binding parameters for Mg$^{2+}$ and Mn$^{2+}$ in terms of the model summarized in Scheme 5 and Equation (6-1). Previously, binding of Mn$^{2+}$ has been monitored by EPR in terms of a model that assumes a strong and a weak binding site, which gave rise to an apparent binding constant of $\sim$ 10 µM (upon addition of TMP which binds at the 3'-5' exonuclease site) [Taylor, et al., 1989]. Kinetic analysis described previously suggests an order of magnitude difference in the binding affinity of the strong and weak sites for the exonuclease domains [Taylor, et al., 1989]. In making comparison to previous work, it is necessary to note that the Mn$^{2+}$ binding constants reported in this work (Table 17) reflect the binding necessary *during turnover* and not binding to the "static" enzyme. In comparison with Mg$^{2+}$, slightly tighter binding to Klenow is observed ($K_{MET}=0.48$ mM from Table 16). Since this is believed to be site A in the crystal structure (the tighter bound metal), Mg$^{2+}$ being slightly smaller ($\sim$ 0.1 Å), possibly fits better into the pocket. The crystal structure shows a metal ion with only one bound water, three enzyme binding and one substrate contact which translates into a well-defined pocket [Steitz, et al., 1993; Beese, et al., 1991]. The inhibition constant ($K_{i}$) reflects population of binding sites on the nucleic acid substrate. In both of the Mn$^{2+}$ and
Mg$^{2+}$ cases, these values are similar to metal ion binding constants to nucleic acids previously measured [Black, et al., 1994b].

Comparison of the Metal Cofactors

Table 14 summarizes the available data for Mn$^{2+}$ binding to DNA polymerase I, the Klenow fragment, and Klenow mutants obtained by EPR measurements [Mullen, et al., 1990]. Since Mg$^{2+}$ is an EPR silent metal ion it is not amenable to this approach. Rather, we have evaluated the metal ion stoichiometry during turnover by use of kinetic approach that has been previously reported from our laboratory. Kinetic activation parameters are summarized in Tables 16 and 17 for Mg$^{2+}$ and Mn$^{2+}$ cofactors. These parameters have not previously been reported in the literature, and so a direct comparison cannot be made. It is seen that Mn$^{2+}$ is slightly more effective than Mg$^{2+}$ in promoting hydrolysis of the substrate oligonucleotide. This is consistent with the observation made with other Mg$^{2+}$-dependent enzymes where Mn$^{2+}$ is used as an in vitro analogue cofactor. Typically, the observed turnover rate (not k$_{cat}$(opt)) tends to increase for Mn$^{2+}$ relative to Mg$^{2+}$-promoted enzymatic hydrolysis [Taylor, et al., 1989], although usually this increase in is accompanied by a loss in specificity [Cowan, et al., 1995]. Table 16 summarizes values for k$_{cat}$(opt) which is the catalytic rate constant that would be obtained under optimal metal and substrate concentrations in the absence of substrate inhibition. Table
15, which is the usual $k_{\text{cat}}$ from substrate dependence, varies by a factor of 10 between the two cofactors while the optimal rate constant is approximately the same within error, and so the difference in rate for the two cofactors appears to arise from differences in the inhibition reaction.

### 6.2.3 Calorimetric Measurements

Calorimetric investigations have been carried out on the Klenow fragment in the presence and absence of thymidine monophosphate. In the absence of this substrate mimic two binding sites are evident. These correspond to the tight binding site in the exonuclease domain, and the weaker site in the polymerase domain, respectively. In each case Mn$^{2+}$ binds with a higher affinity than Mg$^{2+}$. It is noteworthy that in contrast to Mn$^{2+}$ binding, Mg$^{2+}$ interacts with both sites in an endothermic fashion. While this is not unusual for inner-sphere magnesium-phosphate complexes, it is rarely the case for Mg$^{2+}$-protein interactions. In previous examples studied (viz. *E. coli* ribonuclease H and exonuclease III) the enthalpy of binding was exothermic. For each binding site, and for both Mg$^{2+}$ and Mn$^{2+}$, the entropy change was positive, and consistent with loss of coordinated water molecules and solvent displacement from the metal binding pockets.
Fitting Results

To answer the question of metal ion stoichiometry, all of the titration plots were fit to a two site model, representing the polymerase domain and the exonuclease domain (Tables 18-21). Under this two site model, to determine the stoichiometry at the exonuclease site, the constraints \( n_2 = n_1 \) and \( n_2 = 2 \times n_1 \) were used. One stoichiometric possibility, however, is that \( n_1 \) represents a high affinity cation at the exonuclease active site, while \( n_2 \) represents both a cation binding at the polymerase site and a cation at the exonuclease site (assuming a total of 3 cations). While it is difficult to assign binding sites based on thermodynamic constants, for a total of 3 cations, two of those cations must bind with identical thermodynamic parameters. The likelihood of a cation binding at the polymerase site and a cation binding at the exonuclease site, generating the exact same thermodynamic parameters, is extremely improbable. Therefore \( n_1 \) represents, in our model, the polymerase site and \( n_2 \) represents the exonuclease site, which is related to the former by integer values. After fixing \( n_2 \), \( n_1 \) was allowed to vary, obtaining thermodynamic parameters for both cases mentioned above (Tables 18-21). See Chapter 2 for details on all calorimetric methods and equations.

Tables 18 and 19 show the fitting results for Mg\(^{2+}\) and Mn\(^{2+}\) binding to the Klenow fragment. The parameter \( n_{\text{tot}} \) refers to the number of thermodynamically bound metal ions to the entire Klenow fragment. This number therefore encompasses both the polymerase and exonuclease active sites. For Mg\(^{2+}\), the case where \( n_{\text{tot}} = 2 \) (in bold)
matches more closely the kinetic parameters in Table 16 than for the case where \( n_{\text{tot}} = 3 \). This is evidenced insofar as \( K_2 (\text{Mg}^{2+} \text{ binding at the exonuclease site}) \) maintains a dissociation constant of 0.4 mM (Table 18) where the kinetically determined constant is 0.48 mM (Table 16). It is noteworthy that for the \( n_{\text{tot}} = 3 \) case, the polymerase site maintains a slightly higher than usual binding constant (\( K_1 \)) for magnesium binding proteins [Black, et al., 1994a; Cowan, et al., 1995]. Also, comparing the two cases in Table 18, the exonuclease site has an observed binding constant (\( K_2 \)) which does not change significantly between fits (Table 18) and is similar to the kinetically determined binding constant (Table 16). Binding a second metal ion should result in a decrease of the observed binding constant. Therefore we view the case where two \( \text{Mg}^{2+} \) bound at the exonuclease site to have unreasonable binding parameters which are not consistent with the literature or the kinetic data.

From previous work, titration of \( \text{Mn}^{2+} \) to a mixture of thymidine monophosphate (TMP) has been shown to create an additional metal binding site at the exonuclease domain and not the polymerase domain (Table 14) [Mullen, et al., 1990]. To investigate this, titrations were carried out for both \( \text{Mg}^{2+} \) and \( \text{Mn}^{2+} \) cofactors using titration calorimetry. In the presence of TMP, it is seen that one \( \text{Mg}^{2+} \) binds at each of the active sites and the binding constants are slightly augmented (Table 20). In addition, the metal ion bound at the polymerase site (\( K_1 \)) increases its affinity by almost an order of magnitude over the case without TMP and binding at the exonuclease site increases by a
factor of two (comparing Tables 18 and 20). Fitting for two metal ions at the exonuclease site ($n_{\text{tot}}=3$) yields a poorer fit result, with the metal ion at the exonuclease site becoming extremely tight ($8.2 \times 10^3 \text{ M}^{-1}$) and the polymerase site dropping to a low $\mu$M dissociation constant (Table 20).

Due to the prevalence of binding data in the literature using Mn$^{2+}$, titrations were carried out with this cofactor. In comparison to Mg$^{2+}$, the binding constant for the polymerase site ($n_1$) is slightly higher while binding to the exonuclease site ($n_2$) is similar, consistent with the kinetic data. For Mn$^{2+}$ binding to Klenow in the absence of TMP (Table 19), the constants for $n_{\text{tot}}=2$ (in bold) appear more reasonable than for $n_{\text{tot}}=3$. Comparison with the kinetically determined constants listed in Table 17, the case where one Mn$^{2+}$ binds to each active site ($n_{\text{tot}}=2$) shows more consistency. This does not however rule out the case where two Mn$^{2+}$ bind to the exonuclease site, although the binding constants for the polymerase site and the exonuclease site approaches unreasonable limits which do not correlate with the kinetic data. Titration of Mn$^{2+}$ with a mixture of TMP and Klenow yields the fitting constants in Table 21. Here, the binding constants for both cases seem reasonable insofar as Mn$^{2+}$ has been known to bind more strongly than Mg$^{2+}$ in the presence of TMP [Mullen, et al., 1990]. Similar binding is observed with and without the addition of TMP at the polymerase site while binding at the exonuclease site slightly increases. In summary, it is clear that with and without TMP, only one Mg$^{2+}$ thermodynamically binds to the Klenow fragment. The case is
slightly different with Mn$^{2+}$ insofar as without TMP one Mn$^{2+}$ yields the most reasonable fit, while in the presence of TMP it becomes less obvious raising the possibility for two-metal ion binding. Two metal ion binding in the presence of TMP has been observed in the crystal structure and in other binding studies [Mullen, et al., 1990; Han, et al., 1991; Derbyshire, et al., 1988].

In addition to the metal binding data discussed above, enthalpic and entropic components have been estimated as part of the fitting results. For brevity, only the bold-faced results will be discussed from Tables 18-21. The most striking feature in comparing the Mg$^{2+}$ and Mn$^{2+}$ titration plots is that Mg$^{2+}$ binding to Klenow is endothermic while Mn$^{2+}$ binding is exothermically driven. While this is not unusual for inner-sphere magnesium-phosphate complexes, it is rarely the case for Mg$^{2+}$-protein interactions. In previous examples studied (viz. E. coli ribonuclease H and exonuclease III) the enthalpy of binding was exothermic. Although for each binding site, and for both Mg$^{2+}$ and Mn$^{2+}$, the entropy change was positive, and consistent with loss of coordinated water molecules and solvent displacement from the metal binding pockets. It is noteworthy that in the presence of TMP both Mg$^{2+}$ and Mn$^{2+}$ yield enthalpically driven binding at the polymerase site and entropically driven binding at the exonuclease site (Tables 20 and 21). This is consistent with the model developed previously where TMP binds at the exonuclease site replacing metal bound waters with substrate contacts [Derbyshire, et al., 1988; Mullen, et al., 1990].
Figure 67. Calorimetric titration curve for Mg\(^{2+}\) binding to Klenow. The top portion is the raw binding data while the lower is the integrated heats of binding. The fit shown is using an \(n_{\text{tot}}=2\). Conditions were: [Klenow] = 0.3 mM, [Mg\(^{2+}\)] = 12.5 mM and \(T = 25^\circ\text{C}\).
Figure 68. Calorimetric titration curve for Mg$^{2+}$ binding to Klenow. The data points represent the integrated heats of binding. The fit shown is using an $n_{tot}=3$. Conditions were: [Klenow] = 0.3 mM, [Mg$^{2+}$] = 12.5 mM and $T = 25^\circ$C.
Figure 69. Calorimetric titration curve for Mn$^{2+}$ binding to Klenow. The top portion is the raw binding data while the lower is the integrated heats of binding. The fit shown is using an $n_{tot}=2$. Conditions were: [Klenow] = 0.3 mM, [Mn$^{2+}$] = 10 mM and $T = 25^\circ$C.
Figure 70. Calorimetric titration curve for Mn$^{2+}$ binding to Klenow. The data points represent the integrated heats of binding. The fit shown is using an $n_{tot}=3$. Conditions were: [Klenow] = 0.3 mM, [Mn$^{2+}$] = 10 mM and $T = 25^\circ$C.
Table 18. Thermodynamic Binding Constants from Mg$^{2+}$ Binding to the Klenow Fragment.$^a$

<table>
<thead>
<tr>
<th></th>
<th>$\text{Mg}^{2+}$ (n$_{\text{tot}}$=2)</th>
<th>$\text{Mg}^{2+}$ (n$_{\text{tot}}$=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\chi^2$</td>
<td>69</td>
<td>62</td>
</tr>
<tr>
<td>$n_1$</td>
<td>1.02</td>
<td>1.02</td>
</tr>
<tr>
<td>$K_1$ (M$^{-1}$)</td>
<td>$6.1 \times 10^3$</td>
<td>$3.0 \times 10^4$</td>
</tr>
<tr>
<td>$\Delta G^o_1$</td>
<td>-5.2</td>
<td>-6.1</td>
</tr>
<tr>
<td>$\Delta H^o_1$</td>
<td>1.7</td>
<td>0.93</td>
</tr>
<tr>
<td>$\Delta S^o_1$</td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td>$n_2^b$</td>
<td>$n_1$</td>
<td>$2 \times n_1$</td>
</tr>
<tr>
<td>$K_2$ (M$^{-1}$)</td>
<td>$2.5 \times 10^3$</td>
<td>$3.1 \times 10^3$</td>
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<tr>
<td>$\Delta G^o_2$</td>
<td>-4.6</td>
<td>-4.8</td>
</tr>
<tr>
<td>$\Delta H^o_2$</td>
<td>1.0</td>
<td>0.86</td>
</tr>
<tr>
<td>$\Delta S^o_2$</td>
<td>19</td>
<td>19</td>
</tr>
</tbody>
</table>
Both fitting results are shown for comparison purposes. Conditions were: [Klenow] = 0.2 mM, [Mg$^{2+}$] = 12.5 mM at 25°C. All titrations were carried out in 20 mM Tris, pH 7.4, 50 mM KCl and 1 mM DTT. The bold represents the fitting results which best characterize the metal cofactor interactions.

$n_2$ was assigned either $n_1$ or $2n_1$ for the fits due to the fact this will be populated in integer values of $n_1$. The term $n_1$ was varied for all fits.
Table 19. Thermodynamic Binding Constants from Mn$^{2+}$ Binding to the Klenow Fragment.$^a$

<table>
<thead>
<tr>
<th></th>
<th>Mn$^{2+}$ ($n_{tot}=2$)</th>
<th>Mn$^{2+}$ ($n_{tot}=3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\chi^2$</td>
<td>2763</td>
<td>2680</td>
</tr>
<tr>
<td>$n_1$</td>
<td>1.04</td>
<td>0.94</td>
</tr>
<tr>
<td>$K_1$ (M$^{-1}$)</td>
<td>4.9 x 10$^4$</td>
<td>3.4 x 10$^5$</td>
</tr>
<tr>
<td>$\Delta G^o_1$ (kcal mol$^{-1}$)</td>
<td>-6.4</td>
<td>-7.5</td>
</tr>
<tr>
<td>$\Delta H^o_1$ (kcal mol$^{-1}$)</td>
<td>-5.9</td>
<td>-4.3</td>
</tr>
<tr>
<td>$\Delta S^o_1$ (cal K$^{-1}$ mol$^{-1}$)</td>
<td>1.7</td>
<td>11</td>
</tr>
<tr>
<td>$n_2$</td>
<td>$n_1$</td>
<td>2 x $n_1$</td>
</tr>
<tr>
<td>$K_2$ (M$^{-1}$)</td>
<td>1.8 x 10$^3$</td>
<td>4.9 x 10$^3$</td>
</tr>
<tr>
<td>$\Delta G^o_2$ (kcal mol$^{-1}$)</td>
<td>-4.4</td>
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</tr>
<tr>
<td>$\Delta H^o_2$ (kcal mol$^{-1}$)</td>
<td>-1.8</td>
<td>-1.1</td>
</tr>
<tr>
<td>$\Delta S^o_2$ (cal K$^{-1}$ mol$^{-1}$)</td>
<td>8.7</td>
<td>13</td>
</tr>
</tbody>
</table>

$^a$
Both fitting results are shown for comparison purposes. Conditions were: [Klenow] = 0.1 mM, [Mn$^{2+}$] = 10 mM at 25°C. All titrations were carried out in 20 mM Tris, pH 7.4, 50 mM KCl and 1 mM DTT. The bold represents the fitting results which best characterize the metal cofactor interactions.

$n_2$ was assigned either $n_1$ or $2n_1$ for the fits due to the fact this will be populated in integer values of $n_1$. The term $n_1$ was varied for all fits.
Figure 71. Calorimetric titration curve for Mg$^{2+}$ binding to Klenow in the presence of TMP. The top portion is the raw binding data while the lower is the integrated heats of binding. The fit shown is using an $n_{tot}=2$. Conditions: [Klenow] = 0.2 mM, [Mg$^{2+}$] = 12.5 mM, [TMP]=0.2 mM and $T = 25^\circ$C.
Figure 72. Calorimetric titration curve for Mg\(^{2+}\) binding to Klenow in the presence of TMP. The data points represent the integrated heats of binding. The fit shown is using an \(n_{\text{tot}}=3\). Conditions: [Klenow] = 0.2 mM, [Mg\(^{2+}\)] = 12.5 mM, [TMP]=0.2 mM and \(T = 25^\circ\text{C}\).
Figure 73. Calorimetric titration curve for Mn$^{2+}$ binding to Klenow in the presence of TMP. The top portion is the raw binding data while the lower is the integrated heats of binding. The fit shown is using an $n_{	ext{tot}}=2$. Conditions: [Klenow] = 0.2 mM, [Mn$^{2+}$] = 10 mM, [TMP]=0.2 mM and $T = 25^\circ\text{C}$. 
Figure 74. Calorimetric titration curve for Mn$^{2+}$ binding to Klenow in the presence of TMP. The data points represent the integrated heats of binding. The fit shown is using an $n_{tot}=3$. Conditions: [Klenow] = 0.2 mM, [Mn$^{2+}$] = 10 mM, [TMP]=0.2 mM and $T = 25^\circ$C.
Table 20. Thermodynamic Binding Constants from Mg\(^{2+}\) Binding to the Klenow Fragment in the presence of TMP.\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Mg(^{2+}) (n(_{\text{tot}})=2)</th>
<th>Mg(^{2+}) (n(_{\text{tot}})=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\chi^2)</td>
<td>290</td>
<td>263</td>
</tr>
<tr>
<td>(n_1)</td>
<td>0.83</td>
<td>0.70</td>
</tr>
<tr>
<td>(K_1) (M(^{-1}))</td>
<td>(1.2 \times 10^4)</td>
<td>(4.9 \times 10^4)</td>
</tr>
<tr>
<td>(\Delta G^\circ_1) (kcal mol(^{-1}))</td>
<td>-5.6</td>
<td>-6.4</td>
</tr>
<tr>
<td>(\Delta H^\circ_1) (kcal mol(^{-1}))</td>
<td>1.6</td>
<td>1.1</td>
</tr>
<tr>
<td>(\Delta S^\circ_1) (cal K(^{-1}) mol(^{-1}))</td>
<td>24</td>
<td>25</td>
</tr>
<tr>
<td>(n_2)</td>
<td>(n_1)</td>
<td>(2 \times n_1)</td>
</tr>
<tr>
<td>(K_2) (M(^{-1}))</td>
<td>(5.2 \times 10^3)</td>
<td>(8.2 \times 10^3)</td>
</tr>
<tr>
<td>(\Delta G^\circ_2) (kcal mol(^{-1}))</td>
<td>-5.1</td>
<td>-5.3</td>
</tr>
<tr>
<td>(\Delta H^\circ_2) (kcal mol(^{-1}))</td>
<td>0.16</td>
<td>0.50</td>
</tr>
<tr>
<td>(\Delta S^\circ_2) (cal K(^{-1}) mol(^{-1}))</td>
<td>18</td>
<td>19</td>
</tr>
</tbody>
</table>
Both fitting results are shown for comparison purposes. Conditions were: [Klenow] = 0.2 mM, [Mg$^{2+}$] = 12.5 mM, and [TMP] = 0.2 mM at 25°C. All titrations were carried out in 20 mM Tris, pH 7.4, 50 mM KCl and 1 mM DTT. The bold represents the fitting results which best characterize the metal cofactor interactions.

$n_2$ was assigned either $n_1$ or $2n_1$ for the fits due to the fact this will be populated in integer values of $n_1$. The term $n_1$ was varied for all fits.
<table>
<thead>
<tr>
<th></th>
<th>Mn(^{2+}) ((n_{\text{tot}}=2))</th>
<th>Mn(^{2+}) ((n_{\text{tot}}=3))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n_1)</td>
<td>0.81</td>
<td>0.91</td>
</tr>
<tr>
<td>(K_1 \text{ (M}^{-1})</td>
<td>(3.9 \times 10^4)</td>
<td>(5.0 \times 10^4)</td>
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<tr>
<td>(\Delta G_{\text{o},1} \text{ (kcal mol}^{-1})</td>
<td>-6.3</td>
<td>-6.4</td>
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<tr>
<td>(\Delta H_{\text{o},1} \text{ (kcal mol}^{-1})</td>
<td>-6.5</td>
<td>-5.6</td>
</tr>
<tr>
<td>(\Delta S_{\text{o},1} \text{ (cal K}^{-1} \text{ mol}^{-1})</td>
<td>-0.7</td>
<td>2.7</td>
</tr>
<tr>
<td>(n_2^b)</td>
<td>(n_1)</td>
<td>(2 \times n_1)</td>
</tr>
<tr>
<td>(K_2 \text{ (M}^{-1})</td>
<td>(1.4 \times 10^4)</td>
<td>(4.8 \times 10^3)</td>
</tr>
<tr>
<td>(\Delta G_{\text{o},2} \text{ (kcal mol}^{-1})</td>
<td>-5.6</td>
<td>-5.0</td>
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<tr>
<td>(\Delta H_{\text{o},2} \text{ (kcal mol}^{-1})</td>
<td>-0.26</td>
<td>0.47</td>
</tr>
<tr>
<td>(\Delta S_{\text{o},2} \text{ (cal K}^{-1} \text{ mol}^{-1})</td>
<td>18</td>
<td>18</td>
</tr>
</tbody>
</table>
Both fitting results are shown for comparison purposes. Conditions were: [Klenow] = 0.2 mM, [Mn$^{2+}$] = 10 mM, and [TMP] = 0.2 mM at 25°C. All titrations were carried out in 20 mM Tris, pH 7.4, 50 mM KCl and 1 mM DTT. The bold represents the fitting results which best characterize the metal cofactor interactions. In this case, neither fitting result conclusively demonstrates the dominant stoichiometry.

$n_2$ was assigned either $n_1$ or $2n_1$ for the fits due to the fact this will be populated in integer values of $n_1$. The term $n_1$ was varied for all fits.
6.2.4 Comparison with Previous Literature

The data presented in Table 14 is taken from work previously carried out by Mullen, et al. [Mullen, et al., 1990], using Mn$^{2+}$ as a cofactor for metal binding studies to DNA polymerase I, the Klenow fragment and subsequent mutants. While this work is substantial in an attempt to understand metal ion binding, it does not relate either to, Mg$^{2+}$ binding to the "static" enzyme, or to metal cofactor stoichiometry during turnover. The overriding assumption in the work is that Mn$^{2+}$ is an equivalent substitution for Mg$^{2+}$. Data from this work and others have demonstrated that these two cofactors, while similar in many ways, can act very differently. In particular, Mn$^{2+}$ has been found to lower the proofreading capability of DNA polymerase I [El-Deiry, et al., 1988], bind differently than Mg$^{2+}$ to exonuclease III [Casareno, et al., 1996], and effect vastly different cleavage rates for ribozyme cleavage of phosphothiorate derivatives [Piccirilli, et al., 1993]. Differences such as these, on the chemical level, simply reflect physicochemical differences between the divalent cations. Therefore any such substitution should be made with caution. The study by Mullen, et al. also suggests, through proton relaxation rate measurements, that only 1 water is in fast exchange bound to Mn$^{2+}$ in the ternary complex (enzyme-dGTP-Mn$^{2+}$) [Mullen, et al., 1990]. However, when considering Mg$^{2+}$ and Mn$^{2+}$, it is noteworthy that the solvation energies and solvation numbers are different, making intrinsic comparisons between the cation's solvation state in any such system speculative. In large part, the metal binding constants previously determined for Mn$^{2+}$...
binding to DNA polymerase I reflect the highest limit for magnesium binding proteins [Black, et al., 1994a; Cowan, 1995].

The constants determined by Mullen, et al. are obtained through Scatchard analysis of Mn$^{2+}$ EPR signals, where the change in signal intensity for free and bound Mn$^{2+}$ was measured [Mullen, et al., 1990]. Most of the error in this type of analysis is due to the fact that a large portion of the data is collected near $n=1$ or 2, where $n$ reflects the cooperativity in a given system. Since $n$ is determined from the asymptotic limits of the plot, slight differences at high metal:enzyme ratios result in significant differences in $n$. Also the use of Scatchard analysis for this study is questionable since this model applies to systems which exhibit cooperativity in binding metal ions or substrates. Therefore, the value of $n$ reflects the stoichiometric lower limit of metal ion binding to DNA polymerase I and not the metal ion binding stoichiometry as indicated by the authors. We have attempted to treat Mg$^{2+}$ and Mn$^{2+}$ as such: completely different systems. This work is intended to provide new insight on the Klenow fragment as well as reconcile the existing literature using Mn$^{2+}$ as a cofactor.

Following the binding study described above, work from the same lab was undertaken to evaluate the coordination environment of the metal ion binding site(s) using Co$^{2+}$, Mn$^{2+}$, and Mg$^{2+}$ [Han, et al., 1991]. Using a Hill plot analysis of the 3'-5' exonuclease activity it was found for all cofactors that a low affinity site had a Hill coefficient of $-2$, while a higher affinity site maintained a coefficient of $\sim 1$. This was
taken to indicate at least 3 divalent cations were necessary for activation of the 3'-5'
exonuclease site [Han, et al., 1991]. The concentration of cation which yields half-
maximal velocity ($K_{0.5}$) for Mg$^{2+}$ was similar to $K_{\text{MET}}$ in Table 16, while that for Mn$^{2+}$ is
much smaller ($K_{0.5} \sim 0.004$ mM) than the measured $K_{\text{MET}}$ in Table 17 ($K_{\text{MET}} = 0.7$ mM).
The turnover rates were two-fold greater in this work as compared to Han, et al., most
likely due to differences in substrate [Han, et al., 1991]. Again, as noted above, the Hill-
type analysis does not indicate stoichiometry but rather suggests a lower limit in binding
cooperativity. To date there is no evidence from any source suggesting three metal ions
to be necessary for exonuclease activation, making this conclusion unlikely [Han, et al.,
1991; Mullen, et al., 1990; Ollis, et al., 1985; Derbyshire, et al., 1988]. Also, in the Hill
analysis performed by Han, et al., proper consideration for inhibition (either substrate or
metal ion) was not taken into consideration. The half-saturation constant reported by
Han, et al. for Mg$^{2+}$ corresponds closely to the $K_{\text{MET}}$ determined in Table 16. However,
the constant for Mn$^{2+}$ shows significant deviation from the $K_{\text{MET}}$ determined in Table 17.
Hill analysis does not yield a metal binding constant, simply a half-saturation constant,
but this seems to be another indication of the physicochemical differences between the
two cofactors. In order for significant cooperativity to be observed between three metal
ions to be observed, significant changes in protein structure or residue arrangement must
occur to allow strong binding ($K_{0.5} \sim 0.004$ mM). However, this is contradictory to
crystallography carried out on Klenow, where no evidence exists for large local structural
changes upon metal ion binding; in fact, upon dNMP binding to Klenow in the crystal structure no structural change was observed [Ollis, et al., 1985; Beese, et al., 1991]. In addition, it was previously observed with Mg$^{2+}$ binding to xylose isomerase that a 60-fold decrease between $K_1$ and $K_2$ (separate metal binding constants) with both metal ions occupying a binuclear active site [Van Bastelacre, et al., 1992; Lambeir, et al., 1992]. Based on simple electrostatics, this observed dissociation constant, $K_{0.5} \sim 0.004$ mM, appears extremely unreasonable concomitant with the fact that electrostatically the active site does not appear to be able to support 6 positive charges.

Results Summary

Binding Mg$^{2+}$ to the Klenow fragment it was observed that one Mg$^{2+}$ binds to the polymerase site and one binds to the exonuclease site. In the presence of thymidine monophosphate, this stoichiometry for Mg$^{2+}$ does not change. Further examination reveals that both metal ions at the polymerase and exonuclease sites increases binding affinity slightly. Binding Mn$^{2+}$ to the Klenow fragment yields one metal ion bound to the polymerase site and most likely one bound to the exonuclease site. Upon addition of TMP, the results do not clearly show one metal ion at each site, but the more reasonable fitting constants are for such a case.
6.3 Discussion

The mechanism of metal-mediated phosphate ester hydrolysis is an important problem in nucleic acid biochemistry. Such chemistry underlies important biological processes such as DNA replication, transcription and repair, ribozyme activity, and phosphorylation/dephosphorylation steps in signal transduction. The number of metal ions that mediate phosphate ester hydrolysis, condensation, and phosphoryl transfer reactions is a key issue to resolve in addition to the functional roles of these metal ions. In this chapter we have examined the metal dependence of the 3'-5' exonuclease activity of the Klenow fragment of DNA polymerase I. This is an important enzyme for DNA replication and has become a paradigm for a class of metalloenzymes that follow a two-metal-ion mechanism of phosphate ester hydrolysis. Crystallographic studies of the Klenow fragment show two metal ions bound in the exonuclease active site in the presence of the substrate mimic thymidine monophosphate. In the absence of TMP only one metal ion is observed to bind. Subsequent to these studies, several other enzymes have been crystallographically characterized with two active site metal ions. Multiple bound ions have also been identified from solution studies of the Klenow fragment by kinetic and spectroscopic methods. A common feature of each of these studies is the use of Mn²⁺, and occasionally Co²⁺, as analogues for the presumed natural Mg²⁺ cofactor. With a small number of exceptions, almost every crystallographic analysis of a nuclease or polymerase enzyme has employed Mn²⁺ as a probe for Mg²⁺ binding sites. The heavier
manganous ion facilitates discrimination of the metal ion from a solvent water. Increasingly, the two-metal-ion mechanism has served as a paradigm for numerous reactions in nuclease, polymerase, and ribozyme function, but has not received the detailed solution studies with the natural magnesium cofactor that are required to confirm such a model. Recently, several examples of enzymes have been characterized that do not fit the two-metal-ion paradigm. These include the *E. coli* ribonuclease H, where solution and crystallographic studies indicate a one-metal-ion mechanism, and stands in contrast to a two-metal-ion model ascribed to the structurally homologous RNase H domain of HIV reverse transcriptase, which shows two Mn$^{2+}$ ions located among four acidic residues (Asp443, Glu478, Asp498, and Asp549) in a Mn$^{2+}$-doped crystal. These acidic residues are four of the seven conserved residues found in all bacterial and retroviral RNase H domains, including the *E. coli* enzyme. Either the HIV RT RNase H domain does indeed bind two divalent metal ions, or this result reflects the specific use of Mn$^{2+}$ and/or the doping technique employed. Also, the 3'-5' exonuclease domain of *E. coli* exonuclease III shows one metal binding domain in structural studies; a conclusion that is supported by solution kinetic and calorimetric results. It is noteworthy, however, that calorimetric analysis of metal binding shows a different metal ion stoichiometry for Mg$^{2+}$ versus Mn$^{2+}$ binding (1 site for the former and $\geq 2$ sites for the latter). Another example of interest is the Eco RV restriction enzyme. A structural analysis of an Eco
RV-DNA complex showed two metal ions bound at the active site after doping, however, this crystal complex lacked activity. It was concluded in this and in later detailed studies that there is one catalytic metal ion, while at least one other site is required to promote binding of the substrate molecule. Finally, it is well known that hairpin ribozymes enroll metal ions to serve a structural but not a catalytic role, while even the hammerhead ribozyme exhibits substrate cleavage in the absence of a metal cofactor if a sufficiently good leaving group is used. Clearly these examples undermine the generality of a two-metal-ion mechanism in nucleic acid backbone hydrolysis. This fact prompted us to closely examine the reactivity of the Klenow fragment using the natural magnesium cofactor and employing the kinetic and calorimetric approaches that provide an effective means of addressing this important, but difficult problem.

Indeed inspection of the available solution kinetic data for the archetypal two-metal ion system (the 3'-5' exonuclease domain of the DNA polymerase Klenow fragment) [Black, et al., 1996a] suggests that the crystallographic model established from data with Mn$^{2+}$ ions may be illusory. While solution kinetic studies using Co$^{2+}$ and Mn$^{2+}$ cofactors support a second (and even a third) site for the Klenow fragment, the experimental binding constants of ~ 0.6 mM estimated for Mn$^{2+}$ are unlikely to support binding of a second Mg$^{2+}$ [Black, et al., 1996a]. Typically, association constants for Mg$^{2+}$ are significantly lower (5- to 100-fold) than those observed for Mn$^{2+}$ [Black, et al.,]
1996a; Englisch, et al., 1985], (more than 70-fold in the case of the Klenow fragment) [Black, et al., 1996b], and lie outside the range of accessible physiological levels of divalent magnesium (< 1 mM for free non-complexed cation) [Black, et al., 1996b].

In conclusion, while *bona fide* two-metal ion units have been characterized in a number of enzymes (for example, the binuclear iron and zinc sites in acid and alkaline phosphatases [Black, et al., 1994a], and the binuclear iron sites in a variety of redox and O₂ transport enzymes and proteins, the generality of such a model in the case of magnesium-dependent enzymes or ribozymes is less compelling.

### 6.4 Alternative Models

Several possibilities present themselves when analyzing kinetic data from the 3'-5' exonuclease reaction of the Klenow fragment. In an effort to rule out alternative schemes, where the number of metal ions and modes of inhibition seem as likely as the scheme developed and used in the text, two additional mechanisms are considered and equations derived and used for fitting. The goal is to show that the scheme developed in the text is the operating scheme and is also a cofactor independent picture of metal ion interaction in the Klenow fragment.

The symbols for all equations are the same as described in the body of the text except K₁ and K₂ which are used in order to distinguish between two binding constants. The generalized mechanism shown above was applied to the kinetic data as a means of
determining binding constants, stoichiometry and inhibition. On the basis of this model, utilizing both kinetic and calorimetric data, it is concluded that only one Mg\(^{2+}\) ion is necessary for catalytic turnover, while for Mn\(^{2+}\) the question of stoichiometry is ambiguous. The alternative schemes developed below refer to interactions with two metal ions; however, only Mn\(^{2+}\) provided satisfactory fits to the kinetic data. The schemes and derived equations are detailed below as well as the resulting plots from each of the fits based on the equations.

*Alternative Mechanism Two*

One mechanistic possibility for catalysis is that both metal binding sites must be completely saturated before enzymatic turnover can take place (Scheme 6). The resultant metal-saturated complex, E-M\(_{2}\)-S, can then carry out catalysis on DNA substrate. The overriding requirement is that the metal binding sites must be completely saturated before conversion to product can occur. The mode of inhibition is the same as that developed in the main text.
From Scheme 6, using the appropriate manipulations, Equation (6-2) is obtained and was used to fit the plots shown in Figures 75 and 76.

$\frac{v_0}{K_{cat} \cdot K_1 \cdot [Mn^{2+}]^2 \cdot [E^o] \cdot [S^o]} = \frac{k_{cat} \cdot K_1 \cdot [Mn^{2+}] \cdot [E^o] \cdot [S^o]}{K_2 \cdot K_m \cdot (K_1 + [Mn^{2+}]) (K_1 + [Mn^{2+}]) + [Mn^{2+}] \cdot [S^o] \cdot K_1 \cdot (K_2 + [Mn^{2+}])}$ (6-2)

Note in Equation (6-2) the second order dependence of the initial velocity on $[Mg^{2+}]$. 
Alternative Mechanism Three

A third possibility for Klenow activation involves one metal cofactor to be responsible for turnover. This mechanism is similar to Scheme 6 initially, except one metal ion is necessary for activation while binding a second metal ion causes inhibition. The inhibition constant, $K_i$, refers to accommodation of a second metal ion, forming an inactive complex that inhibits product release. Scheme 7 summarizes this possibility:

\[ E + Mn \xrightleftharpoons{K_1} E\cdot Mn \]
\[ E\cdot Mn + S \xrightarrow{K_m} E\cdot Mn\cdot S \]
\[ E\cdot Mn\cdot S + Mn \xrightarrow{K_2} E\cdot Mn_2\cdot S \]
\[ E\cdot Mn_2\cdot S \xrightarrow{K_i(Mn)} E\cdot Mn_2\cdot P \xleftarrow{k_{cat}} E\cdot Mn\cdot P + Mn \]
\[ E\cdot Mn\cdot P \xrightarrow{k_{cat}} E + P \]

Scheme 7
The most notable feature of Scheme 6 (Equations (6-2)) is the inhibition which reflects the binding of a second metal ion to the enzyme at high concentrations of cation. In other words, saturation of the Klenow fragment with two metal ions can result in inhibition of enzyme action. The derived equation is shown as:

\[
\frac{v_0}{K_i} = \frac{k_{cat} \cdot K_i \cdot [Mn^{3+}] \cdot [E^0] \cdot [S^+]}{K_1 \cdot K_2 \cdot [E^0] + K_m \cdot [Mn^{2+}] + [Mn^{2+}] \cdot [S^+](K_2 + [Mn^{2+}])}
\] (6-3)
Table 22. Steady-state kinetic parameters for Mg$^{2+}$ dependence of the 3'-5' exonuclease activity of the Klenow fragment. Fitting results are from Equation (6-2).\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Mg$^{2+}$</th>
<th>Mn$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_\text{m}$ (μM) (fixed)$^b$</td>
<td>80±24</td>
<td>25±7</td>
</tr>
<tr>
<td>$k_{\text{cat(opt)}}$ (sec$^{-1}$)</td>
<td>0.56±0.16</td>
<td>0.50±0.15</td>
</tr>
<tr>
<td>$K_1$ (mM)</td>
<td>0.11±0.03</td>
<td>0.19±0.06</td>
</tr>
<tr>
<td>$K_2$ (mM)</td>
<td>1.2±0.4</td>
<td>0.49±0.15</td>
</tr>
<tr>
<td>$K_1$ (mM)</td>
<td>41±12</td>
<td>42±12</td>
</tr>
</tbody>
</table>

\(^a\) The concentration of DNA oligonucleotide for Mg$^{2+}$ activation was 180 μM while for Mn$^{2+}$ it was 160 μM bases. The concentration of Klenow fragment in both cases was 430 nM.

\(^b\) For the purposes of fitting, $K_\text{m}$, the Michaelis-Menten constant, was fixed. For purposes of fitting, the constants $k_{\text{cat}}$, $K_1$ and $K_2$ were allowed to vary while $K_1$ was fixed. The value of $K_1$ refers to identical inhibition for both Equations (6-1) and (6-2).
Figure 75. Initial velocity dependence on Mg$^{2+}$ for the 3’-5’ exonuclease activity of the Klenow fragment. The fit represents results using Equation (6-2). Constants and conditions are summarized in Table 22.
Figure 76. Initial velocity dependence on Mn$^{2+}$ for the 3'–5' exonuclease activity of the Klenow fragment. The fit represents results using Equation (6-2). Constants and conditions are summarized in Table 22.
Table 23. Steady-state kinetic parameters for Mg$^{2+}$ dependence of the 3'-5' exonuclease activity of the Klenow fragment. Fitting results are from Equation (6-3).$^a$

<table>
<thead>
<tr>
<th></th>
<th>Mg$^{2+}$</th>
<th>Mn$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ (μM) (fixed)$^b$</td>
<td>80±24</td>
<td>25±7</td>
</tr>
<tr>
<td>$k_{cat}(opt)$ (sec$^{-1}$)</td>
<td>4.4±1.3</td>
<td>5.5±1.7</td>
</tr>
<tr>
<td>$K_1$ (mM)</td>
<td>11±3</td>
<td>3.7±1.1</td>
</tr>
<tr>
<td>$K_2$ (mM)</td>
<td>160±48</td>
<td>73±22</td>
</tr>
<tr>
<td>$K_4$ (mM)</td>
<td>41±12</td>
<td>9±3</td>
</tr>
</tbody>
</table>

$^a$ The concentration of DNA oligonucleotide for Mg$^{2+}$ activation was 180 μM while for Mn$^{2+}$ it was 160 μM bases. The concentration of Klenow fragment in both cases was 430 nM.

$^b$ For the purposes of fitting, $K_m$, the Michaelis-Menten constant, was fixed. For purposes of fitting, the constants $k_{cat}$, $K_1$, $K_2$ and $K_4$ were allowed to vary. $K_4$ in Equation (6-3) represents inhibition due to population on Klenow by two metal ions. Therefore, unlike Table 22, this parameter was allowed to float.
Figure 77. Initial velocity dependence on Mg$^{2+}$ for the 3'-5' exonuclease activity of the Klenow fragment. The fit represents results using Equation (6-3). Constants and conditions are summarized in Table 23.
Figure 78. Initial velocity dependence on Mn\textsuperscript{2+} for the 3'-5' exonuclease activity of the Klenow fragment. The fit represents results using Equation (6-3). Constants and conditions are summarized in Table 23.
Fitting Results and Discussion

Scheme 6 (or Equation (6-2)) clearly yields unsatisfactory fits to both sets of data (Figures 75 and 76). This graphically illustrates the inapplicability of the constraint for a binuclear center during catalytic turnover for both Mg$^{2+}$ and Mn$^{2+}$ cofactors in the 3'→5' exonuclease reaction. This is in accord with the kinetic and calorimetric data presented in the main body of text. All kinetic data supports a need for one metal cofactor during turnover (either Mg$^{2+}$ or Mn$^{2+}$), while only the calorimetry data suggests the possibility of multiple bound Mn$^{2+}$ at the exonuclease active site.

Another scenario, where a second metal ion can bind to the active site and is inhibitory rather than catalytic, must be examined. Equation (6-3) accounts for this possibility insofar as a second bound cation acts to form an inactive form of the enzyme. Equation (6-3) (Scheme 7) yields graphical fits which are qualitatively similar to those presented in the main text (Figures 77 and 78). The fitted turnover constants are shown in Table 22. Scheme 7 differs from the previous mechanism 2 in that only one metal ion is necessary for catalytic turnover, while a second metal ion may result in inhibition. From the fitting parameters summarized in Table 22, it is clear Mg$^{2+}$ does not provide a reasonable fit to Scheme 7. Although graphically, the fit is viable, $K_1$ and $K_2$ do not reflect physiologically relevant binding constants. In addition these constants do not correlate with any of the calorimetric data. The data for Mn$^{2+}$ yields a similar trend, however, the constants are slightly more reasonable than the case using Mg$^{2+}$. 
For Mg$^{2+}$, it seems clear that one metal ion activation is relevant to exonucleolytic reaction and also that only one Mg$^{2+}$ binds with reasonable affinity at the exonuclease active site. Alternatively, considering all of the available kinetic data for Mn$^{2+}$, it appears one cation is catalytically relevant for the 3'-5' exonuclease reaction. This does not rule out the possibility of two metal ion activation (Mn$^{2+}$) since thermodynamic data and crystallographic data have demonstrated this possibility [Ollis, et al., 1985; Mullen, et al., 1990]. This question of stoichiometry has received a large amount of attention with a significant amount of data in favor of two metal ion binding. However, this neither translates into stoichiometry during catalytic turnover nor does it extend to a detailed generalization concerning Mg$^{2+}$ activation. Clearly, there is a difference between the two cations that warrants caution in using Mn$^{2+}$ as a chemical probe for Mg$^{2+}$. 
The body of the work presented here has demonstrated the need to reevaluate how magnesium functions \textit{in vivo}. In the past, magnesium has been thought to interact largely through its inner coordination sphere and act as a bulk electrolyte. In the enzyme systems examined here, namely, \textit{E. coli} ribonuclease H, \textit{E. coli} exonuclease III, and \textit{E. coli} DNA polymerase I Klenow fragment, it has been found that magnesium has a rich biological chemistry that is much more detailed than previously thought. In ribonuclease H and exonuclease III, the metal cofactor acts through metal bound waters to stabilize the transition state. Previously, it has been found that the enzyme HIV reverse transcriptase maintains a ribonuclease H domain that is homologous to the enzyme \textit{E. coli} ribonuclease H [Kanaya, et al., 1989; Nakamura, et al., 1991]. Reverse transcriptase was previously crystallized (co-crystallized with MnSO$_4$) and found to maintain a binuclear site on the ribonuclease H domain [Yang, et al., 1990b; Davies, et al., 1991]. Due to the fact that
two Mn$^{2+}$ were observed at the ribonuclease H active site of reverse transcriptase, it was proposed that *E. coli* ribonuclease H also supported a binuclear site of magnesium ions [Davies, et al., 1991]. Concurrent work on the enzyme DNA polymerase I revealed that two Mn$^{2+}$ ions were present at the 3'-5' exonuclease site [Derbyshire, et al., 1988]. Since the RNase H and exonuclease activities function to hydrolytically excise nucleotides, it was proposed that enzymes of this type all function with a binuclear center [Steitz, et al., 1993]. The proposed mechanism is shown in Figure 79. To that end, much of the work has focused on addressing the question of metal ion stoichiometry in the enzymes *E. coli* ribonuclease H, *E. coli* exonuclease III, and *E. coli* DNA polymerase I the Klenow fragment [Katayanagi, et al., 1993b; Mol, et al., 1995; Huang, et al., 1994; Mullen, et al., 1990; Derbyshire, et al., 1988].

Our work provides additional evidence to previous data, that both *E. coli* ribonuclease H and *E. coli* exonuclease III catalyze turnover with one catalytically important metal ion (Chapters 3-5). Applying the methodology developed in conjunction with *E. coli* ribonuclease H and *E. coli* exonuclease III, we have also demonstrated that the two metal ion mechanism proposed in Figure 79 for the DNA polymerase I 3'-5' exonuclease activity is not entirely consistent (Chapter 6). Thus we have successfully shown, first, how the metal catalyzes active turnover in ribonuclease H and exonuclease III, and second, extended our methodology to an existing, well-studied system, DNA polymerase I the Klenow fragment.
Figure 79. The proposed transition state during the 3'-5' exonuclease reaction of *E. coli* DNA polymerase I Klenow fragment. Metal ion A, the tight binding cation, is thought to facilitate attack and formation of OH', while metal ion B is proposed to facilitate the 3'-O leaving group and stabilize the pentacoordinate phosphate (reproduced from Steitz, et al., 1993).
7.1 Ribonuclease H

Initial work with the magnesium-activated enzyme, *E. coli* ribonuclease H, allowed development of a model kinetic scheme (Equation (2-9)) using a novel assay involving the hypochroism inherent in nucleic acids. With this model, metal ion activation and inhibition were characterized through a steady-state kinetic analysis. Inhibition occurred through population of vacant binding sites on the substrate nucleic acid backbone, preventing enzyme binding at sufficiently high metal ion concentrations. This type of inhibition has been seen, but previously not characterized [Kow, 1989]. The analysis provided additional evidence for one metal ion activation of ribonuclease H and that background electrolyte competes with Mg\(^{2+}\) for the enzyme active site. Although, the stoichiometry for one metal ion during turnover had been addressed, the actual function of the cation was still unknown. From Chapter 1, it is known that hydrolytically active cations can act either through the inner coordination sphere or the outer coordination sphere (through bound ligands). To ascertain which was the dominant mode the substitution inert probe, Co(NH\(_3\))\(_6^{3+}\), was used, where the inner sphere ligands cannot be exchanged for protein or substrate contacts. This probe demonstrated that Mg\(^{2+}\) must be acting in an outer sphere mode, through its metal bound waters [Jou, et al., 1991]. Although Co(NH\(_3\))\(_6^{3+}\) demonstrated that an outer sphere mode of catalysis occurred with Mg\(^{2+}\), it did not address how the cation functions, *i.e.* what are the chemical details of catalysis? To understand how the inner sphere ligands modulate reactivity (H\(_2\)O for...
Mg$^{2+}$(aq) and NH$_3$ for Co(NH$_3$)$_6^{3+}$), it is essential to systematically vary the H$_2$O:NH$_3$ around the metal ion. To that end, chromium(III) probes were employed since Co(III) is unstable with high numbers of coordinated waters; a series of substitution inert probes were used of the type, [Cr(NH$_3$)$_{6-x}$(H$_2$O)$_x$]$^{3+}$ (where x = 0 to 6). Activation of ribonuclease H by these probes showed that three metal bound waters were necessary for catalytic turnover and that these waters must be arranged facially around the metal ion. Since the observed binding constant of the substrate (K$_m$) did not change with the different complexes, the metal ion acts to stabilize the transition state (Figure 4).

7.2 Exonuclease III

*E. coli* exonuclease III maintains 4 physiologically relevant activities [Weiss, 1981]. The structure, recently crystallized (Figure 38), maintains one active site thought to carry out these functions. In an effort to apply the methodology developed with ribonuclease H, this system was expressed and purified. Relatively little was known about the function of Mg$^{2+}$ during turnover, making this system very amenable to the aforementioned methodology. The crystal structure unambiguously showed that exonuclease III supported one divalent cation at the active site [Mol, et al., 1995]. The cofactor, from the crystal structure, was tethered to a single Glu-34 and a phosphate oxygen from the substrate (Figure 59).
Application of steady-state kinetic analysis in conjunction with the substitution inert cobalt and chromium probes yielded the same metal-ion mediated mechanism as found for *E. coli* ribonuclease H, namely transition state stabilization. However, use of chromium probes and steady-state kinetic measurements demonstrated that 4 metal-bound waters were essential for catalytic turnover for the RNase H and exonuclease activities, not 3 as in the case of *E. coli* ribonuclease H. This conclusion is supported by the crystal structure insofar as only two inner sphere linkages were observed to the cation (Glu-34 and substrate oxygen). In addition, in contrast to *E. coli* ribonuclease H, there was no preferred geometric arrangement observed for the metal bound waters (Tables 12-13). These waters were found to stabilize the transition state by formation of a hydrogen bonding network with the substrate. Kinetic evidence was also provided for one active site carrying out both RNase H and exonuclease functions. From the dependence of rate on pH it was found that the Cr(H$_2$O)$_6^{3+}$ causes hyper-activity of the enzyme (faster turnover than Mg). This is believed to be due to a bound hydroxide on the chromium probes at the pH of the experiment (Figure 59).

### 7.3 DNA polymerase I Klenow fragment

The Klenow fragment, which is the proteolytically cleaved large subunit, is responsible for the 3'-5' polymerase and 3'-5' exonuclease activities. Both intimately require Mg$^{2+}$ for functional activity. Applying the previously developed methodology
toward elucidation of mechanistic details has provided evidence that two metal ion activation is not necessarily ubiquitous or consistent in this system as previously thought [Steitz, et al., 1993]. Substitution of inert cobalt and chromium complexes yielded no measurable catalytic turnover. Since these complexes can only act through the outer coordination shell, the activating divalent cation must act through its inner coordination sphere, either binding directly to another metal ion, to enzyme residues, substrate, or some combination. Previous studies with mutant protein and crystallography results have shown the binuclear site to act through inner sphere linkages [Ollis, et al., 1985; Derbyshire, et al., 1988; Beese, et al., 1991]. In an attempt to provide further insight into this interaction and mechanism of hydrolysis, kinetic and thermodynamic methods were carried out. Kinetic analysis provided evidence for one catalytically important cation for 3'-5' exonuclease activity with both Mn$^{2+}$ and Mg$^{2+}$ cofactors. This is in disagreement with previous work, although, previous data consisted of Mn$^{2+}$ binding to the "static" enzyme [Mullen, et al., 1990; Derbyshire, et al., 1988]. One study was carried out under turnover conditions but employed Hill plot analysis, concluding 3 Mn$^{2+}$ cations were necessary for activity (see Chapter 6 for a comparative discussion) [Han, et al., 1991]. The binding constants and stoichiometry obtained in this work, in contrast, represents conditions of optimal metal and substrate concentrations and therefore more accurately reflects metal-activated hydrolysis.
Titration calorimetry was carried out in an effort to thermodynamically characterize metal ion binding to the Klenow fragment as well as understand the stoichiometry for both the polymerase and exonuclease active sites. Titration with either Mg\textsuperscript{2+} or Mn\textsuperscript{2+} yielded fits which most reasonably indicated a stoichiometry of one metal ion for each of the two sites. The calorimetric data from these fits was in agreement with the kinetic data described and ruled out multiple ion binding at the exonuclease site. To further clarify metal binding stoichiometry, titrations were carried out with substrate mimic thymidine monophosphate (TMP). In the presence of TMP only one Mg\textsuperscript{2+} ion was observed to bind at each active site. Using Mn\textsuperscript{2+} however, the situation became ambiguous and the fits from either one metal ion at each site or two metal ions at each site were viable. These data are in contrast to previous work concerning metal binding stoichiometry. The most notable difference from previous work is the use of Mg\textsuperscript{2+} in the kinetic experiments and in the thermodynamic analysis. Aside from work by Han, et al., use of Mg\textsuperscript{2+} has been surprisingly absent. What is clear from all data on metal ion binding to the Klenow fragment is: Mg\textsuperscript{2+} interacts and binds differently than Mn\textsuperscript{2+} and therefore should be used with caution as a probe for Mg\textsuperscript{2+}-binding sites.

In addition to stoichiometry and binding constants, titration calorimetry has provided thermodynamic data. One striking contrast between Mg\textsuperscript{2+} and Mn\textsuperscript{2+} binding to the Klenow fragment is the difference in $\Delta H^\circ$. Binding Mg\textsuperscript{2+} to the polymerase site is found to be endothermically driven, while Mn\textsuperscript{2+} is found to be exothermically driven.
Also, in the presence of TMP, metal cofactor is enthalpically driven at the polymerase site, while entropically driven at the exonuclease site.

7.4 Conclusion

Magnesium is a ubiquitous cation, not only in living organisms, but in seawater and minerals. Many enzymes use the various physicochemical properties of the divalent cation to carry out hydrolytic action. The function of these enzymes ranges from breaking down sugars for energy, to neural responses. Why was magnesium chosen for these diverse functions? In addition to its physical properties, magnesium ion is found in high abundance; protein systems have evolved elaborate structures to use this abundant cation for the variety of functions that any living cell demands. The function of magnesium has traditionally been perceived as a background electrolyte and Lewis acid. Simply, the function of magnesium has been thought to maintain electroneutrality and bind substrate molecules such that protein residues can carry out catalysis. This body of work has developed a systematic experimental methodology, and has demonstrated that the chemistry of this cation is more diverse than previously thought. To that extent, outer sphere chemistry has been quantitated and demonstrated for several systems. This clearly demands attention as a mechanistic option in magnesium-dependent biochemical systems.

This body of work illustrates chemically, that Mg\(^{2+}\) can maintain a diverse biochemical role in acting as a bulk electrolyte, as well as in activating enzymes. This
diversity has been probed, and the knowledge base expanded, by providing evidence that metal bound waters on Mg$^{2+}$ are involved in stabilizing the transition state. This idea had not previously been put forward and may constitute an important mechanistic pathway in metal ion activated biochemical systems. As part of our effort in this mechanistic area, the use of chromium and cobalt probes has been developed and shown to be a viable tool in the study of Mg$^{2+}$-activation of enzymes. While these probes do not impart mechanistic equivalence with divalent magnesium, they do provide a measure of the importance of metal-bound waters, charge, and multinuclear centers in the catalytic chemistry of this cofactor.
REFERENCES


Black, C.B.; Foster, M.; Cowan, J.A. (1996a), *manuscript submitted*.

Black, C.B.; Cowan, J.A. (1996b), *manuscript submitted*.


Casareno, R. B. L.; Cowan, J. A. *manuscript submitted*.


