Catalysis by Ribonuclease A: Specificity, Processivity, and Mechanism

by

Stephen Bralley delCardayré

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To My Family

Abstract

Bovine pancreatic ribonuclease A (RNase A) is probably the most studied of all enzymes. Yet, the application of the techniques of protein engineering to RNase A has been limited by problems inherent in the isolation and heterologous expression of its gene. The cDNA that codes for RNase A was recently isolated. This cDNA was inserted into 2 expression plasmids such that the resulting plasmids direct the production of RNase A in *Saccharomyces cerevisiae* (fused to a modified a-factor leader sequence) or *Escherichia coli* (fused to the pelB signal sequence). RNase A secreted into the medium by *S. cerevisiae* was an active but highly glycosylated enzyme that was recoverable at 1 mg per L of culture. RNase A produced by *E. coli* was in an insoluble fraction of the cell lysate. Oxidation of the reduced and denatured protein produced active enzyme, which was isolated at 50 mg per L of culture. The bacterial expression system is ideal for the large-scale production of mutants of RNase A.

RNase A is a non-processive endoribonuclease that catalyzes the cleavage of the P-$O_{5''}$ bond of RNA specifically on the 3' side of pyrimidine nucleotides. Structural data have implicated Thr45 and Phe120 of the B1 subsite as mediating this pyrimidine specificity. To investigate this possibility, RNase A mutants in which all natural amino acids were substituted for Thr45 or Phe120 were screened for the ability to cleave RNA after purine residues. Two mutants were identified as having this ability. T45A and T45G RNase A cleave polyA, polyC, and polyU efficiently and with 10^3– to 10^5–fold increases in purine:pyrimidine specificity. Thus, Thr45 is responsible for the pyrimidine specificity of RNase A.

In addition to preferentially binding pyrimidine nucleotides, the B1 subsite binds to cytidine 50-fold more tightly than to uridine. T45A and T45G maintain this

inherent preference for cytidine nucleotides. Asp83, a residue also in the B1 subsite, forms a hydrogen bond to the β-hydroxyl group of Thr45 when a uridine, but not when a cytidine, is bound in the B1 subsite. To determine whether Asp83 was responsible for the cytidine:uridine specificity of the B1 subsite, the D83A and T45G/D83A mutants were created. The kinetic parameters for cleavage of ribonucleotide substrates by these mutants were then compared to those of the wild-type and T45G enzymes. The results of this comparison indicate that Asp83 enhances the rate of cleavage after a uridine or adenine residue, and that the rate enhancement for cleavage after a uridine residue is dependent on the sidechain of Thr45. The substrate specificity imparted by both Thr45 and Asp83 is manifested predominantly in the chemical transition state for ribonucleotide cleavage. Thus, substrate specificity and catalytic efficiency are related intimately during catalysis by RNase A.

A *processive* enzyme binds a polymeric substrate and catalyzes a series of similar chemical reactions along that substrate before releasing to solvent the extensively modified product. Such catalysis requires an enzyme to remain bound to its substrate after each catalytic event. Engineering RNase A to accept adenine bases in the B1 subsite, allows poly(A) to satisfy the substrate preference of each of RNase A's subsites, B1, B2, and B3. To determine whether T45G and T45A RNase A degrade poly(A) processively, two assays were developed. The results of these two assays suggest that T45A and T45G degrade poly(C) non-processively but degrade poly(A) processively. These results provide a new paradigm: processive enzymes have subsites, each specific for a repeating motif within a polymeric substrate.

The catalytic mechanism of RNase A is well established, yet, the roles of each of the residues in the enzyme's active site have yet to be determined. Structural studies have indicated that the sidechain of Gln11 is intimately associated with the reactive

phosphorous of bound substrate. To investigate the role this residue plays in catalysis, mutants Q11A, Q11N, and Q11H were created. Steady-state kinetic analysis of catalysis by the mutant enzymes shows that the sidechain of Gln11 does not contribute to transition state stabilization. Rather, the primary role of Gln11 is to orient substrate so as to prevent nonproductive binding. Implications to the catalytic mechanism of RNase A are discussed.

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Publications

The work presented in this thesis has been either published or submitted for publication as the following journal articles:

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2. delCardayré, S.B. & Raines, R.T. (1994). Stuctural determinants of enzymatic processivity. *Biochemistry* **33**, 6031-6037.

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Chapter 1

Enzymes, Engineering, and Ribonuclease A

1.1 Background

Organisms are by far the most sophisticated chemists. For billions of years living systems have studied chemistry under the unforgiving tutelage of evolution. The result is an amazing diversity of life forms each uniquely suited to harness the chemical and physical potential of its individual niche. Mediating the success of each organism is a unique portfolio of chemical catalysts, the enzymes. These enzymes accelerate all of the chemical reactions that are intimate to the life processes. Indeed, enzymes catalyze life. The extreme efficiency and specificity of these biological catalysts are extraordinary. Chemical reactions that scarcely occur at ambient temperature and in water can occur at rates approaching that of diffusion and with absolute stereospecificity in the presence of the appropriate enzyme. Many of these catalysts are in addition tightly regulatable. How enzymes achieve these remarkable feats of catalysis is a central question of biochemistry and embodies the field of enzymology.

The study of biochemistry essentially began with the study of enzymology. This early history can be traced to the European breweries of the early nineteenth century and the study of fermentation. The first observed biological catalysts were appropriately termed "ferments", as they catalyzed the fermentation of glucose to carbon dioxide and water. The term "enzyme" (Greek: *en*, in + *zyme*, yeast) was later coined to emphasize that some internal component of yeast, rather than the yeast themselves, was responsible for catalysis. In the early twentieth century the first enzyme was crystallized and was shown to be composed of protein. Since then, and until the recent discovery of catalytic RNA's or "ribozymes," all enzymes were believed to be proteins.

Most identified enzymes are polypeptides constructed largely from twenty natural L-amino acids. In aqueous solutions these polypeptides fold into distinct globular structures concentrating hydrophobic sidechains in their core and polar sidechains on their surface. The final tertiary structure of each polypeptide is determined by its primary sequence of amino acids. Despite the general segregation of hydrophobic and polar residues, hydrophobic, polar, and charged residues are found throughout the tertiary structure of a folded protein. This unique physical and chemical topology is what determines a protein's ultimate biological function. The biological function of an enzyme is to catalyze a chemical reaction that provides some selective advantage for the organism that creates it.

It is no wonder that enzymes have evolved to be the extremely efficient catalysts that they are. If the rate of division of two organisms is limited by a chemical conversion, and one organism creates an enzyme that catalyzes that conversion twice as fast as the other, the faster organism will rapidly proliferate. Enzymes are also metabolically expensive. Each peptide bond formed by standard translation costs the cell three phophoanhydride bonds at a minimum (Voet & Voet, 1990). Most enzymes are at least 100 amino acids in length, and many are much larger. It may thus be energetically more efficient to create one molecule of an enzyme that is a good catalyst than several molecules of an enzyme that is a poor one.

In addition to their efficiency, enzymes are characterized by the extreme specificity with which they catalyze chemical reactions. Enzymes can distinguish substrates that differ by as little as the configuration about a single chiral center. Although there has been debate as to the relationship between specificity and efficiency (Chapter 3), these two aspects of enzymatic catalysis are apparently interdependent. An enzyme having high specificity is likely to be more efficient than a similar enzyme

having broader specificity. This unusual combination of catalytic properties has led enzymes to become both objects of intense research and useful tools in organic synthesis, industry, medicine, and biotechnology.

Most of biochemistry can be reduced to a problem of molecular recognition, and catalysis by enzymes is no exception. The unique chemical topology of enzymes have apparently evolved to be complementary in structure to the chemical transition state of the reactions that they catalyze. Upon binding, enzyme and substrate molecules exchange noncovalent interactions with solvent and solute molecules for interactions with each other. The free energy gained from these noncovalent interactions, the binding energy, is used through a medley of devices to decrease the activation energy for the covalent rearrangement of the bound substrate (Jencks, 1987). The interactions that mediate enzyme-substrate binding energy are extremely precise and are responsible for the efficiency and specificity displayed by enzymes. For example, an enzyme must be somewhat complementary to the ground state structure of its reactants in order to bind them. Yet, in order to effect catalysis, the enzyme must be more complementary to the fleeting structure of the reaction transition state. Finally, the interactions that permit substrate binding and catalysis to occur must be such that neither substrate nor product remain bound to the enzyme, as this would inhibit turnover. Since substrate, product, and transition state will all be similar in structure, the architecture of an enzyme must be finely tuned to discriminate between these species. This fine tuning of an active site is the key to catalysis by enzymes and exemplifies the extraordinary precision of biomolecular recognition.

The study of enzymes has traditionally relied on the techniques of physical organic chemistry. Monitoring the effects of reactants, solvent, pH, salt concentration, and solutes on the kinetics of catalysis and on reaction products have been essential to

elucidating the mechanisms by which enzymes function. X-ray diffraction analysis and nuclear magnetic resonance spectroscopy have permitted an understanding of the three-dimensional structure of proteins, enzymes, and their ligands. Now, the most important goal in protein chemistry is to understand how the amino acid sequence of a protein determines its tertiary structure and how that structure manifests itself in function. The recent explosion in recombinant DNA technology along with advancements in the physical techniques described bring promise that this goal may be realized. Already, the pathways by which individual proteins fold are being mapped, structure prediction algorithms are becoming increasingly accurate, and novel enzymes having engineered activities are being created.

1.2 Recombinant DNA and Protein Engineering

The enzymology of nucleic acid modification permitted the advent of recombinant DNA technology. Recombinant DNA technology has since revolutionized the biological sciences and likewise the field of enzymology. Before the development of modern chromatography and recombinant DNA technology the study of enzymes was primarily limited to the "housekeeping" enzymes, such as those of glycolysis, which are relatively abundant in the cell. The primary structure of a protein was determined by chemically sequencing the protein itself, and structure-function studies were limited to the imprecise technique of chemical modification and proteolysis. Recombinant DNA technology has transposed each of these facets of protein research. Now, large quantities of previously inaccessible enzymes can be obtained by the cloning and overexpression of their genes. In addition, a protein's sequence can be rapidly determined from the enzymatic sequencing of its gene. Advancements in nucleic

chemistry have also provided the means to synthesize genes *de novo* and to engineer precise alterations to a protein's structure by mutating its cloned gene (Winter et al., 1982). This latter technique is known as protein engineering.

Protein engineering is synonymous with both the technique of genetically manipulating protein structure and the scientific field that has blossomed from this technology. Researchers employ protein engineering to study the effect of discrete alterations in amino acid sequence on the folding, structure, stability, and function of proteins. Studies of engineered enzymes have become ubiquitous. Yet, only those investigations that report a thorough study of the mutant enzymes actually prove to be informative. Studies of this nature have greatly advanced our understanding of particular enzymatic mechanisms, of how binding energy is distributed in enzymatic catalysis, and in how the structure of an active site so effectively permits catalysis (Fersht, 1985). In addition, the free energy profiles for entire enzymatic reactions have been determined (Albery & Knowles, 1976; Raines et al., 1986; Avis et al., 1993). The contribution of individual amino acid sidechains to enzymatic catalysis and specificity have been verified and quantitated (Thompson & Raines, 1994). Finally, the role individual amino acids play in the stability and folding of enzymes have been identified, and protein folding pathways have begun to be mapped (Serano et al., 1992).

As the fundamental relationship between protein structure and function becomes more evident, the application of protein engineering becomes more profound. In addition to addressing the academic questions of protein chemistry, protein engineering provides the means to construct new protein molecules. Consequently, efforts to develop efficient, selective catalysts for the synthesis and degradation of complex molecules are turning to enzyme engineering. Most efforts in this area have been aimed at the improvement of natural enzymes through structural modification. Examples of

these are prevalent throughout industry, medicine, and biotechnology (Inouye & Sarma, 1986). Numerous proteases have been engineered to have increased thermal and chemical stabilities in order to be more useful supplements in detergents (Wells et al., 1987). Subtilisin BPN', which is possibly the most engineered enzyme, has also proved to be a useful catalyst in organic synthesis for both acylation and peptide bond formation (Wong, 1989). This has led to numerous attempts to alter the enzyme's substrate specificity (Rheinnecker et al., 1993) and to increase the enzyme's stability in organic solvent (Bryan, 1989). Insulin has been engineered to stabilize its monomeric state at pharmaceutical concentrations thereby improving its rate of absorption and its physiological plasma profile (Brange et al., 1988). Lactate dehydrogenase has shown promise in being useful in organic synthesis, and the substrate specificity (Wilks et al., 1988; Wilks et al., 1990; Wilks et al., 1992) and thermal stability (Kalwass et al., 1992) of this enzyme have also been engineered. Protein engineering is being combined with metabolic engineering in an attempt to produce new secondary metabolites such as antibiotics (Hutchinson, ongoing research). Protein engineering is also appearing in the field of bioremediation (Janssen & Schanstra, 1994). Ligninases and xylanases are being explored for their application to paper bleaching, and the monooxygenases are being examined for their ability to detoxify halogenated hydrocarbons in industrial runoffs and contaminated soils. These are but a few of the current applications for engineered enzymes, and countless others shall certainly arise.

Although recombinant DNA technology has put most any enzyme within reach of the researcher, the effective study of an enzyme by protein engineering still has the following requirements: (1) a gene encoding the protein that is accessible to the manipulations of recombinant DNA technology, (2) an expression system that provides sufficient wild-type and mutant protein for both structural and functional

characterization, and (3) some means of assessing the functional results of a structural alteration. The three-dimensional structure of the protein in question or that of a close homolog is also highly desirable. The elegance of protein engineering is its subtlety and definition. Thus, for better characterized enzymes more detailed questions can be addressed. The answers to these questions, of course, depend on the thorough characterization of the engineered enzyme.

1.3 Ribonuclease A as a Model Enzyme

Ribonucleases, the enzymes that catalyze the degradation of RNA, are an essential component of all living cells. The ribonuclease activity in the pancreas of ruminants is particularly high. This high level of activity has led to the detailed characterization of bovine pancreatic ribonuclease A (RNase A; EC 3.1.27.5). In the early 1940s more than a kilogram of highly pure RNase A was made available by Armour, Inc. This large preparation of known uniformity led to an outbreak of research on this enzyme that continues today and has made RNase A possibly the most studied enzyme.

Just as the early history of biochemistry can be traced to the study of enzymes, much of early enzymology and protein chemistry can be attributed to the study of RNase A. RNase A has been the object of landmark work on the folding, stability, and chemistry of proteins; on enzymology; and on molecular evolution (Richards & Wyckoff, 1971; Karpeisky & Yakovlev, 1981; Blackburn & Moore, 1982; Wlodawer, 1985; Beintema, 1987; Eftink & Biltonen, 1987). RNase A was the first enzyme and the second protein (after myoglobin) to be sequenced completely. It was the third enzyme and the fourth protein (after myoglobin, lysozyme, carboxypeptidase) whose structure was solved by X-ray crystallography. Anfinsen's classic refolding of RNase

A demonstrated that the three-dimensional structure of a protein is determined by its amino acid sequence and provided the widely accepted thermodynamic hypothesis for protein folding. The NMR spectroscopy of proteins (Saunders et al., 1957) and the use of NMR in delineating protein folding pathways (Udgaonkar & Baldwin, 1988) were developed with RNase A. RNase A was also one of the first enzymes to be synthesized completely (Gutte & Merrifield, 1971), and the technology developed during that synthesis has influenced greatly peptide and combinatorial chemistry.

Structure of RNase A. RNase A is a small, extremely stable protein of 124 amino acid residues, a calculated molecular weight of 13.7 kDa, and an isoelectric point of 9.3. RNase A was first sequenced over 30 years ago, and more than 40 other pancreatic RNases from snapping turtle to hippopotamus have since been sequenced (Figure 1.2)(Beintema, 1987). RNase A is the best characterized of a superfamily of pancreatic ribonucleases, which include the pancreatic type ribonucleases, the angiogenins, and the eisonophil derived nuerotoxin and cationic proteins (Benner, 1988). The pancreatic ribonucleases are endoribonucleases that catalyze the transphosphorylation of RNA specifically on the 3' side of pyrimidine bases. The true physiological role of the pancreatic ribonucleases is unknown but is presumably to enable the digestion of RNA produced by stomach microflora (Barnard, 1969). The homology of RNase A to proteins having more intriguing physiological properties (such as the angiogenins), however, suggests that digestion may not be this enzyme's only function (Benner, 1988). The phylogenetic data accumulated for the pancreatic ribonucleases has both provided an interesting story of molecular evolution and been a useful complement to the abundance of chemical and structural data on RNase A

Both RNase A and its subtilisin cleavage product, RNase S, have been the subject of extensive structural studies (Richards & Wyckoff, 1971). The X-ray crystal structures of RNase A and RNase S alone and complexed with numerous substrates, products, and inhibitors have been solved. For an excellent review see Wlodawer (1985). The structure of RNase A is shown in Figure 1.1. The N-terminal 20 residues are an α-helix that make up the small subtilisin cleavage product, the S-peptide. Residues 21-124 are termed the S-protein. The S-peptide and S-protein alone are inactive, but they associate with high affinity to form RNase S, which displays unblemished activity. The combining of S-protein with synthetic S-peptides of altered amino acid sequence provided early structure-function studies on RNase A prior to the advent of protein engineering (Richards & Wyckoff, 1971). Although there are numerous X-ray crystal structures reported, the most detailed is the combined neutron/X-ray structures of RNase A alone and complexed with the inhibitor uridine 2', 3'-cyclic vanadate (U>v) (Wlodawer & Lennart, 1983; Wlodawer et al., 1983) All interatomic distances and bond angles described in this thesis are taken from this structure (Brookhaven Protein Data Bank. 6RSA.ENT).

Although the structure of RNase A has been described thoroughly, a few pertinent details shall be reiterated here. RNase A contains eight half-cystine resides, which form disulfide bonds between residues 26-84, 40-95, 65-72, and 58-110. These residues are conserved in each of the sequenced pancreatic ribonucleases, and provide much of RNase A's notorious stability. There are also four proline residues. In the native enzyme Pro42 and Pro117 are in the *trans* configuration while Pro93 and Pro114 are in the *cis* configuration. The slow isomerization of Pro93 and Pro114 from the *trans* to the *cis* configuration is believed to be responsible for the biphasic kinetics observed for RNase A folding (Kim & Baldwin, 1982). RNase A has four histidine

residues 12, 48, 109, and 119. His12 and His119 are part of the active site and are postulated to contribute acid-base catalysis to RNA cleavage (Thompson & Raines, 1994). Also of interest are the nine lysine and four arginine residues which contribute to the basicity of the protein. Lys41 is of particular interest as it is intimate to the active site and critical for catalysis.

RNase A catalyzes the cleavage of the P–$O_{5''}$ bond of single-stranded RNA specifically after pyrimidine residues. Figure 1.3 depicts a mechanism of catalysis that is consistent with all known data.[For other proposed mechanisms, see; Withzel (1960; 1963), Wang (1968), and Anslyn and Breslow (1989).] In the mechanism in Figure 3, His12 acts as a general base that abstracts a proton from the 2'-hydroxyl of a substrate molecule, and thereby facilitates attack on the phosphorus atom. This attack proceeds in-line to displace the nucleoside (Usher et al., 1972). His119 acts as a general acid that protonates the 5"-oxygen to facilitate its displacement (Thompson & Raines, 1994). Both products are released to solvent. In this mechanism, the reaction apparently passes through a transition state that has a pentacovalent phosphorus atom. Lys41 assists catalysis by stabilizing this transition state. The slow hydrolysis of the 2'-3' cyclic phosphate occurs separately and resembles the reverse of transphosphorylation (Usher et al., 1970; Thompson & Raines, 1994).

Like many enzymes that degrade biopolymers, RNase A binds its substrate in numerous subsites (Figure 1.4)(Parés et al., 1991). The primary binding pocket consists of the B1 and P1 subsites (where B and P refer to base and phosphate). The B1 subsite is responsible for the pyrimidine specificity of RNase A and is constructed from residues Thr45, Asp83, Phe120, and Ser123. Thr45 forms hydrogen bonds from its sidechain hydroxyl and backbone nitrogen to a pyrimidine base (uracil or cytosine) bound to the B1 subsite and is proposed to mediate the primary specificity of RNase A

(Richards & Wyckoff, 1971). The sidechain of Phe120 makes van der Waals contact with a pyrimidine base bound to the B1 subsite and to the sidechain of Thr45. Although this residue is implicated in substrate binding, it may also contribute significantly to the structure and stability of RNase A. The sidechain of Asp83 accepts a hydrogen bond from the sidechain of Thr45 when a uracil base, but not a cytosine base, is bound to the B1 subsite, and thus may be involved in uracil:cytosine specificity (Chapter 3). The sidechain of Ser123 was reported to form a hydrogen bond to a uracil base, but not a cytosine base, bound to the B1 subsite, and apparently improves the turnover of uracil containing substrates (Hodges & Merrifield, 1975; Bruenger et al., 1985). Such a hydrogen bond, however, is not evident in the combined X-ray/neutron diffraction structure of RNase A complexed with uridine 2',3'-cyclic vanadate (Wlodawer et al., 1983).

The P0 subsite is proposed to bind to the 5'-phosphoryl group of nucleotides bound in the primary binding site, and Lys66 is proposed to mediate this binding (Parés et al., 1991). The P1 subsite binds the reactive phosphoryl group of the nucleotide bound to the B1 subsite. All the residues in the P1 subsite, His12, His119, Lys41, Gln11, and Phe120, are thus implicated in catalysis. Although the imidazole sidechain of His12 appears to form a hydrogen bond with a nonbridging oxygen, it is believed that His12 accepts a hydrogen bond from the 2'-hydroxyl as predicted by the notion that His12 is a general base. Consistent with its role as a general acid, His119 donates a hydrogen bond to the 5" oxygen of a substrate phosphoryl group bound to the P1 subsite. Lys41 appears to form a hydrogen bond to the 2'-hydroxyl and is less than 3.6 Å from a non-bridging phosphoryl oxygen. The ammonium group of Lys41 likely stabilizes the transition state by interacting with negative charge that accumulates either on the 2'-hydroxyl, a nonbridging oxygen, or both. The carboxamide sidechain

of Gln11 and the backbone nitrogen of Phe120 each donate a hydrogen bond to different nonbridging phosphoryl oxygens. These interactions could play a number of roles in catalysis (discussed further in Chapter 5). Although not directly in the P1 subsite, the carboxylate sidechain of Asp121 forms a hydrogen bond to the imidizolium sidechain of His119 as well as to the sidechain of Lys66. The former interaction may stabilize this cation in a manner similar to that of the catalytic triad in serine proteases and thereby facilitate catalysis.

The B2 subsite binds to the base 3' to that bound in the B1 subsite and, although binding all bases, shows a strong preference for adenine (Katoh et al., 1986; Parés et al., 1991). Gln69, Asn71, and Glu111 have been proposed to make up this subsite, but recent evidence (see below) suggests that only Asn71 and Glu111 are involved (Tarragona-Fiol et al., 1993). It should be noted that an adenine, base when bound to the B2, subsite appears to stack with the sidechain of His119 (Fontecilla-Camps et al., 1993). As histidine–adenine stacking interactions are prevalent in protein–adenine interactions [for example (Buckle & Fersht, 1994)], His119 may also contribute to the B2 subsite. Further, binding of adenine to the B2 subsite may assist catalysis by the proper alignment of His119, which is believed to exist in two conformations (Eftink & Biltonen, 1987). The P2 site interacts with the 3' phosphoryl group of nucleotides bound to the B2 subsite, and Lys7 and Arg10 have been shown to mediate this interaction (see below)(Parés et al., 1991; Boix et al., 1994). Finally, the B3 subsite binds a base 3' to that bound to the B2 subsite, and both Lys1 and Glu111 have been implicated in this interaction. The structure of crystalline RNase A complexed with $(dAp)_4$ shows that a chain of 12 nucleotides lies across the surface of RNase A, but no additional subsites have been proposed (McPherson et al., 1986).

Structure Function Studies of RNase A. The enormous amount of structural, chemical, and phylogenetic data on RNase A makes this enzyme extremely qualified for investigation by the techniques of protein engineering. Unfortunately, the cloning and heterologous expression of the gene for RNase A has proved to be extremely difficult. Thus, most structure–function studies of RNase A have either employed semisynthetic RNase or have relied on the technique of chemical modification. Most such studies have been reviewed previously (Blackburn & Moore, 1982). Recently, however, expression systems for RNase A have surfaced (discussed extensively in Chapter 2), and protein engineering results are beginning to accumulate. The highlights of these investigations follow.

Folding and Stability. RNase A has been a model for protein folding and stability studies. To study the value of a *cis* peptide bond to the kinetics of folding and the stability of RNase A, Shultz and Baldwin created the mutants P93A, P93S, P114G, P114A, and P93A/P114A. They found that each mutation resulted in a 10° C decrease in the T_m relative to the wild-type enzyme and that the effect was additive for the double mutant. In addition, during folding the double mutant no longer accumulated the slow forms, U_s, observed for the wild-type enzyme, suggesting that the slow folding phase in RNase A is due to the *cis-trans* isomerization of prolyl peptide bonds (Schultz & Baldwin, 1992; Schultz et al., 1992). Scheraga and coworkers created the double mutants C65S/C72S, C40S/C95S, C58S/C110S, and C26S/C84S to assess the importance of the disulfide bonds in the folding and stability of RNase A. They proposed that if all four mutants could fold properly, then no single disulfide bond was necessary for the enzyme's proper folding. Unfortunately, C58S/C110S and C26S/C84S were unstable and could not be purified, and no such conclusion could be made. The C65S/C72S and C40S/C95S mutants, however, were purified, and each

had a 20° C decrease in T_m and 5 – 30% of the catalytic activity of the wild-type enzyme (Laity et al., 1993). Apparently, a disulfide bond contributes the same thermal stability to RNase A as do two *cis* prolyl peptide bonds. These disulfide mutants could prove to be extremely informative if characterized further.

Subsites. Interest in the subsites of RNase A has also lead to several protein engineering studies. Rabin and colleagues were interested in the residues that mediate the specificity of the B2 subsite. They created Q69A, N71A, Q69A/N71A, and E111Q and monitored the cleavage of CpA and C>p and the relative preference for CpN (where N refers to U, C, A, and G) by each mutant. They found that Asn71 plays a major role in catalysis by interacting with all bases bound to the B2 subsite. Altering the sidechain of Asn71 to an alanine residue resulted in a 40-fold decrease in the k_{cat}/K_m for the cleavage of CpA (which binds to the B2 subsite) but less than a 2-fold decrease in the k_{cat}/K_m for the hydrolysis of C>p (which does not bind to the B2 subsite). Interestingly, the mutation effected primarily k_{cat}. A decrease in the catalytic efficiency for the cleavage of CpG, CpC, and CpU was also reported. Glu111 appears to be involved in catalysis as well, but only when there is a guanosine bound to the B2 subsite. Altering the sidechain of Glu111 to a glutamine had little effect on the turnover of CpA, CpC, CpU, or C>p, but resulted in an 8-fold decrease in the turnover of CpG. Contrary to prior suspicions, Gln69 did not appear to contribute to the B2 subsite, as altering Gln69 to an alanine had little effect on catalysis by RNase A (Tarragona-Fiol et al., 1993).

Cuchillo and colleagues have carried out extensive chemical modification studies of the subsites of RNase A, and a review of these efforts has been published (Parés et al., 1991). Their results are convincing—the P2 subsite does exist and that it is formed from residues Lys7 and Arg10. To test further this notion Cuchillo's group created the mutants K7Q, R10Q, and K7Q/R10Q by protein engineering. Each mutant

demonstrated a significant decrease in catalytic efficiency for the cleavage of poly(C), CpA, and 2',3'-cyclic phosphate (C>p). This observation suggests that these residues may form the P2 subsite but that they effect catalysis by other means as well, since C>p and CpA have no phosphate which binds to the P2 subsite. Each mutant also showed a decrease in the rate of reverse transphosphorylation (2C>p ⇒ CpC>p) suggesting that possibly, but not conclusively, an interaction with the phosphate of C>p bound in the secondary subsite had been removed. Finally, each mutant demonstrated a decrease in the efficiency with which it was chemically modified by cl^{6}RMP. This reagent specifically modifies the α-amino group of Lys1 and is effectively blocked by 3'-AMP, which binds to RNase A in the B2, R2, and P2 subsites. These studies along with the earlier chemical modification work support that both Lys7 and Arg10 contribute to substrate binding in the P2 subsite. However, the kinetic analysis of the mutant enzymes suggest that such binding is not necessary for these residues to contribute to catalysis.

Catalysis. Intensive investigation of the role of residues Phe120 and Asp121 have been carried out by Edwards and coworkers using semisynthetic RNase 1-118:11-124, in which residues 1-118 of wild-type RNase A are combined with a synthetic peptide corresponding to residues 111-124. This work complements former studies of Phe120 by Merrifield and coworkers (Lin et al., 1972; Hodges & Merrifield, 1975). Semisynthetic RNase F120L, F120Y, D121N, and D121A RNases have each been constructed and characterized kinetically, and the crystalline structures of each have been determined (deMel et al., 1992; Srini et al., 1994). Altering the sidechain of Phe120 to a leucine has little effect on K_m but an approximately 6-fold decrease in k_{cat} for the hydrolysis of C>p. Replacing Phe120 with a tyrosine residue, however, appeared to slightly affect the ground state binding of C>p, demonstrating a 2-fold

increase in both k_{cat} and K_m. Considering that Phe 120 interacts with both the base (through its sidechain) bound to the B1 subsite and the phosphoryl group (through its backbone nitrogen) bound to the P1, it is difficult to interpret the origins of these small kinetic perturbations. Altering the sidechain of Asp121 also has only small effects on the catalytic activity of the D121N and D121A RNases. These mutants show a 2 – 4-fold increase in K_m and a 5 – 9-fold decrease in k_{cat}, with the D121N mutant being the less active of the two. Again, the sidechain of Asp121 forms interactions with both the imidizolium sidechain of His119 and the sidechain of Lys66 making these small kinetic changes difficult to interpret. Additional characterization of these mutants is necessary if fruitful information is to be gathered.

Numerous groups have reported mutagenesis of the catalytic residue Lys41, but no group has yet carried out a thorough characterization of these important mutants. Benner and coworkers created the K41M mutant and demonstrated that it had an approximately 100-fold decrease in both k_{cat} and k_{cat}/K_m for the cleavage of UpA (Trautwein et al., 1991). Rabin and coworkers duplicated these findings (Tarragona-Fiol et al., 1993). Scheraga's group created the K41G mutant and could detect no activity toward C>p by this mutant (Laity et al., 1993). As has been predicted for over thirty years, the sidechain of Lys41 is important to catalysis by RNase A and appears to function by the selective stabilization of the chemical transition state for RNA cleavage (Mesmore & Raines, unpublished results). How Lys41 stabilizes this transition state, however, has yet to be determined, and will require extensive characterization of Lys41 mutants. Thompson and Raines recently reported an investigation of His12 and His119 using protein engineering. They created the mutants H12A and H119A to determine the contribution of these residues to acid-base catalysis. Both H12A and H119A have greater than a 10^4-fold decrease in k_{cat} and k_{cat}/K_m for the cleavage of UpA and poly(C)

with little effect on K_m. Interestingly, the H119A mutant is not catalytically impaired in the cleavage of the synthetic substrate UpO-C_6H_4-*p*-NO_2. This substrate has an excellent leaving group and does not need the general acid assistance of His119. Apparently, His12 and His119, through acid-base catalysis, each contribute approximately 6 kcal/mol to catalysis by RNase A (Thompson & Raines, 1994). More extensive studies of these mutants and others are currently underway in the Raines laboratory.

Protein engineering of RNase A presented in this thesis. Protein engineering studies on RNase A are limited because the heterologous expression of the gene for RNase A has been so difficult. Described here are two distinct expression systems for the cDNA encoding RNase A (Chapter 2), one of which is ideal for the large scale production of site-directed mutants. The contribution of Thr45 and Asp83 to the substrate specificity of RNase has been investigated by protein engineering and is described in Chapter 3. Some of the altered specificity mutants described in Chapter 3 are shown in Chapter 4 to be processive, illuminating for the first time structural determinants of enzymatic processivity. Finally, the contribution to catalysis by Gln11 is described in Chapter 5. These studies along with the volumes of chemical and structural data that continue to accumulate on RNase A provide further insight into the role that biomolecular recognition plays in protein chemistry.

Figure 1.1 Ribbon diagram of the structure of RNase A. Shown are the two catalytic histidines, His12 and His119, and the four disulfide bonds.

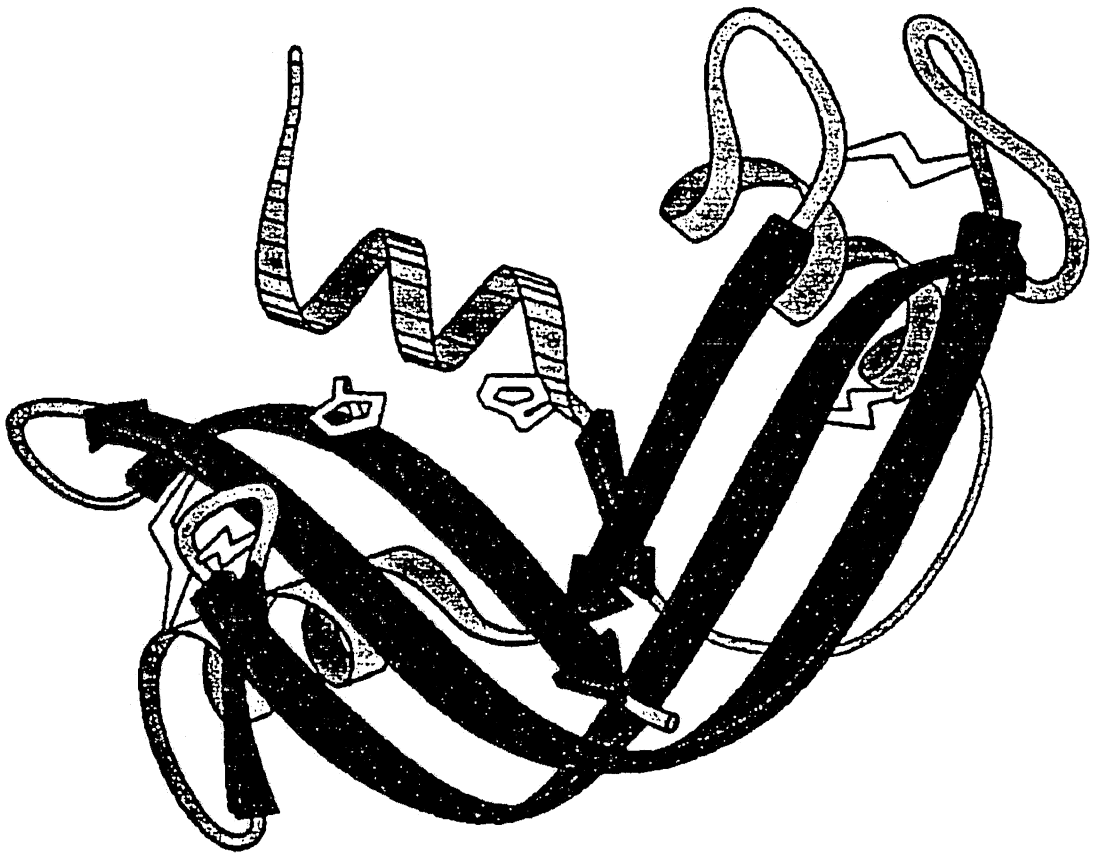


Figure 1.2 Amino acid sequences of 41 pancreatic ribonucleases and bovine seminal ribonuclease in the IUB one letter code (z = Glx; b = Asx). Only differences from the bovine pancreatic sequence are shown. Residues are numbered by homology with the bovine enzyme. Deletions in the sequences are indicated by –, and unidentified residues by x (This figure was transcribed from Beintema's review of the molecular evolution of pancreatic ribonucleases (Beintema, 1987).)

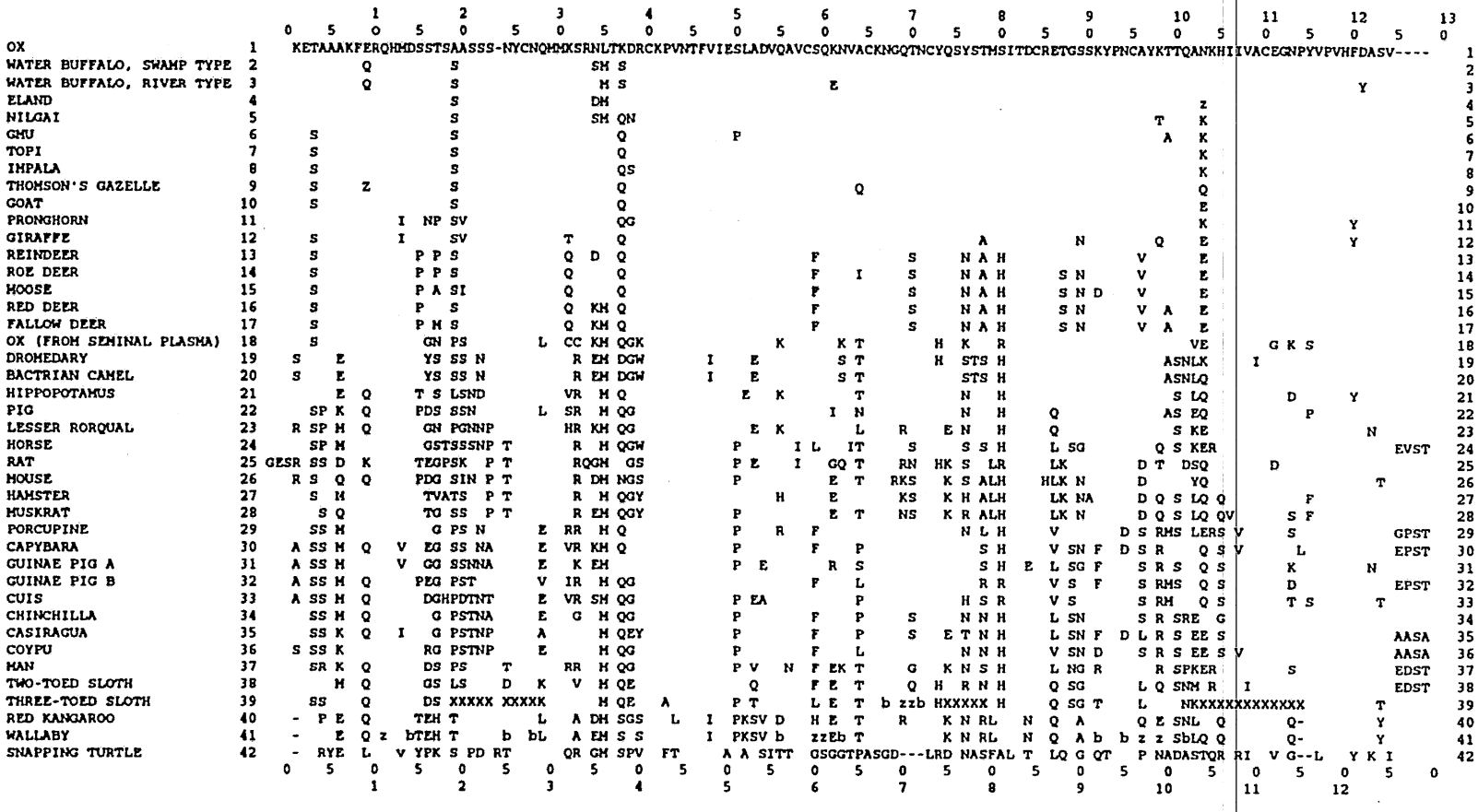
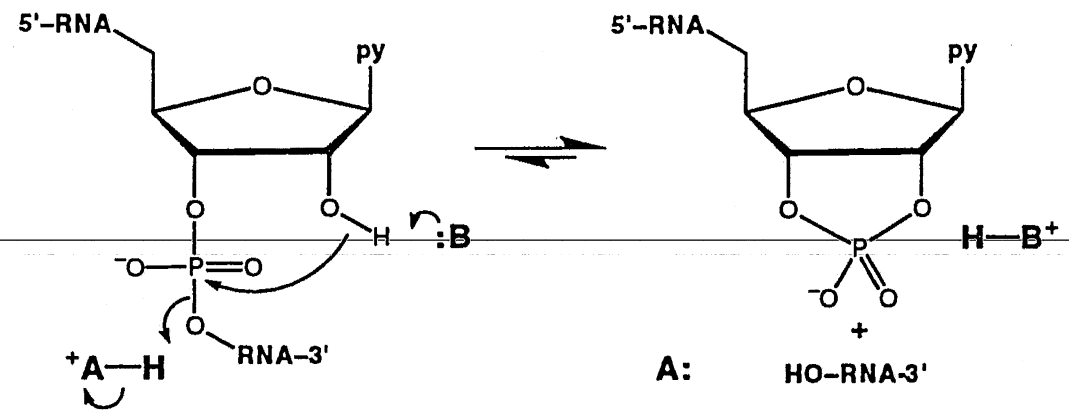
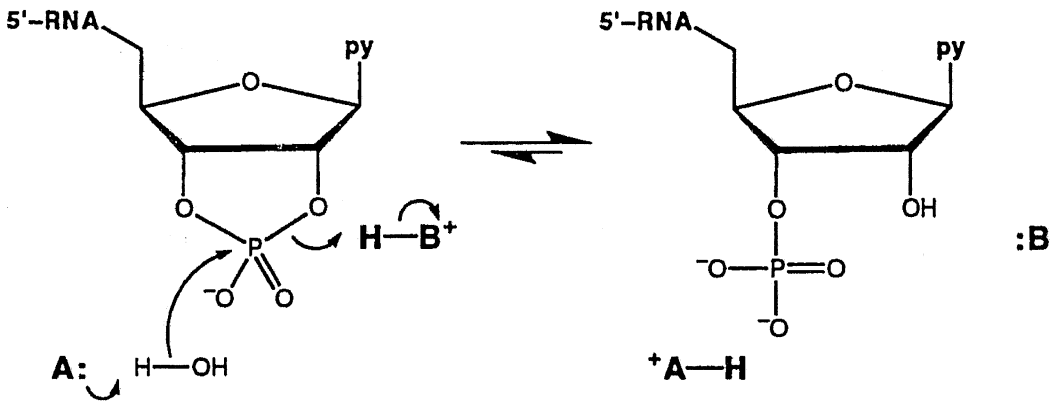

OX
WATER BUFFALO, SWAMP TYPE
WATER BUFFALO, RIVER TYPE
ELAND
NILGAI
GNU
TOPI
IMPALA
THOMSON'S GAZELLE
GOAT
PRONGHORN
GIRAFFE
REINDEER
ROE DEER
MOOSE
RED DEER
FALLOW DEER
OX (FROM SEMINAL PLASMA)
DROMEDARY
BACTRIAN CAMEL
HIPPOPOTAMUS
PIG
LESSER RORQUAL
HORSE
RAT
MOUSE
HAMSTER
MUSKRAT
PORCUPINE
CAPYBARA
GUINAE PIG A
GUINAE PIG B
CUIS
CHINCHILLA
CASIRAGUA
COYPU
MAN
TWO-TOED SLOTH
THREE-TOED SLOTH
RED KANGAROO
WALLABY
SNAPPING TURTLE

Figure 1.3 Mechanism of the reactions catalyzed by RNase A. B is His 12, A is His 119, and py is a pyrimidine base.

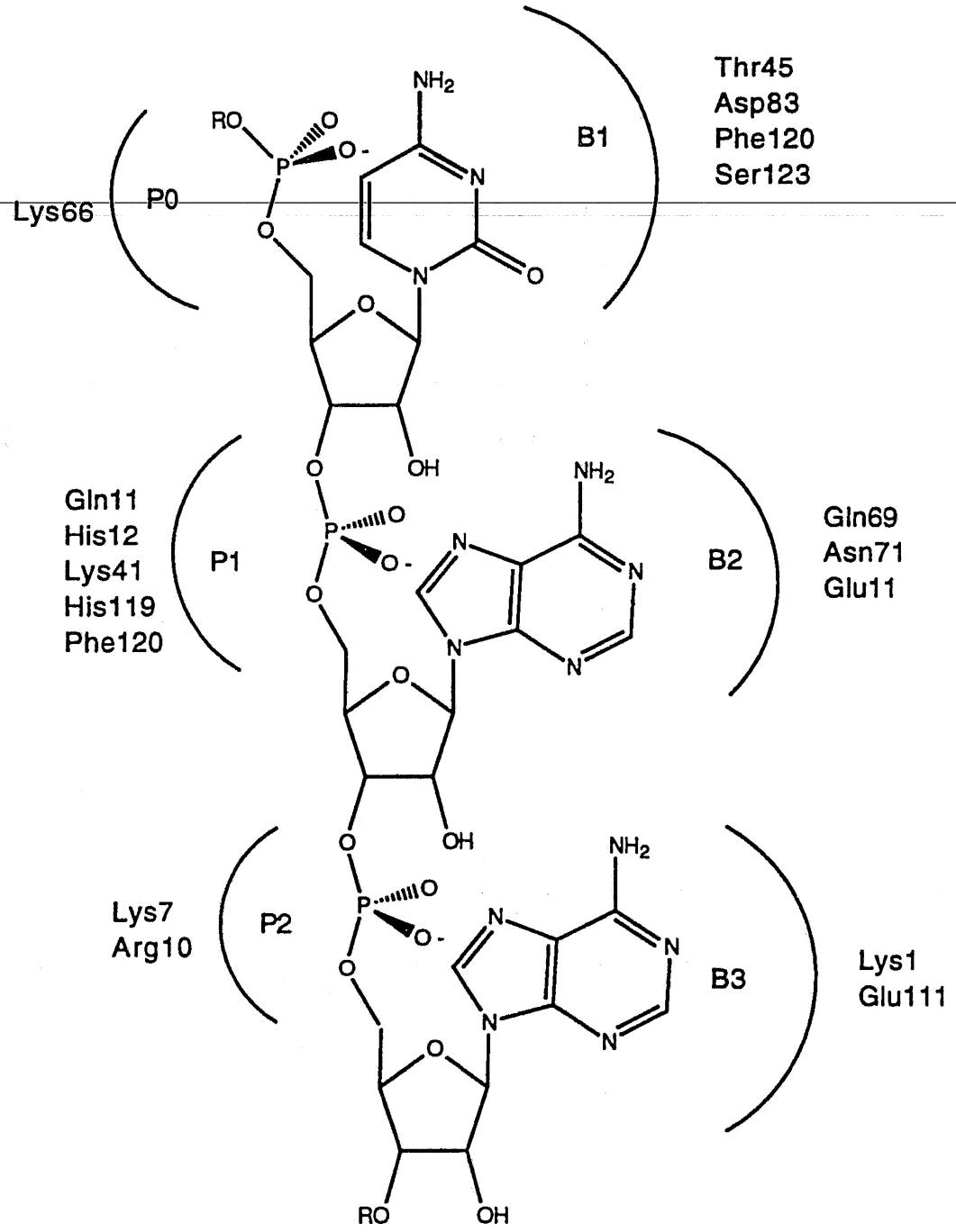


Transphosphorylation



Hydrolysis

Figure 1.4 Interaction of RNA with the subsites of RNase A. The residues proposed to form each subsite are shown.


Thr45
Asp83
Phe120
Ser123
B1
Lys66
P0
Gln11
His12
Lys41
His119
Phe120
P1
Gln69
Asn71
Glu11
B2
Lys7
Arg10
P2
Lys1
Glu111
B3
NH2
RO
OH
N
O

Chapter 2

Expression and Purification of Recombinant RNase A

2.1 Introduction

Ribonuclease A (RNase A) is perhaps the most studied of all enzymes. Yet, the application of the techniques of protein engineering to RNase A has been limited by problems inherent in the isolation and heterologous expression of its gene. Two obstacles have been encountered: (1) cloning of its cDNA—because the RNA that codes for RNase A must be obtained from the pancreas, an organ rich in ribonuclease; and (2) heterologous expression of the recombinant enzyme—because its folding requires the proper formation of four disulfide bonds and unfolded RNase A is susceptible to proteolysis, and because the catalytic activity of ribonucleases can be cytotoxic (Youle et al., 1993). Regardless, several bacterial systems for the expression of synthetic RNase A genes have been developed (McGeehan and Benner, 1989; Schein et al., 1992; Schultz and Baldwin, 1992; Tarragona-Fiol et al., 1992; Laity et al., 1993) and a few studies of RNase A mutants have been reported (Allemann et al., 1991; Trautwein et al., 1991; Schultz et al., 1992; Laity et al., 1993d). Unfortunately, these existing expression systems suffer either from a low isolated yield (making structural studies problematic), or from the presence of a modified N-terminus (making comparisons to the unmodified enzyme unreliable), or both.

The cDNA that codes for RNase A was first reported in 1989 by R.T. Raines (Raines & Rutter, 1989). In order to study RNase A using the techniques of protein engineering an expression system that would make the RNase A cDNA accessible to recombinant DNA manipulations and that would provide means to a high isolated yield of native (unmodified N-terminus) enzyme was necessary. To this end two expression systems were constructed and tested. The first was a novel *Saccharomyces cerevisiae* expression system of our own design and the second was the popular *Escherichia coli*

pET expression system (Studier et al., 1990). The yeast system secretes active ribonuclease to the medium with a low isolated yield of 1 mg per liter of culture. This yeast isolate contains native RNase A along with a mixture of glycosylated and nonmatured forms. The bacterial system produces RNase A in an insoluble form. Oxidation of the reduced and denatured protein produces active enzyme, which can be isolated at 50 mg per L of culture. The kinetic constants obtained for the bacterial isolate are similar to those of RNase A isolated from bovine pancreas, however, those for the yeast isolate vary from these presumably due to its extensive glycosylation.

The Raines laboratory is interested in applying the techniques of protein engineering to study the molecular basis for the substrate recognition and turnover and for the folding and stability of RNase A. The yeast expression system is not useful for this purpose. The genetic manipulations are not easy (due to the large size of the expression plasmid), the protein recovery is low, and isolated protein is heterogeneous. The bacterial expression system, however, is well-suited. The genetic manipulations are easy, protein recovery is the highest available for any recombinant RNase A reported, and the isolated protein is greater than 95% homogeneous. Thus, the bacterial expression system is ideal for the construction and the large-scale production of RNase A mutants.

2.2 Results

***S. cerevisiae* expression cassettes for RNase A cDNA.** The cDNA that codes for RNase A (Figure 2.1) along with two expression cassettes, which direct the expression of the RNase A cDNA when carried by a yeast plasmid and propagated in the yeast *S. cerevisiae*, were previously constructed (Raines & Rutter, 1989). The two cassettes

differ only in the promoter used and the length of the 3'-untranslated region of the RNase A cDNA. The expression of the RNase A cDNA from one cassette is under the transcriptional control of the PHO5 promoter, which is derepressed under conditions of low inorganic phosphate (Hinnen et al., 1989; Schneider & Guarente, 1991). Expression from the other cassette is under the transcriptional control of the hybrid ADH2-GAPDH promoter, which is derepressed by depletion of fermentable carbon sources (Cousens et al., 1987). The PHO5 cassette contains 282 bp of the 3'-untranslated region of the RNase cDNA; the ADH2–GAPDH cassette contains only the 3 bp amber codon of this region. Both cassettes have the RNase A cDNA fused to a gene encoding a modified α-factor leader peptide, a peptide which directs the secretion of mature RNase A to the culture medium. The PHO5 and ADH2-GAPDH cassettes are diagrammed in Figure 2.2.

***S. cerevisiae* expression vector pWL.** Plasmid pWL (where W and L refer to tryptophan and leucine) is a shuttle plasmid designed to facilitate the genetic manipulations required in protein engineering. The plasmid carries regions sufficient for replication (ori) and packaging as a single-stranded phagemid (f1) in *E. coli*, and for replication in [cir$^+$] *S. cerevisiae* (2μ). *E. coli* transformed with pWL can be selected by ampicillin resistance (Amp^r). *S. cerevisiae* transformed with pWL can be selected by recovery of tryptophan (TRP1) or leucine (LEU2-d) prototrophy. The presence of these two *S. cerevisiae* genes allows for both ease of transformation (because a single copy of the TRP1 gene is sufficient to confer tryptophan prototrophy) and high-copy number (because many copies of the 5'-truncated LEU2-d gene are necessary to confer leucine prototrophy)(Erhart & Hollenberg, 1983). A unique *Bam*HI site located between the

Amp^r and TRP1 regions is a convenient site for the insertion of cassettes directing the expression of a gene of interest.

The plasmids YEpWL.RNase A and YEpWL.Pi.RNase A (where YE refers to Yeast Expression and Pi refers to inorganic Phosphate) are simply pWL carrying either the ADH2–GAPDH or the PHO5 *Bam*HI expression cassettes, described above. Yeast harboring either YEpWL.RNase A or YEpWL.Pi.RNase A produce RNase A fused to a modified α-factor leader sequence. Filamentous phage carrying single-stranded copies of either of the two expression plasmids serve as a source of single-stranded DNA useful for oligonucleotide-mediated site-directed mutagenesis and DNA sequencing.

***E. coli* expression plasmid pBXR.** Plasmid pBXR (where BXR refers to Bacterial eXpression of RNase A)(Figure 2.3) was constructed from the cDNA that codes for RNase A and plasmid pET22B(+) (Novagen, Madison, WI) (where E stands for Expression and T stands for T7 RNA polymerase)(Studier et al., 1990). The cDNA for RNase A is fused to the gene that codes for the pelB signal peptide, a peptide which directs the secretion of mature RNase A to the periplasm of *E coli* cells. Expression of the pelB:RNase A fusion protein is mediated by the T7 RNA polymerase promoter and is under the transcriptional control of the lac operator. pBXR carries regions sufficient for replication (ori), packaging as a single-stranded phagemid (f1), and selection by ampicillin resistance (Amp^r) in *E. coli*. In addition pBXR contains the gene for lacI, a protein which represses the transcription of both the gene for T7 RNA polymerase, located in the chromosome of *E. coli* cells carrying the DE3 lysogen (Studier et al., 1990), and the cDNA for RNase A. *E. coli* cells harboring the DE3 lysogen and plasmid pBXR are induced to overexpress RNase A upon addition of IPTG.

Construction of plasmids pWL, YEpWL.RNase A, YEpWL.Pi.RNase A, and pBXR. The *S. cerevisiae* and *E coli* expression plasmids were constructed as in Chapter 6. Maps of plasmids YEpWL.RNase A and YEpWL.Pi.RNase A are shown in Figure 2.2 and a map of plasmid pBXR is shown in Figure 2.3. The genes carried by each of these plasmids were tested for function. All four plasmids were propagated in *E. coli* and conferred ampicillin resistance to transformed cells. Single-stranded DNA was produced by *E. coli* strain CJ236 carrying any of the four plasmids and infected with M13K07 helper phage. Plasmids pWL, YEpWL.RNase A, and YEpWL.Pi.RNase A were also propagated in *S. cerevisiae* and allowed transformed cells to recover tryptophan and leucine prototrophy. *S. cerevisiae* cells carrying YEpWL.RNase A but not those carrying pWL tested positive in the RNase plate assay under low glucose conditions. Similarly, cells harboring YEpWL.Pi.RNase A but not pWL tested positive in the RNase plate assay under low phosphate conditions. *E. coli* BL21(DE3) cells carrying pBXR but not those carrying pET22B(+) tested positive in the RNase plate assay when IPTG was present in the medium. Hence, each gene carried by pWL, YEpWL.RNase A, YEpWL.Pi.RNase A, and pBXR were functioning properly.

Expression and purification of RNase A from *S. cerevisiae*. Plasmid YEpWL.RNase A was designed to direct yeast cells to secrete RNase A to the culture medium. Culture medium from BJ2168 yeast cells harboring YEpWL.RNase A and grown under inducing conditions indeed contained considerable RNase activity. Yeast cells carrying pWL and exposed to the same conditions produced no detectable RNase activity. SDS-PAGE and zymogram electrophoresis demonstrate that the secreted

RNase was in a variety of forms having diverse electrophoretic mobilities (Figure 2.4). This spectrum of RNase species likely arose from disparate post-translational modification of the α-factor~RNase A fusion protein during the secretory process. Cation exchange chromatography allowed the separation of the RNase species into two fractions roughly distinguished by the electrophoretic mobility of the species contained in each. These two fractions were termed RNase A(H) or RNase A(L) (where H refers to high mol wt, and L refers to low mol wt). These fractions were characterized kinetically (Table 2.1) and assayed for sugar content (Figure 2.4).

Glycosylation analysis of recombinant RNase isolated from *S. cerevisiae*. RNase A contains the N-glycosylation sequence (Asn34 - Leu35 -Thr36) and is produced from bovine pancreas in an assortment of glycosylated forms. These various glycosylated forms can be distinguished by their difference in electrophoretic mobility (Ribó et al., 1991; Rudd et al., 1994). RNase from the culture medium of *S. cerevisiae* strain BJ2168 harboring plasmid YEpWL.RNase A was isolated as two fractions, RNase A(L) and RNase A(H), which differed predominantly in their relative electrophoretic mobility. Each fraction contained RNase species that ran at relative molecular weights higher than that expected for RNase A. To discern whether the apparent increase in M_r was due to post-translational glycosylation, these samples were analyzed for the presence of carbohydrate with glycogram electrophoresis. Glycogram electrophoresis shows that the very high but not the high M_r species in RNase A(H) contained carbohydrate, and that only the highest M_r species in RNase A(L) were glycosylated. This result is consistent with an observed decrease in the amount of very high M_r species, but not in that of high and low M_r species, when tunicamycin was added to the growth medium (data not shown). Treatment of RNase A(H) with either O-glycosidase

(which removes O-linked carbohydrate), Endo-F (which removes N-linked carbohydrate), or both, resulted in a decrease in M_r of many of the high M_r species (Figure 2.4). The products from the enzymatic removal of carbohydrate, however, still contained RNase species of a M_r higher than that of RNase A. These data suggest that RNase produced in *S. cerevisiae* is processed inefficiently at its leader sequence, is glycosylated extensively with *N*-linked carbohydrates, and is glycosylated mildly with *O*-linked carbohydrates.

Isolation of RNase A from *E. coli*. Plasmid pBXR was designed to direct the secretion of RNase A to the periplasm of *E. coli* strains containing the DE3 lysogen (Studier & Moffatt, 1986). The majority of the RNase A produced by strain BL21(DE3) carrying pBXR was not released from the periplasm upon cold osmotic shock, nor was it found in the soluble fraction of the cell lysate. Rather, RNase A, in its completely processed form (proteolytically removed from the pelB signal sequence) was produced in an insoluble form. While the partitioning of RNase A to the insoluble fraction of the cell lysate was unexpected, it proved to facilitate purification of the enzyme immeasurably.

Native RNase A is an extremely soluble protein. Methods for the efficient folding/oxidation of denatured/reduced RNase A are well-established. Our isolation strategy was therefore to solubilize RNase A selectively under denaturing conditions, and then to fold and oxidize soluble protein under conditions that were optimized for RNase A (McGeehan & Benner, 1989). The majority of the soluble protein was removed from the induced cells by the cell lysis buffer, which contained a high concentration of urea. RNase A remained in the insoluble fraction along with other insoluble cellular constituents. RNase A was then solubilized from this fraction by the

addition of solubilization buffer, which contained high concentrations of urea, NaCl, and DTT. Removal of urea from the solubilized fraction by dialysis against an acidic buffer left RNase A soluble, but not reoxidized. In contrast, most of the other proteins precipitated during dialysis. Oxidation of the soluble dialysate with a glutathione redox buffer resulted in a greater than 10^3-fold increase in the ribonuclease activity of this fraction. All of this enzymatic activity flowed through an anion exchange column. The activity did bind, however, to a cation exchange column, and RNase A of purity >95% eluted from the cation exchange column when the NaCl concentration was 0.15 – 0.20 M. Fractions from the purification of RNase A from *E. coli* are shown in Figure 2.5. The amino acid sequence of the 14 *N*-terminal residues of the fraction from the cation exchange column (Figure 2.5, lane 7) was identical to that of the enzyme isolated from bovine pancreas.

Comparison of kinetic parameters for UpA hydrolysis by RNase A from different sources. The kinetic constants for the four isolates of RNase A [*E. coli*, *S. cerevisiae*(2), and bovine pancreas] were determined by using an adenosine deaminase coupled assay. In this assay, the conversion of UpA to U>p and inosine is monitored. Previously reported values for the K_m of this reaction had varied from 0.3 mM to 1.8 mM and were measured between pH 5 and 7 (Witzel & Barnard, 1962; Shapiro et al., 1986; Trautwein et al., 1991). The assays described here were performed at pH 6.0, where k_{cat}/K_m appears to be maximal (Richards & Wyckoff, 1971; Shapiro et al., 1986). Although this assay has been used since 1968, no explicit reference to the value of $\Delta\varepsilon_{265}$ for the conversion of UpA to U>p and inosine could be located. We determined this value to be -6.0×10^3 O.D. $M^{-1} cm^{-1}$ in 0.1 M Mes•HCl buffer, pH

6.0, containing NaCl (0.1 M). The kinetic constants for the four isolates of RNase A are shown in Table 2.1.

2.3 Discussion

Expression and purification of RNase A. RNase A has been a challenging enzyme to produce heterologously. This difficulty has arisen from the evasiveness of its cDNA to cloning, its susceptibility to proteolysis when unfolded, and its cytotoxicity when folded. Advances in the technologies of cDNA cloning, heterologous gene expression, and protein renaturation have assisted in overcoming these obstacles and have led to the development of several expression systems for RNase A. The first such system was reported by Benner and coworkers, who synthesized a gene for RNase A and expressed this gene in *E. coli* to produce a fusion protein with β-galactosidase (Nambiar et al., 1987). Purifying RNase A from this system was laborious and inefficient, and these workers later reported an improvement, in which the fusion to β-galactosidase was eliminated (McGeehan & Benner, 1989). The new system produced RNase A with an N-formyl methionine residue. Both of these systems involved denaturation of RNase A followed by its facilitated renaturation in the presence of a reducing agent. Most recently, Schein and coworkers expressed the same synthetic gene fused to a murine signal sequence. The RNase A produced by this system was secreted to the periplasm and was recovered as mature RNase A (Schein et al., 1992). This system had the highest yield described to date, allowing about 5 mg of soluble RNase A and 5 mg of insoluble RNase A to be recovered from each L of fermenter culture.

Other, less effective systems for the heterologous production of RNase A have also been reported. A group at Genex expressed a synthetic gene for RNase A in *Bacillus subtillus*, and isolated small amounts of an N-formyl methionyl RNase A from the culture medium (Vasantha & Fipula, 1989). Recently, Schultz and Baldwin expressed the same synthetic gene in *E. coli*, and recovered small amounts of N-methionyl RNase A from the soluble fraction of the cell lysate (Schultz & Baldwin, 1992). The pINIII system (Masui et al., 1984) that had worked well for the production of RNase T1 (Quaas et al., 1988), failed in the expression of the cDNA for rat pancreatic RNase in *E. coli*, and Miranda had to resort to an inefficient human cell culture expression system (Miranda, 1990). Rabin and coworkers have expressed RNase A in *E. coli*, isolating <0.5 mg of enzyme from the periplasmic fraction of a 1-liter culture (Tarragona-Fiol et al., 1992). Most recently, Scheraga and coworkers expressed the synthetic gene from Genex as a fusion protein with the T7 gene10-II protein. After *in vitro* processing with factor X_a, this group isolated 4 mg of RNase A per liter of culture (Laity et al., 1993).

Here are described two expression systems that direct the expression of the cDNA that codes for RNase A in *S. cerevisiae* and *E. coli*, and the purification of the recombinant enzyme from each organism. The first, YEpWL, is a novel yeast expression plasmid originally designed to facilitate the genetic manipulations required in protein engineering. This system was also designed to facilitate RNase purification by using an α-factor leader peptide to direct the secretion of RNase A to the culture medium. It is the secretory process that is the downfall of this expression system. During the secretory process the RNase A-α-factor hybrid undergoes numerous modifications. Upon translocation into the ER, the signal sequence of the α-factor leader peptide is proteolytically removed by signal peptidase, the polypeptide folds, and

its cysteines are oxidized to disulfides. Then, in the E.R. and golgi, the glycosylation machinery covalently modifies the hybrid enzyme by forming and breaking bonds to a variety of sugar moieties. Finally, upon secretion, the remainder of the α-factor leader peptide is proteolytically removed. Matured and glycosylated RNase is released to the medium. Ribonuclease isolated from the culture medium of yeast cells harboring YEpWL.RNase A is a mixture of RNase species which differ in their extent of post-translational modification. Like RNase secreted from bovine pancreas, RNase secreted from yeast is a mixture of different glycoforms. These glycoforms result from a consensus N-linked glycosylation site at Asn 34 and O-linked sugars on serine residues. This mixture is recoverable at up to 1 mg/L of yeast culture medium and could be separated into two fractions that differ primarily in the extent of glycosylation. Conventional chromatographic procedures, however, failed to separate native RNase A from the glycosylated or unprocessed forms. Thus, the YEpWL expression system is not optimal for producing the homogeneous protein necessary for rigorous chemical or kinetic analysis.

The *S. cerevisiae* expression system is useful for studying cellular biochemistry. For example, coupling expression by YEpWL.RNase A with zymogram electrophoresis allowed us to ascertain readily the effect of tunicamycin on protein glycosylation (data not shown). Also, plasmid pWL and the ribonuclease plate assay has been used to verify that the *RNS2* gene product of *Arabidopsis thaliana* is indeed a ribonuclease (Taylor et al., 1993). In addition, studies that use altered glycosylation patterns as an indicator of the sub-cellular location or secretory history of a protein may benefit from this system.

Plasmid pBXR, derived from plasmid pET (Studier et al., 1990), directs the production of RNase A in *E. coli* (Figure 2.3). BL21(DE3) cells harboring pBXR

produce RNase A in an insoluble form that is recoverable at 50 mg per L of culture. This isolate was >95% pure. Although this expression system is designed to secrete RNase A into the periplasm as a soluble protein, the RNase A produced is insoluble and not recoverable from the periplasm by cold osmotic shock. Interestingly, the insoluble RNase A was mature—it did not contain the pelB signal sequence. This result is unusual because maturation occurs in the periplasm but inclusion bodies usually form in the cytoplasm. This result can be explained if partially folded RNase A aggregated in the periplasm (Mitraki & King, 1989), or if the translocation of RNase A was arrested after signal peptide cleavage. Such inefficient translocation has been observed for lysozyme, which like RNase A has several basic amino acid residues near its N-terminus (Yamane & Mizushima, 1988).

The insolubility of RNase A produced in *E. coli* was useful not only in overcoming the problem of its toxicity but also in facilitating its purification. Whereas others have succeeded in isolating 5 mg of soluble RNase A per L of *E. coli* fermenter culture (Schein et al., 1992), we have isolated RNase A at a 10-fold greater level by using the T7 RNA polymerase promoter and isolating enzyme from the insoluble fraction. This increase in recovery makes studies that require large quantities of protein, such as NMR spectroscopy (Stockman & Markley, 1990) and X-ray diffraction analysis, accessible without the need for fermenter growth. The combination of the T7 RNA polymerase promoter and the murine signal sequence (Schein et al., 1992) could increase the production of RNase A in *E. coli* even further.

Comparison of kinetic parameters for recombinant RNase A. Reported values for the kinetic parameters for cleavage of UpA by RNase A vary greatly, as expected in the absence of a standard value for $\Delta\varepsilon_{265}$. By comparison, the difference in the kinetic

parameters for the enzyme from *E. coli* and from bovine pancreas observed here (Table 2) and elsewhere (Trautwein et al., 1991) is small. This small difference may result from disparate modifications incurred during the various protocols for purification and storage. The k_{cat}/K_m values for the cleavage of UpA are close to that expected for a reaction that is limited by diffusion (Hammes & Schimmel, 1970).

The kinetic parameters for the 2 RNase A fractions isolated from *S. cerevisiae* vary from those of the *E. coli* or bovine pancreas isolates (Table 2.1). Both RNase A(L) and RNase A(H) had an approximately 5-fold decrease in k_{cat} for UpA cleavage. RNase A(L) also had a 3-fold increase in K_m, These changes may arise from an increase in steric crowding and a decrease in dynamic flexibility, as has been observed recently for RNase B (Opdenakker et al., 1994; Rudd et al., 1994). RNase A(H), despite its extensive glycosylation, had a K_m indistinguishable from that of RNase A. RNase A is a basic protein (pI = 9.3) and RNA is an acidic substrate. This reversal of the elevated K_m of RNase A(L) by increased glycosylation may therefore be the result of a decrease in the dielectric constant in the vicinity of the protein imposed by the large layer of carbohydrate (Beintema, 1987).

2.4 Conclusions

The effective study of a protein using the techniques of protein engineering requires several things (Chapter 1): 1) a gene encoding the protein that is accessible to the manipulations of recombinant DNA technology, 2) an expression system that provides sufficient wild-type and mutant protein for both structural and functional characterization, and 3) some means of assessing the functional results of a structural alteration. It is additionally useful to know the three-dimensional structure of the protein

in question or that of a close homolog. RNase A is ideally suited for study by protein engineering as all of the above criteria are now fulfilled.

Table 2.1 Steady-state kinetic parameters for the cleavage of UpA by ribonuclease A isolated from *E. coli*, *S. cerevisiae*, and bovine pancreas.

source	k_{cat} [s^{-1}]	K_m [mM]	k_{cat}/K_m x 10^{-6} [$M^{-1}s^{-1}$]
E. coli	1440 ± 150	0.62 ± 0.09	2.3 ± 0.4
S. cerevisiae			
low Glycosylation	280 ± 140	2 ± 1	0.2 ± 0.1
high Glycosylation	400 ± 70	0.3 ± 0.1	1.4 ± 0.6
bovine pancreas	1920 ± 130	0.33 ± 0.05	5.8 ± 1.0

Figure 2.1 Nucleotide sequence of the cDNA that codes for RNase A, and its translation. Endogenous recognition sites for *Pst*I, *Cla*I, and *Hin*cII; *Eco*R1 linkers used during construction of the cDNA library (Raines & Rutter, 1989); and Asn 34 of the consensus glycosylation site are underlined.

ProSerLeuGly$_{-1}$
GGAATTCCGCCTTCCCTGGGC

LysGluThrAlaAlaAlaLysPheGluArgGlnHisMetAspSerSerThrSerAlaAla$_{20}$
AAGGAAACTGCAGCAGCCAAGTTTGAGCGGCAGCACATGGACTCCAGCACTTCCGCTGCC

SerSerSerAsnTyrCysAsnGlnMetMetLysSerArgAsnLeuThrLysAspArgCys$_{40}$
AGCAGCTCCAACTACTGTAACCAGATGATGAAGAGCCGGAACCTGACCAAAGATCGATGC

LysProValAsnThrPheValHisGluSerLeuAlaAspValGlnAlaValCysSerGln$_{60}$
AAGCCAGTGAACACCTTTGTGCACGAGTCCCTGGCTGATGTCCAGGCCGTGTGCTCCCAG

LysAsnValAlaCysLysAsnGlyGlnThrAsnCysTyrGlnSerTyrSerThrMetSer$_{80}$
AAAAATGTTGCCTGCAAGAATGGGCAGACCAATTGCTACCAGAGCTACTCCACCATGAGC

IleThrAspCysArgGluThrGlySerSerLysTyrProAsnCysAlaTyrLysThrThr$_{100}$
ATCACCGACTGCCGTGAGACCGGCAGCTCCAAGTACCCCAACTGTGCCTACAAGACCACC

GlnAlaAsnLysHisIleIleValAlaCysGluGlyAsnProTyrValProValHisPhe$_{120}$
CAGGCGAATAAACACATCATTGTGGCTTGTGAGGGAAACCCGTACGTGCCAGTCCACTTT

AspAlaSerVal AM
GATGCTTCAGTGTAGGTCTCTACCTAAGGCCAGAGCAGCAAGATGCACCACTTCATCACA
AAGGCACCTGCCTCTCCCCTCATGTTTCCTTGTCCTGGGGGCAATAGCTCAAGTTAGTTA
GGGCTCTTATCTCTGCGCACCTTACCAGAAACACACACACAGGATTCCCTGGCATGAAAG
CAATAACTCAAGCTAGTTAAGTCTTCTATCCAACCCACACTTGCTCCCCTGGCCTGAGTC
TTGCCCCTGGTGGTTTGGGGGGTGAGGAGTGGGTTGTGAGGTGGGACCTGTGTTAACCAA
ATCACTGCTTCTTTCAATAAACATACTTGCAACCACCTGAAAAAAAAAAAAAAAAAAAAA
AAGAAAAAAAAAAAAAAAAGGAATTCC

Figure 2.2 Map of plasmids YEpWL.RNase A and YEpWL.Pi.RNase A. Plasmid pWL is a yeast shuttle plasmid designed to facilitate the genetic manipulations required in protein engineering. YEpWL.RNase A is plasmid pWL containing a cassette that directs the expression of RNase A under the control of the ADH2–GAPDH promoter. YEpWL.Pi.RNase A is pWL containing a cassette that directs the expression of RNase A under the control of the PHO5 promoter. *S. cerevisiae* harboring either of these plasmids and grown under conditions that derepress expression produce RNase A fused to a modified α-factor leader sequence (dashed arrow).

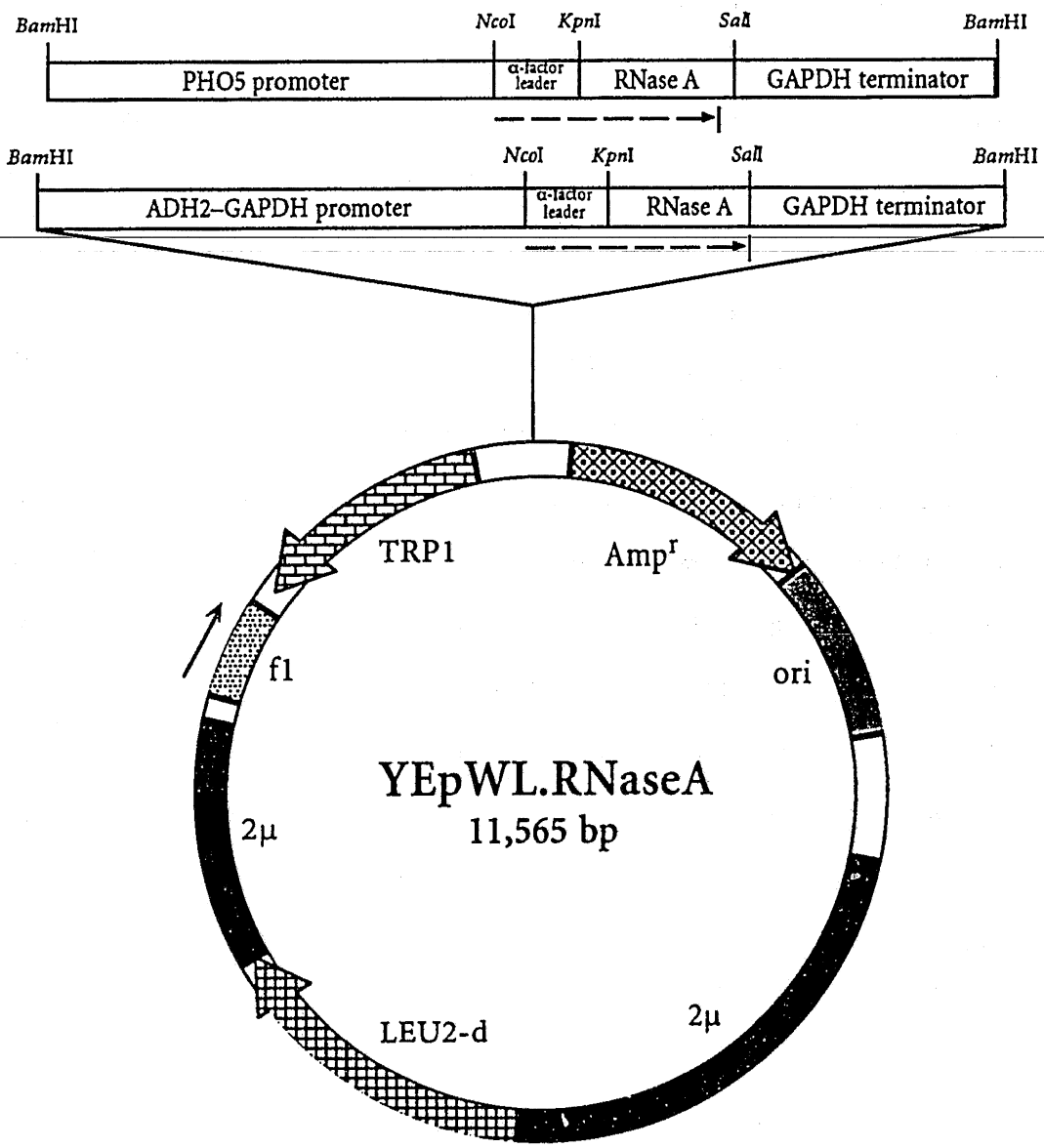

BamHI
NcoI
KpnI
SalI
BamHI
PHO5 promoter
α-factor leader
RNase A
GAPDH terminator
BamHI
NcoI
KpnI
SalI
BamHI
ADH2–GAPDH promoter
α-factor leader
RNase A
GAPDH terminator
TRP1
Amp^r
f1
ori
YEpWL.RNaseA
11,565 bp
2μ
LEU2-d
2μ

Figure 2.3 Map of plasmid pBXR. *E. coli* strain BL21(DE3) harboring pBXR and grown under conditions that derepress expression produces RNase A fused to the pelB signal sequence (dashed arrow).

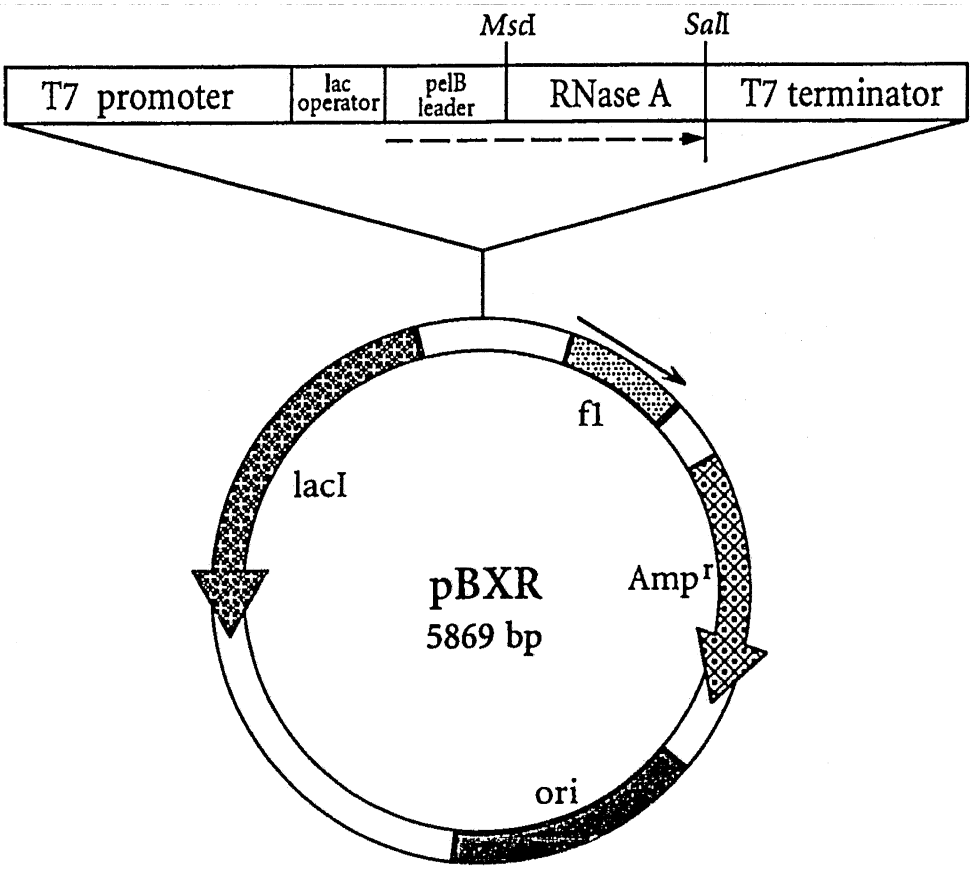

MscI
SalI
T7 promoter
lac operator
pelB leader
RNase A
T7 terminator
f1
lacI
pBXR
5869 bp
Amp r
ori

Figure 2.4 (**A**) Zymogram electrophoresis of purification fractions of RNase A produced by *S. cerevisiae*: RNase A (lane 1), acetone precipitate of concentrated culture media (lane 2), RNase A(H) (lane 3), and RNase A(L) (lane 4). (**B**) Analysis of N and O-linked carbohydrate by zymogram electrophoresis: RNase A (lane 1), mol wt standards (lane 2), RNase A(H) (lane 3), RNase A(H) after treatment with Endo-F (which removes N-linked carbohydrate)(lane 4), *O*-glycosidase (which removes O-linked carbohydrate)(lane 5), or both (lane 6). Enzymatically treated samples have a decreased average M_r, suggesting the presence both N- and O-linked carbohydrate.

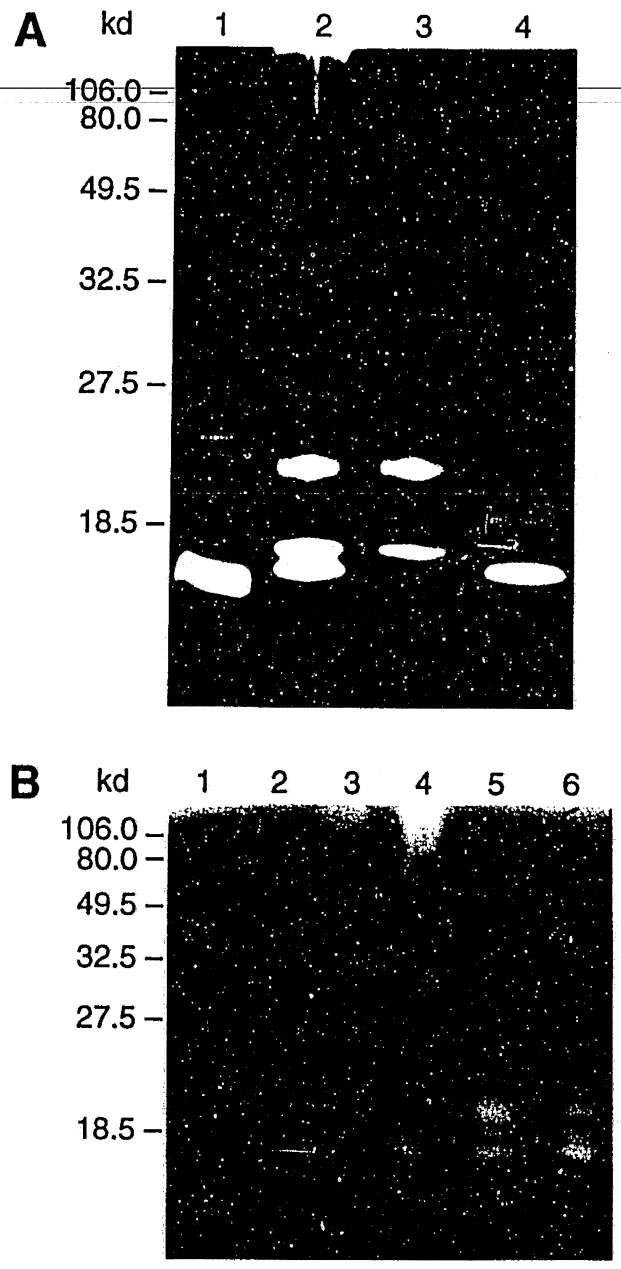
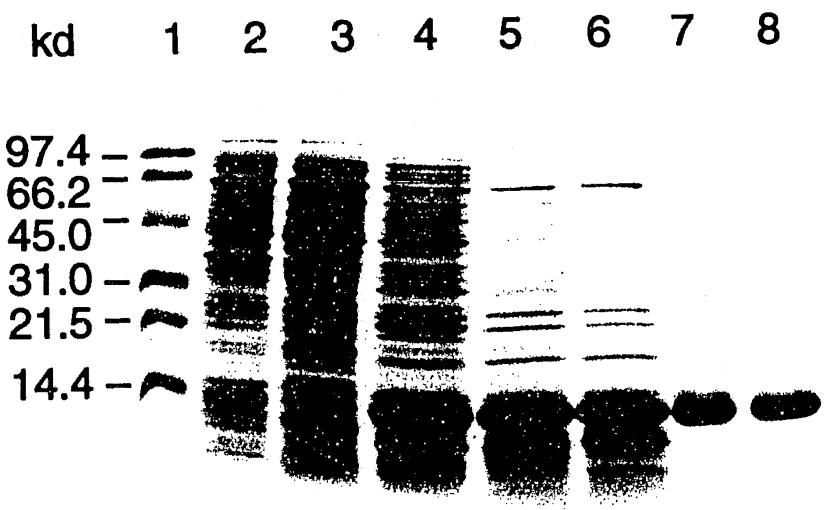

A
kd
1
2
3
4
106.0
80.0
49.5
32.5
27.5
18.5
B
kd
1
2
3
4
5
6
106.0
80.0
49.5
32.5
27.5
18.5

Figure 2.5. (A) SDS-PAGE of fractions from the purification of RNase A produced by *E. coli*: mol wt standards (lane 1), whole cell lysate of uninduced cells (lane 2), whole cell lysate of induced cells (lane 3), after extraction with solubilization buffer (lane 4), dialysate of acid dialysis (lane 5), dialysate of refolding dialysis (lane 6), pooled fractions from cation exchange chromatography (lane 7), and RNase A (lane 8).


kd
1
2
3
4
5
6
7
8
97.4
66.2
45.0
31.0
21.5
14.4

Chapter 3

The Interdependence of Specificity and Efficiency in Catalysis by RNase A

3.1 Introduction

Catalysis by enzymes is characterized by exquisite specificity and extreme efficiency. Yet, the interdependence of these two aspects of catalysis is unclear (Knowles, 1987; Benner, 1989). Enzymes are complementary in structure to the substrates and intermediates of the reactions that they catalyze, and use the free energy gained from substrate binding to effect this catalysis (Jencks, 1987). Understanding the molecular determinants of substrate specificity is thus central to understanding the means by which enzymes achieve their catalytic success.

High-resolution structures determined by X-ray diffraction analysis and NMR spectroscopy have provided detailed pictures of the noncovalent interactions between enzymes and their ligands. The resulting insights have allowed protein engineers to determine the functional role of many enzymic residues. Still, a primary goal of protein engineering remains the creation of new catalysts by the rational manipulation of noncovalent interactions. Many efforts have focused on the changing of an enzyme's substrate specificity, such that the new enzyme catalyzes the same chemical conversion (for example, proteolysis) but with a different preference for substrate (for example, cleavage of the peptide bond of an acidic rather than a basic amino acid residue). Such an alteration requires adjusting interactions that mediate substrate binding but not those critical to substrate turnover, protein structure, or protein stability. Impressive results have been obtained in the redesign of several proteases (Craik et al., 1985; Estell et al., 1986; Wells et al., 1987; Bone et al., 1989; Rheinnecker et al., 1993) and some dehydrogenases (Wilks et al., 1988; Scrutton et al., 1990). For many enzymes, however, binding and turnover are coupled irrevocably, as the same amino acid residue

mediates both. Thus, mutant enzymes of altered specificity are often relatively inefficient catalysts.

Bovine pancreatic ribonuclease A (RNase A; EC 3.1.27.5) is a small protein (14 kDa) that has been an exemplar for studies in all aspects of protein chemistry and enzymology (Chapter 1)(Richards & Wyckoff, 1971; Karpeisky & Yakovlev, 1981; Blackburn & Moore, 1982; Beintema, 1987; Eftink & Biltonen, 1987). The enzyme has also been of particular use in biotechnology (Zuckermann & Schultz, 1988; Kim & Raines, 1993). RNase A is a distributive endoribonuclease that binds the bases of adjacent RNA residues in three enzymic subsites: B1, B2, and B3 (Parés et al., 1991). Catalysis by RNase A results in the cleavage of the P–$O_{5'}$ bond specifically on the 3'–side of pyrimidine nucleotides that are bound in the B1 subsite (Fig. 1.3). The B1 subsite apparently binds only residues having a pyrimidine base (McPherson et al., 1986; Aguilar et al., 1992) and demonstrates an approximately 50-fold preference for a cytosine base. The B2 and B3 subsites bind all residues but with a preference for those having a purine base (Richards & Wyckoff, 1971).

Structural (Wlodawer & Lennart, 1983; Santoro et al., 1993) and phylogenetic (Beintema, 1987) data suggest that the specificity of the B1 subsite is mediated by the sidechains of Thr45 and Phe120. The structure of the B1 subsite is shown in figure 3.1. The hydroxyethyl sidechain of Thr45 forms hydrogen bonds with a pyrimidine base (U or C; Fig. 2)($O_{\gamma 1}$ – N_3 distance = 2.73 Å, $O_{\gamma 1}$ – H – N_3 angle = 147°; N – O_2 distance = 2.65 Å, N – H – O_2 angle = 147°), and excludes sterically the purine bases (A, G, and I). The benzylic sidechain of Phe120 makes van der Waals contact with a pyrimidine base and with the sidechain of Thr45. Asp83 and Ser123 also participate in the B1 binding pocket. Asp83 accepts a hydrogen bond from the sidechain of Thr45

when a uracil base , but not a cytosine base, is bound to the B1 subsite (Wlodawer et al., 1983), and may mediate cytidine:uridine specificity.

RNase A is now suited for study by the techniques of protein engineering (Chapter 2). The molecular determinants of this enzyme's substrate specificity can be investigated, and alterations of this specificity attempted. To determine whether Thr45 and Phe120 were responsible for the pyrimidine specificity of RNase A, codon-45 and codon-120 were subjected to saturation mutagenesis, and cells expressing the resulting mutant libraries were screened for the production of mutants that cleave RNA after purine residues. Mutants T45A and T45G, but no mutant at position-120, demonstrated $10^4 - 10^5$-fold increase in purine:pyrimidine specificity with little compromise to catalytic efficacy. Both T45A and T45G also maintained the preference for cytidine verses uridine demonstrated by wild-type RNase A. To determine whether the carboxyl sidechain of Asp83 was responsible for this inherent preference, mutants D83A and T45G/D83A were created and their substrate specificities were compared to the wild-type and T45G enzymes. Apparently, the sidechain of Asp83 has no effect on the rate of cleavage after cytidine residues, but does effect the cleavage of poly(U) and the hydrolysis of U>p through an interaction that is dependent on the sidechain of Thr45. In addition, the sidechain of Asp83 enhances the rate of cleavage of poly(A), but through an interaction that is not dependent on the sidechain of Thr45. These observations and their implications for the interdependence of substrate specificity and catalytic efficiency are discussed.

3.2 Results

Saturation mutagenesis of codon 45 and 120 of RNase A. The cDNA that codes for RNase A was carried by the vector pBXR, and was expressed in *E. coli* under the control of the T7 RNA polymerase promoter (Chapter 2). Site-directed mutagenesis of plasmid pBXR was used to introduce a unique and translationally silent *Nhe*I site into the cDNA for RNase A on the 3' side of the codon for Thr45, resulting in plasmid pBXR1. The codon for Thr45 was then randomized (changed from ACC to NN(G/C))by cassette-mediated saturation mutagenesis (Reidhaar-Olson et al., 1991) of the *Cla*I/*Nhe*I fragment of pBXR1, resulting in the library pBXR1.Thr45All. The codon for Phe120 was randomized similarly by cassette mutagenesis of the *Bsi*WI/*Sal*I of pBXR1, resulting in the library pBXR1.Phe120All. Individual clones randomly chosen from the Thr45All and Phe120All libraries were sequenced to discern the randomness of codons 45 and 120. These codons were considered to be random because G, A, T, and C were found in each of the first two positions and G and C were found in the third position of each of these codons. pBXR1.Thr45All and pBXR1.Phe120All were transformed into BL21 (DE3) *E.coli* cells and screened for the production of mutants of RNase A having altered substrate specificity.

Screen for mutants of altered specificity. A scheme depicting the methods used to identify mutants of RNase A that cleave poly(R) is shown in figure 3.2. Culture medium from *E. coli* BL21(DE3) cells expressing the cDNA that codes for Thr45All or Phe120All was first screened by zymogram electrophoresis (Blank et al., 1982; Ribó et al., 1991; Kim & Raines, 1993) for production of an enzyme able to cleave poly(A), poly(G), or poly(I). This initial screen determined whether any mutant, but not which

mutant, within the pools was capable of polypurine cleavage. Polypurine–cleaving activity was found only in the Thr45All pool, and this activity was for cleavage of poly(A) but not for cleavage of poly(G) or poly(I). A subsequent screen [by zymogram spot assay (chapter 6)] of 100 individual clones from the Thr45All pool identified 8 clones that produced an enzyme capable of cleaving poly(A) efficiently. Sequencing of plasmid DNA from these clones revealed that each active mutant had either an alanine or a glycine residue at position 45. Poly(A) zymogram electrophoresis of cells expressing the T45A and T45G enzymes further confirmed that the poly(A) cleaving activity was due to these mutants of RNase A (Figure 3.3). These two mutant enzymes were produced and purified to homogeneity using the procedures described in Chapters 2 and 6, and were characterized kinetically.

Construction of aspartate-83 mutants. Asp83 was replaced by an alanine residue by oligonucleotide-mediated site-directed mutagenesis of plasmid pBXR1 and pBXR1.T45G, and the resulting constructs were confirmed by sequencing. D83A and T45G/D83A RNase A were produced and purified to homogeneity using the procedures described in Chapters 2 and 6 and characterized kinetically.

Steady-state kinetics. Steady-state kinetic parameters for the cleavage of the homopolymers poly(A), poly(C), and poly(U), the dinucleotide UpA, and for the hydrolysis of U>p and C>p were determined for wild-type, T45A, T45G, D83A, and T45G/D83A RNase A. As the sidechain of residue 45 became smaller (Thr → Ala → Gly), the value of the specificity constant, k_{cat}/K_m (Fersht, 1985) for poly(A) cleavage became larger (Fig. 3.4). This increase was a result of both an increase in k_{cat} and a decrease in K_m (Table 3.1). The T45G mutant actually cleaved poly(A) more efficiently

than it cleaved poly(U) or poly(C), with $(k_{cat}/K_m)_{poly(A)}/(k_{cat}/K_m)_{poly(U)}$ = 62 ($9x10^4$–fold change in substrate specificity) and $(k_{cat}/K_m)_{poly(A)}/(k_{cat}/K_m)_{poly(C)}$ = 1.2 ($6x10^4$–fold change). This reversal of specificity likely results from a relaxation of the specificity of the B1 subsite and the inherent preference of the B2 and B3 subsites for adenine residues. The T45A mutant exhibited a 20– to 30–fold smaller change in substrate specificity than did the T45G mutant, with $(k_{cat}/K_m)_{poly(A)}/(k_{cat}/K_m)_{poly(U)}$ = 2.5 ($4x10^3$–fold change) and $(k_{cat}/K_m)_{poly(A)}/(k_{cat}/K_m)_{poly(C)}$ = 0.035 ($2x10^3$–fold change). Since the relative preference of the wild-type enzyme for poly(C) and poly(U) is maintained in the T45A and T45G mutants (Fig. 3.4), this preference must not be mediated by Thr45 in RNase A. [The role of the active-site Thr residue in mediating this preference in homologs of RNase A may be more pronounced (Miranda, 1990; Curran et al., 1993).] Although both mutant enzymes cleave polymeric substrates efficiently, both also have a markedly diminished ability to bind and turnover UpA (Table 3.1). This decrease is probably due to the loss of a large fraction of the total binding energy of the enzyme for this small substrate.

The kinetic consequences of the Asp83Ala mutations are considerably less dramatic than those of the Thr45 mutations (Table 3.2). The D83A enzyme showed a decrease in the k_{cat}/K_m for the cleavage of poly(U), poly(A) and the hydrolysis of U>p as compared to wild-type RNase A. This same decrease in k_{cat}/K_m was observed for the cleavage of poly(A) by T45G/D83A relative to the T45G enzyme, but was not observed for the cleavage of poly(U) . The D83A mutant cleaves poly(C) and hydrolyzes C>p with the same kinetic parameters as the wild-type enzyme. However, The T45G/D83A mutant cleaves poly(C) with a 20-fold increase in k_{cat}/K_m relative to the T45G enzyme (Fig. 3.5).

Thermodynamic cycles. Thermodynamic cycles were calculated from values of k_{cat}, K_m, and k_{cat}/K_m for the cleavage of poly(U) by wild-type, D83A, T45G, and T45G/D83A RNase A. The change in free energy ($\Delta\Delta G$) for each side of a thermodynamic box was calculated from the equation

$$\Delta\Delta G = RT\ln f$$

where f was the ratio of a particular kinetic parameter for the two enzymes at each corner of that side of the box under the assumption that the fequencies of formation and braekdown of the chemical transition state were the same for the reactions catalyzed by the wildtype and mutant enzymes. The change in free energy for the binding of the chemical transition state by RNase A was calculated from k_{cat}/K_m, for the apparent binding of the ground state from $1/K_m$ (assuming that $K_S = K_m$), and for the chemical activation energy from k_{cat}. The free energy of interaction ($\Delta\Delta G_{int}$)between Asp83 and Thr45 was

$$\Delta\Delta G_{int} = \Delta\Delta G_{wt \rightarrow T45G/D83A} - \Delta\Delta G_{wt \rightarrow T45G} - \Delta\Delta G_{wt \rightarrow D83A}$$

as described previously (Mildvan et al., 1992). Thermodynamic cycles for the cleavage of poly(U) by wild-type, D83A, T45G, and T45G/D83A RNase A are shown in Figure 3.5. Apparently, the sidechain of Aps83 contributes 1.53 kcal/mol to the binding of the transition state to RNase A. This free energy is divided roughly equally between the binding of the ground state and the chemical transition state. The sidechain of Thr45 contributes 2.73 kcal/mol to the binding of the transition state to RNase A. Only 0.73 kcal/mol of this binding energy is used for binding the ground state, while the

remainder is used to bind the chemical transition state. In the absence of the sidechain of Thr45, the sidechain of Asp83 contributes only little binding energy. The free energy of interaction between Asp83 and Thr45 upon binding of the RNase A to the transition state was 1.36 kcal/mol. Approximately 0.4 kcal/mol were gained upon binding of the ground state and another 0.9 kcal/mol upon chemical activation.

Molecular Modeling. Models of T45A and T45G RNase A, created using the MIDAS molecular modeling program (Ferrin et al., 1988), showed that truncating the sidechain of Thr45 creates a cavity that may accommodate adenine or inosine. Guanine cannot fit in this cavity due to unfavorable steric interactions of N2 with the mainchain of residue 45. The carboxyl group of Asp83, which is also in the B1 subsite (figure 3.1), could exclude inosine by electrostatic repulsion of O6, but as this residue is exposed to the solvent such a scenario is doubtful. The inability of B1 subsite mutants to cleave poly(I)(data not shown) more likely arises from the specificity of the B2 and B3 subsites. In addition, the carboxyl sidechain of Asp83 is within hydrogen bonding distance of the exocyclic amino group of the adenine base modeled in the T45G enzyme. Such a hydrogen bond would favor the binding of an adenine base to the altered B1 subsite and would explain the decrease in adenine affinity observed for both wild-type and T45G RNase A upon replacing Asp83 with an alanine residue (Table 3.2).

3.3 Discussion

Specificity, efficiency, and engineering. What is the relationship between substrate specificity and rate enhancement in enzyme-catalyzed reactions? Two theories have been advanced (Benner, 1988)[1] One theory maintains that a high specificity is incompatible with a high rate. This view follows from the idea that binding the substrate tightly increases the difference in free energy between the ground state and the transition state for the chemical conversion. Interestingly, this idea is analogous to the selectivity – reactivity principle of nonenzymatic reactions: a more reactive reagent is less selective in its reaction (Leffler & Grunwald, 1963) The other theory holds that high specificity is actually required for maximal rate. This view is based on the belief that the chemical interactions between an enzyme and its substrate that provide binding energy for catalysis are maximized at the transition state. Thus, each enzyme – substrate interaction is critical to lowering the free energy of the reaction, and an enzyme's active site can be optimized for only a narrow range of substrates. Although evidence exists in support of each theory, the latter appears to explain the majority of experimental results. In other words, an enzyme having a narrow substrate specificity is likely to be a more efficient catalyst (higher k_{cat}/K_m) than is an enzyme of similar function having a broad substrate specificity. A pertinent corollary to this view is that modifications to enzymes by protein engineering that affect substrate specificity will likely effect catalytic efficiency as well. Indeed, protein engineers have succeeded in creating enzymes of

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1. These 2 theories are reminiscent of the 2 classic models of enzymatic catalysis: Fischer's "lock and key" (Gandour & Schowen, 1978) and Haldane's and Pauling's transition state stabilization (Pauling, 1948).

altered substrate specificity, but only seldom (Wilks et al., 1988; Bone et al., 1989) have these new catalysts had a specific activity comparable to that of the wild-type enzyme.

Altering the substrate specificity of an enzyme by protein engineering involves identifying residues in the active site that mediate substrate binding but not substrate turnover. These residues should bind to parts of the substrate that do not chemically change during catalysis and ideally should be remote from those residues (for example, general acids and general bases) that mediate catalysis. Since small perturbations to a protein's structure can have severe effects on protein function, the residues targeted for engineering should interact with solvent rather than other residues. Most enzymes of engineered specificity have resulted from modifications to residues which meet these criteria (Wilks et al., 1988; Bone et al., 1989). Unfortunately, the residues which mediate substrate binding are often buried or are the same as those that mediate turnover. For example, the coupling of substrate recognition and turnover have severely hampered attempts to alter the specificity of type II restriction enzymes (Inouye & Sarma, 1986).

To avoid the pitfalls of rational design, the alteration of substrate specificity of RNase A described here employed a pseudo-rational strategy. Thr45 and Phe120 were identified as residues that likely mediated the pyrimidine specificity of RNase A. Positions 45 and 120 were randomized by saturation mutagenesis and the mutant enzymes were screened for an activity that could cleave polypurine nucleotides. This type of experiment differs from rational protein engineering in the type of information that is gathered. In the rational approach, particular modifications that *may result* in altered substrate specificity are *tested*, in the pseudo-rational approach, the structural modifications that *do result* in altered substrate specificity are *discovered*. The latter

approach presumes less and allows for surprises. Our results suggest that the pseudo-rational approach, when applicable, may be an improved strategy for the deliberate modification of enzyme function.

Thr45. The sidechain of the residue (Thr45; Fig. 3.1) that is largely responsible for the substrate specificity of RNase A is a solvent exposed residue that is relatively remote from the sidechains of the residues (His12 and His119; Fig. 1.3) that expedite cleavage of the P–$O_{5'}$ bond of RNA. This separation suggests that substrate binding can be uncoupled from substrate turnover in RNase A. Our results indicate that such uncoupling is indeed possible. T45A and T45G RNase A display a 10^3– to 10^5–fold change in purine:pyrimidine specificity with little compromise to catalytic efficacy for the cleavage of homopolymeric RNA (Fig. 3.4). These changes result largely from a 10^2 – 10^3–fold *increase* in the specificity constant for cleavage of poly(A), and a 10 – 10^2- fold *decrease* in the specificity constant for the cleavage of poly(C) and poly(U) (Table 3.1). Thr45 effects both ground state binding and transition state stabilization for the cleavage of both purine and pyrimidine substrates, suggesting that much of the specificity imparted by this residue is manifested in the transition state. Thr45, however, has no effect on the cytidine:uridine specificity, as both T45A and T45G maintain the preference for cytidine verses uridine residues demonstrated by the wild-type enzyme.

Phe120. In contrast to Thr45, no alteration of Phe120 produced an enzyme that catalyzed the efficient cleavage of RNA after purine residues. This result is consistent with several structural features of Phe120 that are apparent in the RNase A–uridine 2',3'-vanadate complex (Wlodawer & Lennart, 1983). First, Phe120 makes numerous

contacts with other amino acid residues near the active site of RNase A and is relatively well situated in the outer core of the protein. According to the criteria discussed above, this makes Phe120 a poor residue to target for protein engineering purposes. Second, the π system of Phe120 appears to interact with that of a pyrimidine base bound in the B1 subsite. The structural difference between a pyrimidine base and a purine base is largely two-dimensional, in the plane of the π system. Hence, the sidechain of Phe120 is likely to enhance substrate binding but not to mediate purine:pyrimidine specificity. Finally, the mainchain nitrogen atom of Phe120 forms a hydrogen bond with a nonbridging oxygen atom of the reacting phosphoryl group. This hydrogen bond has been proposed to be intimate to catalysis (Gerlt & Gassman, 1993). Thus, even if the sidechain of Phe120 did mediate substrate specificity, altering this sidechain may perturb the location of the backbone and hamper catalysis.

Subsites and successful engineering. As was predicted by the theory that high catalytic efficiency is dependent on narrow substrate specificity, T45A and T45G RNase A have both broader substrate specificity and lower catalytic efficiency [no substrate was cleaved by T45A or T45G as well as poly(C) was cleaved by wild-type RNase A]. Remarkable is that the catalytic efficiency of these mutants was not more impaired. This maintained catalytic efficiency may result from RNase A binding RNA in multiple subsites (McPherson et al., 1986; Parés et al., 1991). The binding energy contributed by B1 interactions may only be a small fraction of the total binding energy for the cleavage of a polymeric substrate. For example Asn71 of the B2 subsite has been shown to contribute 2.2 kcal/mol to stabilizing the transition state for cleavage of CpA by RNase A (Tarragona-Fiol et al., 1993). This phenomenon is also demonstrated by the proteases pepsin and elastase, which each show a broader primary specificity for

longer rather than shorter polypeptide substrates (Fersht, 1985). Barnase also has broader primary specificity for longer ribonucleic acid substrates (Fersht, 1989). The importance of subsites explains why T45G cleaves ApA, A>p (data not shown), and UpA so poorly, as these substrates cannot not bind to RNase A as extensively as do polymeric substrates. The specificity of subsite interactions may also explain why T45G cleaved poly(I) so poorly (data not shown) despite the similar structures of inosine and adenine. Thus, B1 subsite of the T45A and T45G enzymes may not bind well to an adenine base. Instead, these engineered subsites may simply provide more space into which an adenine base can be "stuffed", provided there is sufficient binding energy from other subsite interactions. If this phenomena is general, engineering enzymes that modify larger substrates (such as biopolymers) may prove to be easier than engineering those that modify smaller substrates, since the former may have more binding interactions with which to work.

Asp83. The sidechain of Asp83 affects the substrate specificity of RNase A. We had anticipated that the carboxylate of Asp83 could accept a hydrogen bond from the exocyclic amino group of a cytosine base bound in the B1 subsite, and thereby enhance the ability of RNase A to cleave after cytidine residues. Consistent with modeling studies, this was not the case. Replacing Asp83 with an alanine residue had no significant effect on the cleavage of poly(C) or the hydrolysis of C>p (Table 3.2). Rather, Asp83 worked together with Thr45 to enhance the binding and turnover of poly(U) and U>p. Replacing Asp83 with an alanine results in an increase in K_m and a decrease in k_{cat} for both the cleavage of poly(U) and the hydrolysis of U>p. This change in kinetic parameters was not observed, however, between T45G and T45G/D83A. Free energy relationships for the cleavage of poly(U) by wild-type,

T45G, D83A, and T45G/D83A RNase A were analyzed by using a thermodynamic cycle, as shown in Figure 3.6. This analysis indicates the sidechains of Thr45 and Asp83 interact at the rate-limiting transition state with $\Delta\Delta G_{int}$ = -1.38 kcal/mol. This interaction is consistent with the mechanism shown in Figure 3.1. The hydroxyl group on the sidechain of Thr45 must be able to rotate, as it both donates a hydrogen bond to a cytosine base and accepts a hydrogen bond from a uracil base. When a uracil base is bound, the Thr45 hydroxyl group also donates a hydrogen bond to the carboxyl sidechain of Asp83. This hydrogen bond aligns the sidechain of Thr45 so as to accept a hydrogen bond from a uracil base. In the absence of the carboxyl group, a uracil base would have to overcome the rotational entropy of the hyroxyl group in order to bind, and binding energy would be lost. The free energy of rotational entropy about a single bond is worth approximately 4.5 cal/(mol•K) (Page & Jenks, 1971), which at 25 °C corresponds to 1.3 kcal/mol of free energy. This is consistent with the change in free energies observed for the cleavage of poly(U). This scenario also explains the change in chemical shift observed for the resonance of Asp83 upon binding CMP that is not observed upon binding UMP (Bruix et al., 1991). In the free enzyme, Asp83 and Thr45 must share a hydrogen bond. When UMP binds to the enzyme, this hydrogen bond is maintained, but when CMP binds, this hydrogen bond is lost.

The sidechain of Asp83 also affects poly(C) binding in an enzyme lacking the sidechain of Thr45. T45G/D83A demonstrated a 20-fold decrease in K_m for the cleavage of poly(C) as compared to the T45G enzyme. Structural data suggest that the carboxyl sidechain of Asp83 does not interact directly with a cytosine base bound to the B1 subsite of wild-type RNase A. Perhaps, in the T45G enzyme, a cytosine base could slide into the new cavity of the B1 subsite and form a hydrogen bond between its exocyclic amino group and the carboxyl sidechain of Asp83. Such an interaction would

interfere with the proper alignment of a cytidine residue in the active site. By removing the sidechain of Asp83, the undesirable interaction is removed and proper orientation is restored.

The sidechain of Asp83 appears to affect the interaction of RNase A with poly(A). This interaction is manifested as a 3-fold increase in the value of K_m for the cleavage of poly(A) by D83A, and in T45G/D83A, as compared to T45G. Contrary to former suspicions, RNase A must bind adenine residues in the B1 subsite, as the effect of altering Asp83 is the same for the wild-type and T45G enzymes. Modeling studies suggest that this effect could arise from a hydrogen bond between the carboxyl of Asp83 and the exocyclic amino group of adenine.

3.4 Conclusions

Thr45 is the residue predominantly responsible for the pyrimidine specificity of RNase A. Replacing Thr45 with an alanine or glycine residue results in a $10^4 - 10^5$-fold increase in the purine:pyrimidine specificity for homopolymeric substrates with little compromise to catalytic efficiency. The high catalytic efficiency of the mutant enzymes is likely due to binding energy gained from subsite interactions other than those of the B1 subsite, emphasizing the general importance of subsite interactions in enzymatic catalysis. Asp83 also effects the specificity of RNase A by enhancing the binding of substrates containing uracil and adenine bases. Thr45 and Asp83 are remote from the residues that mediate RNA cleavage and interact with substrate moieties that do not undergo covalent modification. Yet, both Thr45 and Asp83 effect ground state and transition state binding. For most substrates transition state binding is more predominant than ground state binding, suggesting that the substrate specificity

imparted by Thr45 and Asp83 is manifested predominantly in the chemical transition state. Thus, the catalytic efficiency and substrate specificity of RNase A are interdependent.

Table 3.1 (**A**) Steady-state kinetic parameters for the cleavage of ribonucleotides by T45A, T45G, and wild-type RNase A.

A

RNase A	Substrate	k_{cat} [s^{-1}]	K_m [mM]	k_{cat}/K_m [10^6 $M^{-1}s^{-1}$]
wild-type				
	UpA	1400 ± 130	0.62 ± 0.09	2.3 ± 0.4
	poly(C)	510 ± 10	0.034 ± 0.001	15 ± 9
	poly(U)	24 ± 15	0.06 ± 0.01	0.4 ± 0.3
	poly(A)	0.023 ± 0.001	0.080 ± 0.009	0.00028 ± 0.00004
T45A				
	UpA	24 ± 13	4 ± 2	0.006 ± 0.005
	poly(C)	500 ± 60	0.48 ± 0.08	1.0 ± 0.2
	poly(U)	1.7 ± 0.2	0.12 ± 0.04	0.014 ± 0.005
	poly(A)	1.4 ± 0.1	0.041 ± 0.005	0.035 ± 0.005
T45G				
	UpA	20 ± 10	6 ± 4	0.003 ± 0.002
	poly(C)	1000 ± 300	4 ± 2	0.2 ± 0.1
	poly(U)	0.86 ± 0.08	0.19 ± 0.04	0.004 ± 0.001
	poly(A)	5.8 ± 0.2	0.023 ± 0.004	0.25 ± 0.04

Table 3.1 (B) Relative specificity constants for the cleavage of ribonucleotides by T45A and T45G RNase.

B

RNase A	$\frac{(k_{cat}/K_m)_{polyA}}{(k_{cat}/K_m)_{polyU}}$	$\left(\frac{(k_{cat}/K_m)_{polyA}}{(k_{cat}/K_m)_{polyU}}\right)_{rel}$	$\frac{(k_{cat}/K_m)_{polyA}}{(k_{cat}/K_m)_{polyC}}$	$\left(\frac{(k_{cat}/K_m)_{polyA}}{(k_{cat}/K_m)_{polyC}}\right)_{rel}$
wild type	$(7.0 \pm 4.7)\times10^{-4}$	1	$(2 \pm 1) \times 10^{-5}$	1
T45A	2.5 ± 1.0	$(4 \pm 3) \times 10^{3}$	0.035 ± 0.009	$(2 \pm 1) \times 10^{3}$
T45G	62 ± 19	$(9 \pm 7) \times 10^{4}$	1.2 ± 0.6	$(6 \pm 4) \times 10^{4}$

Table 3.2. Steady-state kinetic parameters for the cleavage of homopolyribonucleotides and the hydrolysis of mononucleotide 2',3'- cyclic monophosphates by D83A, T45G/D83A, and wild-type RNase A.

RNase A	Substrate	k_{cat} [s^{-1}]	K_m [mM]	k_{cat}/K_m x 10^{-6} [M^{-1} s^{-1}]
wild-type				
	poly(C)	510 ± 10	0.034 ± 0.002	15 ± 1
	poly(U)	20 ± 10	0.06 ± 0.01	0.4 ± 0.3
	poly(A)	0.023 ± 0.001	0.080 ±0.009	0.00028 ±0.00004
	C>p	3.7 ± 0.7	1.0 ± 0.6	0.004 ± 0.003
	U>p	2.9 ± 0.4	3.2 ± 0.5	0.0009 ± 0.0002
D83A				
	poly(C)	240 ± 10	0.022 ± 0.004	11 ± 2
	poly(U)	6 ± 1	0.18 ± 0.09	0.03 ± 0.02
	poly(A)	0.017 ± 0.003.	0.23 ± 0.07	0.0001 ± 0.00003
	C>p	2.5 ± 0.2	1.2 ± 0.3	0.002 ± 0.0005
	U>p	1.0 ± 0.3	11 ± 3	0.0001 ± 0.00003
T45G/D83A				
	poly(C)	380 ± 50	0.18 ± 0.05	2.1 ± 0.6
	poly(U)	1.0 ± 0.4	0.3 ± 0.2	0.003 ± 0.002
	poly(A)	3.5 ± 0.1	0.034 ± 0.007	0.1 ±0.02

Figure 3.1 Hydrogen bonds formed between the pyrimidine bases C (**left**) and U (**right**), and residues of the B1 binding pocket of RNase A (Wlodawer & Lennart, 1983).

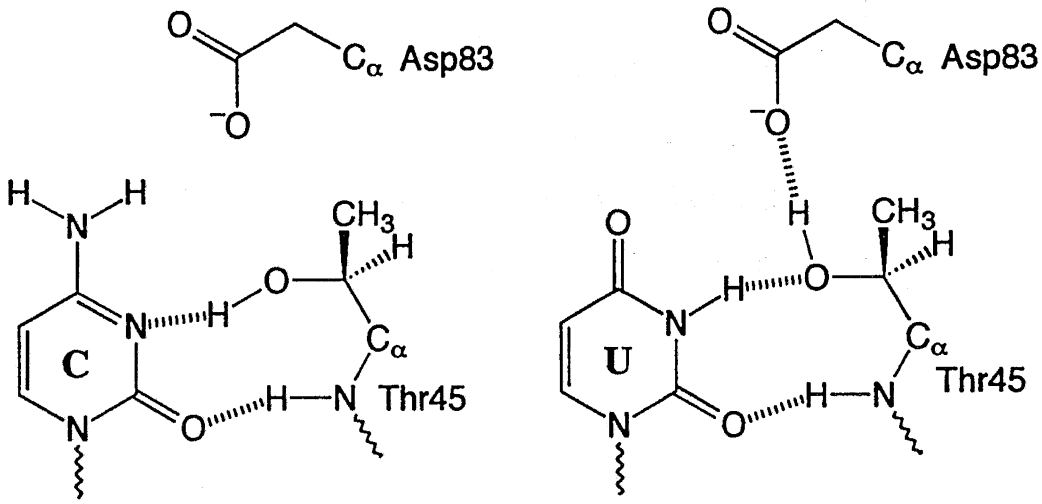

O
Cα Asp83
⁻O
H
N
H
CH3
H
O
N
H
Cα
C
O
H—N Thr45
N
O
Cα Asp83
⁻O
H
CH3
H
O
H
O
N
U
Cα
O
H—N Thr45
N

Figure 3.2 Scheme for the rapid identification of ribonuclease mutants having altered substrate specificity.

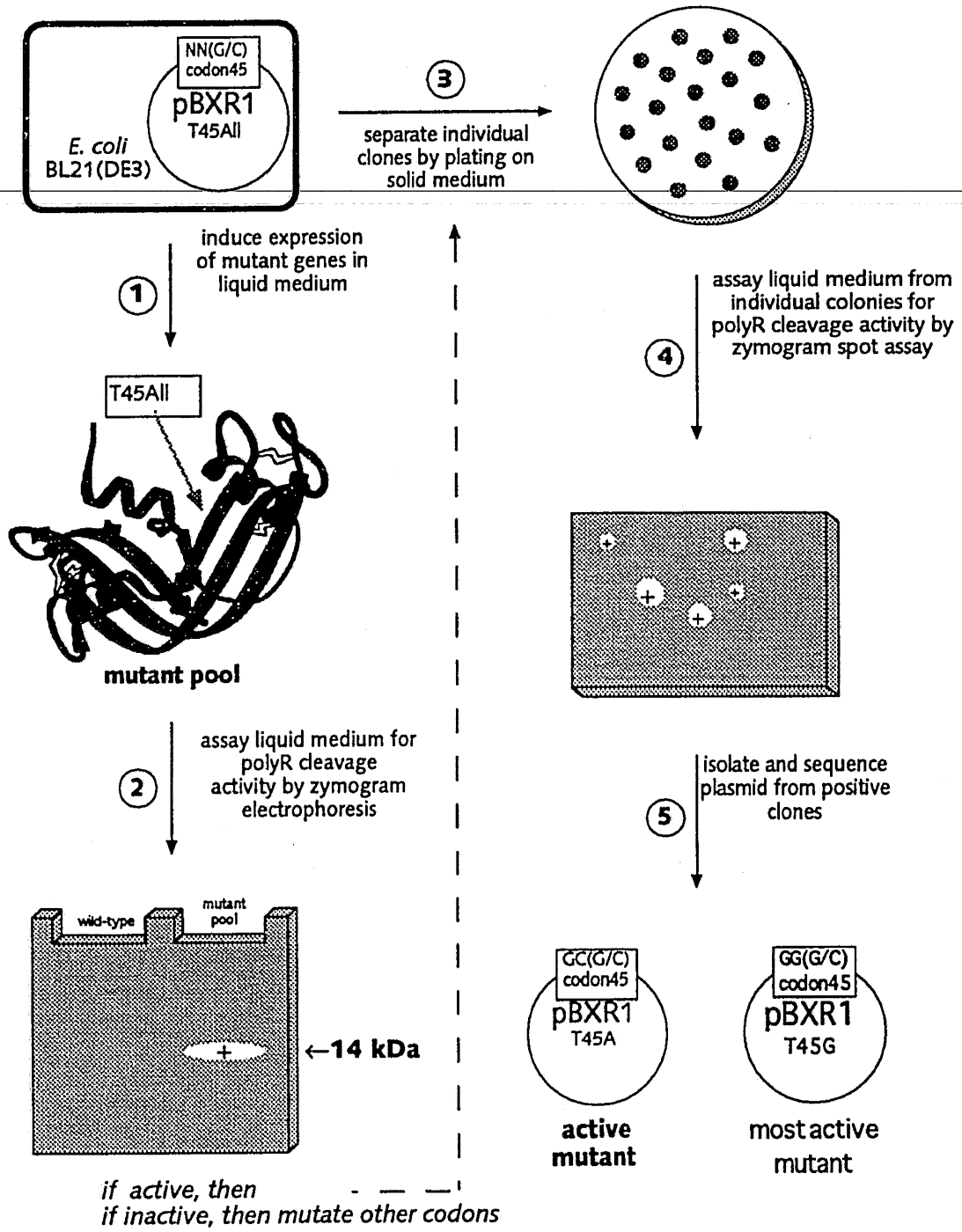

NN(G/C)
codon45
pBXR1
T45All
E. coli
BL21(DE3)
3
separate individual
clones by plating on
solid medium
induce expression
of mutant genes in
liquid medium
1
T45All
mutant pool
2
assay liquid medium for
polyR cleavage
activity by zymogram
electrophoresis
wild-type
mutant
pool
←14 kDa
4
assay liquid medium from
individual colonies for
polyR cleavage activity by
zymogram spot assay
5
isolate and sequence
plasmid from positive
clones
GC(G/C)
codon45
pBXR1
T45A
active
mutant
GG(G/C)
codon45
pBXR1
T45G
most active
mutant
if active, then
if inactive, then mutate other codons

Figure 3.3 Poly(A) zymogram electrophoresis of T45A, T45G, and wild-type RNase A. (1) RNase A (1 ng), and BL21(DE3) cells harboring plasmid (2) pET22B(+), (3) pBXR1, (4) pBXR1.T45G, and (5) pBXR1.T45A.

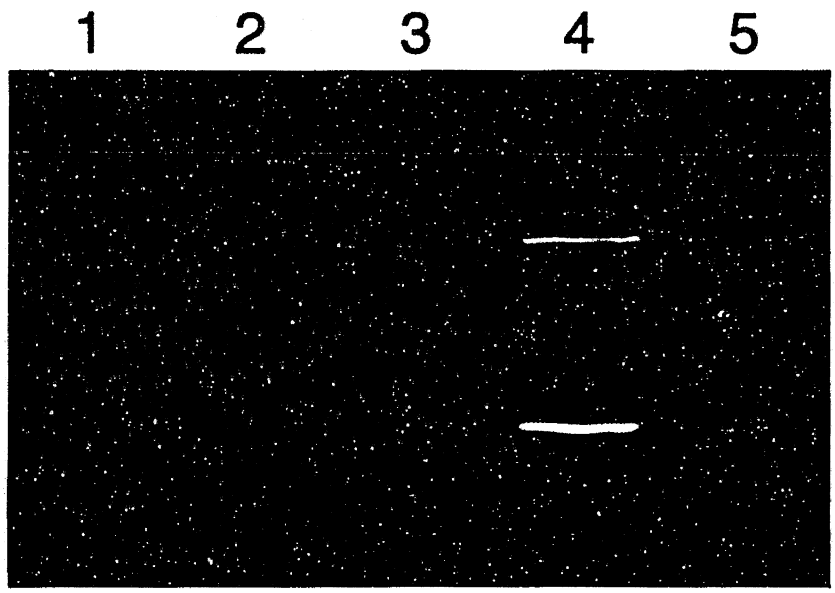

1
2
3
4
5

Figure 3.4 Specificity constants of wild-type and Thr45 mutant RNase A's for the cleavage of homopolymeric substrates.

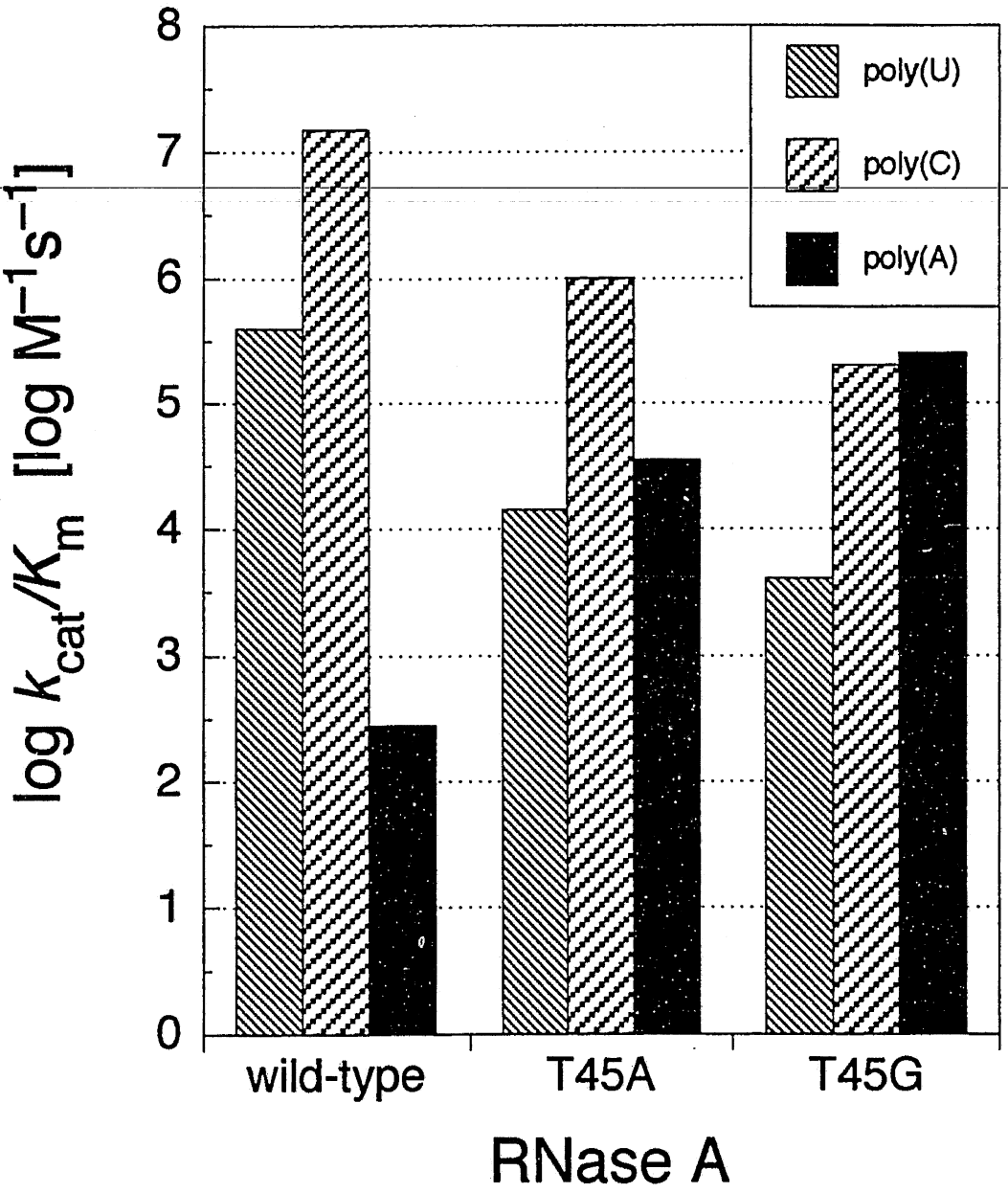

8
7
6
5
4
3
2
1
0
log k_{cat}/K_m [log $M^{-1}s^{-1}$]
poly(U)
poly(C)
poly(A)
wild-type
T45A
T45G
RNase A

Figure 3.4 The effect of altering the sidechain of Asp83 to an alanine residue on the substrate specificity of wild-type and T45G RNase A. Shown are the relative change in specificity constants for the cleavage of homopolymeric substrates by D83A relative to wildtype and T45G/D83A relative to T45G.

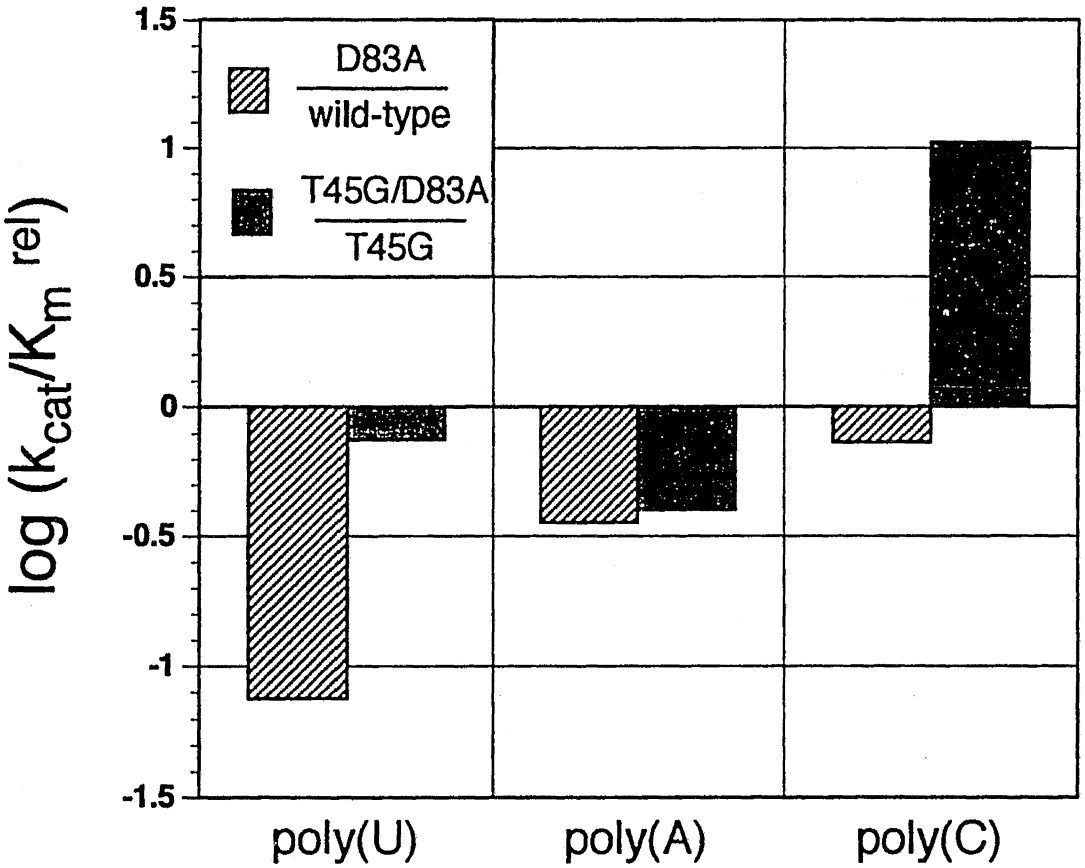
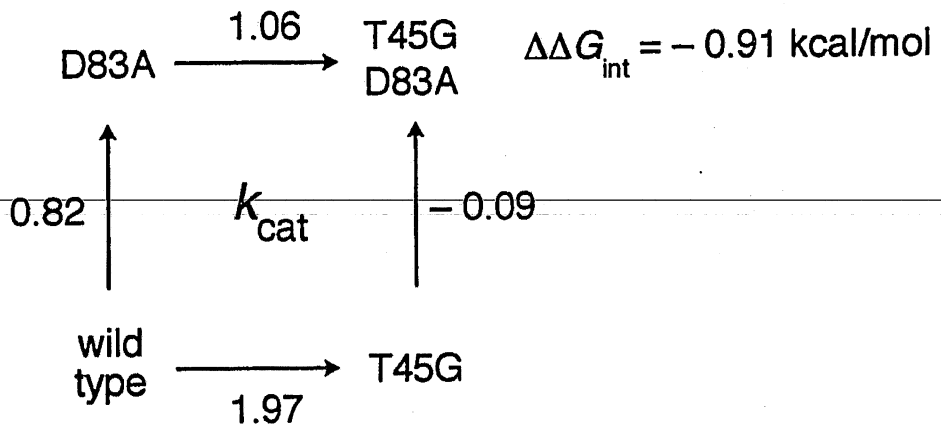
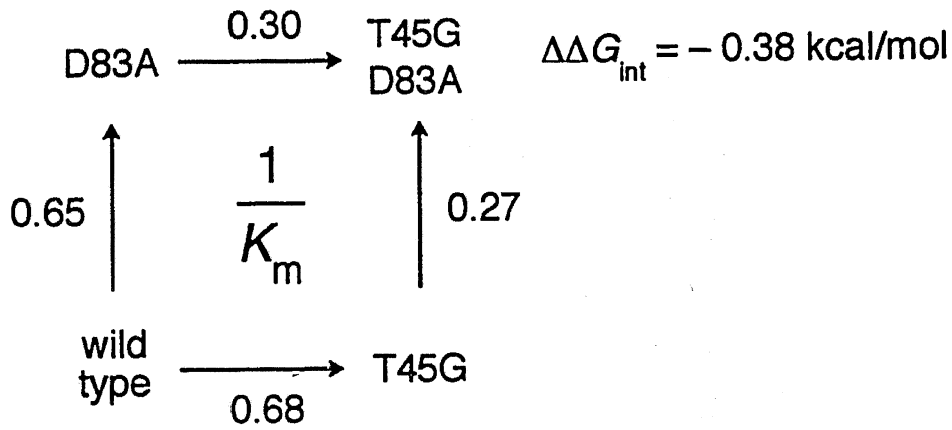
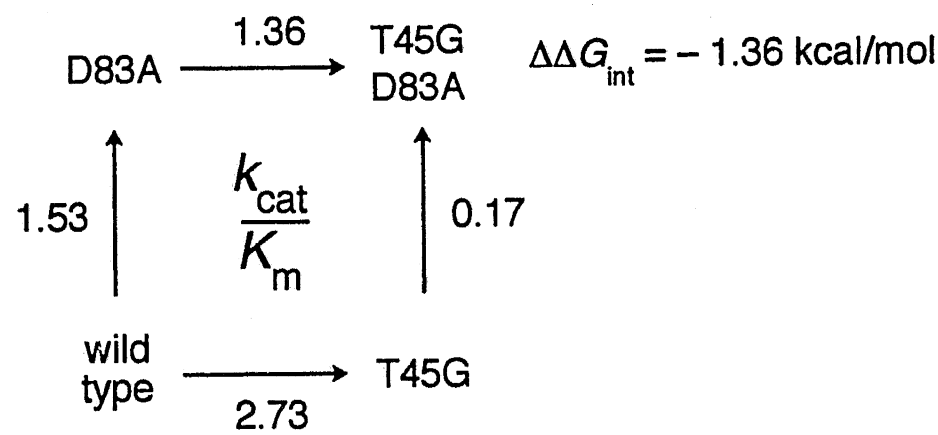

1.5
1
0.5
0
-0.5
-1
-1.5
log (k_{cat}/K_m rel)
D83A
wild-type
T45G/D83A
T45G
poly(U)
poly(A)
poly(C)

Figure 3.5 Thermodynamic cycles for the cleavage of poly(U) by wild-type, T45G, D83A, and T45G/D83A RNase A. The change in free energy for the binding of the chemical transition state by RNase A was calculated from k_{cat}/K_m, for the apparent binding of the ground state from $1/K_m$ (assuming that $K_S = K_m$), and for the chemical activation energy from k_{cat} (Chapter 6).


D83A
1.06
T45G
D83A
ΔΔG int = – 0.91 kcal/mol
0.82
k cat
– 0.09
wild type
T45G
1.97


D83A
0.30
T45G
D83A
ΔΔG int = – 0.38 kcal/mol
0.65
1
K m
0.27
wild type
T45G
0.68


D83A
1.36
T45G
D83A
ΔΔG int = – 1.36 kcal/mol
1.53
k cat
K m
0.17
wild type
T45G
2.73

Chapter 4

Structural Determinants of Enzymatic Processivity

4.1 Introduction

Numerous cellular processes involve the modification of polymers. Most of these reactions entail polymerization or depolymerization, such as replication, transcription, translation, or turnover of RNA, protein, and polysaccharide. Many of the enzymes that catalyze these reactions are *processive*. A *processive* enzyme binds a polymeric substrate and catalyzes a series of similar chemical reactions along that substrate before releasing to solvent the extensively modified product. In contrast, a *distributive* enzyme binds a polymeric substrate, catalyzes a single chemical reaction, and releases to solvent the singly modified product (Kornberg & Baker, 1992). The biological importance of processivity is most evident for replication, transcription, and translation. Each initiation of the these reactions must be taken to completion, or a cell would be overrun by partially replicated genes, truncated RNAs, and useless polypeptides.

The most thoroughly studied processive enzymes are the DNA polymerases. These enzymes are generally made up of a core enzyme, which catalyzes the synthesis of DNA, complexed to numerous accessory factors. Except for the DNA polymerase from phage T5, the core enzymes are essentially distributive, and only become processive once complexed with their respective processivity factors. The processivity of a DNA polymerase is quantitated by its processivity value (PV), which is the number of catalytic events carried out each time substrate is bound. The core enzymes generally demonstrate a PV$\approx$ 10, whereas the holoenzymes have a PV $\approx$ 5, 000 - 20, 0000. Other processive enzymes include exonucleases, helicases, and RNA polymerases [for an excellent discussion see Kornberg's book, DNA Replication (1992)].

Despite the biological importance of processive reactions, little is known at the molecular level about how enzymes achieve processivity. The chemistry of processivity

is essentially one of accessory factors— an enzyme is distributive alone but processive when complexed with some other polypeptide(s). This level of detail is largely the result of little high resolution structural data for processive enzymes. Only the structure of the crystaline dimer of the β subunit (the processivity factor) of DNA polymerase III has been determined (Kong et al. 1992). As put by Arthur Kornberg: "The detailed mechanisms of processivity are largely obscure. Inasmuch as the numerous forces through which the enzyme interacts with a long-chain substrate are still undefined, it is no wonder that the way in which these many contacts are serially released and reformed with each cycle of polymerization must also remain a mystery." While there are processive enzymes that do not rely on processivity factors such as exonuclease I, λ exonuclease (Thomas & Olivera, 1978), T5 DNA polymerase (Kornberg & Baker, 1992), and *E. coli* RNase II (Nossal & Singer, 1968), a molecular picture of processivity by these enzymes also remains obscure.

Even in the absence of structural data, some basic principles governing processive catalysis can be reasoned. Processivity implies that an enzyme 1) must maintain binding interactions with its polymeric product after each catalytic turnover, and 2) must release any non-polymeric products to clear the active site for subsequent turnover. Hence, a processive enzyme must bind its polymeric substrate in at least one enzymic site, a "subsite," that is distinct from the enzyme's active site. Other enzymic subsites may also be required so that the chain would not be lost to solvent during migration. The processivity factors described earlier probably provide these binding interactions to their distributive core enzymes.

RNase A is an endoribonuclease that binds the bases of adjacent RNA residues in three enzymic subsites: B1, B2, and B3 (Parés et al., 1991). Despite having these subsites, catalysis by RNase A is distributive. The distributive behavior of RNase A

may result from the specificity of it subsites. RNase A catalyzes the cleavage of the P–$O_{5'}$ bond specifically on the 3'–side of pyrimidine nucleotides that are bound in the B1 subsite. The B1 subsite binds only residues having a pyrimidine base (as discussed in Chapter 3)(McPherson et al., 1986; Aguilar et al., 1992; Fontecilla-Camps et al., 1993). The B2 subsite binds to the base 3' to that in B1 and, while accepting all bases, has a preference for adenine (Katoh et al., 1986). The B3 subsite binds to the base 3' to that in B2 and, while also accepting all bases, has a preference for purines (Rushizky et al., 1961; Irie et al., 1984). Mutants of RNase A having Thr45 replaced with alanine or glycine bind adenine as well as pyrimidine bases in the B1 subsite (described in Chapter 3) (delCardayré & Raines, 1994).

Engineering RNase A to accept adenine bases in the B1 subsite allows poly(A) to satisfy the base specificity of all three subsites. Once cleavage between the residues occupying the B1 and B2 subsites occurs and one strand is released, the T45A and T45G enzymes could remain bound to the 3' product through interactions with the B2 and B3 subsites. Alternatively, the 5' product could remain bound to the B1 subsite, but as this site has been engineered it likely does not bind substrates well (Chapter 3). The enzymes could then refill the B1, B2, and B3 subsites simply by moving down one (in the 5' → 3' direction; Fig. 4) or two (in the 3' → 5' direction) residues of the poly(A) strand. We therefore reasoned that the T45A and T45G enzymes may degrade a strand of poly(A) in a processive rather than a distributive manner.

Determining whether an enzyme is processive requires an analysis of products isolated during the course of the enzyme-catalyzed reaction. Classically, workers studying degradative enzymes would partially digest a polymeric substrate with enzyme, and then separate the products by gel filtration. If the only products were high molecular weight polymer (that is, starting material) and monomer, then the enzyme

was considered to act processively (Nossal & Singer, 1968). To determine whether T45A and T45G RNase A degrade poly(A) processively, two new assays for nuclease processivity were developed that use modern methods of nucleic acid analysis (delCardayré et al., 1994). The results of these assays demonstrate that both T45A (data not shown) and T45G *distributively* degrade poly(C) but *processively* degrade poly(A). This observation combined with the vast structural and chemical data available for RNase A provides new insight into the structural determinants of enzymatic processivity.

4.2 Results

.c3.**^{31}P NMR Assay for Processivity.** ^{31}P NMR spectroscopy can be used to probe the chemical state of the phosphoryl group during RNA cleavage (that is, acyclic diester → cyclic diester)(Cazzone & Jardetsky, 1977; Thompson et al., 1994). Further, the relative mol wt (strand length) of a phosphodiester can be inferred from its ^{31}P NMR chemical shift. If an acyclic diester [such as poly(A)] were being cleaved processively, then the spectra would show little accumulation of high mol wt polymers containing a cyclic diester [poly(A)A>p]. Instead, the acyclic diester would be converted completely to a monomeric cyclic diester (A>p). If the substrate were not being cleaved processively, then the spectra would show an accumulation of polymeric cyclic diesters.

Spectra for the degradation of poly(A) and poly(C) by T45G and wild-type RNase A are shown in Fig. 4.2. During the degradation of poly(C) by the mutant and wild-type enzymes [or of poly(A) by the wild-type enzyme (Cazzone & Jardetsky, 1977)], the ^{31}P resonance of the acyclic diester shifted from –1.30 ppm to –0.90 ppm.

This downfield shift arises from the deshielding of the phosphorus atom in low mol wt polymers. Concurrent with the shift in the resonance of acyclic diesters was the appearance of two resonances from cyclic diesters, one at 19.7 ppm [from poly(C)C>p] and another at 20.1 ppm (from C>p). The accumulation of poly(C)C>p and the decrease in mol wt of the acyclic diester is consistent with a distributive degradation of poly(C). In contrast, during the degradation of poly(A) by the T45G enzyme, the resonance from the acyclic diester remained at –1.03 ppm, indicating that most strands of poly(A) maintained a constant mol wt. In addition, a single cyclic diester resonance appeared at 19.9 ppm (from A>p). The absence of poly(A)A>p along with the absence of low mol wt acyclic diesters indicates that T45G RNase A degrades poly(A) strands one-at-a-time to A>p, and thus acts processively. Spectra (not shown) for cleavage by T45A RNase A were identical to those for cleavage by T45G RNase A.

Distraction assay for processivity. A ribonuclease that degrades RNA processively binds to an RNA strand and remains associated with that strand until its degradation is complete. Other RNA strands added after the initial association should be protected from degradation for the lifetime of the initial enzyme•polymer complex. An order-of-addition experiment was used to determined whether preincubation with unlabeled RNA could distract T45A, T45G, or wild-type RNase A from degrading [5'-^{32}P]labeled RNA. Unlabeled substrate was exposed to enzyme for time **t'** to allow any processive complex to form. [5'-^{32}P]Labeled substrate was then added, and the mixture was incubated for an additional time **t** before being quenched and separated by polyacrylamide gel electrophoresis.

Results from the distraction assay (Fig. 4.3) show that T45G RNase A was distracted by preincubation with unlabeled poly(A), as evidenced by the absence of

small mol wt species. Yet, neither T45G (data not shown) nor wild-type RNase A was distracted by preincubation with unlabeled poly(C), as evidenced by the accumulation of small mol wt species. These data, along with the data from ^{31}P NMR spectroscopy, demonstrate that T45G RNase A catalyzes the distributive degradation of poly(C) and the processive degradation of poly(A). Gels (not shown) for distraction of the T45A enzyme were identical to those for distraction of the T45G enzyme.

4.3 Discussion

Processive catalysis by T45A and T45G RNase A. Processive catalysis is unique to the enzymes that modify polymers. Processive enzymes use the extensive binding interactions with their polymeric substrate to maintain association after each catalytic event. For many processive enzymes, these binding interactions are mediated by accessory processivity factors. For others, polymer binding and catalysis are accomplished by the same polypeptide. Processivity has arisen in numerous cellular reactions and is critical to the proper functioning of each. Despite the biological importance of processive catalysis, few molecular details have been revealed about this process. The lack of details is due mainly to a shortage of structural data for the enzymes known to be processive. The recent structure of the β subunit dimer of *E. coli* DNA polymerase holoenzyme III provided the first molecular image of a processivity factor. The β subunit dimer apparently forms a tight clamp around duplex DNA that prevents the dissociation of the holoenzyme DNA complex during replication (Kong et al., 1992). The results reported here demonstrate that the T45A and T45G mutants of RNase A processively degrade poly(A). This observation along with the wealth of

structural information available for RNase A further illuminates the molecular determinants of enzymatic processivity.

For a substrate to be acted on processively, it must contain a repeating structural motif. Both poly(C) and poly(A) have repeating motifs, such as a ribosyl group, phosphoryl group, and base. Yet, neither of these polymers is cleaved processively by wild-type RNase A (Fig. 4.2 and 4.3). The distributive behavior of RNase A is likely to arise from the opposing specificities of the B1 subsite [which binds A weakly (Chapter 3)(McPherson et al., 1986)] and the B2 and B3 subsites (which bind C weakly). Our results show that inducing RNase A to degrade poly(A) processively requires simply changing the specificity of the B1 subsite to match that of the B2 and B3 subsites. This change results in mutant enzymes that cleave (at the B1 position) a polymer that can remain bound to the enzyme (presumably at the B2 and B3 positions) after catalysis has occurred (Fig. 4.1).

Model for the structural determinants of enzymatic processivity. Apparently, processive enzymes must bind tightly only to those structural motifs that repeat within their substrate polymer, and must bind those motifs in more than one enzymic subsite. It is not necessary for each subsite to recognize the same repeating motif, only for it to recognize a motif that does indeed repeat. In addition, enzymes that rely on the tight binding of non-repeating motifs are likely to be distributive. Such binding interactions would prohibit processive catalysis. This model is consistent with the modeled interaction of the β subunit dimer with duplex DNA.The dimer forms a cylidrical clamp around a DNA duplex evidently focusing a positive charge toward the substrate's negatively charged phosphate backbone. Thus, the only interactions that this processivity factor makes with its substrate is with repeating structural motifs, the

phosphate backbone. The clamp could further prevent dissociation even in the event of solvation of both enzyme and substrate.

The principles that govern enzymatic processivity can be likened to the interactions of a bicycle chain (the substrate) and its sprocket (the enzyme). A bicycle chain is comprised of numerous links, each identical in its shape and in the size of its hole. A sprocket is comprised of a wheel containing many teeth (subsites), each tooth identical to the next and perfectly shaped and spaced to fit through the hole in each link of the chain. As long as the sprocket and chain remain complementary the chain remains associated with the sprocket. If a link in the chain were to be dented making its hole smaller, then that link could now derail the chain (a distributive behavior), because at that point the chain no longer repeats. Similarly, if one of the teeth on the chain were larger (specific for a larger link) or broken (for a smaller link) the chain would again derail. One could also imagine a similar chain comprised of links having identical hole sizes and spacing to the original, but which contained small spikes protruding out from the exterior sides of *some* of its links. The sprocket would be oblivious to this heterogeneity, and would remain associated with the chain in its original processive manner. Alternatively, if the sprocket relied on the interaction of those spikes to maintain association with the chain, a spikeless link would result in derailing. By considering this analogy and the principles that appear to govern enzymatic processivity, it becomes possible to predict which structural motifs within a substrate must be bound tightly by a processive enzyme. For example, an enzyme that catalyzes the processive cleavage of a heteropolymer of RNA is likely to interact more strongly with the ribose, the phosphate, or the π system of the base (which are similar for each nucleotide) rather than with the functional groups of the base (which are different for

each nucleotide). *E. coli* RNase II, exonuclease I, and λ exonuclease may each act in this way.

The mechanism of processive catalysis by T45A and T45G RNase A. Now that RNase A has been engineered serendipitously to become a processive nuclease, the enzyme provides a unique model for investigating the mechanism of processive catalysis. Once cleavage has occurred between the residues bound to the B1 and B2 subsites, the enzyme must proceed down the poly(A) chain. *How is this migration accomplished?* The structure of crystalline RNase A complexed with the oligonucleotide d(ApTpApApG) (Fontecilla-Camps et al., 1993), may illuminate this question. The structure shows that the adenine bases bound in the B2 and B3 subsites are stacked with both bases residing predominantly in the B2 subsite. The stacking of adenine bases at this locale may be the basis for the processivity of T45A and T45G. Once cleavage has occurred and A>p has been released from the B1 subsite, the "double occupancy" of the B2 subsite may allow the B2 base to migrate to the B1 subsite without leaving the B2 subsite unoccupied. Further, the stacking of adjacent adenines may cause strain in the poly(A) chain that is temporarily released upon migration. Thus, such stacking may contribute to the migration of the chain through the subsites by employing a "spring loaded mechanism," and the poly(A) chain may move across the surface of the enzyme somewhat like an inchworm. Unfortunately, a simple experiment to test this mechanism has yet to be devised. The most obvious experiments would rely on synthetic RNA substrates having abasic residues or residues that were constrained in the stacked configuration. Such substrates, however, would be expensive and of only speculative value.

4.4 Conclusions

In Chapter 3, the importance of subsite interactions to the catalytic efficiency of RNase A was demonstrated—interactions other than those of the B1 subsite were sufficiently strong to keep an adenine base in an engineered B1 subsite to allow the efficient cleavage of poly(A)(Chapter 3). Apparently, these interactions are maintained after the transphosphorylation of poly(A) occurs, resulting in the processive degradation of a poly(A) by T45A and T45G RNase A. These findings provide a new paradigm for the structural determinants of enzymatic processivity: a processive enzyme has subsites, each specific for a repeating motif within a polymeric substrate. In addition it is likely that processive enzymes bind more tightly to motifs that do repeat than those that do not.

Figure 4.1 Models for distributive and processive cleavage of RNA by wild-type and mutant RNase A's. Wild-type RNase A cleaves poly(C) distributively, releasing both RNA cleavage products. In contrast, the T45A and T45G enzymes cleave poly(A) processively, releasing only the A>p product and remaining bound to the polymeric product after each cleavage reaction. The model for processive cleavage implies that the mutant enzymes proceed in the 5' → 3' direction, which is most consistent with the alignment of the subsites and with data not shown.

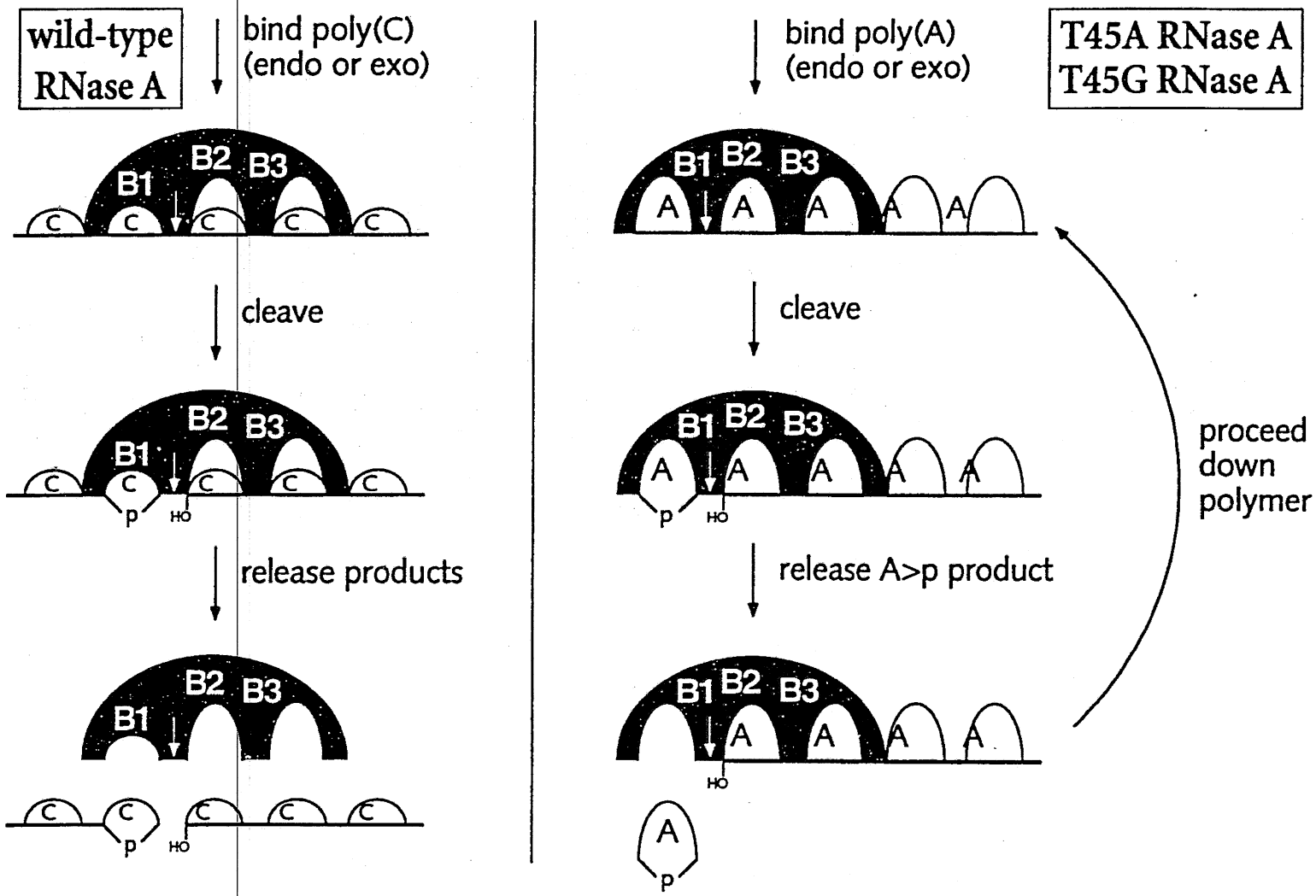

wild-type RNase A
bind poly(C) (endo or exo)
B1
B2
B3
C
cleave
p
HO
release products
bind poly(A) (endo or exo)
T45A RNase A
T45G RNase A
A
release A>p product
proceed down polymer

Figure 4.2 Spectra showing ^{31}P NMR assay for processivity. Panels show spectra recorded during cleavage of poly(C) by wild-type RNase A (**bottom**), poly(C) by T45G RNase A (**middle**), and poly(A) by T45G RNase A (**top**).

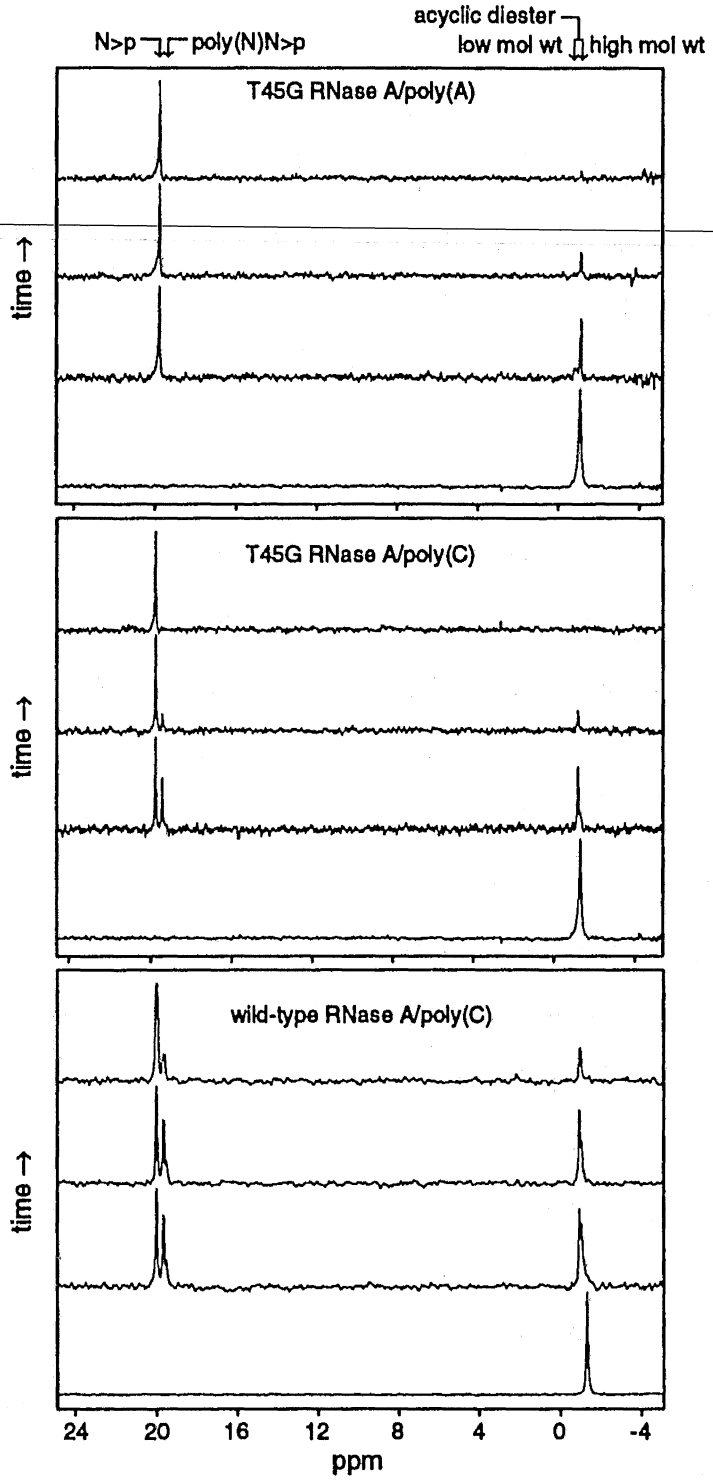

acyclic diester
N>p
poly(N)N>p
low mol wt
high mol wt
T45G RNase A/poly(A)
T45G RNase A/poly(C)
wild-type RNase A/poly(C)
time →
time →
time →
24
20
16
12
8
4
0
-4
ppm

Figure 4.3 Gel showing distraction assay for processivity. Unlabeled substrate was exposed to enzyme for time **t'** to allow any processive complex to form. [5'-^{32}P]Labeled substrate was then added, and the mixture was incubated for an additional time **t** before being quenched.


RNase A: – – wild-type T45G
substrate: polyC polyA polyC polyA
t'[s]: – – – 20 – 20
t[s]: – – 20 20 20 20

Chapter 5

Glutamine-11 and Catalysis by RNase A

5.1 Introduction

The staggering rate enhancements achieved by enzymes are difficult to quantitate. This difficulty arises because the rate of the uncatalyzed reactions are generally too low to measure. For a handful of enzyme-catalyzed reactions an upper limit for the uncatalyzed rate has been determined. According to these studies it is common for an enzyme to achieve a rate enhancement of 10^{10}–10^{15} (Frick et al., 1987; Jencks, 1987). Such rate enhancements are attributed to clever devices such as approximation, strain, desolvation, general acid-base catalysis, covalent catalysis, electrostatic catalysis (Fersht, 1985; Jencks, 1987), and recently the use of low-barrier hydrogen bonds (Cleland, 1992; Cleland & Kreevoy, 1994; Frey et al., 1994). In addition, numerous theories have been coined to explain the phenomena of enzymatic catalysis [for a list of interesting names see (Menger, 1992)], but all converge on the that proposed by Pauling and Haldane which states that enzymes preferentially bind the chemical transition state of the reaction that they catalyze (Pauling, 1948). Preliminary data from the Raines lab measure the background rate of RNA cleavage as approximately 6×10^{-9} s^{-1} (Thompson and Schuster, unpublished results). RNase A catalyzes the cleavage of RNA with a turnover number (k_{cat}) of approximately 3×10^{3} s^{-1} and an apparent second order rate constant (k_{cat}/K_m) of approximately 2×10^{6} $M^{-1}s^{-1}$. This corresponds to a 5×10^{11}-fold rate enhancement and a 20 kcal/mol stabilization of the chemical transition state (Kurz, 1963). We are interested in how each amino acid residue in the active site of RNase A interacts with substrate and in how these interactions contribute to the 10^{11}-fold rate enhancement observed for this enzyme.

Despite extensive study, the precise role of each active-site residue in catalysis by RNase A is uncertain. RNase A catalyzes the cleavage of the P–$O_{5'}$ bond of RNA

specifically after pyrimidine residues. Figure 5.1 depicts a mechanism of catalysis that is consistent with all known data. (For other proposed mechanisms, see: Witzel (1960; 1963), Wang (1968), and Anslyn and Breslow (1989)]. In the mechanism in Figure 5.1, His12 acts as a general base that abstracts a proton from the 2'-hydroxyl of a substrate molecule, and thereby facilitates attack on the phosphorus atom (Thompson & Raines, 1994). This attack proceeds in-line to displace the nucleoside (Usher et al., 1972). His119 acts as a general acid that protonates the 5"-oxygen to facilitate its displacement(Thompson & Raines, 1994). Both products are released to solvent. In this mechanism, the reaction apparently passes through a transition state that has a pentacovalent phosphorus atom. Lys41 assists catalysis by stabilizing this transition-state (Mesmore & Raines, unpublished results). The slow hydrolysis of the 2',3'-cyclic phosphate occurs separately and resembles the reverse of transphosphorylation .(Usher et al., 1970; Thompson & Raines, 1994)

The structure of the complex of RNase A and uridine 2',3'-cyclic vanadate (U>v) obtained at 2.0 Å resolution by joint neutron/X-ray diffraction analysis has provided invaluable insight into the catalytic mechanism of RNase A (Wlodawer et al., 1983), and is shown in Figure 5.2. As expected, this structure shows that the sidechains of His12, Lys41, and His119 are proximal to the vanadyl group of U>v. This structure also shows that 2 other amino acid residues interact intimately with the vanadyl group. The sidechain nitrogen of Gln11 forms a hydrogen bond with the nonbridging oxygen, O_{1v} ($N_{\delta 2} - O_{1V}$ distance = 2.56 Å, $N_{\delta 2} - H - O_{1V}$ angle = 140°), and the mainchain nitrogen of Phe120 forms a hydrogen bond with an nonbridging oxygen, O_{3v} ($N - O_{3V}$ distance = 2.88 Å, $N - H - O_{3V}$ angle = 162°). This arrangement has led to the recent suggestion that these residues stabilize a phosphorane intermediate (Gerlt & Gassman, 1993). A study of semi-synthetic mutants

of RNase S' having various residues at Gln11 have also ascribed a significant role for Gln11 in catalysis (Marchiori et al., 1974). *Do Gln11 and Phe120 polarize the phosphoryl group and thereby make the phosphorous atom more electrophilic? Do they stabilize negative charge that may accumulate on the nonbridging oxygens in the pentacovalent transition state?* Also surprising was the positioning of the sidechain of Lys41, which is often proposed to stabilize the negative charge that may accumulate on the nonbridging phosphoryl oxygens during catalysis. As expected, the sidechain nitrogen of Lys41 is close to O_{1V} ($N_\zeta - O_{1V}$ distance = 3.54 Å, $N_\zeta - H - O_{1V}$ angle = 137°). This nitrogen is much closer, however, to the 2' bridging oxygen ($N_{\varepsilon 2}$ - $O_{2'}$ distance = 2.76 Å ;$N_{\varepsilon 2}$ -H- $O_{2'}$ angle = 142°). *How is the transition state stabilized by Lys 41?* Recently, the techniques of recombinant DNA was used to quantitate the contribution of His12 and His119 to the catalytic turnover (Thompson & Raines, 1994) and of Thr45 to the substrate specificity of this enzyme (Chapter 3)(delCardayré & Raines, 1994). Here, mutant enzymes and synthetic substrates are used to illuminate the role of Gln11 in catalysis by RNase A.

Using the bacterial expression system (Chapter 2) we have created and characterized mutants of RNase A in which Gln11 has been changed to a histidine, asparagine, or alanine residue. Despite the proximity of $N_{\delta 2}$ of the side chain of Gln11 to the vanadium of U>v, altering the sidechain of Gln11 affects the binding of RNA but not of the transition state for transphosphorylation or hydrolysis. The implications to the catalytic mechanism of RNase A are discussed.

5.1 Results

Construction of Q11A, Q11H, and Q11N RNase A. The CAG codon for Gln11 of RNase A was changed to GCG (alanine), AAC (asparagine), or CAT (histidine) by oligonucleotide-mediated site-directed mutagenesis of plasmid YEpWL.RNase A (Chapter 2), and the complete cDNA of each mutant was confirmed by sequencing. The cDNA's encoding these mutant enzymes were then subcloned into the *pst* I/*Sal* I sites of plasmid pBXR (Chapter 2). The resulting plasmids, termed pBXR.Q11A, pBXR.Q11N, and pBXR.Q11H, were transformed into BL21(DE3) cells. Homogenous wild-type and mutant RNase A's were prepared as described in Chapters 2 and 6.

Kinetic Parameters for Q11A, Q11N, and Q11H RNase A. The kinetic parameters for the cleavage of UpA, poly(C), $UpOC_6H_4$-*p*-NO_2, and the hydrolysis of U>p by wild-type, Q11A, Q11N, and Q11H RNase A are shown in Table 5.1. Despite the close approximation of the sidechain nitrogen of Gln11 to the nonbridging oxygen, O_{1v}, of the complex of RNase A with U>v , replacing its sidechain had only a small effect on the binding and turnover of the natural substrates UpA, poly(C), and U>p. Mutation of Gln11 did, however, have a dramatic effect on the kinetic parameters for the cleavage of the synthetic substrate $UpOC_6H_4$-*p*-NO_2. For all substrates, replacing the sidechain of Gln11 resulted in an approximately equal decrease in the values of both k_{cat} and K_m, with no significant change in the value k_{cat}/K_m. Thus, the mutations enhance substrate binding (lower K_m) at the expense of substrate turnover (lower k_{cat}). For the Q11A mutant, the decrease was 2- to 5-fold for the natural substrates, but 100-

fold for $UpOC_6H_4$-*p*-NO_2. The free energy profile shown in Figure 5.3 was constructed assuming that $K_m = K_s$ and that $\Delta G^{\circ\ddagger} = RTln(k_bT/k_{cat}h)$ (Chapter 6).

5.3 Discussion

Role of Gln11 in Catalysis by RNase A. One of the most intriguing residues in RNase A is Gln11, which is conserved in all 41 pancreatic ribonucleases of known sequence (Beintema, 1987). X-ray diffraction analyses show that the sidechain of Gln11 forms a hydrogen bond to substrates, substrate analogs (Howlin et al., 1987), phosphate ions, and sulfate ions bound to the active-site of RNase A (Wlodawer, 1985). NMR spectroscopy provides further evidence for this interaction, as significant changes in the resonances of Gln11 are observed upon binding of pyrimidine nucleotides (Bruix et al., 1990; Bruix et al., 1991). In the combined neutron/X-ray structure of RNase A complexed with U>v, the sidechain nitrogen of Gln11 and the closest oxygen of U>v is 2.56 Å, which is a distance expected to produce a low-barrier hydrogen bond (Cleland, 1992; Gerlt & Gassman, 1993; Cleland & Kreevoy, 1994). Together, these data strongly suggest that Gln11 plays an important role in the catalytic mechanism of RNase A.

To investigate the role of Gln11 in catalysis by RNase A, we created Q11A, Q11N, and Q11H RNase A and measured the ability of these enzymes to catalyze the cleavage of various substrates. As shown in Table 5.1, replacing Gln11 with an alanine, asparagine, or histidine residue resulted in only small changes in the kinetic parameters for the cleavage of poly(C) or UpA, or for the hydrolysis of U>p. Compared to the wild-type RNase A, the mutant enzymes demonstrated a decrease in the values of both k_{cat} and K_m, with no significant change in the value of k_{cat}/K_m. The small effect of altering the sidechain of Gln11 suggests that this residue is not important

in catalysis by RNase A, at least if poly(C), UpA, or U>p is the substrate. These results allot a somewhat lesser role to Gln11 than do results obtained with analogous semi-synthetic mutants of RNase S' (Marchiori et al., 1974).

RNase A has evolved to bind its substrates by various interactions (Parés et al., 1991; delCardayré & Raines, 1994). For example, the phosphoryl group of UpA appears to be oriented by a hydrogen bond from the mainchain nitrogen of Phe120 and by additional constraints imposed by the binding of its uracil and adenosine bases to the B1 and B2 subsites, respectively. The phosphoryl group of poly(C) is oriented by the same interactions that orient UpA, and by additional interactions with the B3 subsite and various basic residues. The phosphoryl group of U>p is constrained by a 5-membered ring as well as through interactions with Phe120 and the B1 subsite. Although structural data suggest that Gln11 forms a hydrogen bond to a nonbridging phosphoryl oxygen, this hydrogen bond may be superfluous for orienting poly(C), UpA, and U>p. If so, only small perturbations in k_{cat} and K_m for the reaction of these substrates would be expected, because the bound phosphoryl group of poly(C), UpA, and U>p would be oriented properly whether or not the sidechain of Gln11 were present.

To unmask the role for Gln11 in catalysis, we determined the contribution of this residue to the cleavage of $UpOC_6H_4$-*p*-NO_2. This substrate does not contain a 5" nucleoside moiety that can bind to the B2 subsite nor is its phosphoryl group constrained by a ring. Bound $UpOC_6H_4$-*p*-NO_2 is therefore limited to interactions with Phe120 and the B1 subsite. Previously, we used this substrate to demonstrate that the sidechain of His119 serves RNase A as a general acid catalyst (Thompson & Raines, 1994). Here, we reasoned that the proper orientation of the phosphoryl group of $UpOC_6H_4$-*p*-NO_2 may rely strongly on a hydrogen bond donated by the sidechain of

Gln11. If so, then altering the sidechain of Gln11 should have a greater effect on the cleavage of $UpOC_6H_4$-*p*-NO_2 than on that of poly(C), UpA, or U>p. As shown in Table 5.1, cleavage of $UpOC_6H_4$-*p*-NO_2 by Q11A RNase A exhibited a 100-fold decrease in the values of both k_{cat} and K_m, but no significant change in that of k_{cat}/K_m. Apparently, Gln11 destabilizes $UpOC_6H_4$-*p*-NO_2 in the same manner as it does the natural substrates but to a greater degree.

The effect of altering the side chain of Gln 11 on catalysis by RNase A is illustrated by the free energy profile shown in Figure 5.3. Apparently, Gln11 does not stabilize the rate-limiting transition state during catalysis by RNase A. Rather, Gln11 serves to increase the free energy of the Michaelis complex. The destabilization of ground state complexes is a common event in the evolution of enzymatic efficiency, and can be the result of several molecular scenarios (Fersht, 1985). Here, the increase in the free energy of the ground state appears to be due to a binding interaction that reduces nonproductive binding. In the absence of the amide sidechain of Gln11, a substrate may bind to the active-site but with its phosphoryl group in an improper conformation for in-line attack by the 2'-hydroxyl (or water molecule). This nonproductively bound substrate would not react until the phosphoryl group assumed a proper conformation. The increase in the number of substrate binding modes causes a decrease in the value of k_{cat}, and an identical decrease in the value of K_m, such that the value of k_{cat}/K_m is unchanged (Fersht, 1985). Thus, a hydrogen bond between the sidechain of Gln11 and a phosphoryl oxygen may not only assist in orienting the substrate for catalysis, but also in preventing it from binding in a nonproductive mode.

Altering the sidechain of Gln11 results in only a small, $\leq$ 0.6 kcal/mol, decrease in the free energy of the Michaelis complexes with substrates constrained about their phosphoryl group. In contrast, altering the sidechain of Gln11 results in an

approximately 2.6 kcal/mol decrease in the free energy of the Michaelis complex with $UpOC_6H_4$-*p*-NO_2 (Figure 5.3), the phosphoryl group of which is not constrained by interaction with the B2 subsite. Page and Jencks (1977)estimated that freezing 1 internal rotation in a ring forming reaction has an entropic cost of 4.5 cal/(mol•K). Thus, the approximately 2 kcal/mol difference between constrained [poly(C), UpA, and U>p] and unconstrained ($UpOC_6H_4$-*p*-NO_2) substrates corresponds to freezing $1-2$ rotations at 25 °C. In bound $UpOC_6H_4$-*p*-NO_2, the unfrozen rotations are likely to arise from the bonds between the phosphorous and the 2 bridging oxygens. A molecular explanation for the ≤ 0.6 kcal/mol decrease in free energy of the Michaelis complexes of the constrained substrates and the Gln11 mutants is more elusive. The sidechain of Gln11 could eliminate residual rotational degrees of freedom in the constrained substrates, or it could activate the ground state by straining its conformation toward that of the transition state.

The interpretation of the kinetic parameters for Q11N and Q11H RNase A is more complex than that for the Q11A enzyme. Catalysis by both Q11N and Q11H RNase A echoes the trends observed with the Q11A enzyme (Table 5.1), but also displays an approximately 2- to 10-fold decrease in k_{cat}/K_m. In RNase A, position 11 is in the heart of the active site. Although asparagine and histidine are relatively conservative replacements for a glutamine residue, both substitutions relocate heteroatoms. This relocation apparently has effects other than that observed for the simple deletion of a sidechain by replacement with an alanine residue.

What remains intriguing about Gln11 is its absolute conservation, despite the presence of the B2 subsite and the negligible contribution of this residue to stabilizing the rate-limiting transition state. The B2 subsite consists of residues Gln69, Asn71, and Glu111. Like Gln11, Asn71 is conserved in all 41 pancreatic ribonucleases of known

sequence. In contrast, Gln69 is conserved in 35/41 (3 Arg, 2 Lys, 1 Asn) and Glu111 is conserved in 38/41 (1 Asp, 1 Gly, 1 Val). Mutation of the residues of the B2 subsite shows that only Asn71 is important for cleavage of dinucleotide substrates (Tarragona-Fiol et al., 1993). RNase A appears to have evolved to catalyze the rapid cleavage of RNA (Thompson et al., 1994). Perhaps the evolution of *both* Gln11 and the B2 subsite has allowed RNase A to cleave a broader spectrum of RNA substrates. $UpOC_6H_4$-*p*-NO_2 is not a natural substrate. Still, natural substrates that retain considerable rotational freedom when bound to Q11A RNase A do exist. For example, pyrimidine dinucleotides such as UpU bind strongly to the B1 subsite but only weakly to the B2 subsite (Wlodawer et al., 1993), which has a strong preference for adenine bases (Parés et al., 1991). Similarly, a pyrimidine residue upstream from secondary structure could bind to the B1 subsite but could not make contact with the B2 subsite. The sidechain of Gln11 provides a third point of contact with acyclic phosphodiester substrates that would otherwise interact only with Phe120 and the B1 subsite. It will be interesting to measure the effects of the Gln11 mutations on the cleavage of UpU.

Implications for Catalysis by RNase A. Possibly the most intriguing effect of altering the sidechain of Gln11 was the absence of any significant decrease in k_{cat}/K_m for RNA cleavage. An important, unresolved aspect of the reactions in Figure 5.1 is the nature of the pentacovalent phosphorus species through which an in-line mechanism must pass. Theoretical arguments have been used to suggest that this species is a pentacovalent transition state (Deakyne & Allen, 1979) or a phosphorane intermediate (Gerlt & Gassman, 1993). Our results suggest that the sidechain of Gln11 does not contribute significantly to the stability of a pentacovalent phosphorous species, regardless of its nature. Still, several viable mechanisms exist for the reactions in

Figure 5.1. The pentacovalent phosphorous species could be an associative transition state or a phosphorane intermediate. Then, negative charge would accumulate on the nonbridging oxygens but would be stabilized largely by residues other than Gln11. While this mechanism is feasible, it is difficult to believe that the partial positive charge of the carboxamide sidechain of Gln11 would not assist in the stabilization of transition state charge. Thus, the reaction may rather proceed by a dissociative, also unlikely, or an S_N2-like transition state in which negative charge would not accumulate on the nonbridging oxygens. This latter mechanism is consistent with all current data. While Lys41 is traditionally relegated to stabilizing negative charge that accumulates in the nonbridging oxygens, the ammonium sidechain of this residue is more appropriately placed to interact with the 2'-hydroxyl of ribose (Wlodawer et al., 1983). Perhaps the difficult step in transphosphorylation is deprotonation of the 2'-hydroxyl. Lys41 may then stabilize negative charge that accumulates on the 2'-hydroxyl or decrease the pK_a of the 2' hydroxyl to approach that of His12 providing the foundation for a low-barrier hydrogen bond. Of course, the placement of Lys41 in the crystalline structure may also not be representative of its role in catalysis, as His12 is also not bound to the 2'-hydroxyl as expected. It will be interesting to see an analysis of nonbridging O^{18} isotope effects for the cleavage of UpA and of the stereospecificity of phosphorothioate cleavage by wild-type, Q11A, and K41A RNase A.

5.4 Conclusions

Despite the apparent approximation of the sidechain of Gln11 of RNase A to the labile phosphoryl group of substrate RNA, this sidechain does not contribute significantly to catalytic efficiency. Evidently, the role of Gln11 is to orient the phosphoryl group in the

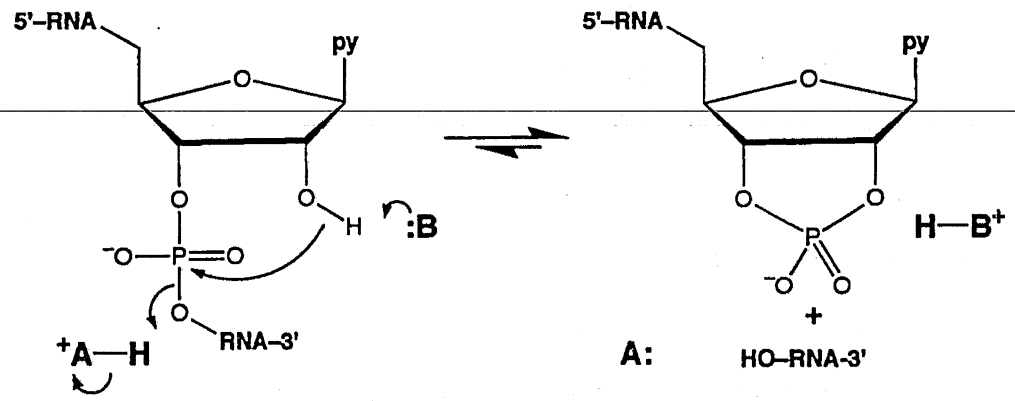
active site. In the absence of the sidechain of Gln11, substrates that can bind to RNase A while maintaining rotation about their phosphoryl group bind non-productively. These substrates, exemplified here by the synthetic substrate $UpOC_6H_4$-*p*-NO_2, bind to the Q11A mutant with much higher affinity but are turned over much more slowly. Finally, the negligible effect of the Gln11 mutations on the catalytic efficiency of RNase A provides additional controversy to the nature of the transition state for RNA cleavage by RNase A.

Table 5.1 Steady-State Kinetic Parameters for the Cleavage of Poly(C) and UpA, and for the Hydrolysis of U>p by Wild-Type, Q11A, Q11N, and Q11H RNase A.

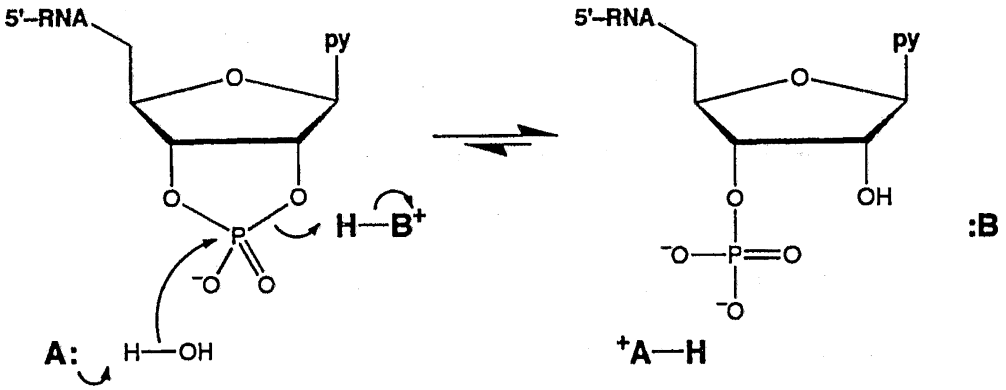
RNase A	Substrate	k_{cat} [s^{-1}]	K_m [mM]	k_{cat}/K_m [10^6 M^{-1} s^{-1}]
wild-type	poly(C) [a]	510 ± 10	0.034 ± 0.002	15 ± 1
	UpA [a]	1400 ± 150	0.62 ± 0.09	2.3 ± 0.4
	$UpOC_6H_4pNO_2$	11 ± 1	0.4 ± 0.1	0.032 ± 0.008
	U>p	2.9 ± 0.4	3.2 ± 0.5	0.0009 ± 0.0002
Q11A	poly(C)	155 ± 2	0.017 ± 0.001	9.0 ± 0.5
	UpA	750 ± 40	0.15 ± 0.02	5.0 ± 0.7
	$UpOC_6H_4pNO_2$	0.106 ± 0.007	0.005 ± 0.002	0.021 ± 0.006
	U>p	1.0 ± 0.4	0.74 ± 0.09	0.0013 ± 0.0005
Q11N	poly(C)	95 ± 2	0.006 ± 0 .001	17 ± 2
	UpA	560 ± 40	0.54 ± 0.07	1.0 ± 0.2
	$UpOC_6H_4pNO_2$	nd	nd	nd
	U>p	nd	nd	nd
Q11H	poly(C)	390 ± 20	0.07 ± 0.01	5.6 ± 0.8
	UpA	290 ± 50	0.8 ± 0.2	0.36 ± 0.04
	$UpOC_6H_4pNO_2$	0.024 ± 0.005	0.08 ± 0.05	0.003 ± 0.001
	U>p	0.21 ± 0.02	0.15 ± 0.09	0.0014 ± 0.0008

[a] Data from delCardayré and Raines (1994) (Chapter 3).

Figure 5.1 Mechanism of the transphosphorylation and hydrolysis reactions catalyzed by RNase A. B: is His 12; $^{+}$A—H is His 119.



Transphosphorylation



Hydrolysis

Figure 5.2 Structure of the complex of RNase A with uridine 2',3'-cyclic vanadate, a transition state analog (Wlodawer et al., 1983).

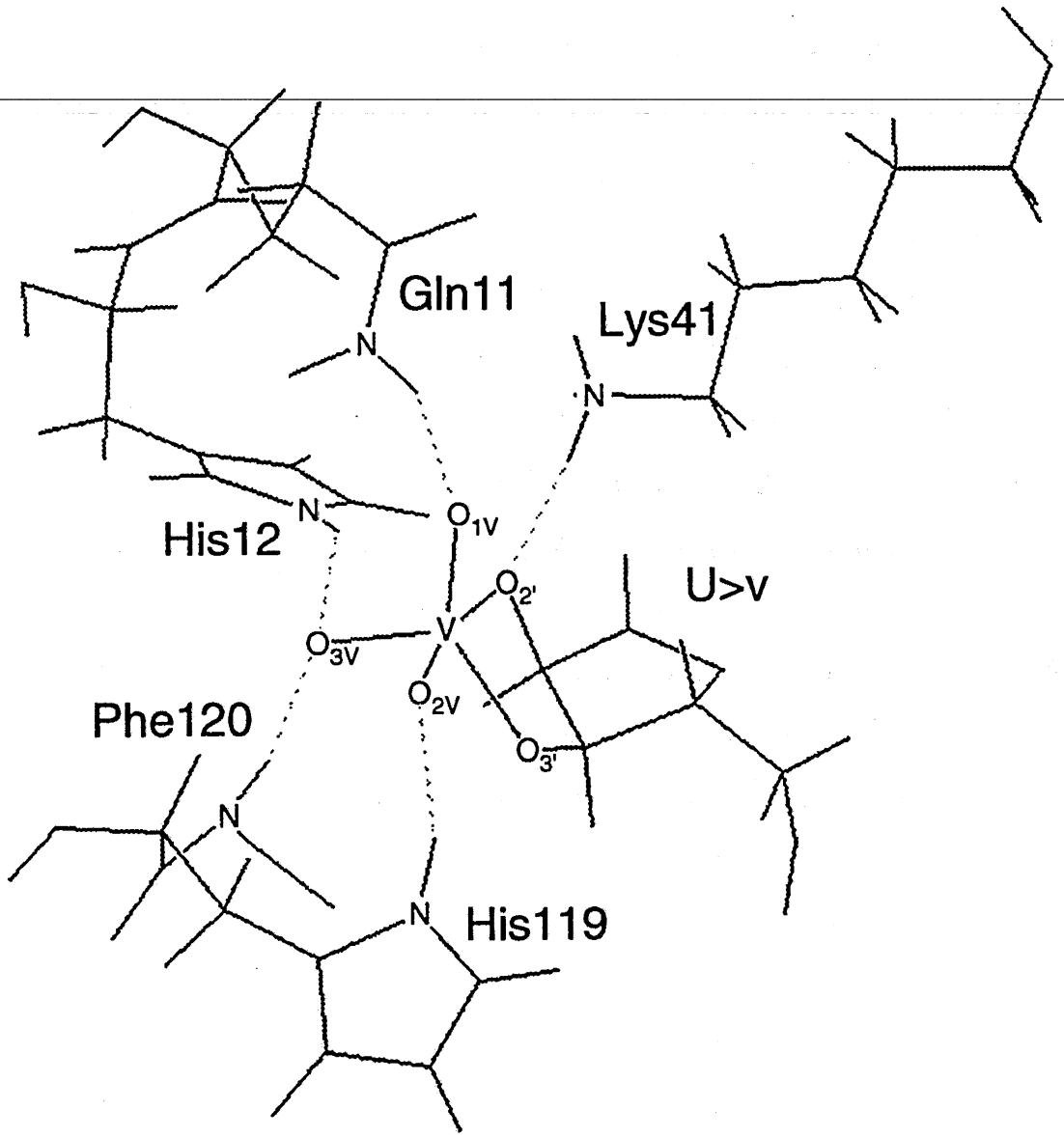
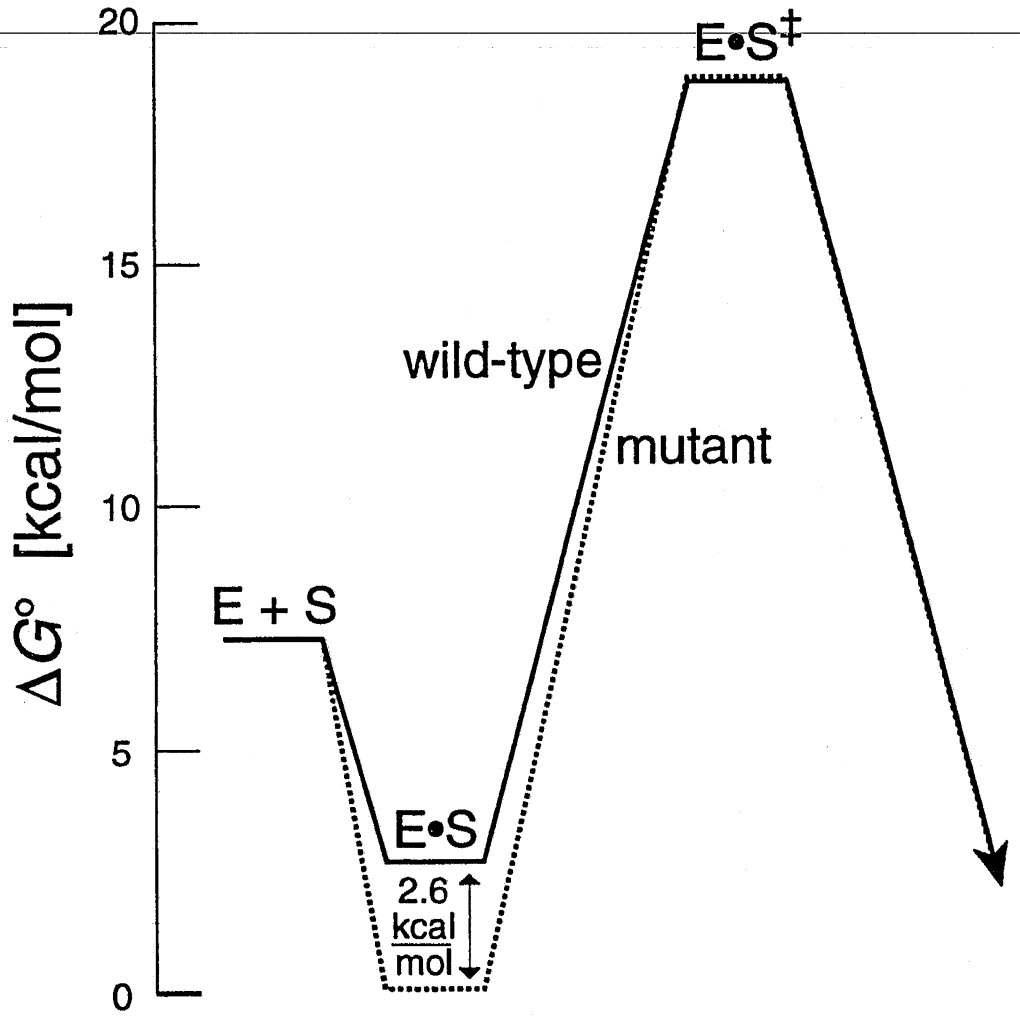

Gln11
Lys41
His12
U>v
Phe120
His119
N
O1V
O2'
V
O3V
O2V
O3'

Figure 5.3. Free energy profiles for catalysis of the transphosphorylation of $UpOC_6H_4$-*p*-NO_2 (1 M) by wild-type (—) and Q11A (---) RNase A. Profiles were constructed by using the data in Table 5.1 and assuming that $K_S = K_m$ and that $\Delta G^{\circ\ddagger} = RT\ln(k_bT/k_{cat}h)$ (Chapter 6).


20
15
10
5
0
ΔG° [kcal/mol]
E•S‡
wild-type
mutant
E + S
E•S
2.6
kcal
mol

Chapter 6

Materials and Experimental Methods

6.1 General

Materials. *S. cerevisiae* strain BJ2168 [*MAT* a prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52) was from the Yeast Genetic Stock Center (Berkeley, CA). *E. coli* strain BL21(DE3) (F- ompT r_B-m_B-) (Studier & Moffatt, 1986) was from Novagen (Madison, WI), *E. coli* strain CJ236 was from Bio-Rad (Richmond, CA), and *E. coli* strain Y1088 (Hanahan, 1983) was the generous gift of Doug Hanahan. Plasmid λgt11 was from New England Biolabs (Beverly, MA). Plasmids pC1/1 (Rosenberg et al., 1984) and pPHO5, pCB124, MP36 (Phillips et al., 1990), and pRS304 (Sikorski & Heiter, 1989) were the generous gifts of Steve Rosenberg, Tony Brake, Meg Phillips, and Robert Sikorski, respectively. Plasmid pET22B(+) was from Novagen.

All enzymes for molecular biology were from Promega (Madison, WI), except for restriction endonucleases *Pvu*II *Stu*I, *Sca*I, and *Bsp*HI, concentrated T4 DNA ligase, which were from New England Biolabs, and T4 RNA kinase which was from United States Biochemical (Cleveland, OH). [γ-^{32}P]ATP was from Amersham (Arlington Heights, IL). Reagents for DNA synthesis were from Applied Biosystems (Foster City, CA), except for acetonitrile, which was from Baxter Healthcare (McGaw, IL). Agarose was from Life Technologies (Gaithersburg, MD). Acrylamide and N, N'-methylene-bis-acrylamide were from Bio-Rad. Spectra/Por dialysis membrane (3,500 mol wt cutoff) was from Spectrum Medical Industries (Los Angeles, CA). DE52 diethylaminoethyl cellulose anion exchange resin was from Whatman (Maidstone, England), and S-Sepharose cation exchange resin was from Pharmacia LKB (Piscataway, NJ).

Bovine pancreatic ribonuclease (type III-A and X-A), toluidine blue, isopropyl-β-D-thiogalactopyranoside (IPTG), and yeast RNA were from Sigma Chemical (St.

Louis, MO). Uridyl-3',5'-adenosine (UpA) was synthesized by Jed Thompson by using the methods of Ogilvie (Ogilvie et al., 1978; Usman et al., 1987), or was from Sigma Chemical. Adenosine deaminase, O-glycosidase, and endo-F were from Boehringer Mannheim (Indianapolis, IN). Poly(A), poly(C), poly(G), poly(I), poly(U), U>p and C>p were from Sigma Chemical (St. Louis, MO). Poly(C) was also from Midland Certified Reagent (Midland, TX). $UpOC_6H_4$-*p*-NO_2 was synthesized by Tatiana Kutateledza. Polymeric substrates were precipitated from aqueous ethanol (70% v/v) before use. Bacto yeast extract, Bacto tryptone, Bacto peptone, Bacto agar, and Bacto yeast nitrogen base without amino acids were from Difco (Detroit, MI). All other chemicals were of reagent grade or better and were used without further purification.

Yeast minimal medium (SD) contained (in 1 L) Bacto yeast nitrogen base without amino acids (YNB, 6.7 g), dextrose (2% w/v), and a supplemental nutrient mix (Ausubel et al., 1989). Variations of SD medium were also used. For example, 2xS4%D–trp medium contained twice the concentrations of YNB and dextrose, and lacked tryptophan in the supplemental nutrient mix. Yeast rich medium (YEPD) contained (in 1 L) Bacto yeast extract (10 g), Bacto peptone (20 g), and dextrose (2% w/v). Yeast rich medium depleted of inorganic phosphate (YEPD–Pi) was prepared as described (Rubin, 1974). Bacterial terrific broth (TB) (Maniatis et al., 1989) contained (in 1 L) Bacto tryptone (12 g), Bacto yeast extract (24 g), glycerol (4 mL), KH_2PO_4 (2.31 g), and K_2HPO_4 (12.54 g). All media were prepared in distilled, deionized water and autoclaved.

Methods. Reactions involving restriction enzymes, T4 DNA ligase, or T4 polynucleotide kinase were performed in the buffers provided by their suppliers. Supplemental ultrapure ATP (Pharmacia) was added to reactions which required this

reagent. Other manipulations of DNA were performed as described (Ausubel et al., 1989). DNA restriction fragments were purified from bands in agarose (0.7% w/v) gels with a GeneClean II kit from BIO-101 (La Jolla, CA). DNA was sequenced with a Sequenase 2.0 kit from United States Biochemicals (Cleveland, OH). Site-directed mutagenesis was performed on single-stranded DNA isolated from *E. coli* strain CJ236 by using the method of Kunkel (Kunkel et al., 1987), and the enzyme Sequenase 2.0 for primer extension.

Oligonucleotides were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer, and purified by either Oligo Purification Cartridges (Applied Biosystems) or polyacrylamide gel electrophoresis (PAGE). Proteins were separated by PAGE performed in the presence of sodium dodecyl sulfate (SDS; 0.1% w/v), as described (Ausubel et al., 1989). Gels were fixed and stained by washing with aqueous methanol (40% v/v), containing acetic acid (10% v/v) and Coomassie Brilliant Blue (0.1% w/v). The mol wt standards were from Bio-Rad: phosphorylase B (97.4 kDa unstained; 106 kDa prestained), serum albumin (66.2; 80.0), ovalbumin (45.0; 49.5), carbonic anhydrase (31.0; 32.5); trypsin inhibitor (21.5; 27.5), and lysozyme (14.4; 18.5).

Ultraviolet and visible absorbance measurements were made with a Cary 3 spectrophotometer equipped with a Cary temperature controller. pH was measured with a Beckman pH meter fitted with a Corning electrode, calibrated at room temperature with standard buffers from Fischer (Chicago, IL). Protein sequences were determined at the University of Wisconsin Biotechnology Center, as described (Rybak et al., 1991).

Steady-state kinetics. The cleavage of UpA was measured using the adenosine deaminase coupled assay (Ipata & Felicioli, 1968). The $\Delta\varepsilon$ for the conversion of UpA

to U>p and inosine in assay buffer was -6000 $M^{-1}cm^{-1}$ at 265 nm. The cleavage of poly(U), poly(C),or poly(A) was monitored by the change in ultraviolet hyperchromicity. The $\Delta\varepsilon$ for these reactions, calculated from the difference in molar absorptivity of the polymeric substrate and the mononucleotide cyclic phosphate product, was 1360 $M^{-1}cm^{-1}$ for poly(U) at 278 nm, 2380 $M^{-1}cm^{-1}$ for poly(C) at 250 nm, and 6400 $M^{-1}cm^{-1}$ for poly(A) at 260 nm. The $\Delta\varepsilon$ for the hydrolysis of U>p was 600 $M^{-1}cm^{-1}$ at 286 nm. The $\Delta\varepsilon$ for the hydrolysis of C>p was 1450 $M^{-1}s^{-1}$ at 287 nm. The $\Delta\varepsilon$ for the cleavage of $UpOC_6H_4$-*p*-NO_2 was 4560 $M^{-1}cm^{-1}$ at 330 nm (Thompson & Raines, 1994).RNase A concentrations were determined by assuming that $A_{277.5}^{0.1\%}$ = 0.72 (Sela et al., 1957). All assays were performed at 25 °C in 0.1 M MES buffer, pH 6.0, containing NaCl (0.1 M), substrate (UpA:10 μM – 0.75 mM; poly(C), poly(U) or poly(A): 10 μM – 0.4 mM; U>p or C>p: 0.05 mM-3mM; $UpOC_6H_4$-*p*-NO_2: 1μM – 1.7 mM), and enzyme (1.0 nM – 1.0 μM). UpA assays contained adenosine deaminase (25 μL of a 22 μg/mL solution). The total reaction volume was 0.8 mL. Samples of excessive absorbance were carried out in 0.2 cm cuvettes. The values for k_{cat} and K_m were determined from the initial velocity data (8-21 independent kinetic assays) using the program HYPERO(Cleland, 1979). Representative Lineweaver-Burke plots of the initial velocity data are shown in Figure 6.1.

Molecular modeling. Molecular models of RNase A mutants were made from the coordinates of the crystalline complex of RNase A with uridine 2',3'-vanadate (Wlodawer & Lennart, 1983) with the program MIDAS PLUS (Ferrin et al., 1988). No energy minimizations were performed.

6.2 Expression and purification of recombinant RNase A

Yeast expression cassettes for RNase A cDNA. The cDNA for bovine pancreatic RNase A was constructed by R.T. Raines (Fig. 2.1)(Raines & Rutter, 1989). Two expression cassettes were designed that would direct the expression of the RNase A cDNA when carried by a yeast plasmid and propagated in the yeast *S. cerevisiae*. These cassettes differed only in the promoter used and the length of the 3'-untranslated region of the RNase A cDNA. The cassettes were constructed by assembling the *Bam*HI/*Nco*I fragment from either pMP36 (which contains the ADH2–GAPDH promoter) or pPHO5 (which contains the PHO5 promoter), the *Nco*I/*Kpn*I fragment from pCB124 (which codes for a modified α-factor leader sequence), a *Kpn*I/*Pst*I adaptor made from oligonucleotides RR9 and RR13; the *Pst*I/*Sal*I fragment from the above M13mp8 clone (which codes for RNase A), and the *Sal*I/*Bam*HI fragment from pPHO5 (which contains the GAPDH terminator). The PHO5 cassette contains 282 bp of the 3'-untranslated region of the RNase cDNA; the ADH2–GAPDH cassette contains only the 3 bp amber codon of this region.

The α-factor leader sequence encoded by the expression cassette contains two mutations: R2G (due to the creation of an *Nco*I site) and S81P (due to the creation of a *Kpn*I site). Also, the C-terminal $(Glu–Ala)_3$ sequence, which is not necessary for efficient protein secretion or processing (Brake et al., 1984; Brake, 1989), has been deleted. The expression cassette therefore codes for a leader sequence of 85 amino acids residues, including the C-terminal Lys–Arg.

Construction of pWL, YEpWL.RNase A, and YEpWL.Pi.RNase A. Plasmid pWL was constructed as follows. The 3,952 base pair (bp) *Stu*I/*Sca*I restriction fragment from pC1/1 carrying the 2μ and LEU2-d regions was isolated and joined by blunt-end ligation with the band-purified 3825 bp *Pvu*II fragment from pRS304 carrying the f1, ori, TRP1, and Amp^r regions. An undesired *Aat*II site within the pC1/1 fragment was eliminated from the resulting plasmid by digestion at the surrounding *Bsp*HI sites and ligating the complementary ends of the modified plasmid. Finally, a *Bam*HI site was positioned between the Amp^r and TRP1 regions by digestion of the plasmid with *Aat*II and insertion of a cassette made from a self-complimentary oligonucleotide, SD1 (Table 1), which contains a *Bam*HI site. This ligation destroyed the *Aat*II site.

YEpWL.RNase A and YEpWL.Pi.RNase A were constructed by the insertion of either the ADH2–GAPDH or the PHO5 *Bam*HI expression cassette into the unique *Bam*HI site of pWL. The orientation of these cassettes was determined by restriction mapping and sequence analysis.

Construction of plasmid pBXR. Plasmid pBXR (where BXR refers to Bacterial eXpression of RNase A) was constructed as follows. A fragment carrying the cDNA for RNase A that could be inserted between the *Msc*I and *Sal*I sites of pET22B(+) was generated from YEpWL.RNase A using the PCR and priming oligonucleotides SD3 and SD4. The amplified fragment was band-purified, treated with T4 DNA polymerase to remove any overhanging bases left by taq polymerase, and digested with *Sal*I. The resulting fragment has the RNase A cDNA flanked on its 5' end by two CG base pairs (which form a blunt end) and on its 3' end by a *Sal*I sticky-end. This fragment was then ligated to the band-purified *Msc*I/*Sal*I fragment of the *E. coli* expression plasmid

pET22B(+) by T4 DNA ligase. The integrity of this construct was assessed by restriction and sequence analysis.

Plate assay for RNase activity. Plate assays have been described that measure the ability of a microbe to secrete an active ribonuclease (Holloman & Dekker, 1971; Quaas et al., 1988). A plate assay was used to test the ability of pBXR, YEpWL.RNase A, and YEpWL.Pi.RNase A to direct the secretion of active RNase A. For bacteria, an aliquot (1 μL) of a culture of BL21(DE3) carrying either pBXR or pET22B(+) and grown overnight in LB medium containing ampicillin (400 mg/mL) was placed on a plate of the same medium containing Bacto agar (1.5% w/v), yeast RNA (2 mg/mL), and IPTG (1 mM). The plate was incubated for 8 h at 37 °C. For yeast, an aliquot (1 μL) of a culture of BJ2168 carrying YEpWL.RNaseA and grown overnight in S4%D–leu was placed on a plate of YEP1%D, containing Bacto agar (2% w/v) and yeast RNA (2 mg/mL). Alternatively, a culture of BJ2168 carrying YEpWL.Pi.RNaseA and grown overnight in SD–leu was placed on a plate of YEPD–Pi, containing Bacto agar (2% w/v) and yeast RNA (2 mg/mL). The plates were incubated at 30 °C for 3 days. The plates were then developed by washing with aqueous perchloric acid (10% v/v), which precipitates high mol wt RNA. Yeast that secrete active ribonuclease produced a clearing in an otherwise foggy background.

Zymogram electrophoresis. Zymogram electrophoresis is an extremely sensitive assay for ribonuclease activity (Blank et al., 1982; Ribó et al., 1991; Kim & Raines, 1993). This technique can detect the activity of 1 pg (10^{-16} mol) of RNase A. Protein samples were separated as usual by SDS-PAGE (Laemmli, 1970) except that the sample buffer contained no reducing agent and the running gel was co-polymerized

with polyC (0.5 mg/mL). After separation, proteins in the gel were renatured by washing the gel twice (for 10 min each) with 10 mM Tris•HCl buffer, pH 7.5, containing isopropanol (20% v/v) to extract the SDS, then twice (for 10 min each) with 10 mM Tris•HCl buffer, pH 7.5, and finally once (for 15 min) with 0.1 M Tris•HCl buffer, pH 7.5. The gel was then stained (for 5 min) with 10 mM Tris•HCl buffer, pH 7.5, containing toluidine blue (0.2% w/v), which binds to high mol wt nucleic acid, and then destained with water. Regions in the gel containing ribonuclease activity appeared as clear bands in a blue background.

Zymogram spot assay. A spot assay was developed to assess rapidly the ribonuclease content of fractions produced during the purification of RNase A. A protein sample (1 μL) was placed on an agarose gel (1% w/v) containing polyC (0.3 mg/mL) and 10 mM Tris•HCl buffer, pH 7.5. The gel was then incubated at 37 °C for 30 min before being stained with 10 mM Tris•HCl buffer, pH 7.5, containing toluidine blue (2 mg/mL) and destained with water. Samples containing ribonuclease activity appeared as a clear spot in a blue background.

Production and purification of RNase A from *S. cerevisiae*. RNase A was purified from S. cerevisiae strain BJ2168 harboring the plasmid YEpWL.RNase A. Transformed cells were stored as –70 °C freezer stocks in glycerol (30% w/v). Frozen cells were rejuvenated by plating onto 2xS 4%D – trp containing Bacto agar (2% w/v) and incubated at 30 °C for 2 days. A liquid culture of 2xS 4%D – trp (25 mL) was inoculated with cells from the plate and shaken at 30 °C until turbid. A liquid culture of 2xS 4%D – leu (25 mL) was then inoculated with the turbid culture (1 mL) and grown

for 24 h or until turbid. This inoculum was added to YEPD medium (1 L), which was then shaken at 30 °C for 96 h.

Cells were removed by centrifugation at 3,000 x g for 10 min, and the supernatant was concentrated to 70 mL with a Minitan™ ultrafiltration system (Millipore, MA) using a 5000 M_r cutoff polysulfone membrane. Acetone was added to the concentrate to a concentration of 60% (v/v), and the resulting precipitate was collected by centrifugation at 27,000 x g for 30 min. The precipitate was resuspended in a minimal volume of water and lyophilized. The lyophilsate was resuspended in and dialyzed exhaustively against 25 mM sodium acetate buffer, pH 5.5.

The diasylate was loaded onto a column (10 cm x 1.8 cm^2) of mono-S cation exchange resin equilibrated with the same buffer. RNase was then eluted with a linear gradient of NaCl (0.00 – 0.35 M) in 25 mM sodium acetate buffer, pH 5.5. Fractions were collected and assayed for ribonuclease activity with the zymogram spot assay. The purity of active fractions was assessed by SDS-PAGE and zymogram electrophoresis. Active fractions were divided into 2 pools, a high M_r pool, which contained RNase species with low electrophoretic mobility, and a low M_r pool, which contained RNase species having electrophoretic mobilities closer to that of native RNase A. The 2 pools were characterized independently.

Production and purification of RNase A from *E. coli*. A freezer stock was prepared of *E. coli* strain BL21(DE3) harboring plasmid pBXR (or its relevant derivitive) from a mid-log phase culture grown in LB medium containing ampicillin (400 μg/mL). This freezer stock was used to inoculate a starter culture (15 mL) of TB medium conatining ampicillin (400 μg/mL). This culture was grown to mid-log phase (A_{600} = 0.5 O.D.) and was used to inoculate a larger culture (1L) of the same medium lacking ampicillin.

The inoculated culture was shaken (250 rpm) at 37 °C until it reached late log phase (A_{600} = 1.9 O.D.), and was then induced to express the cDNA that codes for RNase A by the addition of IPTG (to 2 mM). Shaking at 37 °C was continued for 4 h, before the cells were harvested by centrifugation for 10 min at 5000 x g.

The cell pellet was resuspended in 250 mL of cell lysis buffer, which was 20 mM Tris•HCl buffer, pH 7.8, containing urea (6 M), and EDTA (1 mM), and the suspension was shaken for 20 min at 37 °C. This suspension was then centrifuged for 15 min at 30,000 x g, and the resulting pellet was resuspended in 250 mL of solubilization buffer, which was 20 mM Tris•HCl buffer, pH 7.8, containing urea (6 M), NaCl (0.4 M), DTT (20 mM), and EDTA (1 mM), and the suspension was shaken for 20 min at 37 °C. This suspension was then centrifuged for 15 min at 30,000 x g. The supernatant was collected and to it was added reduced DTT (0.22 g). The resulting solution was stirred at room temperature for 10 min. Glacial acetic acid was added to lower the pH to 5.0, and the resulting solution was dialyzed exhaustively against 20 mM Tris•AcOH buffer, pH 5.0, containing NaCl (0.1 M). The insoluble material that accumulated during dialysis was removed by centrifugation. The soluble fraction was then reoxidized by exhaustive dialysis (>24 h) against refolding buffer, which was 50 mM Tris•AcOH buffer, pH 7.8, containing NaCl (0.1 M), reduced glutathione (1 mM), and oxidized glutathione (0.2 mM). The refolded sample was then dialyzed exhaustively against 20 mM Tris•AcOH buffer, pH 8.0.

The dialyzed sample was passed through a column (15 cm x 4.9 cm^2) of SE-52 anion exchange resin equilibrated with the same buffer. The flow-through was loaded onto a column (15 cm x 1.8 cm^2) of mono-S cation exchange resin equilibrated with Tris•AcOH buffer (20 mM), pH 8.0, and the loaded column was washed with the same buffer (100 mL). RNase A was eluted with a linear gradient of NaCl (0.0 – 0.35M), in

Tris•AcOH buffer, pH 8.0. Fractions were collected and assayed for ribonuclease activity with the zymogram spot assay. The purity of active fractions was assessed by SDS-PAGE and zymogram electrophoresis. Fractions containing RNase A of >95% purity were pooled and characterized.

Detection of protein glycosylation. RNase A contains the N-glycosylation sequence (Asn34 – Leu35 – Thr36) and is produced from bovine pancreas in an assortment of glycosylated forms. To determine whether the recombinant enzyme produced from yeast was similarly glycoylated, the high and low molecular weight samples purified from yeast were analyzed by Glycotrak™ (Oxford Glycosystems, CA). Briefly, a sample was separated by SDS-PAGE and then transfered to a PVDF membrane. Protein-linked carbohydrate were then oxidatively cleaved to a dialdehyde with periodate. Aldehydes generated were then coupled to biotin hydrazine, which was subsequently bound to streptavidin-linked alkaline phosphatase. Incubation of the blot in NBT resulted in the develop of a brown color in areas where the streptavidin-alkaline phosphatase had localized.

To differentiate between N- and O-linked glycosylation 1) cell supernatants from yeast cells expressing RNase A and grown in the presence of tunicamycin (an inhibitor of N-glycosylation) were analyzed by zymogran electrophoresis and 2) RNase A(H) was treated with endoglycosidase-F (EC 3.4.1.96), which removes N- linked sugars, and and O-glycosidase (EC 3.2.1.97), which removes O-linked sugars, and analyzed by zymogram electrophoresis (Fig. 2.4). Decreases in apparent mol wt as a result of any of the above treatments was interpreted as a loss of sugar moities.

6.3 Interdependence of specificity and efficiency in catalysis by

RNase A

Construction of Thr45, Phe120, and Asp83 mutants. The cDNA that codes for RNase A was carried by the vector pBXR, and was expressed in *E. coli* under the control of the T7 RNA polymerase promoter (Chapter 1). Oligonucleotide-mediated site-directed mutagenesis (Kunkel et al., 1987) of plasmid pBXR was used to introduce a unique and translationally silent *Nhe*I site into the cDNA for RNase A on the 3' side of the codon for Thr45, resulting in plasmid pBXR1. The codon for Thr45 was then randomized by cassette-mediated saturation mutagenesis (Reidhard-Olson et al., 1991) of the *Cla*I/*Nhe*I fragment in pBXR1. The codon for Phe120 was randomized similarly by cassette muatgenesis of the *Bsi*WI/*Sal*I in pBXR1. The oligonucleotides used were SD9 and SD10 for Thr45All, and SD11 and SD12 for Phe120All (Table 6.1). Individual clones from the Thr45All and Phe120All cDNA libraries were sequenced to discern the randomness of codons 45 and 120.

Mutants D83A and T45G/D83A were created by oligonucleotide-directed site-directed mutagenesis of plasmids pBXR1 and pBXR1.T45G, respectively. Oligonucleotide SD13 was used as the mutagenic primer. The sequence of each of the constructs was confirmed by sequencing the mutant cDNA.

Screen for altered specificity. Culture medium from *E. coli* BL21(DE3) cells expressing the cDNA that codes for Thr45All or Phe120All was screened by zymogram electrophoresis (Blank et al., 1982; Ribó et al., 1991; Kim & Raines, 1993) for production of an enzyme able to cleave poly(A), poly(G), or poly(I). Culture medium (15 μL) was separated by non-reducing SDS-PAGE in a running gel co-polymerized with poly(A), poly(C), poly(G), or poly(I) (0.5 mg/mL). After electrophoresis,

proteins in the gel were renatured by washing the gel (for 2 x 10 min) with 10 mM Tris•HCl buffer, pH 7.5, containing isopropanol (20% v/v) to extract the SDS, and then (for 2 x 20 min) with 10 mM Tris•HCl buffer, pH 7.5. The gel was stained (for 5 min) with 10 mM Tris•HCl buffer, pH 7.5, containing toluidine blue (0.2% w/v), which binds tightly to high mol wt nucleic acid, and then destained with water. Regions in the gel containing ribonuclease activity appear as clear bands in a blue background.

The mutant enzymes from the Thr45All library that were responsible for the observed poly(A) cleaving activity were identified by using a zymogram spot assay. Zymogram spot assays involved placing samples (1 μL) of culture medium from individual clones on an agarose gel (1% w/v) containing poly(A) (0.3 mg/mL) and 10 mM Tris•HCl buffer, pH 7.5. The gel was then incubated at 37 °C for 4 h before being stained as above. Plasmid DNA from any clones showing poly(A) cleaving activity was isolated and sequenced. Active enzymes were purified to homogeneity from the insoluble fraction of cell lysates as described (Section 6.2).

Thermodynamic Cycles. Thermodynamic cycles were calculated from values of k_{cat}, K_m, and k_{cat}/K_m for the cleavage of poly(U) by wild-type, D83A, T45G, and T45G/D83A RNase A. The change in free energy ($\Delta\Delta G$) for each side of a thermodynamic box was calculated from the equation

$$\Delta\Delta G = RT\ln f$$

where f was the ratio of a particular kinetic parameter for the two enzymes at each corner of that side of the box. These calculations were made assuming that the transmission coeficients (described by the frequency terms for the formation and

brakedown of the chemical transition state) are similar for both the wild-type and the mutant enzymes. The change in frec energy for the binding of the chemical transition state by RNase A was calculated from k_{cat}/K_m, for the apparent binding of the ground state from $1/K_m$ (assuming that $K_S = K_m$), and for the chemical activation energy from k_{cat}. The free energy of interaction ($\Delta\Delta G_{int}$) between Asp83 and Thr45 was

$$\Delta\Delta G_{int} = \Delta\Delta G_{wt \rightarrow T45G/D83A} - \Delta\Delta G_{wt \rightarrow T45G} - \Delta\Delta G_{wt \rightarrow D83A}$$

as described previously (Mildvan et al., 1992).

6.4 Structural determinants of enzymatic processivity

.c3. **^{31}P NMR assay for processivity.** NMR assays were performed at 25 °C in 0.1 M MES buffer, pH 6.0, containing NaCl (0.1 M), RNA (5.0 mM), and enzyme (1.0 – 50 μM). Each reaction was monitored for 50 min. Free induction decays were obtained on a Bruker AM400 spectrophotometer in 10 mm NMR tubes having D_2O inserts using parameters: 4854 Hz spectral width, 90° pulsewidth, 1.69 s acquisition time, 3.2 s relaxation time, 64 scans. The free induction decays were subjected to fourier transformation with a line broadening of 5 Hz. The resulting spectra were phased with the program FELIX (Hare Research; Bothell, WA). Chemical shift values were recorded relative to 0.1 M H_3PO_4.

Distraction assay for processivity. An order-of-addition experiment was used to determined whether preincubation with unlabeled RNA could distract T45A, T45G, or wild-type RNase A from degrading [5'-^{32}P]labeled RNA. Unlabeled substrate was

exposed to enzyme for time **t'** to allow any processive complex to form. [5'-^{32}P]Labeled substrate was then added, and the mixture was incubated for an additional time **t** before being quenched. The molar ratio of enzyme:labeled substrate:unlabeled substrate was 1:10:10. Assays were performed in 0.1 M MES buffer, pH 6.0, containing NaCl (0.1 M), and were quenched by 10-fold dilution into 95% v/v formamide containing xylene cyanol (0.05% w/v). The reaction products were separated by electrophoresis in a gel of polymerized acrylamide [7.5% w/v in 90 mM Tris•H_3BO_3 buffer, pH 7.6, containing EDTA (2 mM) and urea (8 M)], and visualized by autoradiography.

6.5 Glutamine-11 and catalysis by RNase A

Construction of Q11H, Q11N, and Q11A RNase A. The codon for glutamine-11 of RNase A, CAG, was changed to that of histidine,CAT, asparagine, AAC, and alanine, GCG, by oligonucleotide mediated site-directed mutagenesis of plasmid pBXR1 using oligonucleotides SD-7, 8, and 9 (Table 6.1). Sequenase version 2.0 was used for primer extension. The complete cDNA of each mutant was confirmed by sequencing. The resulting plasmids are termed pBXR.Q11H, pBXR.Q11N, and pBXR.Q11A. Mutant enzymes Q11H, Q11N, and Q11A, were purified from BL21(DE3) cells harboring plasmids pBXR.Q11H, pBXR.Q11N, and pBXR.Q11A as described (Section 6.2).

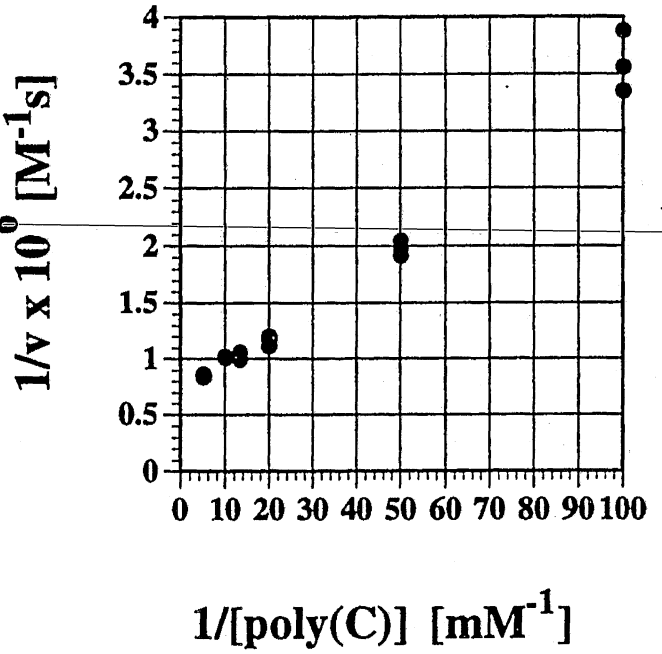
Calculation of the free energy profile for the cleavage of UpO-C_6H_4-*p*-NO_2. The calculation of the free energy of activation of a chemical process in solution, $\Delta G^{\ddagger}$, using transition state theory requires knowledge of the frequency terms that describe the

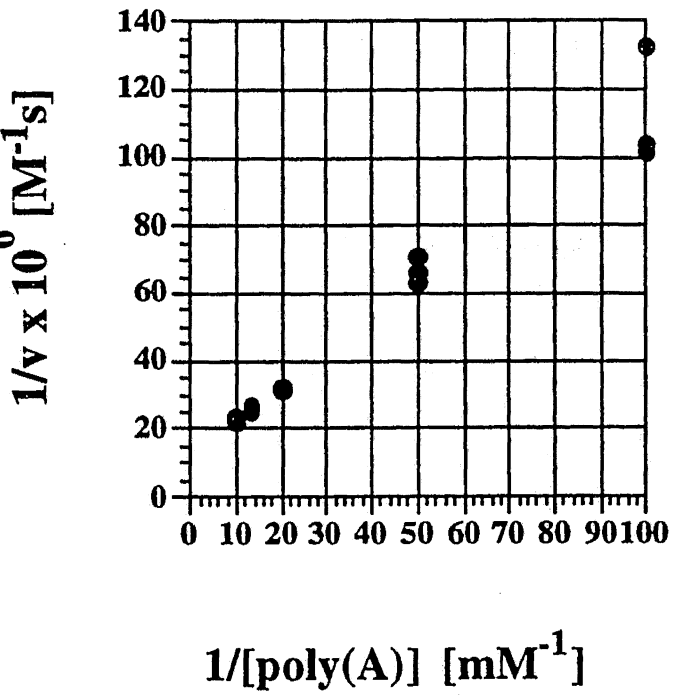
formation and breakdown of the chemical transition state. As chemical transition states in solution have not yet been observed, no experimental information is available for these frequency terms. Calculation of such free energies relies on several assumptions some of which may not be reasonable. In the calculation of the $\Delta G^{\ddagger}$ in Figure 5.3, we assumed that the transmision coeficient (described the frequency terms for the formation and breakdown of the chemical transition state) was unity (Fersht, 1985), and that $\Delta G^{\ddagger} = RT\ln (k_bT/k_{cat}h)$, where k_b is the Boltzmann constant and h is the Planck constant. Thus, the actual $\Delta G^{\ddagger}$ may not be truly represented. The key to the free energy profile in Figure 5.3, however, is the change in the free energy differences, $\Delta\Delta G$, between the wild-type and the mutant enzyme. These $\Delta\Delta G$s were calculated as described above for the thermodynamic cycles and rely on the same more reasonable assumption that the transmision coeficients for catalysis by the wild-type and mutant enzyme were equal.

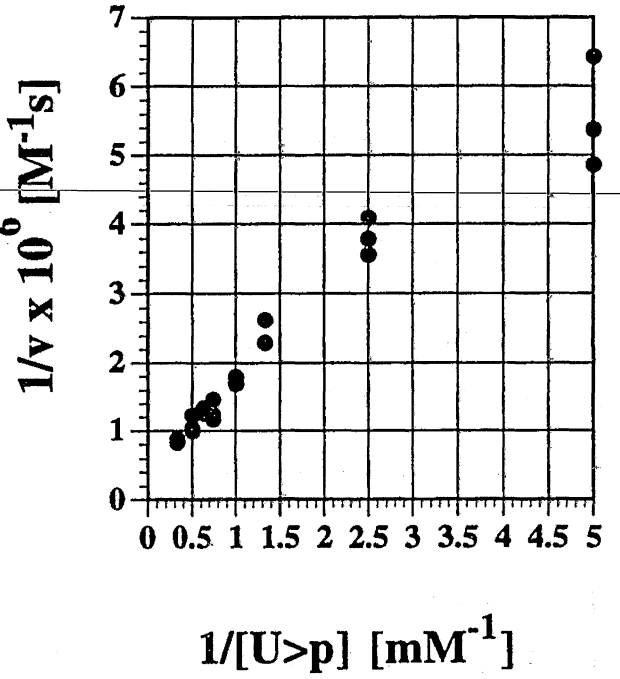
Table 6.1 DNA Oligonucleotides for Cloning, Expression, and Mutation of the cDNA that Codes for RNase A.

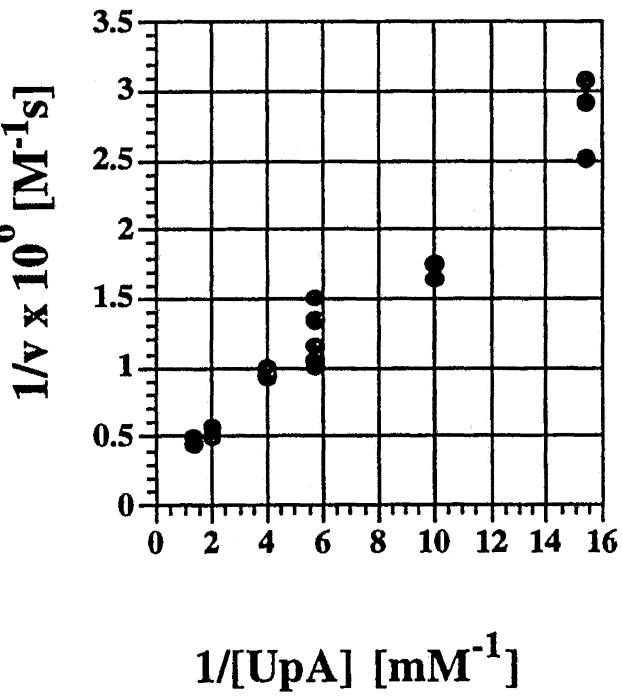
name	sequence (5'→3')	function
JHH16	TTGCCATTGCAGCTGGCAATCGTGG	destroy *Pst*I site
RR1	AACTACTGCAACCAGATGATGAA GAGCAGGAACCTGACCAAGGAC	probe
RR9	CTTTGGATAAAAGAAAGGAAACTGCA	*Kpn*I/*Pst*I adaptor
RR13	GTTTCCTTTCTTTTATCCAAAGGTAC	*Kpn*I/*Pst*I adaptor
RR16	AACACCATGGGATTTCCTT	create *Nco*I site
SD1	GCGGATCCGCACGT	*Bam*HI linker/adaptor
SD2	CGTGCGGATCTCTGGTATGGT	destroy *Bam*HI site
SD3	CCAAGGAAACTGCAGCAGCC	5'PCR primer for RNase A
SD4	GGCCTTAGGTCGACTACTACACTGAAGC	3'PCR primer for RNase A
SD5	CACTACCGGTTCCCCATTTAG	destroy *Kpn*I site
SD6	GCTGGAGTCCATATGATGCCGCTCAAACTT	Q11H, create *Nde*1 site
SD7	GCTGGAGTCCATATGGTTCCGCTCAAACTT	Q11N, create *Nde*1 site
SD8	GCTGGAGTCCATATGCGCCCGCTCAAACTT	Q11A, create *Nde*1 site
SD9	CGATGCAAGCCAGTGAACNN(G/C)TTTGTG CACGAGTCG	Thr45All cassette (coding)
SD10	CTAGCGACTCGTGCACAAA(G/C)NNGTTCAC TGGCTTGCAT	Thr45All cassette (noncoding)
SD11	CTAGCGACTCGTGCACAAA(G/C)NNGTTCACT GGCTTGCAT	Phe120All cassette (coding)
SD12	TCGACTACACTGAAGCATC(G/C)NNGTGGAC TGGCAC	Phe120All cassette (noncoding)
SD13	GGTCTCACGGCACGCGGTGATGCTCAT	D83A, no new site

Figure 6.1 Double reciprocal plots of the initial velocity data for the cleavage of poly(C), poly(A), UpA, and the hydrolysis of U>p by wild-type RNase A.


1/v x 10^6 [M^-1s]
4
3.5
3
2.5
2
1.5
1
0.5
0
0 10 20 30 40 50 60 70 80 90 100
1/[poly(C)] [mM^-1]


1/v x 10^6 [M^-1s]
140
120
100
80
60
40
20
0
0 10 20 30 40 50 60 70 80 90 100
1/[poly(A)] [mM^-1]


1/v x 10^6 [M^-1s]
0
1
2
3
4
5
6
7
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5
1/[U>p] [mM^-1]


1/v x 10^6 [M^-1s]
0
0.5
1
1.5
2
2.5
3
3.5
0 2 4 6 8 10 12 14 16
1/[UpA] [mM^-1]

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