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I. PHYSICOCHEMICAL STUDIES ON THE METAL BINDING
DOMAIN OF \textit{E. coli} RIBONUCLEASE H AND EXONUCLEASE III

II. CLONING AND EXPRESSION OF THE INTRACELLULAR AND
EXTRACELLULAR DOMAINS OF HUMAN FAS AND FAS LIGAND

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

Ruby Leah B. Casareno, B.S.

The Ohio State University

1996

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Department of Chemistry
E. coli ribonuclease H (RNase H) has served as a convenient vehicle for our studies on the metallobiochemistry of divalent magnesium. This enzyme is a small, structurally well characterized endonuclease that functions as a monomer and targets the RNA strand of RNA-DNA hybrids. From previous systematic studies, we have developed a consistent model of the functional role of this cofactor and its interactions with the enzyme and substrate. This study demonstrates the rational redesign of RNase H to form a double mutant protein that exhibits high levels of activity, but lacks the requirement for the magnesium cofactor.

The important structural characteristics of the magnesium binding domain of two metallonucleases, RNase H and exo III are investigated. Isothermal titration calorimetry is used to quantify the thermodynamic parameters of binding of each of these enzymes to a series of group II elements as well as Mn$^{2+}$. In this study, it is demonstrated that there is a distinct difference between magnesium and manganese, not only in terms of stoichiometry but also in thermodynamic parameters of binding. From these results, we strongly suggest caution in the interpretation and generalization of results concerning the location and stoichiometry of magnesium binding from experiments using manganese ions.
Use of the divalent cations gives us an insight on the size of an enzyme's metal binding pocket. Comparison of the configuration around the binding pocket and the plots of free energy of binding versus ionic radius gives us an idea as to what factors contribute to the molecular recognition involved in metal binding. Our results seem to indicate that an interplay of steric, hydration and electrostatic interaction influences metal affinity.

Mutation of one of the carboxylate ligands to the magnesium cofactor (Asp 10) in RNase H did not abolish the metal affinity contrary to expectations. Non-conservative mutation of this residue rendered the engineered enzyme inactive but magnesium binding became tighter. Removal of the negatively charged Asp 10 did not abolish metal binding presumably because the Asp 70 can substitute as a ligand to the metal cofactor in its absence.
To my family
ACKNOWLEDGMENTS

I am greatly indebted to my adviser, Prof. Jimmy Cowan for giving me the chance to pursue my degree under his guidance and support. His expertise in the field of magnesium biochemistry motivated me to carry out this research and his encouraging words uplifted me during the difficult times of experimental work. I would also like to thank Drs. Ross Dalbey, Bill Tschantz and Meesook Sung for teaching me the basics of molecular biology, techniques which proved to be very valuable during my graduate study. Many thanks go to the Department of Chemistry for providing financial assistance during my education.

Six years in OSU had been a long time but thanks to my friends and colleagues who made my stay here very worthwhile. Special thanks go to Vicky, Ohyama and Francis for their friendship; to my colleagues, especially Dr. Dawei Li and Kshama for sharing their expertise and to Elena for entrusting to me her invaluable laptop computer.

Finally, I thank my family for their concern, motivation and support.
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## 3. STRUCTURE AND FUNCTION OF *ESCHERICHIA COLI* RIBONUCLEASE H AND EXONUCLEASE III

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LIST OF ABBREVIATIONS

Arg  Arginine
Asn  Asparagine
Asp  Aspartate
ATP  Adenosine Triphosphate
CD   Circular Dichroism
Che Y Chemotaxis Y
COSY Correlation Spectroscopy
DNA  Deoxyribonucleic acid
ds  Double-stranded
E. coli Escherichia coli
exo III Exonuclease III
FasL  Fas Ligand
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<tr>
<td>Glu</td>
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</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl Thiogalactoside</td>
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<tr>
<td>ITC</td>
<td>Isothermal Titration Calorimetry</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysine</td>
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<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RNase H</td>
<td>Ribonuclease H</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl sulfate</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
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<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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PART I

PHYSICOCHEMICAL STUDIES ON THE METAL BINDING DOMAIN

OF E. COLI RIBONUCLEASE H AND EXONUCLEASE III
Alkaline earth metal-dependent enzymes are ubiquitous in nucleic acid biochemistry. Metal cofactors, especially magnesium ion, are essential for the activation of most enzymes in phosphoryl transfer and phosphodiester hydrolysis. However, the role of these metal cofactors in orchestrating enzyme structure and activity is poorly understood; most likely because the commonly used cations (Mg$^{2+}$, Ca$^{2+}$) are, for the most part, spectroscopically invisible and are therefore difficult to study.

Research efforts in our laboratory have successfully evaluated the role of metal cofactors. We have demonstrated the use of physicochemical methods to monitor magnesium binding chemistry by isothermal titration calorimetry (ITC), and nuclear magnetic resonance (NMR) spectroscopy ("Mg" NMR, 'H NMR) (Cowan, 1992; Cowan, 1991; Reid & Cowan, 1990; Huang & Cowan, 1994), while inert inorganic complexes of defined coordination state have been used as probes for inner-and outer-sphere chemistry of hydrated magnesium ion (Jou & Cowan, 1991; Black & Cowan, 1994a).
In this study, two magnesium-dependent nucleases, *E. coli* ribonuclease H (RNase H) and exonuclease III (exo III) are investigated. Recent literature concerning the structure and function of these enzymes is reviewed in Chapter 3. The aim of this study is to investigate the important structural characteristics of the magnesium binding domain that regulates the kinetics and thermodynamics of metal cofactor binding and catalytic activity. A combination of two approaches has been taken: (1) by selectively engineering the active site through recombinant molecular biology techniques, and (2) by probing the size of the active site through the use of divalent metal cations of various ionic radii.

Previous research efforts in our laboratory have resulted in the proposal of RNase H-catalyzed magnesium-mediated hydrolysis of a nucleotide backbone: through stabilization of a transient intermediate by formation of an outer-sphere complex with the positively-charged metal cofactor (Jou & Cowan, 1991; Black & Cowan, 1994a). This observation suggested to us the interesting hypothesis that mutation of active site carboxylate residues to positively-charged Lys or Arg might provide sufficient positive charge density and hydrogen bonding propensity in the active site domain to mimic the role of the hydrated divalent magnesium. Chapter 4 presents the results of a study to rationally engineer the active site to remove the metal dependence of RNase H.

In the structural study of enzymes, manganese ion is often substituted for the magnesium cofactor not only in kinetic studies but most especially in crystallographic data generation. In Chapter 5, we evaluated the metal binding behavior in solution of
these two metallonucleases by titrating each with salt solutions of magnesium, calcium and manganese. Through calorimetric measurements, we show that there is a distinct difference between manganese and magnesium, not only in terms of stoichiometry but also in thermodynamic parameters of metal binding. From these results, we strongly suggest caution in the interpretation and generalization of results concerning the location and stoichiometry of magnesium binding from experiments using manganese ions.

Magnesium-binding enzymes have been shown to have two or three carboxylate ligands to the metal cofactor. The two enzymes involved in this study, RNase H and exo III, have two and one magnesium-binding carboxylates, respectively. Che Y, a chemosensory protein, show three metal-binding carboxylates (Stock, et. al., 1989, 1993; Volz & Matsumura, 1991). Both the coordination environment and charge density in the metal-binding pockets for these magnesium-binding enzymes are distinct; nevertheless, the binding affinity for $\text{Mg}^{2+}$ is similar ($-10^3$ to $10^4 \text{ M}^{-1}$) (Huang & Cowan, 1994; Needham, et. al., 1993; Casareno & Cowan, 1996). In this work, we characterize the factors that regulate metal recognition by RNase H and exo III through a series of calorimetric measurements. A comparison of the metal binding profiles reflects the factors which dominate metal recognition and binding. Comparison of these profiles in Chapter 6 permits a comprehensive overview of structure/recognition features for this family of magnesium-dependent enzymes.
Since RNase H is a low molecular weight protein that has been extensively studied by X-ray crystallography (Katayanagi, et. al., 1990, 1992, 1993b), NMR spectroscopy (Yamazaki, et. al., 1991, 1993; Oda, et. al., 1991, 1992, 1994), and site-directed mutagenesis (Kanaya, et. al., 1990a, 1990b, 1991a, 1991b), it serves as a useful model for systematic studies of mutant proteins and the influence of these mutations on metal binding characteristics. We believe that hydration factors do indeed dominate metal binding to the native enzyme. By engineering mutations that selectively increase or decrease the accessible volume in the binding pocket, it should be possible to change the relative steric and solvation contributions to binding energy, resulting in an ability to fine-tune the metal binding selectivity. Calorimetric determination of the thermodynamic parameters of magnesium ion binding to Asp 10 mutants is also presented in Chapter 6. Contrary to expected results, removal of one of the two carboxylate ligands to the metal cofactor did not abolish the metal affinity.
CHAPTER 2

EXPERIMENTAL PROCEDURES

2.1. Bacterial Strains and Plasmids

Plasmid pET 21 b(+) and *E. coli* strains BL 21 (DE3) and XL1B were purchased from Novagen. *E. coli* strain RZ 1032 was obtained from Dr. Ross E. Dalbey's laboratory. *E. coli* XL1B cells transformed with pET 21 b(+) carrying the *rnha* gene, with Nde I and Sal I restriction sites, was kindly provided by Mr. Anton Tevelev. The plasmid pSGR3 containing the *xth* gene coding for exo III was a generous gift from Dr. B. Weiss.

2.2. Materials.

Restriction enzymes and polynucleotide modifying enzymes (T4 Polynucleotide kinase, T4 DNA ligase, T4 DNA Polymerase) were purchased from BRL. Poly (A.dT) was bought from Pharmacia/LKB. \[\alpha^{35}\text{S}]dATP was obtained from Amersham Co.
Sephadex G-50 was purchased from Pharmacia Co. while P-11 and DE 52 were obtained from Whatman. The Ni-NTA resin was purchased from Novagen. Agarose gel and Phast gels were purchased from NuSieve and Pharmacia Co., respectively. DNase I and streptomycin sulfate were bought from Boehringer Mannheim. All other reagents were purchased from Sigma or Aldrich (reagent grade). pH measurements were done with an Accumet model 910 pH-meter equipped with a Corning semi-micro combination pH-electrode from Fischer Scientific. Deionized water used in all procedures was obtained by filtering distilled water through a Barnstead nanopure system. Concentration measurements using uv spectroscopy were determined on a Hewlett-Packard 8452A diode-array spectrophotometer (run by software from On-Line Instrument Systems).

2.3. Methods.

2.3.1. Gene Subcloning and Protein Engineering

2.3.1.1. pET vectors. The pET vectors, originally constructed by Studier and colleagues (Studier and Moffatt, 1986; Rosenberg, et. al., 1987; Studier, et. al., 1990), were improved by Novagen for subcloning, detection and purification of target proteins. λDE3 lysogens of strain BL21 (FompT r6 m8) is the most widely used host for target gene expression. BL 21 strain lacks the lon protease and the ompT outer membrane
protease that can degrade proteins during purification (Grodberg and Dunn, 1988). This strain contains a chromosomal copy of the gene for the T7 RNA polymerase. In this lysogen, the only promoter known to direct transcription of the T7 RNA polymerase gene is the lacUV5 promoter, which is inducible by IPTG. However, it is known that there is background expression of T7 RNA polymerase from the lacUV5 promoter in λDE3 lysogens. If target gene products are sufficiently toxic, this basal level can prevent the establishment of plasmids in λDE3 lysogen. One solution to this problem was to incorporate the T7 lac promoter into the pET vector (Studier, et. al., 1990; Dubendorff and Studier, 1991). pET 21b(+) contains a lac operator sequence just downstream of the T7 promoter (Fig. 1). It also carries the natural promoter and coding sequence for the lac repressor (lac I), oriented so that the T7 lac and lacI promoters diverge. The lac repressor acts both at the lacUV5 promoter in the host chromosome to repress transcription of the T7 RNA polymerase gene by the host polymerase and at the T7lac promoter in the vector to block transcription of the target gene by any T7 RNA polymerase that is made. Also incorporated at the C-terminus of the multiple cloning site of pET 21 b(+) is the codon for six consecutive histidine residues which can be used as a tag for target protein purification.
Figure 1. pET 21 b(+) plasmid used for subcloning.
2.3.1.2. Ribonuclease H with histidine tag and active site mutants. The plasmid pET 21 b(+) containing the \textit{rnha} gene was subjected to oligonucleotide-directed mutagenesis using Kunkel’s method (1985). For the recombinant ribonuclease H with histidine tag, the stop codon and 30 base pairs before the six consecutive codons for histidine were deleted by mutagenesis. This deletion of 33 base pairs included the removal of the incorporated Sal I site. For the active site single mutants, degenerate primers were used to generate different mutants in a single mutagenesis reaction. Briefly, a uracil-containing single-stranded DNA template for native ribonuclease H was isolated and purified from an \textit{E. coli ung} \textit{dut} strain (RZ1032) using published protocols in Sambrook, et. al. (1989). The mutagenic primer (Table 1), ordered from IDT was phosphorylated and annealed to the template. Synthesis of the opposite strand was commenced by the addition of T4 DNA Polymerase. The resulting double-stranded DNA template was ligated and transformed into \textit{E. coli} XL1B (\textit{dut} \textit{ung}+) for isolation and purification of the mutated DNA. Fig. 2 shows the results of Nde I- and Ndel-Sal I cut plasmids for RNase H. For the native RNase H, excision of $\sim$0.5 kb fragment was observed with Nde I-Sal I restriction (lane 2) while RNase H 6X His tag had no small fragment since the Sal I site was deleted by mutagenesis. The desired deletion and point mutations were confirmed by single-stranded dideoxynucleotide
<table>
<thead>
<tr>
<th>primers</th>
<th>sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>D10N/S</td>
<td>GCC CAG ACA CGA ACC (G(TC)T)^10 GGT GAA AAT TTC TAC</td>
</tr>
<tr>
<td>D10E/G/R</td>
<td>GCC CAG ACA CGA ACC ((TA)(TC)(CT))^10 GGT GAA AAT TTC TAC</td>
</tr>
<tr>
<td>D10K</td>
<td>CAG ACA CGA ACC (TTT)^10 GGT GAA AAT TTC</td>
</tr>
<tr>
<td>E48R/K</td>
<td>AGC GGC CAT CAA (T(CDT)^48) CAT ACG GTT GTT</td>
</tr>
<tr>
<td>D70R/K</td>
<td>GAC ATA CTG GCT (T(CDT)^70) GGT ACT CAA AAT</td>
</tr>
<tr>
<td>N100</td>
<td>TTG CCA GAG ATC GAC ATT^100</td>
</tr>
<tr>
<td>H124R/K</td>
<td>GTG TCC GGC (T(TCT)^{124}) GCC TTT AAC</td>
</tr>
<tr>
<td>C^4(His)</td>
<td>GTG GTG GTG GTG AAC^155 TTC AAC TTG GTT</td>
</tr>
</tbody>
</table>

**Table 1.** Primers used to construct and confirm the sequence of the ribonuclease H mutants. Codon for the replaced amino acid residue is underlined.
Figure 2. Restriction digestion results for wild type and recombinant RNases H and wild type exo III. The plasmids were incubated with the restriction enzymes for two hours at 37°C. Lane (1) RNase H + Nde I + Sal I (2) RNase H + Nde I (3) 1 kb marker (4) RNase H 6x His tag + Nde I (5) RNase H 6x His tag + Nde I + Sal I (6) Exo III + Nde I + Xho I (7) Exo III + Nde I (8) 1 kb marker.
Figure 3. DNA sequencing of ribonuclease H using Sanger’s method. An anti-sense mutagenic primer (5'-AGCGGCCATCAAT<TTCTTTTACATCGGTTGTT) was used. (a) wild type RNase H (b) Arg 10 mutant.
Figure 4. DNA sequencing results for RNase H 6X Histidine Tag using the T7 termination primer.

5'- CAA GTT GAA GTT CAC CAC CAC CAC CAC CAC TGA GAT-3'
Gln Val Glu Val His His His His His His His Stop
<table>
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<tr>
<th>primers</th>
<th>sequence</th>
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<tbody>
<tr>
<td>5' - Nde I</td>
<td>GCG <strong>CAT ATG</strong> AAA TTT GTC TCT TTT AA</td>
</tr>
<tr>
<td></td>
<td>Nde I</td>
</tr>
<tr>
<td>3' - Xho I</td>
<td>TTT <strong>CTC GAG</strong> GTT AAT TCT CCT GAC CC</td>
</tr>
<tr>
<td></td>
<td>Xho I</td>
</tr>
<tr>
<td>H62</td>
<td>CGC CAT AAT GGC CT</td>
</tr>
<tr>
<td>K140</td>
<td>GAT TAT CAC GTT TG</td>
</tr>
<tr>
<td>D221</td>
<td>CCA CGG TTA TCG TC</td>
</tr>
</tbody>
</table>

**Table 2.** Primers used to subclone and sequence the *xth* gene. The restriction sites are underlined.
For a 100 μL reaction mix:

100 μM dNTP’s

0.5 μM primers

160 ng template DNA

10 μL reaction buffer

2.5 U Pfu DNA Polymerase

Mixed gently; spun down briefly; overlaid with mineral oil.

PCR cycle:  

- denatured at 94°C for 20 sec
- annealed at 45°C for 20 sec
- extended at 72°C for 30 sec

30 cycles

- extension at 72°C for 5 min.
- incubated at 4°C, then stored at -20°C

Table 3. Polymerase Chain Reaction (PCR) components.
sequencing using Sanger's method (Figs. 3 and 4). For the double mutants, uracil-containing single-stranded templates were isolated from Arg 10 mutant. The corresponding primer was then added and mutagenesis performed to generate the respective double mutants desired. Each engineered plasmid was then transformed into \textit{E. coli} BL21 (DE3) strain for protein expression. The production of native and mutant RNases H in cells was examined by analyzing whole cell extracts using SDS-PAGE method by Laemmli (1970). The level of production was estimated from the intensity of the band visualized by Coomassie brilliant blue staining (Fig. 6).

\textbf{2.3.1.3. Exonuclease III.} The plasmid pSGR3 containing the \textit{xth} gene coding for the exo III was purified using published protocols in Ausubel, et. al. (1992). Two primers were designed incorporating the Nde I site for the 5'-end and the Xho I site for the 3'-end (Table 2). The gene was amplified by PCR using 0.5 \textmu M each of these two primers (Table 3) and subsequently subcloned into the pET 21 b(+) vector using the Nde I and Xho I restriction sites (Fig. 2). The entire \textit{xth} gene was sequenced using three internal primers to confirm that no mutation was introduced by PCR. Fig. 5 shows the results of the dideoxynucleotide sequencing of the \textit{xth} gene. The resulting plasmid was then introduced into the expression host, BL 21 (DE3) for protein production which was examined by SDS-PAGE stained with Coomassie brilliant blue staining (Fig. 7).
<table>
<thead>
<tr>
<th>(a)</th>
<th>(b)</th>
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<tr>
<td>G</td>
<td>T</td>
</tr>
<tr>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td>C</td>
<td>A</td>
</tr>
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</table>

Figure 5. Partial sequencing results for Exo III using antisense primers: (a) GATTATCACGTTTG-3' and (b) 5'-CCACGGTTATCGTC-3'
Fig. 5 (cont.)

(a) ACC AAA GAG ACG CCG ATT GCC GTG CGT CGC GGC TTT CCC GGT GAC
    Thr Lys Glu Thr Pro Ile Ala Val Arg Arg Gly Phe Pro Gly Asp

    GAC GAA GAG GCG CAG CGG CGG ATT
    Asp Glu Glu Asp Gln Arg Arg Ile

(b) ATT ATG GGC GAT ATG AAT ATC AGC CCT ACA GAT CTG GAT ATC GGC
    Ile Met Gly Asp Met Asn Ile Ser Pro Thr Asp Leu Asp Ile Gly

    ATT GGC GAA GAA AAC CGT AAG CGC TGG
    Ile Gly Glu Glu Asn Arg Lys Arg Trp
Figure 6. SDS-PAGE gel stained with Coomassie brilliant blue. Total cell lysates containing (1) RNase H D10G and (2) RNase H D10R/E48R. (3) low MW protein markers.
Figure 7. SDS-PAGE gel stained with Coomassie blue. Total cell lysate from two colonies which expressed the exo III protein upon IPTG induction. Lane 1) MW marker, 2) exo III-1, 3) exo III-2.
2.3.2. Bulk growth of *E. coli* Ribonucleases H and Exonuclease III proteins.

For each native and mutant protein generated, a glycerol stock was prepared by adding 150 μL of sterile 50% glycerol to 850 μL of cell culture which was frozen immediately by immersing in liquid nitrogen or in dry ice-ethanol bath. Hundred μL of the frozen cell stock was added to 20 mL of LB supplemented with 120 μg/mL Amp and incubated at 37°C until cloudy. This starter culture was then transferred to 1L LB/Amp and shaken at 37°C until OD₆₀₀ was 0.6 (about 3-4 hours). At this point, IPTG was added to a final concentration of 1 mM to induce target protein production. The cells were incubated at 37°C for 4-5 hours more. The cells were harvested by centrifugation at 8000 rpm using a refrigerated Sorvall RC2B centrifuge. The tubes were inverted upside down after discarding the supernatant to drain the pellet well. The pellet was resuspended in minimum amount of buffer and stored at -80°C until needed.

2.3.3. Protein Isolation

2.3.3.1. Ribonucleases H

2.3.3.1.1. Cell Lysis. The frozen cell paste from 2 L culture (~5 g) resuspended in buffer 1 (50 mM Tris-HCl, pH 7.0, 0.2 M KCl, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM 2-mercaptoethanol) was thawed at room temperature. Once thawed, the solution was placed on ice, sonicated using a Fisher discmembranator with big probe at 90%
efficiency in 10 sec bursts followed by 1 minute incubation on ice. This process was repeated as many times as deemed necessary until the solution was homogeneous. DNase I was added (20 μg/mL) and the solution was incubated on ice for 15 minutes until it turned less viscous. The solution was then centrifuged at 15000 rpm using Sorvall RC2B centrifuge for 30 minutes to remove the cell debris. The supernatant was transferred to another tube and centrifuged at 20000 rpm for 2 hours. The cleared supernatant was transferred to a chilled tube and diluted 3 fold with buffer 2 (50 mM Tris-HCl, pH 7.0, 0.1 mM EDTA, 1 mM 2-mercaptoethanol).

2.3.3.1.2. DE-52 column chromatography. The sample was applied to a DE-52 (30 cm x 3.5 cm) column previously equilibrated with buffer 3 (50 mM Tris-HCl, pH 7.0, 50 mM NaCl, 0.1 mM EDTA, 1 mM 2-mercaptoethanol). As soon as the last trace of the sample entered the top of the column, 20 mL of buffer 3 was used to wash off the protein solution stuck to the sides of the column. The RNase H was eluted by passing buffer 3 into the column. Ten mL fractions were collected and the absorbance at 280 nm of each eluent was monitored. The elution profile is shown in Fig. 8. The tubes corresponding to the first major peak were pooled together.

2.3.3.1.3. P-11 column chromatography. The pooled fraction containing RNase H was loaded onto a P-11 column (30 cm x 3.5 cm) previously equilibrated in buffer 3. The column was washed with 300 mL of 0.25M NaCl/buffer 3. The bound RNase H was
Figure 8. DE-52 elution profile for ribonuclease H using buffer 3. The eluents corresponding to the first major peak contained RNase H.
Figure 9. P-11 elution profile for RNase H using a linear gradient of 0.25-0.6 M NaCl/buffer 3. The protein came off the column at ~0.45 M NaCl/buffer 3.
then eluted from the column using a linear gradient of 0.25 M -0.60 M NaCl/buffer 3 and ten mL fractions were collected using a fraction collector. The absorbance at 280 nm for each tube was monitored and the elution profile is shown in Fig. 9.

2.3.3.2. Ribonuclease H with histidine tag.

2.3.3.2.1 The Ni-NTA protein purification system. This is a powerful and versatile tool for isolating recombinant proteins. The Ni-NTA resin binds nickel ions through 4 chelating sites (Fig. 10). This property makes it superior compared to other metal chelate resins which have fewer chelating sites. This resin holds the nickel ions more tightly which then prevents leaching of the metal ion and results in much greater binding capacity. The Ni-NTA resin has a remarkable selectivity for histidine residues. Thus, proteins with an affinity tag of six consecutive histidine residues are isolated easily in one-step purification under native or denaturing conditions. The 6X his tag often does not affect protein structure or function, and need not be removed from the purified protein.

A cleared lysate is prepared, and the tagged protein is bound to the Ni-NTA resin (Fig. 11). Contaminants are rapidly washed away, and the pure protein is eluted under mild conditions, by increasing the amount of imidazole as a competitor.

26
Figure 10. The Ni-NTA resin.

**Ni-NTA resins**

**Most metal chelate resins**
2.3.3.2. Preparation of Ni-NTA column. A 2-mL resin column was packed in a small disposable column (Invitrogen). The following sequence of washes was done to charge and equilibrate the column:

a) 3 volumes of sterile distilled water
b) 5 volumes of 1x Charge buffer
c) 3 volumes of 1x Binding buffer

The resin turned light green after charging and equilibration.

2.3.3.2.3 Cell Extract Preparation. The cell paste was thawed on ice. The cells (~5g) were sonicated using a Fisher sonic dismembranator Model 300 with power set at 90%. The solution was cooled down on ice. The cells were sonicated again until the solution was not viscous anymore. DNase I was added and the solution was incubated on ice for 15 min or until the solution was more flowing. The solution was aliquoted into ice-cold microcentrifuge tubes and spun down at 14000 rpm in a benchtop microcentrifuge at 4°C. The supernatant was transferred to another tube and was centrifuged again for another 15 min. The supernatant was pooled together and filtered using a 0.45 μm membrane attached to a plastic syringe. The solution was kept on ice.

The filtered solution was applied to the previously equilibrated Ni-NTA resin and the flow through was collected. The column was washed with 10 volumes of 1x Binding buffer (5 mM imidazole), followed by 6 volumes of 1x Wash buffer (60 mM imidazole). The bound RNase H protein was eluted with 80-95 mM imidazole/binding buffer (Table 4).
**Figure 11.** The Ni-NTA purification scheme for 6X Histidine Tagged RNase H.
Table 4. Buffers for RNase H-6X His Purification
2.3.3.3. Exonuclease III

2.3.3.3.1 Cell lysis. The thawed cells were broken by sonication at 90% power setting in 20 second bursts followed by 1 min incubation on ice. Streptomycin sulfate was added and the solution was incubated on ice for 15 min. The cell debris were spun down at 15000 rpm and the supernatant was transferred to another tube. Ammonium chloride was added (0.1g/mL sup). After making sure that all of the solid had been dissolved, the solution was centrifuged at 15000 rpm for 30 min. To the supernatant, 0.5 g ammonium chloride was added per mL of solution. The precipitated protein was pelleted by centrifugation at 15000 rpm. The pellet was resuspended slowly in 30 mL of 10 mM potassium phosphate buffer, pH 6.5.

2.3.3.3.2. P-11 column chromatography. The protein sample was then applied to a P-11 column previously equilibrated with 20 mM potassium phosphate buffer, pH 6.5. The column was washed of impurities by passing 200 mL of 100 mM potassium phosphate buffer, pH 6.5. Bound exo III protein was eluted from the column by using a linear gradient of 100 to 400 mM potassium phosphate buffer, pH 6.5. Ten mL fractions were collected and the absorbance at 280 nm monitored. The elution profile is shown in Fig. 12.

2.3.4. Protein purity.

The purity of the protein purified was judged by Coomassie Blue staining of the 20% homogeneous SDS-PAGE gel run on a Phast electrophoresis system (Pharmacia/LKB) at 15°C. Molecular mass determinations were referenced to
commercially available standards (BRL). Most of the time, the purity was >99%. Figs. 12 and 13 shows the purity of the RNase H 6X His tag, exo III and RNase H wild type and mutant enzymes isolated.

2.3.5. Pre-steady state Stopped-flow Kinetics

2.3.5.1 Sample preparation. The purified RNase H was concentrated using a 10K membrane in an Amicon concentrator. This was then passed through a Sephadex G-50 column previously equilibrated in kinetics buffer (20 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM EDTA, 1 mM 2-mercaptoethanol). The protein was eluted using the same buffer, 1 mL fractions were collected and the absorbance of the protein at 280 nm was monitored. The protein came out at tubes 2-4.

2.3.5.2 Instrumentation and Methods. Stopped-flow kinetics were carried out using an OLIS (On-Line Instrument Systems, Inc.) stopped-flow apparatus (Fig. 14). 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 for a slit width of 0.5 mm. A photomultiplier tube (Homatsu) was mounted linearly from the source to detect the loss of hypochromicity of the hybrid substrate at 260 nm. One syringe contained 570 nM RNase H in reaction buffer (50 mM Tris, pH 7.5, 50 mM KCl, 1 mM DTT) while a second syringe contained saturating amounts of poly (A.dT) hybrid substrate in the same reaction buffer. Each of the two syringes were loaded with the same concentration of metal cofactor (10 mM) to
Figure 12. Phosphocellulose-11 elution profile for exo III. The protein came off the column at 0.3 M KP buffer, pH 6.5.
Figure 13. SDS-PAGE gel stained with Coomassie Blue showing the purity of the isolated 6X Histidine Tagged RNase H using the Ni-NTA resin. Lane (1) flow through (2) 1X Binding Buffer (3-5) 1X Wash buffer (6) 85 mM imidazole buffer.
Figure 14. SDS-PAGE gel showing the purity of the isolated (A) exo III and (B) RNases H enzymes. Lane 1) D10R/D70K, 2) D10N, 3) D10S, 4) D10E, 5) D10R, 6) D10R/E48R, and 7) native.
Figure 15. Schematic diagram for the stopped-flow apparatus (Burgess, 1988)
minimize the effects of secondary binding chemistry. Reactants were pre-equilibrated at 37°C for at least 5 min. prior to mixing. At least 3 data sets were averaged for each v. 

2.3.6. Isothermal Titration Calorimetry.

2.3.6.1 Sample preparation.

2.3.6.1.1. Ribonuclease H with histidine tag. The purified recombinant ribonuclease H concentrate was dialyzed extensively in the calorimetry buffer (20 mM Tris-HCl, pH 6.5, 2-mercaptoethanol) at 4°C immediately before use. Dialysis tubing with 15000 MW cut off were prepared by boiling in distilled water with 1 mM EDTA for at least 10 minutes. Protein concentrations between 0.1-0.2 mM were used for the titration. The pH of the protein solution was carefully adjusted to pH 7.0 with NaOH immediately before each measurement.

2.3.6.1.2 Exonuclease III. The concentrated protein was dialyzed extensively in the calorimetry buffer (20 mM Tris-HCl, pH 7.5, 2-mercaptoethanol) at 4°C immediately before use. Protein concentrations between 0.1-0.2 mM were used for each titration.

2.3.6.2 Instrumentation and Methods. The block diagram for the Microcal OMEGA ultrasensitive titration calorimeter (Microcal Inc.) is shown in Fig. 15, and a drawing of the injector system, adiabatic shield, and matched reference and sample cells is shown in Fig. 16. During an experiment, a small, constant power of less than a milliwatt is dissipated in the heater of the reference cell, which activates the cell feedback circuit to drive ΔT, back to 0. In the absence of a reaction, the feedback power will be
Figure 16. Block diagram for the Microcal isothermal titration calorimeter (Wiseman, et. al., 1989).
Figure 17. The injector system, adiabatic shield, matched reference and sample cells of the Microcal OMEGA isothermal titration calorimeter (Wiseman, et. al., 1989).
constant at the resting baseline value. Exothermic reactions will temporarily decrease and endothermic reactions temporarily increase feedback power; the reaction heats are readily obtained by computer integration of these deflections from the baseline.

An injection schedule (number of injections, volume per injection and time between injections) was set up with interactive software, and this schedule was carried out with no operator involvement; all data were stored on disk. Areas for all injections were determined simultaneously by computer integration. Deconvolution was based on interactive nonlinear, least-squares, using either automatic or manual initialization parameters (n, ΔH, and K for either one or two sets of sites).

For each experiment, 12.5 μL of a 2 mM-10 mM metal solution were delivered over a 10 s time interval with 5-10 min between injections to allow complete equilibration. A total volume of 250 μL was added over a time period of 2-3 hours. Data points were collected every 2s. Background buffer was the same for both solutions (20 mM Tris, pH 7.0, 1 mM 2-mercaptoethanol) to minimize heat changes from mixing. Control experiments in the absence of protein were used to determine and, if necessary, correct for background heats of dilution or (de)protonation of the Tris buffer. To facilitate faster equilibration time, the solutions were pre-equilibrated at 25°C before loading. Each solution was thoroughly degassed under vacuum for 20 minutes prior to data acquisition to obtain better baseline stability. During data fitting, all parameters (number of binding sites, n; heat of binding, ΔH; and binding constant, K) were floating.
2.3.7. NMR Spectroscopy

2.3.7.1 Sample preparation.

Native and double mutant RNases H were exchanged into 10 mM sodium acetate, pH 5.5, 100 mM KCl buffer by ultrafiltration (Amicon). The protein solution was reduced to a volume of 0.5 mL, and the final sample contained 0.3-1 mM RNase H. Samples contained 10% (v/v) D$_2$O for the lock.

2.3.7.2 Instrumentation and Methods.

NMR spectra were acquired at 500.13 MHz on a Bruker AM-500 (Billerica, MA) spectrometer with Aspect 300 data station. All measurements were carried out in H$_2$O at 298K. The COSY spectra were recorded in magnitude mode with 2048 data points in the $t_2$ dimension and 512 points in $t_1$ dimension with 64-128 transients per $t_1$ increment. The water signal was suppressed by low power saturation of the H-O-D resonance during the relaxation period. An unshifted sine-bell function and a zero-filling of $t_1$ dimension to 2048 data points were made prior to Fourier transformation.

2.3.8. Circular Dichroism Spectroscopy.

2.3.8.1 Sample Preparation.

Purified protein samples were exchanged into 10 mM sodium citrate, 10 mM sodium borate, 10 mM sodium phosphate, pH 7.0, 50 mM KCl buffer by ultrafiltration (Amicon).
2.3.8.2. Instrumentation and Methods.

All CD spectra were collected at room temperature (20°C) in a JASCO 500 CD spectrophotometer. A quartz cuvette with 1 cm path length was used for the measurement. The parameters used were: step resolution at 1.0 nm, scan speed at 20 nm/min., band width of 2.0 nm, sensitivity at 10 mdeg/FS, and time constant at 4 sec. A total of 4 scans were collected per sample measurement.
Figure 18. Circular dichroic spectrum of 40 mg/mL exo III in 10 mM sodium citrate, 10 mM sodium borate, 10 mM sodium phosphate, pH 7.0, 50 mM KCl.
Figure 19. Circular dicroic spectrum of 30 mg/mL RNase H 6X His tag in 10 mM sodium citrate, 10 mM sodium borate, 10 mM sodium phosphate, pH 7.0, 50 mM KCl.
Figure 20. Circular dicroic spectrum of 30 mg/mL RNase H D10R/E48R in 10 mM sodium citrate, 10 mM sodium borate, 10 mM sodium phosphate, pH 7.0. 50 mM KCl.
3.1. RIBONUCLEASE H

Ribonuclease H (RNase H, EC 3.1.26.4), a magnesium-dependent endonuclease that specifically recognizes a DNA-RNA hybrid duplex, catalyzes the hydrolysis of the phosphodiester linkages in only the RNA strand to produce short oligoribonucleotides containing the 5'-phosphate residue and the 3'-hydroxyl function (Crouch & Dirksen, 1982).

3.1.1. Distribution and physiological role

The enzyme was first isolated from calf thymus (Stein and Hausen, 1969; Hausen and Stein, 1970). Since then, RNase H has been shown to be distributed widely from E. coli to humans, including retroviruses such as the human immunodeficiency virus (HIV; Johnson et. al., 1986; Hansen, et. al., 1988). Although the physiological role of this enzyme has not been conclusively determined, it’s function has been implicated with

### 3.1.2. Enzymatic Activity

The enzyme exhibits little base specificities. It can cleave the phosphodiester bond adjacent to pyrimidines (Robertson and Dunn, 1975) as well as those adjacent to purines as well (Inoue, et. al., 1987; Shibahara, et. al., 1987; Metelev, et. al., 1988). These studies, as well as that of Donis-Keller's (1979), suggest that the minimum size of the DNA/RNA hybrid recognized by the enzyme is a tetramer. The complete digestion of poly (A.dT) yields oligonucleotides with various chain lengths ranging from monomer to hexamer (Berkower, et. al., 1973) or dimer to nonamer (Crouch, 1981). The major products are (pA)_1 and (pA)_2 in both cases. AMP was detected, with poor yield (4%), only by Berkower, et. al., (1973), suggesting that the enzyme does not exhibit exonuclease activity.

The enzyme possesses a broad pH optimum between 7.5 and 9.1 (Berkower, et. al., 1973). It requires 2-4 mM MgCl₂ and 50-100 mM NaCl for maximal activity. The requirement for Mg²⁺ is only partially replaced by Mn²⁺. The enzyme exhibits 2-3% of the maximal activity in the presence of 0.2 mM MnCl₂ (Berkower, et. al., 1973). The enzymatic activity is inhibited by dextran (Dirksen and Crouch, 1981).

Various methods have been described to determine RNase H activities (Crouch and Dirksen, 1982). Hydrolysis of a radioactively labeled substrate with the enzyme followed by measurement of the radioactivity in acid (ethanol) soluble or insoluble...
materials is generally used for RNase H assay (Kanaya, et. al., 1990a; Uchiyama, et. al.,
1994a). In our laboratory, we used the pre-steady state stopped-flow kinetics method
using poly (A.dT) as the hybrid substrate to monitor the enzymatic activity. Metal
activation of RNase H has been monitored by the change in hypochroism of the
DNA/RNA substrate upon hydrolysis. This methodology obviates the inaccuracies
inherent in the use of radiolabeled substrates (Black & Cowan, 1994a).

3.1.3. Physicochemical Properties

The solubility of the enzyme is highest at around pH 5 and dramatically decreases
as the pH is raised beyond 7. The molar absorption coefficient, ε, of the enzyme is 39500
M\(^{-1}\) cm\(^{-1}\) (Kanaya, et. al., 1990b). The enzyme consists of a single polypeptide chain with
155 amino acid residues and a molecular weight of 17,580 (Kanaya and Crouch, 1983a).

The enzyme is stable between pH 1.66 and pH 10.12 at 10°C, as judged by the
circular dichroic (CD) spectra (Kanaya, et. al., 1993). The unfolding of the enzyme with
thermal denaturation is fully reversible between pH 1.66 and 4.02. The temperature of
the midpoint of the transition, T\(_m\), is 65-66°C at pH 4.02, but decreases as the pH is
lowered (Kimura, et. al., 1992a,b; Kanaya, et. al., 1993). At pH 5.5, the enzyme is more
stable, but it reversibly unfolds in a single cooperative fashion in the presence of 1M
guanidine hydrochloride (GdnHCl). The T\(_m\) of the enzyme is 50.2-52.7°C under these
conditions (Kanaya, et. al., 1991a, 1993; Kimura, et. al., 1992b). At an alkaline pH, the
unfolding of the enzyme is not reversible, even in the presence of denaturants, and
therefore the T_m cannot be determined. However, the apparent T_m values decrease as the pH is raised.

3.1.4. Three Dimensional Structure

RNase H is functionally active as a monomer. A synthetic gene has been cloned and expressed in *E. coli* (Kanaya & Crouch, 1983; Kanaya, et. al., 1989; Yang, et. al., 1990). High resolution structural data are available from both X-ray crystallography, now at 1.48 Å resolution (Yang, et. al., 1990; Katayanagi, et. al., 1992), and NMR studies (Yamazaki, et. al., 1991, 1993).

These structural studies reveal a single domain α + β protein consisting of five-stranded β-sheet and five α-helices (Fig. 21). The single binding site for Mg^{2+}, which is required for enzymatic activity, has been identified by soaking RNase H crystals in a solution containing Mg^{2+} ion followed by calculation of the difference Fourier maps (Katayanagi, et. al., 1990). The position of the Mg^{2+} binding site was confirmed by determining the three-dimensional structure of the enzyme in complex with Mg^{2+} at 2.8 Å resolution (Katayanagi, et. al., 1993b). The backbone structure of the enzyme was basically unchanged on binding of Mg^{2+}. According to the crystal structure of the enzyme-Mg^{2+} complex, the side chain O atom of Asp 10, the main chain carbonyl oxygen of Gly 11, the side chain O atom of Glu 48, and the side chain O atom of Asn 44 are located close to the Mg^{2+} with distances of 2.1, 2.4, 2.4, and 3.0 Å, respectively, indicating that these residues, except for Asn 44, directly coordinate with the Mg^{2+}. The localizations of the Mg^{2+} and the amino acid residues that provide the coordinates for the
Figure 21. Overall structure and the active site of *E. coli* RNase H (Katayanagi, et. al., 1993a).
binding of Mg\[^{2+}\] are shown in Fig. 22. A large cleft-like depression, in which a DNA/RNA hybrid is expected to bind, is located in the middle of the molecule. The amino acid residues included in \(\alpha_{II}, \alpha_{III}, \) the C-terminal portion of \(\beta_{A}, \) the N-terminal portion of \(\alpha_{I}, \) the loops between \(\beta_{C} \) and \(\alpha_{I}, \) between \(\alpha_{III} \) and \(\alpha_{IV}, \) and between \(\beta_{E} \) and \(\alpha_{V}, \) constitute this cleft-like depression. The extensive hydrophobic core is formed by amino acid residues located in \(\beta_{A}, \beta_{D}, \beta_{E}, \alpha_{I}, \alpha_{II} \) and \(\alpha_{IV}. \) Two parallel \(\alpha\)-helices, \(\alpha_{I} \) and \(\alpha_{IV}, \) are tightly associated by a ladder of leucine and isoleucine interactions. The \(\alpha_{III} \) helix and the following loop form the relatively independent structure termed the handle region (Yang, et. al., 1990) or the basic protrusion (Katayanagi, et. al., 1992). This densely charged basic protrusion, which is located at the edge of the cleft-like depression, is in sharp contrast to the region rich in negative charges around the Mg\[^{2+}\] binding site. Three tryptophan residues clustered in this region, as well as Trp 104 in the \(\alpha_{IV} \) helix, seem to fix the conformation of this basic protrusion through aromatic interactions. The high values in the mean temperature factors in the \(\alpha_{III} \) helix suggest that this helix is rather flexible.

The catalytic mechanism of RNase H seems to be similar to that of DNase I (Suck & Oefner, 1986) and exo III, and some similarities have been found in the architecture of the \(\beta\)-sheets around the suggested active site residues (Fig. 23). It has also been confirmed by x-ray crystallography that Mg\[^{2+}\] binds close to this region. The amino acid residues of this enzyme responsible for substrate binding and catalytic reaction have been identified by site-directed mutagenesis (Kanaya, et. al., 1990a).
Figure 22. Active site of the Mg$^{2+}$-bound enzyme (Katayanagi, et al., 1993b).
Figure 23. Directional relationship comparison of β-strand topologies between RNase H and DNase I (Katayanagi, et. al., 1990).
3.1.5. NMR Studies

The backbone nuclei of *E. coli* RNase H have been completely assigned by heteronuclear three-dimensional NMR spectroscopy, with the help of amino acid-specific $^{15}$N labeling (Nagayama, et. al., 1990; Yamazaki, et. al., 1991). Amino acid-selective $^1$H labeling of a fully deuterated protein sample also allowed the assignments of the backbone nuclei in the labeled residues (Oda, et. al., 1992). More recently, all types of $^1$H, $^{15}$N and $^{13}$C magnetic resonances for the enzyme have been completely assigned by double and triple-resonance two- and three-dimensional NMR spectroscopy (Yamazaki, et. al., 1993).

3.1.5.1. pK$_a$ values

The pK$_a$ values for the imidazole groups of all the His residues of *E. coli* RNase H were determined by analyzing the pH titration shifts of the C2H proton resonances of the imidazole groups (Oda, et. al., 1993a). The pK$_a$ values for the side-chain carboxyl groups of all the Asp and Glu residues have also been determined (Oda, et. al., 1994). Among four conserved residues, Asp 10 and Asp 70 show unusual ionization behaviors. The pK$_a$ values of 6.1 for Asp 10 and 2.6 for Asp 70 are considerably higher and lower, respectively, than the normal value of 4.0 (Fersht, 1985). These pK$_a$ values, however, shift to 4.2 for Asp 10 and 3.4 for Asp 70, but that of Glu 48 does not, when Mg$^{2+}$ binds to the enzyme. These results suggest that Glu 48 is not involved in the Mg$^{2+}$ binding site, which seems surprising because determination of the crystal structure of the enzyme-Mg$^{2+}$ complex clearly shows that Glu 48 directly coordinates with Mg$^{2+}$ ion.
3.1.5.2. Binding of Metal Ions and Substrates

The metal ion binding site of *E. coli* RNase H and the binding constant were determined by monitoring chemical shift changes during the titration of the enzyme with divalent cations, using $^1$H-$^{15}$N heteronuclear two-dimensional NMR (Oda, et. al., 1991). From the titration analysis, a single Mg$^{2+}$, Ca$^{2+}$ or Ba$^{2+}$ ion has been shown to bind to the enzyme at pH 5.5, in the absence of the substrate, with dissociation constants of 4.6 mM, 2.2 mM, and 2.9 mM, respectively. These values are considerably higher than those determined by NMR or calorimetry at pH 7.6 (Huang & Cowan, 1994) probably because Asp 10, which directly coordinates with Mg$^{2+}$ ion, is protonated at pH 5.5 (Oda, et. al., 1994).

The amino acid residues that interact with the substrate have also been identified by titration of the chimeric nonanucleotide duplex, while changes in the chemical shifts of the nitrogen and hydrogen nuclei of the backbone amides were monitored (Nakamura, et. al., 1991b). The amino acid residues with backbone amide chemical shifts that were affected by the addition of this substrate are clustered around the Mg$^{2+}$ binding site of the enzyme.
3.1.6. Role of Amino Acid Residues

3.1.6.1. Site-Directed Mutagenesis

The role of each amino acid residue in the function and stability of *E. coli* RNase H has been extensively investigated by site-directed mutagenesis experiments. The relative $k_{cat}$ and $K_m$ values of the various mutant proteins with single amino acid substitutions are summarized in Table 5 (Kanaya & Ikehara, 1995).

3.1.6.2. Conserved residues

Among the various highly conserved amino acid residues with functional side chains, Asp 10, Glu 48 and Asp 70 were identified as active-site residues, because the replacement of any of these residues with Asn or Gln and with Ala almost abolishes the enzymatic activity (Kanaya, et. al., 1990a). His 124 and Asp 134 have not been considered to be catalytic residues, because the His 124 to Ala mutation reduced both the substrate-binding affinity and the hydrolysis rate and the Asp 134 to Asn mutation has no effect on the enzymatic activity (Kanaya, et. al., 1990a). However, further mutagenesis experiments revealed that the imidazole group of His 124 is not essential for the enzymatic activity, but is required to enhance the catalytic efficiency of the enzyme (Oda, et. al., 1993a). Likewise, Asp 134 has also been shown to contribute to the acceleration of the hydrolysis rate of the enzyme (Haruki, et. al., 1994). The Asn 44 to Ala mutation decreased the substrate-binding affinity without affecting the hydrolysis rate (Table 5). The mutagenesis experiment suggests that Asn 44 is involved in the substrate binding instead of the Mg$^{2+}$ binding.
<table>
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*Asterisk indicates relative specific activity.

Table 5. Relative kinetic parameters of E. coli RNase H mutants (Kanaya and Ikehara, 1995).
3.1.6.3. Basic Protrusion

In the basic protrusion of the enzyme, seven basic amino acid residues are clustered. Alanine-scanning mutagenesis, followed by the determination of the kinetic parameters of each mutant protein, strongly suggests that the cluster of positive charges in this region is important for the effective binding of the substrate (Kanaya, et. al., 1991c).

3.1.6.4. Cysteine Residues

The enzyme contains three free Cys residues at positions 13, 63 and 133. Site-directed mutagenesis and chemical modification experiments have been clearly shown that the inactivation of the enzyme arises not from the loss of the thiol groups but from the steric hindrance by the modifying group incorporated at Cys 13 or Cys 133 (Kanaya, et. al., 1990b).

3.1.7. Mechanisms for Substrate-Binding and Hydrolysis

3.1.7.1. Model for the Enzyme-Substrate Complex

A model for the complex between the enzyme and a DNA/RNA hybrid was originally constructed by Yang, et. al. (1990b). They docked a hybrid model (decamer) constructed from an A-type mixed hybrid (Wang, et. al., 1982) onto the free structure of the enzyme by simply superimposing a phosphate from the DNA strand onto the sulfate position and allowing the RNA strand to interact with the active site of the enzyme. According to this model, a DNA/RNA hybrid fits well into the large groovelike depression of the protein molecule (Fig. 24).
Figure 24. RNase H-substrate complex model built by docking the hybrid oligomer by molecular graphics (Katayanagi, et. al., 1993c).
3.1.7.2. Proposed Mechanism for Catalytic Reaction

Based on the findings that a single Mg\(^{2+}\) ion binds to the enzyme (Katayanagi, et. al., 1990, 1992), and that the three carboxylates, Asp 10, Glu 48 and Asp 70, are essential for the catalytic activity (Kanaya, et. al., 1990a), a carboxylate-hydroxyl relay mechanism has been proposed for *E. coli* RNase H activity (Nakamura, et. al., 1991b; Oda et. al., 1993a; Kanaya and Ikehara, 1993) (Fig. 25). According to this mechanism, at least one carboxyl group is required to bind the Mg\(^{2+}\) ion, which can attract the phosphate group of RNA electrostatically. For cleavage of the P-O3' bond, one carboxyl group acts as a proton acceptor for H\(_2\)O to release a hydroxyl ion, which subsequently attacks the phosphate group nucleophilically, and one carboxylate acts as a proton donor. Because Asp 70 is the only carboxylate that does not coordinate with the Mg\(^{2+}\) ion and is essential for the catalytic activity of the enzyme, this residue is currently proposed to activate a H\(_2\)O molecule as a proton acceptor for the cleavage of the P-O3' bond (Katayanagi, et.al., 1993a,b).

Recently, a revised model was proposed by Kanaya and Ikehara (1995) and is shown in Fig 26. According to this mechanism, Glu 48 accepts a proton from an attacking H\(_2\)O molecule. Asp 70 governs the conformation of Asp 10 through electrostatic repulsion. His 124 accelerates the proton transfer by accepting a proton from the resultant-COOH group of Glu 48 and acting as a proton pump, and thereby enhances the catalytic efficiency of the enzyme (Oda, et. al., 1993a). Asp 134 may be required to hold the attacking H\(_2\)O molecule or a H\(_2\)O molecule that supplies a proton to the 3'-oxygen released after the cleavage of the P-O3' bond of the RNA.
Figure 25. Proposed mechanism for the hydrolysis of hybrid substrate by magnesium-bound RNase H.
Figure 26. Revised mechanism for the RNase H-catalyzed hydrolysis of the hybrid substrate (Kanaya & Ikehara, 1995).
A two-metal ion mechanism, which is basically proposed for the exonuclease activity of *E. coli* DNA polymerase I (Beese and Steitz, 1991), has been proposed for RNase H activity (Yang, et. al., 1990b), because of the similarity in the geometrical conformations of the catalytically essential carboxylates between these two enzymes. However, no evidence to support this mechanism has so far been provided. Crystallographic, NMR, kinetic and calorimetric studies clearly indicate that only one Mg$^{2+}$ ion binds to the free-form of the enzyme (Katayanagi, et. al., 1993; Huang & Cowan, 1994; Black & Cowan, 1994a; Casareno & Cowan, 1996).

### 3.2. EXONUCLEASE III

Exonuclease III is the major apurinic/apyrimidinic (AP) DNA repair endonuclease in *E. coli*, accounting for over 80% of the cellular AP endonuclease activity (Weiss, 1981). Exo III (EC 3.1.11.2) is the product of the *xth* gene of *E. coli* K-12 (Saporito, et. al., 1988). This enzyme is responsible for the repair of many types of DNA damage that arise spontaneously under normal growth conditions. In addition to being a class II AP endonuclease (it hydrolyzes the phosphodiester bond 5' to the AP site), exo III exhibits other catalytic activities: (1) 3'-5' exonuclease activity (Richardson and Kornberg, 1964; Weiss, 1981), (2) 3'-phosphomonoesterase activity (Richardson & Kornberg, 1964; Weiss, 1981), (3) urea endonuclease activity (Kow & Wallace, 1985), and (4) ribonuclease H activity (Weiss, 1981). Thomas & Olivera (1977) demonstrated that exo III is a nonprocessive enzyme, that is, it dissociates from the DNA after every catalytic event.
In order to explain these multiple, apparently unrelated activities of exo III, Weiss (1981) proposed that the active site recognizes a space between the two DNA strands generated by either the loss of a base (AP endonuclease activity), the presence of a small fragmented base (urea endonuclease activity), or simply the 3' breathing end (the 3'phosphomonoesterase, the 3' phosphodiesterase and the 3'-5' exonuclease and RNase H activities) (Kow, 1989). Apparently, all of these different activities require Mg$^{2+}$ (Weiss, 1981).

To understand the structure, function and regulation of exo III, the xth gene of *E. coli* K-12 has been sequenced (Saporito, et. al., 1988) (Fig. 27). The enzyme is a monomeric protein with a molecular weight of 30,921 based on the DNA sequence. Saporito et. al. (1988) obtained a native MW of 25,000 from gel filtration and a MW of 32,000 in the presence of SDS. Using the same methods, Weiss (1976) reported values of 27,400 and 28,500, respectively.

3.2.1. Three Dimensional Structure

Recently, the structure of exo III has been determined to 1.7 Å resolution (Mol, et. al., 1995). The enzyme is a compact, globular protein of rough dimensions 55 x 50 x 45 Å consisting of two six-stranded $\beta$-pleated sheets flanked by four $\alpha$-helices (Fig. 28). It revealed a 2-fold symmetric, four-layered $\alpha\beta$ fold with similarities to deoxyribonuclease I (DNase I) (Oefner & Suck, 1986) and RNase H (Katayanagi, K. et. al., 1990). Figure 29 shows the polypeptide topology schematic for DNase I and exo III. Inspite of the multiple biochemical and biological activities exhibited by exo III, there is
Figure 27. Nucleotide sequence of the xth gene encoding the exo III (Saporito, et al., 1988).
Figure 28. Three dimensional structure of exo III (Mol. et. al.. 1995).
Figure 29. Polypeptide topology comparison for a) exo III and b) DNase I (Mol. et. al. 1995).
no evidence for the presence of more than one active site. The proposed active site is lined with charged and polar residues that interact with dCMP in the ternary complex determined at 2.6 Å resolution. Mn\(^{2+}\) and dCMP bind to exo III at one end of the αβ-sandwich, in a region dominated by positive electrostatic potential (Mol. et. al., 1995).

3.2.2. Proposed Mechanism

The active site of exo III is located at the bottom of the groove between the two β-sheets. In the ternary complex, the single Mn\(^{2+}\) ion is bound (~2.2 Å) to the O atom of the side chain of Glu 34. Other potential ligands lack the correct orientations or bond lengths for direct metal ion ligation, but movements of Asp 258 could allow metal ion binding (Mol, et. al., 1995). The possibility of binding two metal ions appear unlikely. Native crystal soaked in 10 mM Zn\(^{2+}\) exhibited no notable peak while crystals soaked in Mn\(^{2+}\) or Sm\(^{2+}\) had peaks only at the same single site. The structural data suggest phosphate-bond cleavage at AP sites through a nucleophilic attack facilitated by a single bound metal ion. Figure 30 illustrates the proposed reaction mechanism for the hydrolytic cleavage of the P-O3' bond. This proposed Asp-His-H\(_2\)O proton acceptors and donors are thus similar to the Asp-His-Ser catalytic triad observed in serine proteinase. The authors proposed that the positively-charged metal ion may serve a structural role to orient the phosphate group, stabilize the transition state, and also polarize the P-O3' bond. The Asp 151 carboxylate may serve as the Lewis acid, which protonates the O3' leaving group.
**Figure 30.** Proposed mechanism for exo III (Mol, et. al., 1995).
CHAPTER 4

RATIONAL REDESIGN OF THE ACTIVE SITE OF MAGNESIUM-DEPENDENT RNASE H TO FORM AN ACTIVE METAL-INDEPENDENT NUCLEASE.

4.1. Introduction

Recent advances in the understanding of structure-activity correlations have fueled interest in the *de novo* design or modification of functional properties of enzymes by use of site-directed mutagenesis (Jung, et. al., 1995; Bocanegra, et. al., 1993; Jenkins, et. al., 1992; Willett, et. al., 1995). In this chapter we demonstrate how a critical appraisal of molecular mechanism, in this case the role of an essential metal cofactor in enzyme catalysis, can provide the insight required for rational reconstruction of active site residues to produce a metal-independent enzyme exhibiting significant levels of activity. The enzyme selected for study was the magnesium-dependent endoribonuclease H, which hydrolytically cleaves the ribonucleotide backbone of RNA/DNA hybrids to...
produce 5'-phosphate and 3'-hydroxyl oligonucleotides (Yang, et. al., 1990). The *E. coli* enzyme is structurally homologous to the RNase H domain of HIV reverse transcriptase and shows retention of key active site residues (Davies, et. al., 1991). Recent studies suggest that hydrolysis is promoted by one essential metal cofactor (Katayanagi, et. al., 1993; Huang, & Cowan, 1994; Black, & Cowan, 1994a; Uchiyama, et. al., 1994a; 1994b), which is typically divalent magnesium. Intensive research efforts in our laboratory have resulted in the proposal of an unusual mechanism for enzyme-catalyzed metal-mediated hydrolysis of a nucleotide backbone: through stabilization of a transient intermediate by formation of an outer-sphere complex with the positively-charged metal cofactor (Jou, & Cowan, 1991). This observation suggested to us the interesting hypothesis that mutation of active site carboxylate residues to positively-charged Lys or Arg might provide sufficient positive charge density and hydrogen bonding propensity in the active site domain to mimic the role of hydrated divalent magnesium ion. These experiments also provide firm support for the mechanism of metal-mediated hydrolysis described in earlier works from our laboratory (Black, & Cowan, 1994a; Jou, & Cowan, 1991; Kim, et. al., 1992; Black, et. al., 1994b; Black, et. al, 1994c).
4.2. Data and Results

Our design of the engineered active site was guided by recent crystallographic studies of the Mg\(^{2+}\)-bound enzyme (Katayanagi, et. al., 1993). Of the three principal active site carboxylate residues, crystallographic evidence suggests that both Glu48 and Asp10 are bound to Mg\(^{2+}\), while Asp70 is proposed to serve as a catalytic base that is required for deprotonation of water prior to hydrolysis of the backbone (Katayanagi, et. al., 1993). We selected Asp10, Glu48, and Asp70 for mutational studies. The structures of the amino acid residues substituted for positions 10, 48 and 70 are shown in Fig. 31. Oligonucleotide-directed mutagenesis was done using Kunkel’s method (1985). The mutation was confirmed by Sanger’s dideoxynucleotide sequencing method (Sanger, et. al., 1977). The wild type as well as the mutant proteins were purified as described in Experimental Procedures. Flow diagrams for protein isolation and sample preparations are outlined in Figs. 32 and 33.

Kinetic activity was evaluated using the rapid (stopped-flow) kinetics method developed in our laboratory (Black, & Cowan, 1994a). Absorbance versus time plots for the hydrolysis of poly (A.dT) hybrid substrate catalyzed by wild type or mutant RNase H in the presence or absence of added magnesium ion are shown in Figs. 34-36. Kinetic traces were fitted using Origin software. Since we were looking at the pre-steady state kinetics, the initial linear part of each absorbance trace was fitted to a straight line to
Figure 31. Structures of amino acids substituted at positions 10, 48 and 70.
Figure 32. Flow diagram for the isolation of RNase H for stopped-flow kinetics measurements.
cell lysate

↓

ammonium sulfate precipitation

↓

P-11 column

↓

concentrate
exchange buffer

↓

10 mM sodium acetate-d<sub>3</sub>, pH 5.5
100 mM potassium chloride

**Figure 33.** Flow diagram for the preparation of NMR samples.
Figure 34. Absorbance vs. time plots for hydrolysis of a hybrid substrate catalyzed by RNase H in the a) absence and b) presence of Mg$^{2+}$. 
Figure 35. Kinetic traces for the enzyme catalyzed hydrolysis of hybrid substrate in the presence of 10 mM MgCl₂: a) D10E. b) D10N/D10S/D10G/D10R.
Figure 36. Kinetic traces for the enzyme-catalyzed hydrolysis of a hybrid substrate using a) D10R/D70K and b) D10R/E48R (-Mg^{2+}), pH 5.5.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (substr s$^{-1}$)</th>
<th>Enzyme</th>
<th>$k_{cat}$ (substr s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>native</td>
<td>28 ± 8</td>
<td>Asp10Glu</td>
<td>14 ± 4</td>
</tr>
<tr>
<td>native (-Mg$^{2+}$)</td>
<td>0</td>
<td>Asp10Asn/Ser/Arg/Gly</td>
<td>0</td>
</tr>
<tr>
<td>Glu48Asp$^b$</td>
<td>1.1 ± 0.3</td>
<td>Asp10Arg/Asp70Lys</td>
<td>0</td>
</tr>
<tr>
<td>Glu48Gln$^b$</td>
<td>0</td>
<td>Asp10Arg/Glu48Arg (-Mg$^{2+}$)</td>
<td>24 ± 7$^c$</td>
</tr>
<tr>
<td>Asp70Asn$^b$</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Unless otherwise indicated, all reactions were carried out in 20 mM Tris base, pH 7.5, 50 mM KCl, 0.1 mM DTT, 0.1 mM EDTA, saturating poly(A.dT), 10 mM Mg$^{2+}$, 570 nM RNase H, and 25 ± 1°C. Data was obtained by use of an OLIS (On-Line Instrument Systems, Inc.) stopped-flow apparatus. At least 3 data sets were averaged for each $v_o$.

Data adapted from Kanaya, et al., 1990.

For the Asp10Arg/Glu48Arg mutant the optimal activity obtained at pH ~ 5.5 is reported.

Table 6. Kinetic parameters for RNase H digestion of hybrid substrate.
determine the initial velocity for each reaction. Hydrolysis of the hybrid substrate by RNase H followed Michaelis-Menten kinetics. Kinetic parameter ($k_{cat}$) was then determined using the initial velocity and known concentrations of enzyme. Results are tabulated in Table 6.

4.3. Discussion of Results:

Mutation of the essential catalytic base (Asp70Asn) resulted in inactive enzyme. Single point mutations of the magnesium-binding residues, Asp10 and Glu48, yielded inactive enzyme either with or without added Mg$^{2+}$ (Table 6), with the expected exception of the Asp10Glu and Glu48Asp mutants which retained 50% and 4% activity, respectively. Introduction of a single positive charge (for example, Asp10Arg) is insufficient for activation, while also serving to inhibit binding of magnesium cofactors as observed by calorimetry. In contrast, at pH 7.5 the double mutant D10R/E48R demonstrated about 7% of the maximum activity of the native enzyme, even in the absence of added Mg$^{2+}$. To pursue this, we evaluated the kinetic profile of this double mutant vs. pH and surprisingly, at pH 5.5 this active mutant demonstrated up to 87% of the maximum activity of the native enzyme, all in the absence of added magnesium ion (Fig. 36b). Substrate activation by native enzyme is promoted through transition state stabilization, which is dominated by hydrogen bonding from waters of solvation. In the
Figure 37. Variation of activity with pH for the D10R/E48R mutant (•). Data for the native RNase H is shown for comparison (o) (Black & Cowan, manuscript submitted).
double mutant, the role of the hydrogen bond donor is accommodated by the guanidinium centers of the mutant sidechains, since there is only a small increase in transition state energy (~ 0.1 kcal mol⁻¹) relative to the reaction promoted by hydrated magnesium.

The pH dependence of activity for the D10R/E48R mutant is shown in Fig. 37 and is distinct from that of the native Mg²⁺-promoted activity. Each can be fit to yield similar ionization constants (pKₐ's ~ 6.5 and 6.6, respectively). Loss of activity at lower pH for the native enzyme reflects protonation of Asp10, a Mg²⁺-binding residue. In contrast, the activity of the D10R/E48R mutant is optimal at pH values immediately below the pKₐ and we ascribe the loss of activity at higher pH to deprotonation of one of the three basic residues in the active site of the mutant enzyme (Arg10, Arg48, and His124). A significant decrease in pKₐ for arginine is possible, by analogy with the increase in pKₐ observed for Asp10 in the native enzyme, but, this has not yet been established.

Two-dimensional NMR analysis of the active double mutant indicated minimal conformational change relative to the native protein (Fig. 38-39). Published crystallographic data obtained from the native enzyme with and without bound Mg²⁺, and from active site mutants, have demonstrated localized perturbations in sidechain orientations and hydrogen bonding patterns in the Mg²⁺-binding pocket. However, the striking result reported herein, demonstrating up to 87% recovery of native activity by the
Figure 38. Aliphatic region of the 2D COSY spectrum of a) native RNase H with Mg$^{2+}$ and b) double mutant D10R/E48R without Mg$^{2+}$.

to be continued
Fig. 38 (cont.)

(b)
Figure 39. Aromatic region of the 2D COSY spectrum of a) native RNase H with Mg$^{2+}$ and b) double mutant D10R/E48R without Mg$^{2+}$. 

to be continued
Fig. 39 (cont.)

(b)
double mutant in the absence of metal cofactor, does not support a role for these minor structural changes in catalytic activation.

4.4. Conclusion

Our results demonstrate how a critical appraisal of molecular mechanism, in this case the role of an essential metal cofactor, can provide the insight required for rational engineering of an enzyme's active site. In contrast to previous studies, the net enzymatic reaction has not been altered. Rather, the active site has been modified to provide the important catalytic components as an integral structural element of the enzyme's catalytic apparatus.
CHAPTER 5

STOICHIOMETRY AND THERMODYNAMIC PARAMETERS OF DIVALENT METAL ION BINDING TO RNASE H AND EXO III

5.1. Introduction

The mechanism of metal-ion-mediated phosphate ester hydrolysis by enzymes and ribozymes is an important issue in nucleic acid biochemistry (Black, et. al, 1994b; Cowan, 1995; Cowan, 1993). The stoichiometry of metal cofactor required to effect hydrolysis has been the subject of speculation (Black & Cowan, 1994a; Steitz, et. al., 1993; Davies, et. al., 1991; Katayanagi, et. al., 1993b; Katayanagi, et. al., 1992), with much of the current thinking on this subject being inferred from analysis of a rather small body of crystallographic data (Steitz, et. al., 1993; Davies, et. al., 1991), with relatively few results from solution experiments (Black & Cowan, 1994a). Inspite of the "common awareness" that the metalllobiochemistry of transition metal ions (typically Mn\(^{2+}\) or Co\(^{2+}\)) may differ from Mg\(^{2+}\)(Cowan, 1992), these analog cofactors are commonly used without
a critical appraisal of the significance of the results. We have carried out a comparative
study of the binding of Mg$^{2+}$, Ca$^{2+}$ and Mn$^{2+}$ to *E. coli* RNase H and exo III enzymes.

These isothermal titration calorimetry experiments characterize the
thermodynamics of metal ion binding and selectivity, and clearly show a 1:1
stoichiometry for metal binding to RNase H for Mg$^{2+}$, Ca$^{2+}$ and Mn$^{2+}$. However, exo III
binds at least two Mn$^{2+}$ ions, in sharp contrast to results obtained for Mg$^{2+}$ and Ca$^{2+}$
which show a single site. Inasmuch as crystallographic and mechanistic studies of
magnesium-dependent nucleases often substitute Mn$^{2+}$ for Mg$^{2+}$, these results carry clear
implications for the interpretation of such experiments, and for the important issue of
one-metal-ion versus two-metal-ion mechanisms for nucleic acid hydrolysis.

5.2. Protein Isolation and Preparation.

The *rnhA* gene coding for RNase H had been cloned in pET 21b(+) plasmid. To
facilitate easier and faster purification, we utilized the his-tag incorporated at the C-
terminus of the multiple cloning site. Oligonucleotide-directed mutagenesis was done
using Kunkel’s method as described in the Experimental Procedures. Fig. 40 illustrates
the steps in the isolation of pure protein. The recombinant RNase H protein was purified
using the Ni$^{2+}$-NTA resin and was >99% pure. The protein was concentrated using the
amicon concentrator and dialyzed extensively at 4°C in 20 mM Tris-HCl, pH 6.5, 1 mM
2-mercaptoethanol. The pH of the enzyme solution was carefully adjusted to pH 7.0 by the addition of NaOH immediately before the calorimetry measurement.

On the other hand, the xth gene coding for the exo III was PCR amplified and subcloned in pET 21b(+) plasmid. Production of protein was induced by addition of IPTG and protein purification was done as described in the Experimental Procedures. Fig. 41 shows the steps for protein isolation and sample preparation. The purified protein was dialyzed extensively at 4°C in 20 mM Tris-HCl, pH 7.5, 1 mM 2-mercaptoethanol.

5.3. Results and Discussion

Metal ion binding to E. coli RNase H and exo III was evaluated by isothermal titration calorimetry as described in the Experimental Procedures. Table 7 summarizes the thermodynamic parameters obtained for metal binding to RNase H. In each case, the fit yielded one metal ion binding site (Figs. 42-44). The binding entropy is positive for both Mg$^{2+}$ and Mn$^{2+}$ (9.7 and 5.7 cal K$^{-1}$ mole$^{-1}$, respectively), consistent with the release of several water molecules as suggested by crystallographic data. The carboxylates of Asp10 and Glu48, and the backbone carbonyl of Gly11 have been shown to coordinate to Mg$^{2+}$ (Katayanagi, et. al., 1993b). In contrast, the binding entropy for Ca$^{2+}$ is negative (-10.4 cal K$^{-1}$ mole$^{-1}$). Most likely the larger Ca$^{2+}$ ion can coordinate to the catalytic base Asp70 (Yang, et. al., 1990; Katayanagi, et. al., 1990). Subsequent ordering of residues in
Figure 40. Flow diagram for isolation of recombinant RNaseH protein for calorimetry.

- cell lysate
  - \( \downarrow \)
  - \( \text{Ni}^{2+}-\text{NTA resin} \)
  - \( \downarrow \)
  - concentrate
  - \( \downarrow \)
  - dialyze
  - \( \downarrow \)
  - 20 mM Tris-HCl, pH 6.5
  - 1 mM 2-mercaptoethanol
cell lysate

↓

ammonium sulfate precipitation

↓

P-11 column

↓

20 mM Tris-HCl, pH 7.5
1 mM 2-mercaptoethanol

Figure 41. Flow diagram for the isolation of exo III used in the calorimetry measurements.
Figure 42. Calorimetric titration of 0.13 mM RNase H with 20 x 12.5 μL injections of 10.0 mM Mg^{2+} at 298 K in 20 mM Tris, pH 7.0, 1 mM 2-mercaptoethanol. The raw data is shown above and the integrated heats and fitted data are below.
**Figure 43.** A similar experiment, and conditions, with 0.17 mM RNase H and 20 x 12.5 μL injections of 2.7 mM Ca²⁺.
Figure 44. A similar experiment and conditions, with 0.22 mM RNase H and 19 x 12.5 μL injections of 10.0 mM Mn²⁺.
Data from the calorimetric titration of RNase H (0.17 mM) with 20 x 12.5 nL injections of metal ion (2 to 10 mM) at 298 K in 20 mM Tris, pH 7.0, 1 mM 2-mercaptoethanol, obtained from the average of at least two independent experiments. Experiments were carried out with appropriate controls, correcting for heats of dilution when necessary.

Table 7. Thermodynamic parameters for metal ion binding to *E. coli* RNase H.

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>K (M⁻¹)</th>
<th>ΔH (kcal mol⁻¹)</th>
<th>ΔG (kcal mol⁻¹)</th>
<th>S (cal mol⁻¹ K⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg²⁺</td>
<td>1.1 (± 0.2) x 10⁴</td>
<td>-2.6 ± 0.1</td>
<td>-5.5 ± 0.1</td>
<td>9.7 ± 0.7</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>4.4 (± 1.2) x 10⁵</td>
<td>-10.8 ± 0.2</td>
<td>-7.7 ± 0.1</td>
<td>-10.4 ± 0.6</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>5.9 (± 0.6) x 10⁴</td>
<td>-4.8 ± 0.2</td>
<td>-6.5 ± 0.1</td>
<td>5.7 ± 0.8</td>
</tr>
</tbody>
</table>
the active site can presumably compensate for the release of up to four water molecules displaced from hydrated Ca$^{2+}$, and would explain both the negative binding entropy and the negligible level of activity observed for the Ca$^{2+}$-activated enzyme (Jou & Cowan, 1991). The Mn$^{2+}$ binding stoichiometry that we have obtained for *E. coli* RNase H contrasts with crystallographic data obtained with 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 (Davies, et. al., 1991). 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.

The issue of metal cofactor stoichiometry is important and of general relevance to the understanding of metal-mediated nucleic acid hydrolysis. The possibility of distinct metal binding stoichiometries, according to the selection of cofactor is clearly demonstrated for a metallonuclease enzyme in this study. Table 8 summarizes calorimetry plots and parameters for metal binding to exo III. The structure of this DNA repair enzyme has recently been established (Mol, et. al., 1995). In contrast to RNase H, there appears to be only one metal-coordinating ligand (Glu 34) in the active site. The
Figure 45. Calorimetric titration of 0.13 mM exo III with 20 × 12.5 μL injections of 10.0 mM Mg²⁺ at 298 K in 20 mM Tris, pH 7.5, 1 mM 2-mercaptoethanol. The raw data is shown above and the integrated heats and fitted data are below.
Figure 46. A similar experiment and conditions, with 0.17 mM exo III and 16 x 12.5 µL injections of 10.0 mM Ca²⁺.
Figure 47. A similar experiment and conditions, with 0.14 mM exo III and 20 x 12.5 μL injections of 10.0 mM Mn²⁺.
### Table 8: Thermodynamic parameters for metal ion binding to *E. coli* exo III.

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>K (M(^{-1}))</th>
<th>ΔH (kcal mol(^{-1}))</th>
<th>ΔG (kcal mol(^{-1}))</th>
<th>ΔS (cal mol(^{-1}) K(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg(^{2+})</td>
<td>9.1 (± 0.2) x 10(^3)</td>
<td>-4.6 ± 0.1</td>
<td>-5.4 ± 0.3</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>1.8 (± 0.3) x 10(^4)</td>
<td>-5.3 ± 0.2</td>
<td>-5.8 ± 0.1</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>Mn(^{2+}) (type 1)(^h)</td>
<td>1.6 (± 0.2) x 10(^4)</td>
<td>-8.9 ± 0.2</td>
<td>-7.1 ± 0.3</td>
<td>-6.0 ± 0.8</td>
</tr>
<tr>
<td>Mn(^{2+}) (type 2)(^h)</td>
<td>2.5 (± 0.3) x 10(^4)</td>
<td>1.0 ± 0.1</td>
<td>-6.0 ± 0.3</td>
<td>16.8 ± 0.9</td>
</tr>
</tbody>
</table>

\(^a\) Data from the calorimetric titration of exo III (0.17 mM) with 20 x 12.5 mL injections of metal ion (10 mM) at 298 K in 20 mM Tris, pH 7.5, 1 mM 2-mercaptoethanol. Other procedures are described in the legend to Table 7.

\(^h\) These parameters are from one of several possible fits to the data. At least two classes of site are required; one displaying exothermicity and the other endothermicity in Mn\(^{2+}\) binding.
binding parameters for Mg\(^{2+}\) and Ca\(^{2+}\) are similar (Table 8, Figs. 45-47), with positive entropy terms that are consistent with the release of a water ligand. For both of these ions one binding site was available. In contrast, the binding profile for Mn\(^{2+}\) was distinct with clear evidence for (at least) two classes of site, one showing exothermic binding and the other showing endothermic binding (Fig. 47). While the X-ray structure of Tainer and coworkers shows only one bound Mn\(^{2+}\); presumably the weaker site(s) is not populated under the crystallization conditions employed (Mol, et. al., 1995). The distinct peaks observed at each injection suggest that binding to the endothermic sites is considerably faster than binding to the exothermic sites. The number of ions at each type of site can vary, resulting in distinct binding parameters; however, a combination of endothermic and exothermic sites are always obtained, and clearly demonstrate the distinct binding behavior of Mn\(^{2+}\) relative to Mg\(^{2+}\).

5.4. Summary

Since divalent manganese is commonly used as a probe for Mg\(^{2+}\) chemistry in solution and crystallographic studies, our data suggest a note of caution against the generalization and interpretation of results concerning the location and stoichiometry of metal binding from such experiments. This conclusion is particularly pertinent to the issue of one-metal versus two-metal ion mechanisms in phosphate ester hydrolysis.
CHAPTER 6
SIZE SELECTIVITY OF THE Mg(II) BINDING SITE OF
RNASE H AND EXO III

6.1. Introduction

The ligand environment for Mg(II) binding sites in many magnesium-dependent proteins often exhibit a carboxylate cluster site which consists of 3-4 side chain carboxylates grouped in a solvent exposed cleft (Stock, et. al., 1992; Davies, et. al., 1991; Katayanagi, et. al., 1993; Kim & Wyckoff, 1991). The bound magnesium ion is essential for phospho-substrate binding, catalysis, or both. Key features of the metal binding characteristics of these carboxylate cluster remain undetermined. To shed light on this area, Falke and colleagues have systematically studied the Ca(II) site specificity of the D-galactose and D-glucose receptor protein (Snyder, et. al., 1990; Falke, et. al., 1991) as well as the *E. coli* galactose binding protein (GBP) (Drake & Falke, 1996; Drake, et. al., 1996). Recently, they have characterized the ion specificity of the carboxylate cluster site of Che Y, a phosphorylation-activated signaling protein of the bacterial chemotaxis pathway (Needham, et. al., 1993).
The Che Y Mg(II) site is illustrated in Fig. 48a, where Asp 12, Asp 13, and Asp 57 comprise the carboxylate cluster (Stock, et. al., 1989; Volz & Matsumura, 1991). The bound ion was crystallized in complex with the protein and was found to be coordinated by the monodentate side chain carboxylates of Asp 13 and Asp 57, the backbone carbonyl of Asn 59, and three water molecules, one of which is stabilized by outer-sphere coordination to Asp 12 (Stock, et. al., 1993). One hemisphere of the bound ion is coordinated by three protein oxygens, while the other hemisphere is coordinated by three solvent molecules at the protein surface. Interestingly, this is very similar to the coordination of the protein carboxylate side chains of RNase H to the bound Mg(II) ion (Fig. 48b). It has been shown by X-ray crystallographic study that one hemisphere of the bound ion is coordinated by three protein oxygens (Katayanagi, et. al., 1993) while the other hemisphere is bound to three water molecules based on kinetics study (Black, et. al., 1995). Exo II (Fig. 48c), on the other hand, has only one carboxylate ligand to the metal cofactor (Mol, et. al., 1995).

Both the coordination environment and charge density in the Mg(II) binding pockets are distinct for each of these magnesium-binding proteins. Nevertheless, the binding affinity for Mg(II) is similar (~10^3 to 10^4 M^-1) (Huang & Cowan, 1994; Needham, et. al., 1993, Casareno & Cowan, 1996). Clearly, there is no relationship between local charge density in the binding pocket and the binding affinity for the metal ion. In a quantitative analysis of the ion specificity of magnesium dependent Che Y protein and the calcium binding galactose receptor protein, Falke and coworkers demonstrated a
Figure 48. The magnesium binding domain of (a) Che Y, (b) RNase H and (c) exo III.
distinct binding free energy versus size relationship for a variety of metal ions (Snyder, et. al., 1990; Needham, et. al., 1993). These data quantitatively demonstrated the generally held tenet that proteins may discriminate between Mg\(^{2+}\) and Ca\(^{2+}\) using their distinct size and ligand geometries. However, the similarity in Mg(II) binding affinities just noted suggests that even for a specific metal ion, a variety of factors can be used to tune metal binding. The mechanism employed by each enzyme is most likely distinct.

In this chapter, the thermodynamic parameters of divalent metal ion binding to RNase H and exo III are presented. Plots of the effective binding free energy as a function of ionic radius for each of the two enzymes studied will be compared to the published data on Che Y. Comparison of these plots will reflect the factors that dominate metal recognition and binding for each enzyme. This study permits a comprehensive overview of structure/recognition features for this family of magnesium-dependent enzymes. As noted for the Che Y protein, weak size specificity is also observed for exo III. RNase H, on the other hand, has a slight preference for binding Ca\(^{2+}\) over Mg\(^{2+}\).

Crystallography data for RNase H shows that the carboxylate of Asp 10 is one of the three protein ligands that bind the divalent magnesium ion. This is supported by NMR pH titration studies which show the largest change in \(^{13}\)C chemical shift and pKa following Mg\(^{2+}\) binding (Oda, et. al., 1994). By engineering mutations that selectively increase or decrease the accessible volume in the binding pocket, it should be possible to change the relative steric and solvation contributions to binding energy. It was therefore
expected that nonconservative mutation of this residue would lead to a reduced binding affinity for magnesium. However, our results proved otherwise.

6.2. Data and Results

6.2.1. Approach. In order to determine the binding parameters for each metal ion, protein samples were titrated with the salt solutions of each of the divalent metal ions. Isothermal titration calorimetry allows evaluation of the thermodynamic parameters of binding by taking advantage of the heat changes that accompany such binding reactions (Wiseman, et. al., 1989). Since it measures heat directly, it is the only technique which allows direct evaluation of binding parameters (e.g. n, K, ΔH) in a single experiment. Both enthalpic and entropic contributions to the binding free energy can be evaluated by using eqn. (1).

\[
\Delta G^o = -R \ T \ \ln K = \Delta H^o - T \Delta S^o
\]  

The calorimetric titration data were analyzed using eqn. 1, yielding binding free energy for each metal ion describing the equilibrium

\[
R \ast (H_2O)_m + M \ast (H_2O)_j \leftrightarrow R \ast \ast M \ast (H_2O)_k + (m + j - k)H_2O
\]  

where R is the metal binding site, M is the metal ion, and R* represents the metal-bound enzyme which may have undergone a change of conformation. By measuring the binding affinities for a series of chemically-related metal ions that differ in size, the only two variables are the metal hydration energy for M\ast(H_2O)_j and the energy of the R\ast\ast M\ast(H_2O)_k species, since the energy of R\ast(H_2O)_m is constant. Since the hydration energies for M\ast(H_2O)_j are known, they can be factored out of the binding energy to
leave a direct measure of the energy of binding by the partially hydrated series of metal ions and complexes to yield useful information on molecular recognition of these metal cofactors.

6.2.2. Metal Affinity and Size Specificity of the Carboxylate Cluster Site.

Table 9 and 10 summarizes the ionic radii, binding constants, enthalpy, entropy and free energies of binding of the chosen divalent metal ions. Monovalent ions are known to be essentially excluded from the site, exhibiting very weak binding constants and thus would not be measurable by calorimetry. Trivalent cations, on the other hand, have solubility problems at the condition that the titrations were done. The solubility problem can be alleviated by carrying out the experiments at pH 6. However, at this lower pH, the carboxylate ligands are protonated resulting in a weaker interaction between the metal ion and the protein. The only feasible measurements were that of the divalent cations which exhibited measurable affinities, with $K_a$ values from $10^4$ to $10^5$ M$^{-1}$.

By comparing free energy versus ionic radii plots, the carboxylate cluster site for exo III exhibits a striking lack of size specificity as observed for the Che Y (Fig. 49). There is no stable free energy minimum observed indicating that the site prefers no specific ion size. The free energy curves are relatively flat. Fig. 48 shows the weak size selectivity of RNase H to cations of approximately 1 Å in radius.
<table>
<thead>
<tr>
<th>cofactor</th>
<th>ionic radius (Å)</th>
<th>K (M⁻¹)</th>
<th>ΔH (kcal mol⁻¹)</th>
<th>ΔG (kcal mol⁻¹)</th>
<th>ΔS (cal mol⁻¹ K⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg²⁺</td>
<td>0.72</td>
<td>1.1 (±0.2) × 10⁻²</td>
<td>-2.6 ± 0.1</td>
<td>-5.5 ± 0.1</td>
<td>9.7 ± 0.7</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1.00</td>
<td>4.4 (±1.2) × 10⁻¹</td>
<td>-10.8 ± 0.2</td>
<td>-7.7 ± 0.1</td>
<td>-10.4 ± 0.6</td>
</tr>
<tr>
<td>Sr²⁺</td>
<td>1.18</td>
<td>8.0 (±1.6) × 10⁻²</td>
<td>-1.7 ± 0.4</td>
<td>-5.2 ± 0.1</td>
<td>11.8 ± 0.4</td>
</tr>
<tr>
<td>Ba²⁺</td>
<td>1.35</td>
<td>4.8 (±0.6) × 10⁻²</td>
<td>-2.9 ± 0.2</td>
<td>-5.0 ± 0.7</td>
<td>7.2 ± 0.6</td>
</tr>
</tbody>
</table>

Table 9. Thermodynamic parameters for metal ion binding to *E. coli* RNase H.
Table 10. Thermodynamic parameters for metal ion binding to *E. coli* exo III.

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>Ionic Radius (Å)</th>
<th>$K$ ($M^{-1}$)</th>
<th>$\Delta H$ (kcal mol$^{-1}$)</th>
<th>$\Delta G$ (kcal mol$^{-1}$)</th>
<th>$\Delta S$ (cal mol$^{-1}$ K$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg$^{2+}$</td>
<td>0.72</td>
<td>9.1 ($\pm$ 0.2) x 10$^3$</td>
<td>-4.6 $\pm$ 0.1</td>
<td>-5.4 $\pm$ 0.3</td>
<td>2.7 $\pm$ 0.3</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>1.00</td>
<td>1.8 ($\pm$ 0.3) x 10$^4$</td>
<td>-5.3 $\pm$ 0.2</td>
<td>-5.8 $\pm$ 0.1</td>
<td>1.7 $\pm$ 0.2</td>
</tr>
<tr>
<td>Sr$^{2+}$</td>
<td>1.18</td>
<td>9.2 ($\pm$ 1.8) x 10$^3$</td>
<td>-2.8 $\pm$ 0.2</td>
<td>-5.4 $\pm$ 0.1</td>
<td>8.4 $\pm$ 0.9</td>
</tr>
<tr>
<td>Ba$^{2+}$</td>
<td>1.35</td>
<td>5.0 ($\pm$ 0.5) x 10$^3$</td>
<td>-4.4 $\pm$ 0.2</td>
<td>-5.0 $\pm$ 0.1</td>
<td>2.0 $\pm$ 0.6</td>
</tr>
</tbody>
</table>
6.2.3. Magnesium Binding Affinity of the Asp 10 Mutants. The thermodynamic parameters of magnesium binding to the Asp 10 mutants of RNase H were quantitatively determined by calorimetry. The results outline in Table 11 indicate that removal of the carboxylate ligand to the magnesium cofactor did not abolish metal binding as expected, with the exception of the D10R mutant. The thermodynamic data for the Asp10Glu mutant is very similar to the native enzyme, with a modest two-fold increase in binding affinity (K). More surprisingly, the binding affinities for all the mutants studied are increased relative to the native. In most cases, this increase in binding arises from entropic factors since the enthalpy terms become less negative.

Crystallographic data on the Asp10Asn mutant indicate that the amide nitrogen forms a new hydrogen bond with the carboxyl group of Asp 70 in the absence of the metal cofactor (Kanaya, et. al., 1990). The authors proposed that this mutation abolishes activity since the loss of the negative charge precluded Mg\(^{2+}\) binding. However, our results show that this is not the case. The mutants studied actually exhibit higher binding affinity to the magnesium ion.

6.3. Discussion

Exo III, known to have only Glu 34 as the protein ligand to the metal cofactor, exhibits no size selectivity for the spherical group IIA ions studied. This is not surprising considering that the binding site is located at the surface of the molecule (Mol, et. al., 1995). And since there is only one ligand from the protein, it is quite possible that it can
<table>
<thead>
<tr>
<th>enzyme</th>
<th>K</th>
<th>ΔH</th>
<th>ΔG</th>
<th>ΔS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(M⁻¹)</td>
<td>(kcal mol⁻¹)</td>
<td>(kcal mol⁻¹)</td>
<td>(cal mol⁻¹ K⁻¹)</td>
</tr>
<tr>
<td>native</td>
<td>1.1 (± 0.2) x 10⁴</td>
<td>-2.6 ± 0.1</td>
<td>-5.5 ± 0.1</td>
<td>9.7 ± 0.7</td>
</tr>
<tr>
<td>D10E</td>
<td>2.2 (± 0.6) x 10⁴</td>
<td>-2.7 ± 0.1</td>
<td>-5.9 ± 0.2</td>
<td>11.0 ± 0.7</td>
</tr>
<tr>
<td>D10N</td>
<td>1.1 (± 0.1) x 10⁵</td>
<td>-1.7 ± 0.1</td>
<td>-6.9 ± 0.1</td>
<td>17.2 ± 0.4</td>
</tr>
<tr>
<td>D10S</td>
<td>1.3 (± 0.2) x 10⁵</td>
<td>-1.6 ± 0.1</td>
<td>-7.0 ± 0.1</td>
<td>18.2 ± 0.4</td>
</tr>
<tr>
<td>D10G</td>
<td>1.5 (± 0.3) x 10⁵</td>
<td>-2.0 ± 0.1</td>
<td>-6.8 ± 0.1</td>
<td>16.1 ± 0.9</td>
</tr>
</tbody>
</table>

*Table 11.* Thermodynamic parameters of magnesium (II) binding to *E. coli* native and mutant RNase H.
easily accommodate ions of various sizes. In this site, the ions of various sizes can also be free to optimize their coordination by changing the number and spacing of inner-sphere solvent oxygens, requiring little or no rearrangement of the protein to accommodate large ions. Interestingly, the observed binding characteristics of RNase H and Che Y are significantly different considering that the ligands to the bound Mg\(^{2+}\) are very similar. Both of these proteins have solvent exposed Mg (II) sites. The difference in their metal binding suggests that a variety of factors can be used to fine tune metal binding.

RNase H had been crystallized using different cations. The difference Fourier maps indicated a single, common, significant peak for Ba\(^{3+}\), Ca\(^{2+}\) and Co\(^{2+}\) at about the same position as that of Mg\(^{2+}\) (Katayanagi, et. al., 1990). It has been found that the Mg\(^{2+}\) position was shifted further inward within the metal binding cavity than those of Ba\(^{2+}\) or Ca\(^{2+}\). In the case of Ba\(^{3+}\), Katayanagi, and colleagues (1990) have found that two water oxygens lie in a possible range of coordination. Taking this data into consideration, the fact that Ca\(^{2+}\) exhibits higher affinity to the binding pocket implies that this arises not because this cation can fit more snugly into the pocket. Rather, it could possibly interact directly with the negatively-charged side chain of Asp 70. This interaction would then lead to a tighter interaction and as mentioned in an earlier chapter, would reduce the efficiency of the catalytic base. In the case of Ba\(^{2+}\), it is most likely that the larger size of this cation prevents it from going deep into the binding pocket. The weaker interaction could stem from constraints within the Mg (II) site.
Figure 49. Free energies of divalent metal ion binding to exo III (●), RNase H (▲) and Che Y (■) as a function of effective ionic radius.
Then why does the Che Y Mg(II) binding site exhibit no size selectivity at all considering that the binding motif is similar to that of RNase H? Presumably, the Mg(II) site in Che Y does not provide a constrained ion cavity such that ions of different sizes can easily be accommodated. Steric factors around the cavity, and not only the electrostatic interactions provided by the ligands could also influence the metal affinity of these Mg(II) sites.

There are several possible reasons for the retention of the high binding affinity for the engineered Asp 10 mutants. Perhaps in the absence of the carboxylate side chain of Asp 10, Asp 70 could serve as the carboxylate donor. In the native enzyme, it could be that the positioning of the metal binding residues serves the important function of preventing disadvantageous interactions between the bound cofactor and other catalytic residues in the active site. It is also possible that favorable steric factors for smaller side chains may facilitate metal binding. And third, perhaps this carboxylate does not contribute significantly to binding. It has been demonstrated that exo III binds Mg$^{2+}$ with an affinity comparable to RNase H, despite the fact that it possesses only one metal binding carboxylate in the active site.
CHAPTER 7
SUMMARY

In the study of the metal-binding domains of *E. coli* RNase H and exo III, we have demonstrated that there is a distinct difference between the two divalent metals, magnesium and manganese, not only in terms of stoichiometry but also in thermodynamics of binding. Through calorimetric measurements, we have proven that there is a only one Mn$^{2+}$ bound to RNase H contrary to crystallographic results obtained for the homologous RNase H domain of HIV RT where two Mn$^{2+}$ ions were observed. Another case in point is exo III. Mol et. al. (1995) observed a single Mn$^{2+}$ tethered to Glu 34 in exo III. Calorimetric data however, indicated that there are at least two sets of sites, one endothermic and the other exothermic. Therefore, extension of crystallographic results based on Mn$^{2+}$ as a probe of magnesium binding sites should be done with caution.

We have also demonstrated in this study that we can gain insight on the size of an enzyme's metal binding pocket with the use of divalent metal ions of various sizes. Plots of free energy of binding versus ionic radii gives us an idea as to which size of spherical ion is best accommodated in the pocket. Comparison of the configuration around the
binding pocket and the plots generated can give us an idea as to what factors contribute to
the molecular recognition involved in metal binding. In this study, we have shown that
exo III exhibits no size selectivity at all. This is not surprising since the pocket is known
to be on the surface of the protein. Besides, with only one ligand from the protein it is
not hard to imagine that this site can be flexible to accommodate metals of various sizes.

The metal binding domain of RNase H is quite more constrained compared to that
of exo III. The protein ligands (Asp 10, Gly 11, Glu 48) comprise one hemisphere of the
coordinated ion while three inner-sphere water molecules occupy the opposite
hemisphere of the bound ion. Larger ions (Sr\(^{2+}\) and Ba\(^{2+}\)) are sterically hindered to fit
well into the site although they are still observed to be bound while smaller, more
hydrated ions (Mg\(^{2+}\) and Mn\(^{2+}\)) are accommodated fairly well. The catalytically-
inefficient Ca\(^{2+}\) ion is bound the tightest. Presumably, it could coordinate directly not
only to Asp 10, Gly 11 and Glu 48 but also to the proposed catalytic base, Asp 70. This
study has shown that there is no single major factor that controls metal affinity. Rather,
it is a combination of hydration, electrostatic and steric constraints that determine the
protein's binding affinity to metals.

RNase H is a very well characterized enzyme both structurally and catalytically.
However, there is still a debate as to what role each of the active site residue and the
magnesium cofactor plays. In this study, we have proven that Asp 70 is important as the
catalytic base since its removal renders the enzyme inactive at all times. Glu 48 and
Figure 50. Proposed mechanism for RNase H.
Asp 10 serve as ligands to the magnesium cofactor which in turn interacts by H-bonding through the inner-sphere water molecules with the phosphate substrate. From our rational redesign study we have shown that substitution of positively charged Arg provided the structural component necessary to maintain the structural integrity of the active site to effect catalysis. Presumably, the magnesium cofactor is there to neutralize the negatively-charged carboxylates as well as provide the H-bonding propensity required to coordinate and position the substrate for catalysis. Removal of Asp 10 alone did not abolish the metal affinity contrary to expectations. Nonconservative mutation of this residue to Ser, Gly and Asn rendered the engineered enzyme inactive but magnesium binding became tighter. Removal of the negatively charged Asp 10 did not abolish metal affinity presumably because the negatively-charged asp 70 can take its place as ligand to the metal.
PART 2

CLONING OF THE INTRACELLULAR AND EXTRACELLULAR DOMAINS
OF HUMAN FAS AND FAS LIGAND
CHAPTER 8
INTRODUCTION

8.1. Apoptosis

Homeostasis of multicellular organisms is maintained through a balance between cell proliferation and cell death. The regulation of this process occurs through the induction of apoptosis, a process which is characterized by autodigestion of the cell.

Apoptosis research was born when Kerr, Wyllie and Currie proposed that there are two major types of cell death (Kerr, et. al., 1972). The first type, necrosis (passive or accidental cell death), results from physical damage to cells, involves groups of cells and has the deleterious effect of causing tissue inflammation. The second, which they termed apoptosis, is undergone by individual cells that are surrounded by healthy neighbors (Earnshaw, 1995). This programmed cell death proceeds through the activation of endogenous proteases which results in nuclei condensation and segmentation, cell shrinkage, membrane blebbing, cytoskeletal disruption and extensive chromosomal DNA fragmentation (Nagata & Golstein, 1995; Thompson, 1995). This process does not result in inflammation (Wylie, et. al., 1980).
Many of the signals that induce apoptosis are now well defined. Two molecular mechanisms of T-cell mediated cytotoxicity have emerged (Kagi, et. al., 1994a). Interactions between Fas and its ligand have emerged as the major mechanism for the deletion of activated peripheral T cells and autoreactive B cells. (Osborne, 1996). The other mechanism, perforin-based (Henkart, 1985; Podack, et. al., 1985) was confirmed by using a perforin-deficient mice obtained by gene targeting (Kagi, et. al., 1994b). This review emphasizes recent work on the Fas-based mechanism, and in particular, on the elucidation of the role of Fas and its ligand on induction of apoptosis.

8.2. Fas and Fas Ligand

In 1989, two groups independently isolated mouse-derived antibodies that were cytolytic for various human cell lines (Trauth, et. al., 1989; Yonehara, et. al., 1989). They designated the cell surface proteins recognized by these antibodies as Fas/CD 95 and APO-1. From the cDNA clone isolated, human Fas was found to have 325 amino acids with a signal sequence at the NH₂-terminus and a membrane-spanning region in the middle of the molecule (Fig. 51). Fas/APO-1 belongs to a member of conserved membrane-bound receptors known as the tumor necrosis factor receptor (TNFR) superfamily (Itoh, et. al., 1991; Oehm, et. al., 1992; Watanabe-Fukunaga, et. al., 1992). Members of this family have 3-6 cysteine-rich domains in the extracellular region. The cytoplasmic region's amino acid sequence is not conserved, except for some similarity between Fas and TNF-R1 (Itoh, et. al., 1991; Oehm, et. al., 1992). Mutational analyses of Fas and TNF-R1 indicated that a span of 70 amino acid residues conserved in the
Figure 51. The TNF and NGF receptor family. (Nagata & Goldstein, 1995).
The cytoplasmic domain of these two proteins is necessary and sufficient to transduce the apoptotic signal (Itoh & Nagata, 1993; Tartaglia, et. al., 1993). This domain has been designated the death domain.

The human Fas gene comprises 9 exons. The protein product has been shown to be abundantly expressed in mouse thymus, liver, heart, lung, kidney and ovary (Watanabe-Fukunaga, et. al., 1992). Fas is highly expressed in activated mature lymphocytes (Trauth, et. al., 1989) or lymphocytes transformed with human T cell leukemia virus (HTLV-1), Human immunodeficiency virus (HIV), or Epstein-Barr virus (EBV) (Debatin, et. al., 1994; Kobayashi, et. al., 1990; Falk, et. al., 1992).

Fas ligand (FasL) was purified by means of affinity-chromatography with the soluble form of Fas fused to human IgG (Suda & Nagata, 1994). The purified protein has a MW of 40 kDa and showed strong cytotoxic activity against Fas-expressing cells. Just like Fas, Fas L is a transmembrane protein. However, unlike Fas, it has no signal sequence at the NH₂-terminus and the COOH-terminus is outside the cytoplasm. This indicates that it is a type II membrane protein. A stretch of about 150 amino acid residues in the extracellular region has significant homology to the corresponding region of other members of the TNF family which includes TNF, lymphotoxin (LT), CD40 ligand, CD27 ligand, CD30 ligand and OX40 ligand (Fig. 52). Expression of recombinant FasL on the cell surface of COS cells was sufficient to induce apoptosis in Fas-expressing target cells within a few hours (Suda, et. al., 1993), indicating that FasL is a death factor and Fas is
Figure 52. The TNF Family. (Nagata & Golstein, 1995).
8.3. Mechanisms of Fas-FasL Interactions

Three mechanisms for a cell to die via Fas in the immune system have been proposed (Golstein, et. al., 1995) and is illustrated in Fig. 53 (Nagata & Golstein, 1995). When Fas and FasL are expressed in different cells, they could interact leading to Fas-based death in \textit{trans} through a cytotoxicity-like mechanism. “Suicide” in \textit{cis} can occur when both Fas and FasL are expressed in the same cell, leading to death of this cell. The third intermediate model stems from recent observations that the FasL can be released into the extracellular medium (Dhein, et. al., 1995; Tanaka, et. al., 1995) and could thus effect \textit{cis} or \textit{trans} Fas-based death.

8.4. The Reaper Connection

Golstein and his colleagues (1995) have considered the possibility that the Fas and FasL may derive from a chimeric ancestor molecule. From the point of view of both topology and molecular structure, they are considering the possibility that upon emergence of multicellularity, an intracellular protein can shift to a membrane or extracellular location. Alternatively, they also propose that a rearrangement through exon shuffling could have led to a chimeric molecular ancestor of Fas. Fas and TNFR1 are homologous in their cytoplasmic regions for a stretch of 60-70 amino acids, called the death domain, which makes them different from other members of this family. It is interesting to note that this domain is encoded in the last exon of their respective genes.
Figure 53. The three mechanisms of Fas and FasL interaction: a) trans, b) cis, and c) soluble FasL can act cis or trans. (Nagata & Golstein, 1995).
Figure 54. Amino acid sequence homology between reaper and the part of the cytoplasmic domain or related proteins. (Golstein, et. al., 1995).
(Behrmann, et. al., 1994). This suggests the possibility that both of these proteins may derive from a chimeric ancestor molecule.

Reaper, a cytoplasmic 65 amino acid peptide discovered by White, et. al. (1994) can also induce apoptosis in *Drosophila*. Amino acid sequence alignment showed homology between reaper and the death domains of TNFR1 and, to a lesser extent, of Fas (Fig. 54). This finding is significant because it involves *Drosophila* and mammalian molecules and because all of these are able to signal cell death (Golstein, et. al., 1995). With the discovery of reaper and its homology to the death domain in Fas, we are convinced that the intracellular domain of Fas can be expressed as a soluble protein. Dividing this membrane protein into its extracellular and intracellular domains and expressing them separately might simplify future structural characterization. Removal of the transmembrane domain would make this domains soluble and thus easier to purify for NMR studies. Also, we can study how the extracellular domain of Fas can possibly interact with the extracellular domain of Fas L *in vitro* from the structural point of view. Attempts to clone, express and isolate the extracellular and intracellular domains of Fas and FasL will be presented.
9.1. Introduction

The immediate availability and proven efficiency of pET 21b(+) vector in overexpressing recombinant proteins made it a very likely choice for the task of cloning the domains of the human Fas and FasL genes. The strategy was to design primers with engineered unique restriction sites to be used to amplify the desired gene fragments by PCR. The primers were designed so as to eliminate the transmembrane domain in each protein as well as separate the intracellular from the extracellular domain for each gene.

9.2. Materials and Methods


The pET 21b(+) vector and *E. coli* strains XL1B and BL 21 (DE3) were bought from Novagen. A plasmid carrying the human Fas receptor gene was a generous gift from Dr. Nagata. Another plasmid carrying the human Fas ligand gene was also provided by Dr. Nagata. Restriction enzymes and polynucleotide modifying enzymes were purchased
from BRL. The double stranded DNA sequencing cycle kit was purchased from USB. P-32 ATP was bought from Dupont-NEN.

9.2.2. Methods

9.2.2.1. Amplification of the Gene Fragments.

Oligonucleotide primers with engineered Nde I site at the 5'-end and Eco R1 at the 3'-end for each domain were ordered from IDT (Table 12). Amplification reactions were performed using 100 pmol of each primer and 100 ng of template DNA in a total of 30 cycles at 55°C and 72°C as the annealing and extension temperature, respectively. All reactions were completed with a final extension cycle of 72°C for 5 min. Following amplification, aliquots of each sample were subjected to electrophoresis in 1% agarose gel and analyzed by ethidium bromide staining and visualized with shortwave uv illumination (Fig. 55).

9.2.2.2. Cloning into pET vector

Twenty five μL of each PCR reaction mixture was digested with Nde I and Eco R1 at 37°C. The cut DNA fragments were purified by phenol extraction and ethanol precipitation. These fragments were then ligated to the doubly-cut dephosphorylated pET 21 b(+) plasmid and incubated at 16°C overnight. Half of the ligated mixture was transformed into XL1B competent cells and plated on Amp plates. The surviving colonies were screened for inserts by restriction digest.
<table>
<thead>
<tr>
<th>Upstream primer</th>
<th>Downstream primer</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(5'-3')</td>
<td>(5'-3')</td>
<td></td>
</tr>
<tr>
<td>FGI</td>
<td>GGGCATATGACACTAAGTCAAGTTAAA</td>
<td>GGGCGAATCCCTAGACCAAGCTTGGGA</td>
</tr>
<tr>
<td>FGE</td>
<td>GGGCATATGAGATTTCGCTCCCAAAGT</td>
<td>GGGCGAATCTCTAGTTAGCATCTGGATC</td>
</tr>
<tr>
<td>FLI</td>
<td>GGGCATATGCAGCAGCCCTTCAATTAC</td>
<td>GGGCGAATCTTACGCTTGTGCTGTGTT</td>
</tr>
<tr>
<td>FLE</td>
<td>GGGCATATGCACACAGCATCTCTTTCG</td>
<td>GGGCGAATCTTACGCTTATATAAGC</td>
</tr>
</tbody>
</table>

Table 12. PCR primer sets used in amplification of human Fas and FasL genes.
Figure 55. Amplification of FGI and FGE by PCR. Lane 1) 1 kb marker, 2) FGE and 3) FGI.
9.2.2.3. Expression.

Four colonies with the correct size of insert was isolated for the intracellular domain of Fas (FGI). Each of these plasmids were transformed into BL21(DE3) for protein expression. Small scale expression experiment was performed by inoculating a 5 mL LB media supplemented with Amp and inducing protein production when the OD$_{600}$ reaches 0.6. The cells were induced for 4 hours at 37°C. After harvesting the cells, protein expression was determined by running an SDS-PAGE gel. Results are shown in Fig. 56. A slightly over-expressed band was observed for each candidate. However, the size of each of these band varied.

For the case of the FasL, only two colonies had inserts corresponding to the intracellular domain of Fas L (FLI). Small scale expression yielded no distinguishable over-expressed band at all.

9.2.2.4. Sequencing

Sequencing of the whole insert was done to determine any mutation introduced by PCR. Figure 57 shows the nucleotide base sequence for the intracellular domain of FGI. The first amino acid corresponds to position 224 in the full length human Fas protein. FGI-1 was found to have one base deletion at position 50 (residue 273 in the full length protein). FGI-2 and 4 had one base deletion at position 96 (residue 319 in the mature full length protein) which would introduce a frameshift mutation and resulted in the expression of extra 20 amino acids. FGI-4, on the other hand, had one A → G substitution resulting in Ala to Gly mutation at position 50.
Figure 56. Over-expression of FGI clones. Lane 1) MW marker, 2) FGI-1, 3) FGI-2, 3) FGI-3 and 4) FGI-4.
ATG ACA CTA AGT CAA GTT AAA GGC TTT GTT CGA AAG AAT GGT

10

GTC AAT GAA GCC AAA ATA GAT GAG ATC AAG AAT GAC AAT GTC CAA

20

GAC ACA GCA GAA CAG AAA GTT CAA CTG CTT CGT AAT TGG CAT CAA

30

40

CTT CAT GGA AAG AAA GAA GCG TAT GAC ACA TTG ATT AAA GAT CTC

50

AAA AAA GCC AAT CTT TGT ACT CTT GCA GAG AAA ATT CAG ACT ATC

60

70

ATC CTC AAG GAC ATT ACT AGT GAC TCA GAA AAT TCA AAC TTC AGA

80

AAT GAA ATC CAA AGC TTG GTC TAG

90

Figure 57. Nucleotide sequence of the cloned intracellular domain of FGI.
ATG CAG CAG CCC TTC AAT TAC CCA TAT CCC CAG ATC TAC TGG

GTG GAC AGC AGT GCC AGC TCT CCC TGG GCC CCT CCA GGC ACA GTT

CTT CCC TGT CCA ACC TCT GTG CCC AGA AGG CCT GGT CAA AGG AGG

CCA CCA CCA CCA CCG CCA CCA CCA CCA CTA CCA CCT CCG CCG CCG

CCG CCA CCA CTG CCT CCA CTA CCG CTG CCA CCC CTG AAG AAG AGA

GGG AAC CAC AGC ACA GGC TAA

Figure 58. Nucleotide sequence of the cloned intracellular domain of FasL.
Sequencing of the FLI insert showed one base mutation at position 76 (Fig. 58). This resulted in the conversion of Asn to Asp.

9.3. Discussion of Results.

Only the FGI and FLI were cloned into the pET 21 b(+) vector. In both cases, we observed single base mutation in all of the candidates screened. PCR amplification of DNA using Taq DNA Polymerase is known to be error-prone. Two FGI clones had a single base mutation while the other two clones had a frameshift mutation due to a missing base in position 96. This frameshift mutation led to about 20 extra amino acids being expressed.

Attempts to over-express the cloned gene after the successful small scale expression experiment failed due to undetermined reason. BL 21 (DE3) pLys S was also tried since this strain was reported to handle toxic genes better. However, no over-expression was observed. Possibly this insert is unstable to be expressed on its own and so we resorted to the use of a fusion vector and will be discussed in the next chapter.
CHAPTER 10

CLONING OF FAS AND FAS LIGAND DOMAINS INTO pTRX FUS VECTOR

10.1. Introduction

Efforts to express the Fas and FasL intracellular domains failed using the pET 21b(+) vector from Novagen. With the possibility that these protein products might be unstable on their own, we resorted to try a fusion vector instead. Invitrogen markets the pTrx Fus vector which has the full length thioredoxin gene incorporated before the multiple cloning site (Bayer, 1968; Holmgren, 1985). The enterokinase recognition sequence had also been added to facilitate removal of the fused protein for later in vitro studies (Fig. 59). This plasmid has the added advantage of making the fusion protein most likely to be a soluble protein as proven with other fusion constructs (Holmgren, 1985; La Vallie, et. al., 1992).

The plasmid used to create thioredoxin fusions, pTrxFus, uses the P_L promoter from bacteriophage \( \lambda \) to drive expression. This promoter is tightly regulated. The bacteriophage \( \lambda \) cl repressor binds to the operator region in front of the P_L promoter and controls the level of transcription from this promoter. Expression of the cl repressor is also regulated. pTrxFus is propagated in E. coli GI 724 cells where the cl repressor gene
Figure 59. The pTrxFus expression vector.
is under control of the trp promoter. Tryptophan is added to induce protein production instead of the more expensive IPTG commonly used for the pET vectors.

10.2. Materials and Methods

10.2.1 Materials

The pTrxFus expression system kit was bought from Invitrogen. Casamino acid was purchased from Sigma. Restriction enzymes and polynucleotide modifying enzymes were purchased from BRL. P-32 ATP used for sequencing was obtained from Dupont-NEN. DNA purification kit was purchased from Qiagen.

10.2.2. Recombinant DNA Techniques

Amplification reactions were performed using 100 pmol of each primer with engineered Kpn 1 site (Table 13) and 100 ng of template DNA. The template DNA was denatured at 95°C for 1 min. before the PCR cycle (denaturation at 95°C for 20 sec; annealing at 55°C for 20 sec and extension at 72°C for 30 sec) was started. After 30 cycles, a final extension at 72°C for 5 min was added. Following amplification, aliquots of each sample were subjected to electrophoresis on a 1% agarose gel and final products were analyzed by ethidium bromide staining and visualized with shortwave uv illumination (Fig. 60).

Each of the amplified DNA fragments was incubated with Kpn1 at 37°C. The pTrx Fus vector was also cut with Kpn 1 and dephosphorylated using alkaline phosphatase. Each of the cut DNA fragments and vector was purified using the purification kit from Qiagen. The ligation mixtures were set up and incubated at 16°C.
<table>
<thead>
<tr>
<th>Upstream primer</th>
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<td>GGGGGGTACCTTAGTTAGATCTGGATCCT</td>
</tr>
<tr>
<td>FG</td>
<td>GGGGGTACCCAGATTATCGTGCCAAAAGT</td>
<td>GGGGGGTACCCCTAGAAGCTTTGGATT</td>
</tr>
<tr>
<td>FLI</td>
<td>GGGGGTACCCATGCAGCAGCCTTCAAT</td>
<td>GGGGGGTACCTTAGCCTGTGCTGGTTC</td>
</tr>
<tr>
<td>FLE</td>
<td>GGGGGTACCCATGCACACAGCATCATCT</td>
<td>GGGGGGTACCTTAGCTTATAAAGCCG</td>
</tr>
<tr>
<td>FL</td>
<td>GGGGGTACCCATGCAGCAGCCTTCAAT</td>
<td>GGGGGGTACCTTAGCTTATAAAGCCG</td>
</tr>
</tbody>
</table>

**Table 13.** PCR primer sets used in amplification of the human Fas and Fas ligand genes.
overnight. Half of the ligated mixtures was transformed into GI 742 competent cells and plated on RMG-Amp plates.

10.2.3. Analysis of Transformants

10.2.3.1 Kpnl Digests. Preliminary screening for inserts was done with Kpnl digestion of the plasmid isolated for each clone. Since both primers used to amplify the gene fragments had Kpnl site, there are different ways that the DNA fragments can be ligated to the vector. With Kpnl digests, we can determine if there is an insert and whether there are multiple copies of the insert ligated together before getting inserted into the vector.

Figure 61 shows the Kpnl digestion results for FLE and FL. Lane 14 contains the 1 kb molecular marker. Lane 1 has the pTrxFus vector cut with Kpnl. Lanes 2-7 shows the results for the FLE candidates while lanes 8-13 shows the results for the FL candidates. As shown in the gel, there are two FLE clones which had the correct size of insert. For the case of FL, there were three with a single copy of the FL gene while two other clones had two FL gene fragments connected to each other before being ligated to the vector. There is still the possibility that the three FL clones thought to have only a single copy of the gene might also have duplicate copies. However, this could not be the case since equal amounts of enzyme were added to each tube.
Figure 60. PCR amplification of the Fas and FasL domains. lane 2) FGE, 3)FGI, 4)FG, 5)FLE and 7)FL.
Figure 61. Kpn1 digests of FLE and FL clones. Lane 1) pTrxFus vector, 2-7) FLE candidates, 8-13) FL candidates and 14) 1 kb molecular weight marker.
Figure 62. Hind III digest of FGI clones. Lane 1) 1 kb molecular weight marker, lane 2) FGI-1 (Kpn1-EcoR1), lane 3) FGI-2 (Kpn1) and lane 4) FGI-4 (Kpn1-EcoR1).
3.2.2.2. Determination of the Correct Orientation of the Inserts.

**FGI.** In order to determine if the clones were in the correct orientation, additional restriction digestion experiment should be done. Hind III digest of FGI clones would excise a 114 bp fragment if inserted correctly and a 375 bp fragment if it is in the opposite orientation. Figure 62 shows the appearance of ~100 bp Bam H1 fragment in a 1% agarose gel for all three FGI clones isolated.

**FGE.** A Bam H1 digest would yield a 50 bp fragment if inserted correctly since Bam H1 site in the FGE is 50 bp away from the Bam H1 site in the vector. Figure 63 shows the results of a Bam H1 digestion of two plasmids. The plasmid in lane 1 has FGE insert as shown previously by KpnI digest and the plasmid in lane 2 has no insert. The 50 bp fragment is hardly legible from the gel but it is obvious that the two plasmids tested run differently. The plasmid in lane 1 is definitely larger than that in lane 2.

**FLI.** Since Bgl II is unique in the FLI gene and Hind III is in the vector, a Bgl II-Hind III digest would determine the correct orientation of the insert by giving a 300 bp fragment. The result is shown in lane 10 of Fig. 64.

**FL.** The analysis of FL involved Bgl II and Hind III digestion. For the single copy of insert, a 900 bp fragment would indicate that the FL is inserted correctly (lanes 5 and 6, Fig. 64) while generation of a 130 bp would indicate the wrong orientation (lane 3 and 4, Fig. 64). For the duplicate copies of the gene, analysis of orientation is illustrated in Figure 65. Since Kpn I has overhangs, the DNA fragments were ligated together in two ways. Illustration (a) would give rise to an 850 bp, 900 bp and ~3.6 kb fragments while illustration (b) would generate a 100 bp, 850 bp and a 4.4 kb fragments.
Figure 63. Bam H1 digests of FGE clone. Lane 1) with insert, and 2) no insert.
Figure 64. Restriction digestion results for FL and FLI clones. Lane 1) 1 kb marker, 2-9) FL clones and 10) FLI clone.
Figure 65. Analysis of the orientation of the FL clones.
Figure 66. Hinc II digest for the FLE clone. Lane 1) FL clone, 2) FLE clone and lane 3) 1 kb molecular weight marker.
**FLE.** To analyze the correct orientation of the FLE clone, a Hinc II digestion should be done. There are two Hinc II sites in the vector (2161 and 3104). These sites will be ~1.5 kb apart with the insertion of the FLE gene. FLE has a unique Hinc II site, about 75 bp from its 3’-end. So, a Hinc II digest would generate a 78 bp, 1.4 kb and 2.4 kb fragments if oriented correctly. Results are shown in lane 2 of Figure 66.

**10.3. Summary.**

As illustrated above, analyses of the transformants were done by restriction digests. The strategy involved determination of unique restriction sites in both the inserts and the vector. Using these restriction sites, we deduced the orientation of the constructs by the sizes of DNA fragments generated by the restriction digests. We have cloned the FGI, FGE, FL, FLI and FLE domains separately into the pTrx Fus vector. Efforts to clone the full length Fas receptor gene in the correct orientation had been unsuccessful. The next chapter will present results on the expression and isolation of the thioredoxin-FGI fusion protein.
CHAPTER 11

EXPRESSION AND ISOLATION OF

THE THIOREDOXIN-FGI FUSION PROTEIN

11.1. Introduction

In the preceding chapter, we have isolated the FGI, FGE, FLI, FLE and FL clones. The focus of this chapter is to express and isolate the thioredoxin-FGI fusion protein. We are interested in this particular protein since it has been shown to share some amino acid homology to the apoptosis-inducing reaper protein (Golstein, et. al., 1995). Reaper, a cytosolic protein, is reported to have 65 amino acid residues.


11.2.1. Sequencing

DNA sequencing was performed using the ds DNA cycle sequencing kit from USB. The primers (Table 14) were end-labeled with P-32 ATP using T4 polynucleotide kinase. To the end-labeled primer, the template DNA, Taq sequencing buffer and Taq DNA polymerase were added (pre-reaction mixture). Each of the dideoxynucleotide was distributed into four separate tubes. Each tube was mixed with the pre-reaction mixture,
overlaid with oil and placed on ice. The tubes were then placed on the thermal cycler set at 95°C for 3 minutes. The first amplification reaction (denaturation at 95°C for 30 sec. Annealing at 55°C for 30 sec, extension at 70°C for 60 sec) with 20 cycles was started followed by 10 cycles of denaturation at 95°C for 30 sec and extension at 70°C for 60 sec. The whole process took approximately two hours. The reaction was terminated by the addition of 5 μL of stop solution. The samples were placed on ice or stored at -20°C if not used immediately. The tubes were heated at 95°C for 5 min before loading 4 μL of the reaction mixture to a denaturing PAGE gel.

11.2.2. Small scale optimization of expression.

Five mL induction medium was inoculated with 100 μL of overnight cells and shaken at 30°C until OD_{590} reaches 0.6 (~4h). One mL aliquot was withdrawn and the cells were pelleted and stored at -20°C. To the remaining 4 mL culture, tryptophan was added to induce protein production at 37°C. After 2h, one mL aliquot was withdrawn and processed as stated earlier. Another mL of aliquot was withdrawn after another 3h had elapsed. Finally, one mL aliquot was withdrawn after 20h of incubation at 37°C.

The cell pellet in each sample tube was resuspended in 30 μL of osmotic shock solution #2, sonicated, frozen, thawed and sonicated again. Aliquots from each sample was run in an SDS-PAGE gel and stained with Coomassie blue. Results are shown in lanes 6-8 in Fig. 67.
### Table 14.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trx forward</td>
<td>5'-TTCCCTCGACGCTAACCTG-3'</td>
</tr>
<tr>
<td>Trx reverse</td>
<td>5'-TGTAAACGACGCGCCAGTG-3'</td>
</tr>
</tbody>
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*Table 14. Primers used to sequence the FGI clones.*
To optimize the amount of tryptophan added for induction, the same procedure was employed but instead of varying the length of induction time, the amount of added tryptophan was varied instead. All of the sample were induced for 5h. Results are shown in lanes 2-5 in Fig. 67.

11.2.3. Bulk growth of cells.

Twenty mL of RM medium supplemented with Amp is inoculated with cells from a single colony or from a frozen glycerol stock of FGI and incubated at 30°C overnight with shaking at 225 rpm. This overnight culture was diluted to 1L of induction medium and grown to OD$_{550}$ of 0.6 at 30°C. Protein production was induced by the addition of tryptophan (final conc. = 100 µg/mL). The inoculum was then transferred to 37°C and shaken at 250 rpm for 3-4 h. Cells were harvested by pelleting them down at 8000 rpm for 15 minutes in a refrigerated Sorvall 2B centrifuge. The cell pellet was then resuspended in a minimum amount of TE buffer (50 mM Tris-HCl, pH 7.4, 3 mM EDTA) and kept frozen at -80°C until needed.

11.2.4. Purification

11.2.4.1. Thiobond resin. This resin is composed of phenylarsine oxide (PAO) bound to an agarose support (Fig. 68). The dithiol group in the active site of the thioredoxin protein (-Cys-Gly-Pro-Cys-) binds to the PAO group. The 2-mercaptoethanol reduces the dithiols and elutes the protein from the PAO.
Figure 67. Optimization of expression for the fusion protein. Lane 1) MW marker, 2) before induction, 3) 1x Trp after 5h of induction, 4) 3x Trp, 5) 5x Trp, 6) 4x Trp induced for 2h, 7) 4x trp induced for 5h, and 8) induced for 20h.
Figure 68. The thiobond resin.
The resin was activated with 20 mM 2-mercaptoethanol in TE buffer. The lysate was prepared by sonicating the cells. The cleared lysate was loaded onto the activated resin after filtering it using a 0.45 μm membrane. The bound protein was eluted with increasing amount of 2-mercaptoethanol in the buffer.

11.2.4.2. Osmotic shock. The GI 724 strain containing the construct was growing a small culture volume at 30°C. Expression of the fusion protein was induced by the addition of tryptophan transferred to 37°C for 4h. The cells were harvested and osmotically shocked by transferring from a high ionic strength buffer (osmotic shock solution #1) to a low ionic strength buffer (osmotic shock solution #2). The shock fluid as well as the cell pellet were analyzed by SDS-PAGE gel. Results are shown in lanes 4 and 5 in Fig. 69.

11.2.4.3. Heat Treatment. The cells were grown as stated above. The cells were harvested after the optimal point was reached. The cells were resuspended in osmotic shock solution #2 and the cell lysate was prepared by sonicating several times. The cleared lysate was fractionated into three different tubes labeled t=0, t=2, and t=5 indicating the time that the sample was subjected to an 80°C water bath. The heated tubes were immediately placed on ice. The heat-treated samples were then centrifuged at 4°C to remove denatured proteins. The supernatant was transferred to new tubes and placed on ice. The pellet was resuspended in TE buffer and kept on ice until ready to be assayed on an SDS-PAGE gel. Results are shown in lanes 3-7 in Fig. 70.
Figure 69. Osmotic shock purification of FGI construct. Lane 1) MW marker, 4) shock fluid, and 5) pellet.
Figure 70. Heat treatment of the cell lysate. Lane 1) MW marker, 2) total cell lysate, 3) t=0, 4) t=2, supernatant, 5) pellet of t=2, 6) supernatant of t=5, and 7) pellet of t=5.
11.2.5. Conventional way of purification.

The thawed cell paste was sonicated using a big probe of the Fisher discmembranator set at 90% efficiency until the mixture was homogeneous. The solution was then quickly frozen in dry ice-ethanol bath or under liquid N$_2$. Afterwards, it was allowed to thaw at room temperature. This process was repeated two more times. The solution was centrifuged at 15000 rpm for 30 minutes. The pellet was resuspended in 5 mL of fresh TE buffer and sonicated until homogeneous. The cell debris was pelleted down at 15000 rpm for 30 minutes. The supernatant from the first and second centrifugation steps were combined and centrifuged again at 20000 rpm for 2h. To each mL of cleared supernatant, 0.3 g of ammonium sulfate was added. This mixture was stirred at 4°C overnight.

On the next day, the precipitated proteins were collected after spinning at 15000 rpm for 30 minutes. The precipitate was redissolved in 3 mL of 20 mM potassium phosphate (KP), pH 7.4, 3 mM EDTA and desalted in a G-25 column (4 x 8 cm) pre-equilibrated in 20 mM KP, pH 7.4, 3 mM EDTA buffer. The protein/DNA-containing fractions were pooled together and concentrated using an amicon concentrator equipped with a 10K membrane. The sample was diluted to 50 mL with 20 mM KP, pH 7.4, 3 mM EDTA and applied to a pre-equilibrated DE-52 column (4 x 23 cm). The flow through was collected and the column was washed with 20 mL of KP buffer. The bound proteins were eluted using a linear gradient of 20 mM-200 mM KP, pH 7.4 buffer. Eight mL fractions were collected and the absorbance of each fraction was monitored at 280 nm.
Figure 71. DE-52 eluents in the purification of Thioredoxin-FGI protein. Lane 1) MW marker, 2) before loading, 3-4) flow-through, 5) tube 4, 6) tube 14, 7) tube 36, 8) tube 56. * indicates the position of the fusion protein.
Five hundred μL aliquots from selected fractions were TCA precipitated and protein samples were run in a 20% Phast homogeneous gel. Results are shown in Fig. 71.

11.3. Discussion of Results.

11.3.1 Nucleotide Sequence. As mentioned in Chapter 10, three FGI clones were isolated. Two of these three clones were generated using the Kpn1-EcoR1 set of primers (FGI-1 and FGI-4). FGI-2 has Kpn1 site at both ends. The two sets of primers were used to increase the probability of getting the correct construct. Sequencing of both strands of the constructs showed that FGI-1 and FGI-4 have no base mutation introduced by PCR. FGI-2, on the other hand, has a single base substitution at position 94 (corresponding to position 317 in the full length protein) resulting in a Leu → Phe mutation.

11.3.2. Expression. The thioredoxin protein is about 16 kDa and the FGI has 96 amino acids. The fusion protein seems to be approximately 28 kDa based on the protein gel.

11.3.3. Preliminary isolation of the fusion protein. Three different ways of purifying thioredoxin fusion protein has been reported: 1) by thiobond resin, 2) by osmotic shock, and 3) by heat treatment. In this study, all of these suggested purification protocols were tried but none proved to be applicable for the thioredoxin-FGI fusion protein. Resuspending the cell paste in osmotic shock solutions did not release the fusion
protein into the supernatant. Subjecting the cleared lysate in an 80°C water bath precipitated out the fusion protein together with the majority of the proteins.

After consulting published purification protocols for thioredoxin, a combination of three steps was found to solubilize the majority, if not all of the fusion protein expressed. This process involves (1) sonication, (2) immediate freezing under liquid N₂ or in an ethanol-dry ice bath, and (3) thawing at room temperature. This series of steps were repeated three times. The remainder of the fusion protein that still remained in the pellet can be released into the supernatant by sonicating the pellet after the first centrifugation step. An ammonium sulfate fractionation step was added as reported in the isolation of thioredoxin alone. To the cleared lysate, 0.3 g of ammonium sulfate was added per mL of solution. This step precipitated out the contaminating proteins (lane 1 in Fig. 72). To the supernatant, another 0.3 g of ammonium sulfate was added per mL. The fusion protein came out of solution at this step (lane 2 in Fig. 72).

The protein sample was desalted in a G-25 column before applying to a DE-52 column. DE-52 is an anion exchanger and thus binds negatively-charged moieties. Since the fusion protein has a very low pl, it is negatively-charged at pH 7.4 and would be expected to bind tightly to the DE-52 column. However, it was observed that because of the abundance of DNA/RNA in the crude lysate, much of the protein can not compete for binding to the resin. Some of the contaminating nucleic acids were gotten rid of by using an amicon concentrator with a 10K membrane. Since the fusion protein has a MW of approximately 28 kDa, it was retained and anything below 10 kDa came out of the amicon.
Figure 72. Ammonium sulfate fractionation step. Lane 1) first pellet, and 2) second pellet. (*) indicates the fusion protein.
The fusion protein seemed to be bound weakly to the resin since it eluted at about 50-70 mM KP. Thioredoxin alone was reported to come off the column at about 70 mM KP. Results indicated that the isolated fusion protein was < 50% pure (Fig. 71).

The major problem encountered in the isolation of the protein was the abundance of nucleic acids that can compete for binding to the resin. It is highly recommended that future isolation should include addition of streptomycin sulfate to the supernatant before ammonium sulfate fractionation step. Addition of the antibiotic would precipitate out the DNA.

11.4. Summary.

At this point, clones of FGI, FGE, FL, FLE and FLI have been isolated. The thioredoxin-FGI fusion protein had been isolated to almost 50% purity. There is still a long way to go to achieve our ultimate goal of isolating the individual domains of the Fas and FasL proteins but the work presented here definitely laid out the foundation for this major task.
REFERENCES


Kagi, D., Lederman, B., Brki, K., Seiler, P., Odermatt, B., Olsen, K. J., Podack, E. R.,


Kanaya, S., Kohara, A., Miyagawa, M., Matsuzaki, T., Morikawa, K. and Ikehara, M.


Katayanagi, K., Miyagawa, M., Matsushima, M., Ishikawa, M., Kanaya, S., Nakamura,

Katayanagi, K., Miyagawa, M., Matsushima, M., Ishikawa, M., Kanaya, S., Nakamura,

Katayanagi, K., Ishikawa, M., Okumura, M., Ariyoshi, M., Kanaya, S., Kawanos, Y.,

Katayanagi, K., Okumura, M., Morikawa K. (1993b) *Proteins: Structure, Function, and


Tanaka, M., Suda, T., Takahashi, T., Nagata, S.


