RIBONUCLEASE A HOMOLOGS: CATALYSIS AND CYTOTOXICITY

by

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A dissertation submitted in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

(Biochemistry)

at the

UNIVERSITY OF WISCONSIN–MADISON

2006



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i

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Bovine pancreatic ribonuclease (RNase A) has been a popular model system in studies of protein structure and function. Lately, several homologs of RNase A have been found to display interesting biological activities. Onconase (ONC), an amphibian ribonuclease, and bovine seminal ribonuclease (BS-RNase) are selectively toxic to cancer cells. Basic biochemistry as well as biomedical applications of these proteins is an attractive subject for biochemists. This dissertation describes advances in our understanding of ONC and BS-RNase.

Chapter Two describes the function of the active-site residues of ONC. The contribution of the active-site residues to catalysis is studied by using a novel fluorogenic substrate; their contribution to cytotoxicity is also investigated. The results provide information on the origin of low catalytic activity of ONC.

In Chapter Three, the molecular basis for the substrate specificity of ONC is described, along with the first three dimensional structure of an ONC•nucleic acid complex. These findings could be used in developing ONC variants with enhanced cytotoxicity.

Chapter Four describes the contribution of inhibitor evasion to ribonuclease-mediated cytotoxicity. BS-RNase is cytotoxic because its dimeric structure enables it to evade the ribonuclease inhibitor protein (RI) present in the cytosol. We created BS-RNase variants that evade RI even as a monomer based on previous *in silico* analyses. Some of these variants had greater cytotoxicity than any known RNase A variant or homolog including onconase, a benchmark toxin now in clinical trials as a cancer therapeutic.

Together, this dissertation provides new insights into the mechanism of the catalysis and cytotoxicity of ONC and BS-RNase. The progress made in this work advances both basic scientific understanding and medical applications of these enzymes.

Acknowledgments

This work would not have been possible without help of many people. First, I would like to thank my thesis advisor, Ron Raines, for opportunity to work for him on many exciting projects. I especially thank him for providing me an utmost freedom in developing my research projects. Thanks to him, I learned how to think creatively and critically as a scientist. I also thank him for putting up with my irregular working hours.

I appreciate help from many members of the Raines laboratory. I thank Bryan Smith for his friendship and advice on various aspects of my projects. Brian Miller was a great mentor. His sincere attitude towards science was tremendous influence on me. I am grateful to Betsy Kersteen for reading many of my crude manuscripts. She is a great writer and editor. I thank Chiwook Park for inspiring me to come to Madison for graduate study and helping me adjust to the unfamiliar American culture during my first several months here. I appreciate many contributive discussions from Steve Fuchs, Tom Rutkoski, Jeet Kalia, and Jeremy Johnson. I thank Parit Plainkum, Kenneth Woycechowsky, and Kimberly Dickson for their assistance and advice.

I am grateful to my present and former committee, including Professors George Phillips, Mike Cox, Marv Wickens, Sam Butcher, John Markley, and Pete Belshaw for their advice on my research projects. I especially thank Professor George Phillips, Euiyoung Bae, and Craig Bingman for the solution of onconase structure. I appreciate Darrell McCaslin's assistance in the Biophysics Instrumentation Facility.

I would like to thank my parents and my brother for supporting and encouraging me to pursue a scientific career 7000-miles away from home. I am grateful to Jiwon for her

iii

love, friendship, and continuous support. Last 18 months we spent together in Madison was an unforgettable experience. I also thank the members of the Korean Biology Club, especially Jaeyoung Yun and Kyounwon Kim, for many interesting discussions about biology and their friendship. Finally, I am grateful to the NIH and the Steenbock Predoctoral Fellowship for their financial support.

Table of Contents

Abstract		i
Acknowledgments		iii
Table of Contents		v
List of Figures		vii
List of Tables		ix
List of Abbreviations		X
Chapter One		
Introduction	••••••	1
1.1 Overview	· · · · · · · · · · · · · · · · · · ·	2
1.2 Onconase		
1.3 Mechanism of Ononase-Mediated Cytotox	icity	7
1.4 Bovine Seminal Ribonuclease		
1.5 Conclusions		
Chapter Two		
Function of the Active-Site Residues of Onconas	e	20
2.1 Abstract		
2.2 Introduction	·····	
2.3 Experimental Procedures	· · · · · · · · · · · · · · · · · · ·	
2.4 Results	·····	
2.5 Discussion		
2.6 Conclusions		

Chapter Three

te Speci	ficity of	Onconase	• • • • • • • • • • • • •	••••••	
•••••			••••••	•••••	56
•••••	•••••		••••		57
••••••	• • • • • • • • • • • • • • •	••••••	••••	•••••	
	•••••		••••	•••••	63
	· · · · · · · · · · · · · · · ·		••••	•••••	69
	•••••				
Ribonu	clease: N	Ionomers	<i>versus</i> Din	ners	83
		• • • • • • • • • • • • • • • • • • • •		•••••	
		, *		••••••	85
			•••••		
•••••••••			•••••		
•••••	•••••		••••		
•••••		• • • • • • • • • • • • • • • • • • • •	••••		
		· · · · · · · · · · · · · · · · · · ·	••••		108
	te Speci	te Specificity of Ribonuclease: N	te Specificity of Onconase	Ribonuclease: Monomers versus Dim	Ribonuclease: Monomers versus Dimers

vi

List of Figures

Figure 1.1	Flow of chemical information in biology12
Figure 1.2	Three-dimensional structures and sequence alignment of RNase A,
	BS-RNase, and Onconase [®] 14
Figure 1.3	Structure of the crystalline porcine ribonuclease inhibitor protein16
Figure 1.4	Putative mechanism of ribonuclease-mediated cytotoxicity
Figure 2.1	Structure and function of Onconase [®] 41
Figure 2.2	Circular dichroism spectra of wild-type Onconase [®] and its variants43
Figure 2.3	Chemical structure of the substrate 6-carboxyfluorescein-
	dArUdGdA-6-carboxytetramethylrhodamine45
Figure 2.4	Chemical structure of the ligand 6-carboxyfluorescein-dAdUdGdA47
Figure 2.5	Binding isotherms for wild-type Onconase [®] and its variants with the
	ligand 6-FAM-dAdUdGdA49
Figure 2.6	Effect of Onconase [®] , its variants, and ribonuclease A on the
	proliferation of human leukemia cell line K-56251
Figure 2.7	Thermodynamic cycle of $k_{\text{cat}}/K_{\text{M}}$ for the cleavage of 6-FAM–
	dArUdGdA-6-TAMRA by Onconase® upon replacing Pca1 or Lys9
	(or both) with alanine
Figure 3.1	Amino acid sequence and structure of Onconase [®]
Figure 3.2	Structure and electron density diagram of 9-methylguanine and 9-
	methyladenine

Figure 4.1	Amino acid sequences and three-dimensional structures of RNase A		
	and BS-RNase102		
Figure 4.2	Effect of BS-RNase, its variants, RNase A, and ONC on the		
	proliferation of cultured K-562 cells104		
Figure 4.3	Putative mechanism for the cytotoxic activity of wild-type BS-RNase. 106		

List of Tables

Table 2.1	Values of $k_{\text{cat}}/K_{\text{M}}$, m/z , and T_{m} for ONC and its Variants	40
Table 3.1	Summary of Data Collection and Refinement Statistics	76
Table 3.2	Values of k_{cat}/K_M , m/z , and IC ₅₀ for ONC and its Variants	77
Table 3.3	Values of <i>K</i> _i for ONC and its Variants	78
Table 4.1	Properties of Wild-Type BS-RNase and its Dimeric and Monomeric	
	Variants	100
Table 4.2	Molecular Charges of RNase A and its Monomeric Homologs	101

List of Abbreviations

3	extinction coefficient
6-FAM	6-carboxyfluorescein
6-TAMRA	6-carboxytetramethylrhodamine
5'-AMP	adenosine 5'-monophosphate
5'-GMP	guanosine 5'-monophosphate
BIS-TRIS	bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane
BS-RNase	bovine seminal ribonuclease
CCD	charge-coupled device
CD	circular dichroism
ddH ₂ O	distilled deionized water
DNA	deoxyribonucleic acid
DTT	dithiothreitol
<e< td=""><td>pyroglutamic acid</td></e<>	pyroglutamic acid
E	enzyme
EDTA	ethylenediaminetetraacetic acid
EF	elongation factor
F	fluorescence
FBS	fetal bovine serum
FPLC	fast performance liquid chromatography
FRET	fluorescence resonance energy transfer
GSH	reduced glutathione

GSSG	oxidized glutathione
h	hour
HCl	hydrochloric acid
HeLa	an immortal cell line derived from cervical cancer cells
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HIV	human immunodeficiency virus
hRI	human ribonuclease inhibitor
I	inhibitor
IPTG	isopropyl-1-thio-β-D-galactopyranoside
<i>k</i> _{cat}	first-order enzymatic rate constant
K _d	equilibrium dissociation constant
kDa	kilodalton
Ki	inhibitor dissociation constant
K _m	Michaelis constant
LB	Luria–Bertani broth
MALDI-TOF	matrix-assisted laser desorption ionization-time-of-flight
MEPEG	polyethylene glycol monomethyl ether
MES	2-(N-morpholino)-ethanesulfonic acid
min	minute
mRNA	messenger ribonucleic acid
MW	molecular weight
MWCO	molecular weight cutoff

xi

NaCl	sodium chloride
NMR	nuclear magnetic resonance
OD	optical density
ONC	Onconase [®] (a registered trademark of Alfacell, Inc)
OVS	oligo(vinylsulfonic acid)
PBS	phosphate-buffered saline
PCA	pyroglutamic acid
PCR	polymerase chain reaction
PDB	protein data bank
PEG	polyethylene glycol
pK _a	log of the acid dissociation constant
PVS	poly(vinylsulfonic acid)
RC-RNase	ribonuclease from Rana catesbeiana
RI	ribonuclease inhibitor protein
RMSD	root-mean-square deviation
RNA	ribonucleic acid
RNase A	unglycosylated bovine pancreatic ribonuclease
RNase T1	ribonuclease from Aspergillus oryzae
rRNA	ribosomal ribonucleic acid
S	second
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA	small interfering RNA

t	time
TB	terrific broth
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
tRNA	transfer ribonucleic acid
UpA	Carboxyfluorescein-dArUdAdA-6-carboxytetramethylrhodamine
UpG	Carboxyfluorescein-dArUdGdA-6-carboxytetramethylrhodamine

xiii

Chapter One

1

Introduction

Portions of this chapter will be published as a review solicited by *BioDrugs*: Lee, J. E. and Raines, R. T. (2007) Engineering ribonucleases as novel cancer therapeutics. *BioDrugs*, in preparation.

1.1 Overview

RNA functions as a medium between DNA and protein in the flow of biological information (Figure 1.1). Thus, the action of genes is modulated by the synthesis and degradation of RNA. Accordingly, intervening in the metabolism of RNA can be an attractive therapeutic strategy. For example, the degradation of mRNA by the RNA interference machinery can serve as the basis for a new class of drugs against diverse diseases (Mahanthappa, 2005; Bumcrot *et al.*, 2006). Sequence-specific antisense oligonucleotides that form hybrids with RNA are being explored as potential chemotherapeutics for the treatment of cancer, inflammatory disorders, and cardiovascular diseases (Kurreck, 2003). Ribozymes (RNA enzymes) capable of cleaving RNA *in trans* are being pursued as novel therapy for HIV infection (Rossi, 2000).

In addition, there are a growing number of secretory ribonucleases that display potential therapeutic utility. These ribonucleases are homologs of ribonuclease A (RNase A), which has been known for its remarkable ability to catalyze the cleavage of RNA. Onconase (ONC), a recently discovered homolog from an amphibian source, is in clinical trials for the treatment of malignant mesothelioma. Bovine seminal ribonuclease (BS-RNase) is endowed with antitumoral and antiviral effects. Our understanding of these ribonuclease toxins is still incomplete. A greater understanding gained from the structure–function studies described in this thesis could aid in their development as chemotherapeutics.

In this Chapter, I review the literature on ONC and BS-RNase. Chapter Two describes the function of the active-site residues of ONC. In Chapter Three, the crystal

structure of ONC•nucleic acid complexes is described, along with the molecular basis for the substrate specificity of ONC. Chapter Four describes the contribution of the quaternary structure of BS-RNase and consequent inhibitor evasion in cytotoxicity. Overall, Chapters Two, Three, and Four present a molecular mechanism for the catalytic activity and cytotoxic activity of ONC and BS-RNase, and provide principles that can be used to develop ribonucleases with enhanced cytotoxicity.

1.2 Onconase

Onconase (ONC) was isolated originally from the oocytes of the Northern leopard frog, *Rana pipiens* (Darzynkiewicz *et al.*, 1988). ONC belongs to the bovine pancreatic ribonuclease (RNase A; EC 3.1.27.5) superfamily of vertebrate proteins. RNase A, the prototype of the superfamily from bovine pancreas, was the first enzyme whose aminoacid sequence was determined and the third whose three-dimensional structure was solved. RNase A was also the subject of much early research on protein folding. Indeed, four researchers were awarded the Nobel Prize in chemistry for their landmark works on this enzyme¹. Lately, much of the interest in the field has shifted toward the members of the superfamily that display interesting biological activities. For example, human RNase 2 (eosinophil-derived neurotoxin (EDN)) and human RNase 3 (eosinophil cationic protein (ECP)) possess neurotoxic and antiviral activity. Angiogenin is a plasma protein

¹ The 1972 Nobel Prize for Chemistry was awarded jointly to Christian Anfinsen, Stanford Moore, and William Stein for their contribution in understanding of protein folding, and the connection between protein structure and catalytic activity. In 1984, Bruce Merrifield was awarded the Nobel Prize for Chemistry for the development of chemical synthesis on a solid matrix.

that enhances the formation of new blood vessels, and bovine seminal ribonuclease exhibits immunosuppressive and antitumoral activities. ONC and other ribonucleases from amphibian source are selectively toxic to tumors.

History. Antitumoral activity in early embryo extracts from Rana pipiens was discovered in the 1970's². It was determined a decade later that most of the activity can be attributed to a major basic protein (ONC). Further studies showed that ONC was both cytotoxic and cytostatic toward cultured tumor cells and caused the regression of xenograft tumors in mice (Darzynkiewicz et al., 1988). The complete amino acid sequence of ONC was determined in 1991 (Ardelt et al., 1991), and its three-dimensional structure was reported 3 years later (Mosimann et al., 1994). Cytotoxic amphibian ribonucleases are mainly localized with yolk proteins in the oocytes (Liao and Wang, 1994). It has been postulated that ONC is synthesized in the liver of female frogs in a seasonal manner, followed by secretion into the blood and its deposit in the maturing oocytes (Chen et al., 2000). ONC has been speculated to play physiological roles in host defense (Liao and Wang, 1994). Currently, ONC in combination with doxorubicin is in Phase III clinical trials for the treatment of unresectable malignant mesothelioma, an asbestos-related lung cancer (Pavlakis and Vogelzang, 2006). The trials recently obtained "fast-track" designation from the U.S. Food and Drug Administration (FDA). ONC is also in Phase I/II trials for the treatment of non-small cell lung cancer (NSCLC) (Mikulski et al., 1995). In addition, ONC has been shown to be effective for the treatment

² Presented at the 27th Annual Eastern Colleges Science Conference, Pennsylvania State University, April 28, 1973 (Shogen, K. and Yoan, W. K., unpublished study).

of multi-drug resistance cancers (Rybak *et al.*, 1996; Schein, 1997). Renal toxicity is the major side effect in ONC therapy (Vasandani *et al.*, 1999; Costanzi *et al.*, 2005).

Biochemical Characteristics. Like other members of the RNase A superfamily, ONC catalyzes the cleavage of P–O^{5°} bond on the 3° side of a pyrimidine base of an RNA molecule. The active site of ONC contains the catalytic triad (His10, Lys31, and His97) that is characteristic of the superfamily, implying that ONC possesses a catalytic mechanism similar to that of RNase A. Besides the catalytic triad, ONC seems to possess two additional active-site residues: Lys9 and an N-terminal pyroglutamic acid (Pca1). The ribonucleolytic activity of ONC is important for its cytotoxicity. Decrease in ribonucleolytic activity leads to a concomitant reduction in cytotoxicity (Ardelt *et al.*, 1991). Chapter Two describes the contribution of these active-site residues to the function of ONC.

Although ONC resembles RNase A in tertiary structure and possesses the key catalytic residues, the ribonucleolytic activity of ONC is five orders-of-magnitude lower than that of RNase A (Leland *et al.*, 2000). NMR and molecular dynamic simulation studies have shown that ONC has an extremely rigid β -sheet (Gorbatyuk *et al.*, 2004; Merlino *et al.*, 2005). This rigidity could deter an "induced fit" necessary for substrate binding and catalysis (Koshland, 1958). As described in Chapter Two, the binding of a single-stranded nucleic acid to ONC is 10³-fold weaker than to RNase A. Chapter Three describes attempts to enhance the weak catalytic with rational design.

Another interesting feature of ONC is its extraordinary conformational stability. ONC is thermally hyperstable ($T_m = 87$ °C at pH 7) and resists degradation by various

proteases (Notomista *et al.*, 2000). The exceptional conformational stability of ONC is due largely to it tethered C-terminus, created by a C-terminal disulfide bond (Leland *et al.*, 2000). This synapomorphic C-terminal disulfide bond is conserved in amphibian ribonucleases but absent from the mammalian homologs. The elimination of the Cterminal disulfide bond dramatically decreases the conformational stability of ONC. It has been reported that the blocked N-terminus created by the hydrogen bond network also contributes to the conformational stability of the enzyme (Notomista *et al.*, 2000). The conformational stability of ONC is critical for its cytotoxicity — the variants of ONC with reduced conformational stability are only partially cytotoxic (Leland *et al.*, 2000; Notomista *et al.*, 2000).

Substrate Specificity. The substrate specificity of ONC can be considered on two levels. On the nucleobase level, ONC prefers to cleave the phosphodiester bond on the 5' side of a guanine nucleobase. This preference is in marked contrast to that of RNase A, which does not differentiate between guanine and adenine at this position (unpublished results). In terms of the identity of the RNA substrate in the cell, it has been reported that tRNA is the main target for ONC (Saxena *et al.*, 2002). A recent study demonstrated that the cleavage sequence in tRNA is the guanosine–guanosine bond in the variable loop or D-arm (Suhasini and Sirdeshmukh, 2006). The basis for this substrate specificity has been largely unknown, due to the lack of structural information on the ONC–substrate interaction. Chapter Three describes the solution of the structure of ONC•nucleic acid complex, along with an analysis of the molecular basis for the substrate specificity of ONC.

1.3 Mechanism of onconase-mediated cytotoxicity

As a cytotoxin, onconase (ONC) is administered extracellularly. To exert an antitumoral effect, ONC must reach the cytosol and there cleave RNA substrates. The generally accepted mechanism of ONC-mediated cytotoxicity is divided into two major stages: cytosolic internalization and catalytic degradation of RNA.

7

Cytosolic Internalization. The first step for the cytosolic internalization of ONC is its binding to the cell surface. The existence of an unidentified ONC receptor on the cellular membrane has been reported (Wu *et al.*, 1993), but other findings contradict the existence of such a receptor (Haigis and Raines, 2003). The cell surface is negatively charged due to the phosphoryl and sulfuryl groups of carbohydrates and phospholipids. It is probable that the highly cationic character of ONC (calculated pI = 9.7) facilitates its nonspecific binding to the cell surface through favorable Coulombic interaction. It is known that random or rational cationization of RNase A increases its cellular uptake (Futami *et al.*, 2001). In addition, ONC does not bind to cells deficient in enzymes for heparan sulfate proteoglycan synthesis (unpublished results from our laboratory).

After binding to the cell surface, ONC is internalized by energy-dependent endocytosis. Small-molecule inhibitors that block ATP synthesis and metabolic inhibitors negate ONC cytotoxicity (Haigis *et al.*, 2003). A comprehensive mechanism for this endocytosis is unknown. Nonetheless, it appears that the productive endocytic pathway is the dynamin-independent one, because HeLa cells expressing the dominant-negative

dynamin mutant are more susceptible to ONC than are cells expressing wild-type dynamin.

It is not understood how a hydrophilic ONC crosses the lipid bilayer. To facilitate successful entry into the cell, the protein toxins diphtheria toxin and ricin contain two distinct domains: a translocation domain and a catalytic domain. These domains dissociate upon cytosolic entry. In contrast, ONC is a hyperstable, single-domain protein, which is believed to remain intact during its endocytosis. It is difficult to pinpoint precisely where ONC translocates to the cytosol. Drugs that disrupt retrograde transport from the Golgi to the endoplasmic reticulum (ER) potentiate the cytotoxicity of ONC (Wu *et al.*, 1995; Haigis *et al.*, 2003). These results imply that the Golgi is an inefficient site for the translocation of ONC, unlike for that of diphtheria toxin and ricin. Hence, it can be concluded that ONC reaches the cytosol by translocation from a pre-ER compartment.

Degradation of Cellular RNA and Induction of Apoptosis. Once ONC reaches the cytoplasm, ONC degrades cellular RNA. ONC is a unique ribonuclease in that it completely evades the cytosolic ribonuclease inhibitor protein (RI). RI is a 50-kD protein present in every surveyed mammalian cell. RI is composed of 15 leucine-rich repeats (LRRs) (Figure 1.2), a motif that often participates in protein—protein interactions (Kajava, 1998). RI binds to certain members of the RNase A superfamily with femtomolar affinity, and renders them inactive. The complex formed by RI and ribonuclease is among the tightest known in biology. The crystalline structure of the porcine RI•RNase A complex reveals a vast surface area (2900 Å²) buried at the complex

interface (Kobe and Deisenhofer, 1995; Kobe and Deisenhofer, 1996). The ability of ONC to evade RI seems to be a critical factor for its cytotoxic activity. It has been demonstrated that non-cytotoxic RNase A become cytotoxic by incorporating residues that enable RI evasion (Leland *et al.*, 2001). Moreover, the cytotoxicity of the RNase A variants was shown to correlate with their RI-evading ability (Rutkoski *et al.*, 2005).

ONC predominantly cleaves tRNA in the cell, leaving rRNA and mRNA undamaged (Saxena *et al.*, 2002). The basis for this specificity is poorly understood, but it is possible that proteins associated with rRNA an mRNA protect them from ONC cleavage. The susceptibility of non-coding RNA such as microRNA or small interfering RNA (siRNA) to ONC cleavage is unknown.

Degradation of cellular RNA by ONC inhibits protein synthesis in the cell, and leads to apoptosis (Deptala *et al.*, 1998; Iordanov *et al.*, 2000). The ONC-induced apoptosis pathway in HeLa cells is initiated with the activation of the stress-activated c-Jun N-terminal kinase (JNK), followed by the activation of caspase-9, which activates the executioner caspases-3 and -7. Caspase-8 or the tumor suppressor protein p53 are not required in this pathway (Iordanov *et al.*, 2000).

Basis for Therapeutic Index. ONC is more toxic to tumor cells than to normal cells *in vivo* and *in vitro*. The mechanism for this selectivity is unknown. A promising hypothesis is that ONC is selectively internalized by tumor cells. Tumor cells are more negatively charged than are homologous normal cells (James *et al.*, 1956). The amount of sialic acid-rich ganglioside is greater and phosphoplipid contents are altered in certain tumor cells (Fredman, 1993; Kojima, 1993). The elevated anionic character of tumor cells

would likely enhance the interaction with highly cationic ONC. Other hypotheses include a different and more efficient intracellular routing of ONC to the cytosol in tumor cells, and a greater susceptibility of rapidly growing tumor cells to RNA degradation.

1.4 Bovine seminal ribonuclease

History. In 1967, ribonucleolytic activity was found in bull seminal fluid (Farina *et al.*, 1967). This activity was ascribed to bovine seminal ribonuclease (BS-RNase), a homolog of RNase A. BS-RNase shares 83% amino acid sequence identity with RNase A. Unlike RNase A, which is catalytically active only towards single-stranded RNA, BS-RNase is additionally capable of cleaving double-stranded RNA and the RNA strand in DNA•RNA hybrids (Libonati and Floridi, 1969; Taniguchi and Libonati, 1974). The enzymatic parameters of BS-RNase are indistinguishable from that of RNase A (Irie and Hosokawa, 1971; Floridi *et al.*, 1972). The physiological function of BS-RNase is thought to suppress immune responses from the female organism toward noneself sperm cells (Tamburrini *et al.*, 1990). BS-RNase was the first RNase A homolog shown be cytotoxic (Dostal and Matousek, 1973). Moreover, BS-RNase displays a diverse array of biological activities including aspermatogenetic, embryotoxic, antiviral, immunosuppressive, and antitumoral activity. BS-RNase has been tested for the treatment of multi-drug resistant cancers and thyroid carcinomas (Cinatl *et al.*, 1999; Kotchetkov *et al.*, 2001).

Quaternary Structure and Cytotoxicity of BS-RNase. The most notable feature of BS-RNase is its quaternary structure. BS-RNase is the only member of the RNase A superfamily that exists in nature as a dimer, here, a homo-dimer (Figure 1.2C). The dimer is cross-linked by two disulfide bonds between Cys31 of one subunit and Cys32 of the other. Due to its dimeric structure, BS-RNase is resistant to RI (Murthy and Sirdeshmukh, 1992). The cytotoxic activity of BS-RNase is largely the result of its RIevading capability, because the monomeric BS-RNase is inhibited by RI and not cytotoxic (Vescia *et al.*, 1980; Murthy and Sirdeshmukh, 1992; Kim *et al.*, 1995)

The antitumoral activity of BS-RNase is significantly lower than that of ONC *in vivo* and *in vitro* (Matousek *et al.*, 2003), even though BS-RNase possesses much greater ribonucleolytic activity. One hypothesis to explain the puzzling observation is that the intersubunit disulfide bonds of BS-RNase are severed in the reducing environment of the cytoplasm. Consequently, the resulting monomers will be inhibited by RI and lose cytotoxicity. In Chapter Four, this hypothesis is tested using *in silico*-designed BS-RNase variants capable of evading RI as monomers. These variants actually surpassed ONC in cytotoxic activity.

1.5 Conclusions

ONC and BS-RNase are promising antitumoral therapeutic candidates. Understanding the biochemistry of these cytotoxic ribonucleases is critical for the development of these chemotherapeutic agents. This dissertation provides new insights into the mechanism of the catalysis and cytotoxicity of ONC and BS-RNase. The progress made in this work advances both basic scientific understanding and medical applications of these enzymes.

Figure 1.1Flow of chemical information in biology. RNA serves as an active
medium between DNA and protein. Ribonucleases can be useful
chemotherapeutics because their degradation of RNA renders genetic
information indecipherable.



Three-dimensional structures and sequence alignment of RNase A, BS-Figure 1.2 RNase, and Onconase[®]. (A) Photograph of the Northern leopard frog, Rana pipiens. (B) Amino acid sequence alignment of RNase A, BS-RNase, and ONC. Helices, sheets, and turns are indicated by h, s, and t. < E denotes a pyroglutamate residue. The catalytic triad of the superfamily is in black boxes. Additional active-site residues in ONC are in red boxes. Residues conserved among the three ribonucleases are in gray boxes. Cysteine residues that form intramolecular disulfide bonds are in yellow boxes. Cysteine residues in BS-RNase that participate in intermolecular disulfide linkages are in green box. Cysteine residues that form the synapomorphic disulfide bond in ONC are in orange boxes. (C) Structurefunction diagram of RNase A, ONC, and BS-RNase. The model protein RNase A is an efficient catalyst but not cytotoxic. In contrast, ONC is a potent cytotoxin despite its low catalytic activity. Lastly, BS-RNase is cytotoxic because of its unique quaternary structure. Yet, BS-RNase is only modestly cytotoxic. Ribbon diagrams of the proteins were created with the programs MOLSCRIPT (Avatar Software AB, Stockholm, Sweden) and RASTER3D (Merritt and Murphy, 1994).



hhhhhhhh hhh hhhhhhh hhhhtttt sssssss h hhhhhhhhh ssssttt KETAAAKFER OHMOSSTSAA SSSNYCNIMM KSRNLTKORC KPVKITVHES LADKOVCSQ KNVACKNGQ KESAAAKFER OHMOSGNSPS SSSNYCNIMM KRKNTQKKC KPVKITVHES LADKAVCSQ KKVTCKNGQ DWLT-RC KEITNTRDVD -----CDNIM STNL--FHC KDKWIIYSR PEPIKAIC-K GIIASKNVI 70 RNase A BS-RNase 70 60 ONC sssss ss ssssssttt ttttsssss ss ssssss stttt sss sss NCYQSYSTMS ITTCRETGSS KYPNCAYKTT QANKHIIVAC EGNPYVINE DASV--NCYQSKSTMR ITTCRETGSS KYPNCAYKTT QVEKHIIVAC GGKPSVINE DASV--T----EFY LSDCNVT--S R-P-CKYKLK KSTNKFVTC ENQA--NE VGVGS 124 RNase A BS-RNase 124 ONC VGVGS 104

Α

В

С

RNase A EFFICIENT CATALYST NO CYTOTOXICITY



ONC INEFFICIENT CATALYST POTENT CYTOTOXICITY



BSR EFFICIENT CATALYST **MODEST CYTOTOXICITY** Figure 1.3 Structure of the crystalline porcine ribonuclease inhibitor protein (PDB entry 1BNH (Kobe and Deisenhofer, 1996)). The ribbon diagram was created with the program MOLSCRIPT (Avatar Software AB, Stockholm, Sweden) and RASTER3D (Merritt and Murphy, 1994).



Putative mechanism of ribonuclease-mediated cytotoxicity. (A) Ribonuclease first binds to the surface of the target cell. The cationic character of ribonucleases facilitates interaction with the anionic cell surface. (B) Ribonuclease is internalized by energy-dependent endocytosis and crosses a lipid bilayer, probably from an endosome. (C) Certain ribonucleases evade the cytosolic RI and are thus active catalyst in the cytosol (ONC). Cleavage of cellular RNA by these ribonucleases leads to apoptosis. (D) On the contrary, ribonucleases such as wild-type RNase A bind RI tightly. Inhibited ribonucleases are not able to degrade cellular RNA, averting apoptosis.

Figure 1.4



Chapter Two

Function of the Active-Site Residues of Onconase

Portions of this chapter were published as:

Lee, J. E. and Raines, R. T. (2003) Contribution of active-site residues to the function of onconase, a ribonuclease with antitumoral activity. *Biochemistry* **42**, 11443–11450.

2.1 Abstract

Onconase (ONC), a homolog of ribonuclease A (RNase A), is in clinical trials for the treatment of cancer. ONC possesses a conserved active-site catalytic triad, which is composed of His10, Lys31, and His97. The three-dimensional structure of ONC suggests that two additional residues, Lys9 and an N-terminal lactam formed from a glutamine residue (Pca1), could also contribute to catalysis. To determine the role of Pca1, Lys9, and Lys31 in the function of ONC, site-directed mutagenesis was used to replace each with alanine. Values of k_{cat}/K_{M} for the variants were determined with a novel fluorogenic substrate, which was designed to match the nucleobase specificity of ONC and gives the highest known k_{cat}/K_{M} value for the enzyme. The K9A and K31A variants display 10³fold lower k_{cat}/K_M values than the wild-type enzyme, and a K9A/K31A double variant suffers a $>10^4$ -fold decrease in catalytic activity. In addition, replacing Lys9 or Lys31 eliminates the antitumoral activity of ONC. The side chains of Pca1 and Lys9 form a hydrogen bond in crystalline ONC. Replacing Pca1 with an alanine residue lowers the catalytic activity of ONC by 20-fold. Yet, replacing Pca1 in the K9A variant enzyme does not further reduce catalytic activity, revealing that the function of the N-terminal pyroglutamate residue is to secure Lys9. The thermodynamic cycle derived from k_{cat}/K_M values indicates that the Pca1...Lys9 hydrogen bond contributes 2.0 kcal/mol to the stabilization of the rate-limiting transition state during catalysis. Finally, binding isotherms with a substrate analog indicate that Lys9 and Lys31 contribute little to substrate binding, and that the low intrinsic catalytic activity of ONC originates largely
from the low affinity of the enzyme for its substrate. These findings could assist the further development of ONC as a cancer chemotherapeutic.

2.2 Introduction

Ribonuclease A (RNase A; EC 3.1.27.5) was perhaps the most studied enzyme of the 20th century (Cuchillo *et al.*, 1997; Raines, 1998) Although RNase A is still a popular model system for enzymologists and protein chemists, much interest in RNase A has shifted to its variants and homologs that have remarkable biological activities (Youle and D'Alessio, 1997; Leland and Raines, 2001; Matousek, 2001; Makarov and Ilinskaya, 2003). For example, several frog homologs of RNase A are endowed with potent antitumoral and antiviral activity. The ability of these ribonucleases to enter cells and cleave cellular RNA leads to apoptosis (Saxena *et al.*, 2002; Haigis and Raines, 2003). One of these frog ribonucleases, Onconase[®] (ONC), is in Phase III clinical trials for the treatment of unresectable malignant mesothelioma, an asbestos-related lung cancer (Mikulski *et al.*, 2002).

ONC is an 11.8-kDa protein from the oocytes and early embryos of the Northern leopard frog, *Rana pipiens* (Ardelt *et al.*, 1991). Although ONC and RNase A have 30% amino acid sequence identity and a similar three-dimensional structure (Ardelt *et al.*, 1991; Mosimann *et al.*, 1994), the ribonucleolytic activity of ONC with known substrates is 10^4 - to 10^5 -fold lower than that of RNase A. This low ribonucleolytic activity appears to be paradoxical, as the ribonucleolytic activity of ONC is essential for its cytotoxicity, yet RNase A is not cytotoxic (Wu *et al.*, 1993). Apparently, the low catalytic activity of ONC is offset by other attributes (Dickson *et al.*, 2003), including its ability to evade the cytosolic ribonuclease inhibitor protein (RI) (Wu *et al.*, 1993; Haigis *et al.*, 2003) and its extraordinary conformational stability ($T_m = 87 \text{ °C}$) (Leland *et al.*, 2000; Notomista *et al.*, 2001).

The active site of ONC lies in the cleft of its kidney shape (Mosimann *et al.*, 1994). This active site contains the catalytic triad that is characteristic of the RNase A superfamily, preserved as His10, Lys31, and His97 in ONC (*cf.*: His12, Lys41, and His119 in RNase A (Kartha *et al.*, 1967)). Two other residues, Pca1 and Lys9, are conserved in the active sites of frog but not mammalian ribonucleases (Beintema *et al.*, 1997; Irie *et al.*, 1998). The N-terminal pyroglutamic acid (Pca or \leq E) of ONC is an uncommon residue found in a variety of proteins and hormones (Busby *et al.*, 1987; Fischer and Spiess, 1987; Bateman *et al.*, 2001), including human RNase 4 and RNase 5 (*i.e.*, angiogenin). A pyroglutamate residue is a lactam formed by the spontaneous or enzyme-catalyzed cyclization of an N-terminal glutamine with the loss of ammonia (Lechan *et al.*, 1986; Bateman *et al.*, 2001). In crystalline ONC, Pca1 and Lys9 are linked by a hydrogen bond (Figure 2.1).

Little is known about catalysis by ONC. The few previous studies have employed heterogeneous substrates and variants of ONC having an additional N-terminal methionine residue, which lies near the active site and precludes the cyclization of Gln1 to form Pca1 (Boix *et al.*, 1996; Newton *et al.*, 1998). Here, we have produced active-site variants of ONC without any additional residues at the N-terminus. We have also developed a novel homogeneous substrate for ONC that has allowed us to measure

kinetic parameters accurately, even for ONC variants of low catalytic activity. We use these tools to address the following mechanistic issues: (1) How do the active-site lysine residues of ONC contribute to transition-state and ground-state binding? (2) What is the role in catalysis of the hydrogen bond between Pca1 and Lys9? and (3) What is the origin of the low ribonucleolytic activity of ONC?

2.3 Experimental Procedures

Materials. Human RI (as RNasin[®]) was from Promega (Madison, WI). 6-Carboxyfluorescein–dArUdAdA–6-carboxytetramethylrhodamine (6-FAM–dArUdAdA– 6-TAMRA), 6-FAM–dArUdGdA–6-TAMRA, and 6-FAM–dAdUdGdA were from Integrated DNA Technology (Coralville, IA). 2-(N-Morpholino)ethanesulfonic acid (MES) was from Sigma Chemical (St. Louis, MO). MES was purified further by anionexchange chromatography prior to its use so as to eliminate oligo(vinylsulfonic acid), which is a potent inhibitor of ribonucleases (Smith *et al.*, 2003). [methyl-³H]Thymidine was from PerkinElmer Life Sciences (Boston, MA). Phosphate-buffered saline (PBS) contained (in 1 liter) 0.20 g of KCl, 0.20 g of KH₂PO₄, 8.0 g of NaCl, and 2.16 g of Na₂HPO₄·7H₂O. All other chemicals and reagents were of commercial grade or better, and were used without further purification.

K-562 cells, which derive from a continuous human chronic myelogenous leukemia line, were from the American Type Culture Collection (Manassas, VA). Cell culture medium and supplements were from Invitrogen (Carlsbad, CA).

Instruments. Mass was measured by matrix-assisted laser desorption ionization-timeof-flight (MALDI-TOF) mass spectrometry using a Voyager-DE-PRO Biospectrometry Workstation Applied Biosystems, Foster City, CA) and a 3,5-dimethoxy-4hydroxycinnamic acid (sinapinic acid) matrix (Sigma Chemical). Fluorescence measurements were performed with a QuantaMaster 1 Photon Counting Fluorometer equipped with sample stirring (Photon Technology International, South Brunswick, NJ). Fluorescence anisotropy measurements were made with a Beacon 2000 Fluorescence Polarization System (Panvera, Madison, WI). Circular dichroism (CD) experiments were performed with a Model 62A DS CD spectrophotometer (Aviv, Lakewood, NJ) equipped with a temperature controller. Radioactivity was quantitated with a Microbeta TriLux liquid scintillation and luminescence counter (PerkinElmer, Wellesley, MA).

Production of ONC and its Variants. Wild-type ONC was produced in *E. coli* with pONC, a pET-22b(+)-based vector described previously (Leland *et al.*, 1998). DNA encoding variants of ONC was made from pONC with the QuikChange[®] Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA). All vectors encoded a *pelB* leader sequence at the 5' end of the ONC coding sequence. The *pelB* leader sequence directs proteins to the periplasmic membrane of *E. coli*, where the sequence is removed by *pelB* peptidase. This expression system enabled us to produce enzymes without any additional amino acids at the N-terminus. *E. coli* strain BL21(DE3) transformed with pONC or plasmid encoding variants of ONC was grown to an OD of 1.8 at 600 nm in terrific broth medium containing ampicillin (0.40 mg/mL). Expression of the ONC cDNA was induced by addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG; to 0.5 mM). Cells were

collected 4 h after induction and lysed with a French pressure cell. Inclusion bodies were recovered by centrifugation and resuspended for 2 h in 20 mM Tris-HCl buffer, pH 8.0, containing guanidine-HCl (7 M), dithiothreitol (DTT; 0.10 M), and EDTA (0.010 M). The protein solution was diluted 10-fold with aqueous acetic acid (0.020 M), subjected to centrifugation to remove any precipitate, and dialyzed overnight against aqueous acetic acid (0.020 M). Any precipitate was removed again by centrifugation. The supernatant was diluted to a protein concentration near 0.5 mg/mL in a refolding solution of 0.10 M Tris-HCl buffer, pH 8.0, containing NaCl (0.10 M), reduced glutathione (0.001 M), and oxidized glutathione (0.00020 M). ONC was refolded for 16 h and concentrated by ultrafiltration with a YM10 membrane (10,000 M_r cut-off; Millipore, Bedford, MA). Concentrated ONC was applied to a Superdex G-75 gel filtration FPLC column (Pharmacia, Piscataway, NJ) in 0.050 M sodium acetate buffer, pH 5.0, containing NaCl (0.10 M) and NaN₃ (0.02% w/v). Protein from the major A_{280} peak was collected and applied to a Mono S cation-exchange FPLC column (Pharmacia, Piscataway, NJ). ONC was eluted from the column with a linear gradient of NaCl (0.2–0.3 M) in 0.050 M sodium acetate buffer, pH 5.0. Protein concentration was determined by UV spectroscopy using $\varepsilon = 0.87 \text{ mL mg}^{-1} \text{ cm}^{-1}$ at 278 nm (Leland *et al.*, 1998).

Assays of Ribonucleolytic Activity. Ribonucleolytic activity was measured with a hypersensitive assay based on the cessation of fluorescence quenching (Kelemen *et al.*, 1999). Briefly, the increase of fluorescence at 515 nm was measured upon adding enzyme to 0.020 M MES–NaOH buffer (pH 6.0) containing NaCl (0.010 M), 6-FAM– dArUdGdA–6-TAMRA (50 nM), and human RI (0.1–1.6 nM) at 23 ± 2 °C. The addition

of RI eliminates artifacts that could arise from unintentional contamination by RNase A or its human homolog, which bind tightly to RI. Values for k_{cat}/K_{M} were calculated with the equation:

$$k_{\rm cat}/K_{\rm M} = \left(\frac{(\Delta F/\Delta t)}{F_{\rm max} - F_0}\right) \frac{1}{[\rm E]}$$
(Eq. 2.1)

In Equation 2.1, $\Delta F/\Delta t$ is the initial slope of the reaction, F_0 is the initial fluorescence intensity, F_{max} is the fluorescence intensity once the reaction is brought to completion, and [E] is the concentration of the enzyme.

Assays of Thermal Stability. CD spectroscopy was used to assess the thermal stability of wild-type ONC and its variants (Leland *et al.*, 2000). A solution of ONC (0.2 mg/mL in PBS) was heated from 25 to 95 °C in 1-°C increments, and the change in molar ellipticity at 204 nm was monitored after a 6-min equilibration at each temperature. CD spectra were fitted to a two-state model for denaturation to determine the value of $T_{\rm m}$.

Thermodynamic Cycle. A thermodynamic cycle was constructed from the values of k_{cat}/K_M for the cleavage of 6-FAM–dArUdGdA–6-TAMRA by wild-type ONC, and its <E1A, K9A, and <E1A/K9A variants. The effect of a particular mutation on the change in the contribution of free energy ($\Delta\Delta G$) was determined with Equation 2.2:

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

(Eq. 2.2)

In Equation 2.2, *R* is the gas constant, *T* is temperature in K, and *f* is the ratio of the $k_{\text{cat}}/K_{\text{M}}$ values for the two enzymes being compared (delCardayré and Raines, 1995; Radzicka and Wolfenden, 1995). The free energy of the interaction between Pca1 and Lys9 ($\Delta\Delta G_{\text{int}}$) was calculated with Equation 2.3 (Mildvan *et al.*, 1992):

$$\Delta \Delta G_{\text{int}} = \Delta \Delta G_{\text{wt} \to \leq E1A/K9A} - \Delta \Delta G_{\text{wt} \to K9A} - \Delta \Delta G_{\text{wt} \to \leq E1A}$$
(Eq. 2.3)

Assays of Nucleic Acid Binding. The ability of ONC and its variants to bind to singlestranded DNA was assessed by fluorescence anisotropy (Fisher *et al.*, 1998; Park *et al.*, 2001). All measurements were carried out at 23 ± 2 °C. Protein (~20 mg) was dissolved in 190 µL of 0.020 M MES–NaOH buffer (pH 6.0) containing NaCl (0.010 M). Half of the protein solution was mixed 1:1 with buffer solution in a new test tube. Serial dilutions were made so as to prepare protein solutions with a wide range of concentrations. 6-FAM–dAdUdGdA (5 µL of a 20 nM solution) was added to each dilution. After 30 min, the fluorescence anisotropy at 520 nm was measured. Anisotropy (*A*) was defined by the equation:

$$A = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$
(Eq. 2.4)

In Equation 2.4, I_{\parallel} and I_{\perp} are the emission components that are parallel and perpendicular to the polarized excitation, respectively. Values of the equilibrium dissociation constant (K_d) were obtained by fitting the anisotropy values at each protein concentration to

Equation 2.5, which describes the binding of 6-FAM–dAdUdGdA to a single site on a ribonuclease:

$$A = \frac{(\Delta A)[\text{Protein}]}{K_{\text{d}} + [\text{Protein}]} + A_{\text{min}}$$
(Eq. 2.5)

Values of K_d were obtained by a nonlinear least-squares analysis, using the program DELTAGRAPH 4.0 (DeltaPoint, Monterey, CA).

Assays of Cytotoxic Activity. The effect of ONC, its variants, and RNase A on cell proliferation was determined by measuring the incorporation of [methyl-³H]thymidine into cellular DNA (Leland *et al.*, 2000; Dickson *et al.*, 2003; Haigis *et al.*, 2003). K-562 cells were grown in RPMI 1640 medium (Moore *et al.*, 1967) containing fetal bovine serum (10% v/v), penicillin (100 units/mL), and streptomycin (100 µg/mL). Cytotoxicity studies were performed using asynchronous log-phase cultures grown at 37 °C in a humidified incubator containing $CO_2(g)$ (5% v/v). To assay toxicity, cells (95 µL of a solution of 5×10^4 cells/mL) were incubated with a 5-µL solution of a ribonuclease or PBS in the wells of a 96-well plate. Cells were then grown for 44 h. Cell proliferation was monitored with a 4-h pulse of [methyl-³H]thymidine (0.25 µCi/well). Cells were harvested onto glass fiber filters using a PHD Cell Harvester (Cambridge Technology, Watertown, MA). Filters were washed with water and dried with methanol, and their ³H content was quantitated with liquid scintillation counting.

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2.4 Results

Production of ONC and its Variants. The yields of purified ONC and its variants were \geq 30 mg per L of culture and comparable to that from a previous study (Leland *et al.*, 2000). Purified proteins appeared as a single band after electrophoresis in a polyacrylamide gel in the presence of sodium dodecyl sulfate (data not shown) and had the expected mass to within 0.05% according to MALDI–TOF mass spectrometry (Table 2.1).

Development of a Novel Substrate for ONC. Despite ONC sharing a similar three-dimensional structure with RNase A (Figure 2.1), the ribonucleolytic activity of ONC is 10^4 - to 10^5 -fold lower than that of RNase A using conventional substrates (Boix *et al.*, 1996). The low activity of ONC makes measuring accurate kinetic parameters of low-activity variants problematic. 6-FAM–dArUdAdA–6-TAMRA, was designed to match the nucleobase specificity of RNase A, and is the best known substrate for RNase A (Kelemen *et al.*, 1999) and its homolog angiogenin (Leland *et al.*, 2002). This substrate contains a single ribonucleotide embedded within three deoxyribonucleotides. RNase A and its homologs do not catalyze the cleavage of DNA (Brown and Todd, 1952; Richards and Wyckoff, 1971). Hence, 6-FAM–dArUdAdA–6-TAMRA is cleaved by RNase A and angiogenin only between its uridine and adenosine residues.

Unlike RNase A and angiogenin, ONC and other frog ribonuclease homologs prefer to cleave the P– $O^{5'}$ bond of RNA between uridine and guanosine residues (Okabe *et al.*, 1991; Boix *et al.*, 1996; Leu *et al.*, 2003). Accordingly, 6-FAM–dArUdGdA–6-TAMRA was designed such that ONC could efficiently cleave the P– $O^{5'}$ bond between the uridine

and guanosine residues (Figure 2.3). This design was successful, as the $k_{cat}/K_M = 2.5 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ value for cleavage of 6-FAM–dArUdGdA–6-TAMRA by wild-type ONC (0.10 M MES–NaOH buffer, pH 6.0, containing 0.10 M NaCl) was at least 10^2 -fold greater than that with known substrates (including 6-FAM–dArUdAdA–6-TAMRA) under similar conditions. The use of 6-FAM–dArUdGdA–6-TAMRA enables convenient continuous assays of ONC as well as the acquisition of accurate k_{cat}/K_M values for variants with low ribonucleolytic activity.

Although 6-FAM–dArUdGdA–6-TAMRA is an optimized substrate for ONC, the ribonucleolytic activity of ONC is still much less than that of RNase A. Under the same conditions (0.10 M MES–NaOH buffer, pH 6.0, containing 0.10 M NaCl; 23 ± 2 °C), the value of k_{cat}/K_M for the cleavage of 6-FAM–dArUdGdA–6-TAMRA by ONC is 10^3 -fold less than that for the cleavage of 6-FAM–dArUdAdA–6-TAMRA by RNase A (data not shown).

Ribonucleolytic Activity. As listed in Table 2.1, K9A ONC and K31A ONC were 10^3 -fold less active catalysts than wild-type ONC. The K9A/K31A variant did not have any measurable activity, even with 6-FAM–dArUdGdA–6-TAMRA as the substrate (k_{cat}/K_M < 10 M⁻¹s⁻¹). Replacing Pca1 with an alanine residue resulted in an enzyme, <E1A ONC, which was 20-fold less active than wild-type ONC. <E1A/K9A ONC had 10^3 -fold less activity than the wild-type enzyme.

In RNase A, residue 9 is a glutamine rather than a lysine. K9Q ONC was created in an attempt to increase the low ribonucleolytic activity of the wild-type enzyme. The

 k_{cat}/K_{M} value of K9Q ONC was, however, 10³-fold less than that of wild-type ONC (Table 2.1).

The <E1A/K9A variant of ONC lacks the amino group of Lys9 and has low ribonucleolytic activity. Adding a residue to the N-terminus of this variant could in theory put an amino group in approximately the same position as the missing amino group of Lys9 (*cf*.: Figure 2.1). The presence of Gly(-1) did not, however, increase the k_{cat}/K_M value of <E1A/K9A ONC (data not shown), suggesting that the α -amino group of Gly(-1) in this variant is not in a position to enhance catalysis.

Thermal Stability. The decrease in the catalytic activity of the ONC variants could be due to a decrease in conformational stability. Accordingly, thermal denaturation studies were performed on each variant. The $T_{\rm m}$ value of the variants did not vary by more than 10 °C from that of the wild-type enzyme ($T_{\rm m} = 85$ °C in PBS). These $T_{\rm m}$ values (Table 2.1) along with CD spectra (Figure 2.2) suggest that the overall structure of each variant is similar to that of wild-type ONC.

Thermodynamic Cycle. Pca1 and Lys9 form a hydrogen bond in crystalline ONC (Figure 2.1). To discern the free energy of this interaction, the k_{cat}/K_M values for wild-type ONC and its <E1A, K9A, and <E1A/K9A variants were used to construct a thermodynamic cycle for the cleavage of 6-FAM–dArUdGdA–6-TAMRA (Figure 2.7). The side chain of Lys9 contributes 4.4 kcal/mol to catalysis, whereas that of Pca1 contributes 1.9 kcal/mol. The near zero value of $\Delta\Delta G_{K9A\rightarrow}$ <E1A/K9A = -0.09 kcal/mol indicates that Pca1 does not contribute to catalysis if Lys9 is absent from the active site.

Finally, the value of $\Delta\Delta G_{int} = -2.0$ kcal/mol reveals that the hydrogen bond between Pca1 and Lys9 stabilizes the rate-limiting transition state during catalysis by 2.0 kcal/mol.

Nucleic Acid Binding. Ribonucleases bind to single-stranded DNA but do not cleave this nucleic acid (Brown and Todd, 1952; Richards and Wyckoff, 1971). Hence, binding to single-stranded DNA can be used to assess the affinity of a ribonuclease for its substrate (Fisher *et al.*, 1998; Park *et al.*, 2001). Isotherms for the binding to 6-FAM– dAdUdGdA (Figure 2.4), which contains the same nucleobases as the 6-FAM– dArUdGdA–6-TAMRA substrate, were obtained by measuring fluorescence anisotropy, and are shown in Figure 2.5. Unexpectedly, these data reveal that replacing Lys9 or Lys31 (or both) with alanine had little effect on the affinity of ONC for a single-stranded nucleic acid, even in a solution of low salt concentration (0.010 M NaCl) that enables the manifestation of Coulombic interactions.

Cytotoxicity Activity. The toxicity of each ribonuclease was measured with the K-562 human leukemia cell line. Wild-type ONC had an IC₅₀ value of 0.8 μ M (Figure 2.6), which is similar to IC₅₀ values reported previously (Leland *et al.*, 2000; Dickson *et al.*, 2003; Haigis *et al.*, 2003). Like wild-type RNase A, the K9A, K31A, and K9A/K31A variants of ONC were not cytotoxic at protein concentrations of \leq 50 μ M.

2.5 Discussion

ONC was discovered 15 years ago (Darzynkiewicz *et al.*, 1988). Since then, more effort has been made to understand its biological actions than its enzymology. We believe that

further development of ONC as an antitumoral drug would benefit from a detailed understanding of its catalysis of RNA cleavage.

The three-dimensional structure of ONC is known (Mosimann et al., 1994), though not in a complex with a nucleic acid. We have used its well-studied homolog, RNase A, as a guide for important active-site residues (Cuchillo et al., 1997; Leland et al., 1998). Although ONC and RNase A possess only 30% amino acid sequence identity, their overall three-dimensional structures are remarkably similar (Figure 2.1; C^{α} RMSD = 1.7 Å (Shindyalov and Bourne, 1998)). A structural comparison of ONC and RNase A indicates that the active site of ONC is most likely composed of His10, Lys31, and His97, which are conserved within the RNase A superfamily, and Pca1 and Lys9, which are conserved among frog homologs (Beintema et al., 1997; Irie et al., 1998). ONC residues His10 and His97 correspond to RNase A residues His12 and His119, which function as the base and acid in catalysis of RNA cleavage (Findlay et al., 1961; Thompson and Raines, 1994) and contribute to nucleic acid binding (Park et al., 2001). Chemical modification of His10 and His97 of ONC showed that these residues are likewise critical for catalysis (Wu et al., 1993). In contrast, the role of the other three active-site residues of ONC is less clear. To elucidate that role, we made variants of ONC in which Pcal, Lys9, and Lys31 are replaced with an alanine residue.

A problem that arises in the study of enzymes of low intrinsic catalytic activity, such as ONC, is assay sensitivity. To determine accurate kinetic parameters for active-site variants of ONC, a new substrate was necessary. Previous work had shown that frog ribonucleases prefer to cleave RNA between uridine and guanosine residues (Okabe *et*

al., 1991; Boix *et al.*, 1996; Leu *et al.*, 2003). Our new substrate, 6-FAM–dArUdGdA–6-TAMRA, is cleaved at least 10²-fold faster by ONC than any substrate described previously. By using 6-FAM–dArUdGdA–6-TAMRA, we were able to obtain kinetic parameters for low-activity variants.

Role of Lysine Residues in Catalysis. The side chain of Lys41 in RNase A is known to enhance catalysis by forming a hydrogen bond with a non-bridging phosphoryl oxygen during catalysis (Messmore et al., 1995; Messmore and Raines, 2000). The corresponding residue in ONC, Lys31, could play a similar role. Interestingly, ONC contains an additional lysine residue in its active site, Lys9. Most frog ribonucleases have a lysine residue at this position, whereas other RNase A homologs have a conserved glutamine residue (Beintema et al., 1997; Irie et al., 1998). Replacing this glutamine residue in RNase A with alanine decreases k_{cat} and K_M , but does not affect k_{cat}/K_M , indicating that the glutamine residue contributes to catalysis by promoting the productive binding of a substrate (delCardayré et al., 1995). To illuminate the role of Lys9 and Lys31 of ONC, we replaced these two lysine residues with alanine. Table 2.1 shows that replacing either Lys9 or Lys31 with an alanine residue decreases the value of k_{cat}/K_{M} by 10^3 fold. The catalytic activity of the K9A/K31A double variant was below the sensitivity limit of the assay ($k_{cat}/K_M < 10 \text{ M}^{-1}\text{s}^{-1}$). Thus in ONC, *two* lysine residues, instead of the single lysine residue in RNase A, are critical for catalysis. From the overall decrease in the $k_{\text{cat}}/K_{\text{M}}$ value of the K9A/K31A variant, we conclude that Lys9 together with Lys31 stabilize the rate-limiting transition state by ≥ 5.9 kcal/mol. We tried to increase the intrinsically low catalytic activity of ONC by replacing a residue of ONC with one from

RNase A. Specifically, we replaced Lys9 with glutamine, only to obtain an enzyme with 10^3 -fold lower k_{cat}/K_M than wild-type ONC (Table 2.1). Apparently, the role of Lys9 in ONC differs from that of Gln11 in RNase A.

Single-stranded DNA can bind to a ribonuclease but is not hydrolyzed by the enzyme, enabling binding constants to be obtained without catalytic turnover (Fisher et al., 1998; Park et al., 2001). In RNase A, the K_d value acquired by this method does not differ significantly from the $K_{\rm M}$ value for analogous substrates (Fisher *et al.*, 1998). To facilitate comparisons, we made use of a DNA ligand that has the same nucleobase sequence as the 6-FAM-dArUdGdA-6-TAMRA substrate. The results were surprising (Figure 2.5). Deleting positive charges in the active site did not have a major effect on nucleic acid binding by the enzyme. The K_d values of the variants were all within twofold of that of wild-type ONC. We conclude that the primary function of the two lysine residues in catalysis is to accelerate substrate turnover rather than to enhance substrate affinity. Another interesting aspect of the binding data is the unusually high K_d value of the enzyme • nucleic acid complexes. Indeed, we had to adopt a low salt concentration (0.010 M NaCl) for assays of binding and catalysis, as we were not able to measure accurate binding constants otherwise. The value of K_d for an ONC • nucleic acid complex is $>10^2$ -fold greater than that of an analogous RNase A \cdot nucleic acid complex under the same conditions (Fisher et al., 1998). This discrepancy indicates that the low intrinsic catalytic activity of ONC originates largely from the low affinity of the enzyme for its substrate.

Role of Lysine Residues in Cytotoxicity. ONC is cytotoxic by virtue of its ability to degrade cellular *t*RNA or *r*RNA (Saxena *et al.*, 2002). We tested the cytotoxicity of wild-type ONC and its K9A, K31A, and K9A/K31A variants on a leukemia cell line. None of the variants are cytotoxic, even at a protein concentration of 50 μ M, whereas wild-type ONC has an IC₅₀ value of 0.8 μ M (Figure 2.6). Thus, the loss of catalytic activity corresponds to a loss of the cytotoxicity, as has been reported for cytotoxic ribonucleases (Wu *et al.*, 1993; Kim *et al.*, 1995; Boix *et al.*, 1996; Dickson *et al.*, 2003; Leu *et al.*, 2003). The loss of positive charge(s) could, of course, disrupt other attributes that determine the cytotoxicity, including binding to the cell surface, uptake into vesicles, and translocation into the cytosol. Regardless, both active-site lysine residues are required for the cytotoxicity of ONC.

Function of Pyroglutamate Residue. An N-terminal pyroglutamate is found in a variety of enzymes and protein hormones. Its side-chain lactam can form by the enzyme-catalyzed or spontaneous cyclization of an N-terminal glutamine residue (Cummins and O'Connor, 1998; Bateman *et al.*, 2001). The chemical mechanism of cyclization likely involves the nucleophilic attack of the α -amino group of glutamine on the amidic carbon of the side chain with the release of ammonia (Bateman, 1989). In crystalline ONC, the side-chain oxygen of Pca1 forms a hydrogen bond with the side-chain amino group of Lys9 (Mosimann *et al.*, 1994) (Figure 2.1). (Pca1 likewise forms a hydrogen bond with Lys9 in a crystalline ONC homolog from the frog *Rana catesbeiana* (Leu *et al.*, 2003). An ONC variant having a methionine residue at the –1 position has 10-fold lower catalytic activity than does wild-type ONC (Boix *et al.*, 1996). This additional

methionine residue precludes the cyclization of Gln1 to produce pyroglutamate, as did Gly(-1) installed in our <E1A/K9A ONC (vide supra). Because we found that the side chain of Lys9 provides an important amino group to the active site, we hypothesized that the Pca1...Lys9 hydrogen bond could limit the rotation of the Lys9 side chain and thereby position its amino group properly for catalysis. To test this hypothesis, we replaced Pcal with an alanine residue to give <E1A ONC. Loss of the Pca1...Lys9 hydrogen bond results in a 20-fold decrease in ribonucleolytic activity (Table 2.1), which suggests that the acquisition of rotational freedom by Lys9 undermines catalysis. To verify that the decreased activity was caused by the elimination of the hydrogen bond, rather than the deletion of the Pca1 itself, we prepared a double variant, replacing Pca1 and Lys9 with alanine to give <E1A/K9A ONC. Interestingly, <E1A/K9A ONC has the same catalytic activity as does K9A ONC (Table 2.1), indicating that once Lys9 is eliminated from the active site, Pca1 does not contribute to the catalysis. These data support our hypothesis that the function of Pca1 is to position Lys9 properly for catalysis through the formation of a hydrogen bond (Figure 2.1), and reveal a mechanistic imperative for having a pyroglutamate residue at the N-terminus of a protein. Finally, the thermodynamic cycle in Figure 2.7 and its value of $\Delta\Delta G_{int} = -2.0$ kcal/mol reveals that the Pca1...Lys9 hydrogen bond contributes 2.0 kcal/mol to the stabilization of the rate-limiting transition state during catalysis.

2.6 Conclusions

We have investigated the function of the active-site residues of ONC, the RNase A homolog and possible cancer chemotherapeutic from the Northern leopard frog. We developed a novel fluorogenic substrate for the ribonucleolytic activity assay of ONC, which will facilitate future studies of catalysis. We find that Lys9 and Lys31 of ONC are critical for both catalytic and cytotoxic activity. We reveal that the function of a pyroglutamate residue, Pca1, is to form a hydrogen bond that anchors the side chain of Lys9. Finally, we propose that much of the low intrinsic ribonucleolytic activity of ONC arises from the low affinity of the enzyme for a single-stranded nucleic acid.

Acknowledgments. We are grateful to Dr. B. –M. Kim for help in the initial stages of this work. We thank Prof. E. A. Craig, P. Huang, and P. D'Silva for assistance with fluorescence anisotropy, Drs. J. A. Hodges and D. R. McCaslin for assistance with CD spectroscopy, and Dr. B. G. Miller, E. A. Kersteen, Dr. P. A. Leland, and B. D. Smith for contributive discussions. CD spectroscopy and mass spectrometry were performed at the University of Wisconsin–Madison Biophysics Instrumentation Facility, which was supported by the University of Wisconsin–Madison and grants BIR-9512577 (NSF) and S10 RR13790 (NIH).

Table 2.1 . Values of $\kappa_{cat}/K_{\rm M}$, m/Z , and $T_{\rm m}$ for ONC and its variants					
ONC	$k_{\rm cat}/K_{\rm M} ({ m M}^{-1}{ m s}^{-1})^a$	relative activity (%)	m/z^b		T (0C) ^C
			expected	observed	$I_{\rm m}({}^{\circ}{\rm C})$
Wild-type	$(1.7 \pm 0.3) \times 10^5$	100	11820	11820	85
K9A	$(1.2 \pm 0.1) \times 10^2$	0.071	11763	11763	80
K9Q	$(1.7\pm0.2)\times10^2$	0.10	11820	11824	>75
K31A	$(1.6 \pm 0.2) \times 10^2$	0.094	11763	11767	85
K9A/K31A	$< 1.0 \times 10^{1}$	< 0.006	11706	11711	78
<Ė1A	$(7.2 \pm 0.7) \times 10^3$	4.2	11780	11783	82
<e1a k9a<="" td=""><td>$(1.4 \pm 0.2) \times 10^2$</td><td>0.082</td><td>11723</td><td>11727</td><td>>75</td></e1a>	$(1.4 \pm 0.2) \times 10^2$	0.082	11723	11727	>75

<E1A/K9A $(1.4 \pm 0.2) \times 10^2$ 0.0821172311727> a Values of k_{cat}/K_M are for the cleavage of 6-carboxyfluorescein–dArUdGdA–6-
carboxytetramethylrhodamine in 0.020 M MES–NaOH buffer (pH 6.0) containing NaCl

(0.010 M) at $23 \pm 2 \text{ °C}$.

^bValues of m/z were determined by MALDI–TOF mass spectrometry.

^cValues of $T_{\rm m}$ were determined in PBS by CD spectroscopy.

Figure 2.1

Structure and function of Onconase[®]. (A) Three-dimensional structure of ONC (PDB entry 1ONC (Mosimann *et al.*, 1994)). (B) Three-dimensional structure of RNase A (PDB entry 7RSA (Wlodawer *et al.*, 1988)), which is a homolog of ONC. (C) Active-site residues of ONC. His10, Lys31, and His97 are conserved in the RNase A superfamily (Beintema *et al.*, 1997). Pca1 and Lys9 are absent from RNase A and its mammalian homologs (Irie *et al.*, 1998). Images were created with the program MOLSCRIPT and rendered with the program RASTER3D (Merritt and Murphy, 1994). (D) Putative mechanism of catalysis of RNA cleavage by ONC.



Figure 2.2 Circular dichroism spectra of wild-type Onconase[®] and its variants.
 Spectra are shown as mean residue molar ellipticity ([Θ]) at different
 wavelengths, and were recorded on proteins (0.2 mg/mL) in PBS at 25 °C.



Figure 2.3 Chemical structure of the substrate 6-carboxyfluorescein–dArUdGdA–6carboxytetramethylrhodamine (6-FAM–dArUdGdA–6-TAMRA). The scissile bond is indicated. *B* and *P* refer to nucleobase and phosphoryl group binding subsites in ONC, respectively.



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Figure 2.4 Chemical structure of the ligand 6-carboxyfluorescein–dAdUdGdA (6-FAM–dAdUdGdA). Note that the uridine residue contains a deoxyribose moiety.



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Figure 2.5 Binding isotherms for wild-type Onconase[®] and its variants with the ligand 6-FAM–dAdUdGdA. The increase in fluorescence anisotropy was measured in 0.020 M MES–NaOH buffer (pH 6.0) containing NaCl

measured in 0.020 M MES–NaOH buffer (pH 6.0) containing NaCl (0.010 M) at 23 ± 2 °C. Values of K_d were obtained by fitting the data points to Equation 2.5 using the program DELTAGRAPH 4.0.



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Figure 2.6

Effect of Onconase[®], its variants, and ribonuclease A on the proliferation of human leukemia cell line K-562. Cell proliferation was determined by incorporation of [methyl-³H]thymidine into cellular DNA after a 44-h incubation with a ribonuclease. Each data point (\blacksquare , wild-type ONC; O, K9A ONC; \triangle , K31A ONC; \Box , K9A/K31A ONC; \bullet , RNase A) is expressed as a percentage of the PBS control.



Figure 2.7

Thermodynamic cycle of k_{cat}/K_M for the cleavage of 6-FAM–dArUdGdA– 6-TAMRA by Onconase[®] upon replacing Pca1 or Lys9 (or both) with alanine. Values of $\Delta\Delta G$ (in kcal/mol) were calculated with Equation 2.2 and the k_{cat}/K_M values in Table 2.1. $\Delta\Delta G_{int}$ is the free energy of interaction between the side chains of Pca1 and Lys9, and was calculated with Equation 2.3 and the data in Table 2.1.





Chapter Three

Molecular Basis for the Substrate Specificity of Onconase

Prepared for submission to the Journal of Molecular Biology as:

Lee, J. E.^{*}, Bae, E.^{*}, Bingman, C. A., Phillips, G. N., Jr., and Raines, R. T. (2006) Molecular Basis for the Substrate Specificity of Onconase.

* These authors contributed equally to this work. I was responsible for variant design, protein purification, substrate preparation, cytotoxicity measurements, and kinetic/binding studies, and I assisted Dr. Bae with the screening of the protein crystals.

Dr. Bae solved the structure of the crystalline ONC complexes.

3.1 Abstract

Onconase (ONC), a homolog of ribonuclease A (RNase A), is from the eggs and the early embryos of the frog Rana pipiens. ONC displays antitumoral activity, and is in clinical trials for the treatment of cancer. ONC prefers to cleave the phosphodiester bond 5' to a guanine base of RNA, and its catalytic activity is at least three orders of magnitude lower than that of RNase A. We have determined the x-ray crystal structure of two ONC-nucleic acid complexes: the structure of a nonproductive T89N/E91A ONC-5'-AMP complex was solved at 1.7 Å resolution; the structure of a productive wild-type ONC•d(AUGA) complex was determined at 1.9 Å resolution, and used for further analyses. In the latter structure, Glu91 makes two H-bonds with the guanine moiety of the nucleic acid, and Thr89 is in close proximity to the nucleobase. Replacing Glu91 with a residue whose side chain is either uncharged or positively charged decreased the guanine preference of ONC by more than 12-fold. T89N ONC showed a 50-fold lowered guanine preference. Combining the mutations (T89N/E91A) resulted in an enzyme that favors adenine over guanine. In contrast, addition of a positive charge at position 89 increased the guanine preference more than 6-fold. Taken together, ONC utilizes H-bond network and Coulombic interaction to discriminate between guanine and adenine. Elongating the two loops linking the β -strands in ONC did not enhance catalytic activity. The T5R substitution, designed to promote a favorable Coulombic interaction between ONC and a phosphoryl group in RNA, increased catalytic activity by twofold. Lastly, ONC did not cleave a guanosine-guanosine bond in vitro, indicating an involvement of an unidentified structural element in the *in vivo* targeting of ONC.

3.2 Introduction

Onconase (ONC) is a ribonuclease found in the eggs and early embryos of the frog *Rana pipiens*. ONC is a homolog of bovine pancreatic ribonuclease (RNase A), and the two proteins possess 30% amino acid sequence identity and share a similar three-dimensional structure. ONC is in Phase III clinical trials for the treatment of mesothelioma (Mikulski *et al.*, 2002; Favaretto, 2005), and was recently granted Fast Track status by the Food and Drug Administration. ONC is also toxic to HIV-infected human cells (Youle *et al.*, 1994)

ONC is a unique ribonuclease. It exhibits remarkable conformational stability ($T_m = 87 \text{ °C}$) (Leland *et al.*, 2000; Notomista *et al.*, 2000; Notomista *et al.*, 2001). Blocked Nand C-termini are known to contribute to this stability. It completely evades the cellular ribonuclease inhibitor protein (RI), to which other ribonucleases bind with femtomolar affinity (Lee *et al.*, 1989; Lee and Vallee, 1989; Vicentini *et al.*, 1990; Shapiro and Vallee, 1991; Boix *et al.*, 1996; Haigis *et al.*, 2003). The exceptional conformational stability and the RI-evading ability are thought to facilitate its cytotoxic function (Boix *et al.*, 1996; Leland *et al.*, 2000).

ONC is a poor catalyst. The ribonucleolytic activity of ONC is three to five orders of magnitude lower than that of RNase A (Lee and Raines, 2003). Although the catalytic activity of ONC is low, it is nevertheless required for the cytotoxicity. (Wu *et al.*, 1993). In Chapter Two, I report that the low catalytic activity is due, in part, to poor substrate binding. ONC displays a distinct preference for a guanine base 3' to the cleaved phosphodiester bond (B_2 subsite; see Figure 2.3). This guanine preference is also found in
other frog ribonucleases, but not in mammalian homologs (Witzel and Barnard, 1962; Okabe *et al.*, 1991). tRNA appears to be the major cellular substrate for ONC (Saxena *et al.*, 2002). A recent study revealed a rather unconventional cleavage sequence in tRNA— ONC predominantly cleaved the guanosine–guanosine bond in the variable loop or the Darm in tRNA (Suhasini and Sirdeshmukh, 2006). In general, homologs of RNase A favor a pyrimidine base 5' to the cleaved phosphodiester linkage, due to the small nucleobasebinding pocket (Rushizky *et al.*, 1961; Wlodawer *et al.*, 1983; delCardayre and Raines, 1994; Kelemen *et al.*, 2000).

The development of ONC as a cancer therapeutic will benefit from an in-depth understanding of its catalysis. Herein, we report on the crystalline structure of two ONC•nucleic acid complexes. We use this structural information to address key issues in ONC catalysis. First, we determine the molecular basis for the nucleobase specificity of ONC through a systematic site-directed mutagenesis study. Next, we ask whether the low catalytic activity of ONC can be improved by a rational design approach. Finally, we try to confirm the cellular target sequence of ONC *in vitro* using two novel fluorogenic substrates.

3.3 Experimental Procedures

Materials. Human RI (as RNasin[®]) was from Promega (Madison, WI). RNase T1 was from Amibon (Austin, TX). 6-Carboxyfluorescein–dArUdAdA–6carboxytetramethylrhodamine (6-FAM–dArUdAdA–6-TAMRA), 6-FAM–dArUdGdA– 6-TAMRA, 6-FAM–dUrGdGdA–6-TAMRA, and 6-FAM–dArGdGdA–6-TAMRA were

from Integrated DNA Technology (Coralville, IA). 2-(N-Morpholino)ethanesulfonic acid (MES), adenosine 5'-monophosphate (5'-AMP), and guanosine 5'-monophosphate (5'-GMP) were from Sigma Chemical (St. Louis, MO). MES was purified further by anionexchange chromatography prior to its use so as to eliminate contaminating oligo(vinylsulfonic acid), which is a potent inhibitor of ribonucleases (Smith *et al.*, 2003). [methyl-³H]Thymidine was from PerkinElmer Life Sciences (Boston, MA). Phosphatebuffered saline (PBS) contained (in 1 liter) 0.20 g of KCl, 0.20 g of KH₂PO₄, 8.0 g of NaCl, and 2.16 g of Na₂HPO₄·7H₂O. All other chemicals and reagents were of commercial grade or better, and were used without further purification.

K-562 cells, which derive from a continuous human chronic myelogenous leukemia line, were from the American Type Culture Collection (Manassas, VA). Cell culture medium and supplements were from Invitrogen (Carlsbad, CA).

Instruments. Mass was measured by matrix-assisted laser desorption ionization-timeof-flight (MALDI-TOF) mass spectrometry using a Voyager-DE-PRO Biospectrometry Workstation Applied Biosystems, Foster City, CA) and a 3,5-dimethoxy-4hydroxycinnamic acid (sinapinic acid) matrix (Sigma Chemical). Fluorescence measurements were performed with a QuantaMaster 1 Photon Counting Fluorometer equipped with sample stirring (Photon Technology International, South Brunswick, NJ). Radioactivity was quantitated with a Microbeta TriLux liquid scintillation and luminescence counter (PerkinElmer, Wellesley, MA).

Production of ONC and its Variants. Wild-type ONC and its variants were produced in *E. coli* strain BL21(DE3) as described in Chapter Two. The yields of purified wild-

type ONC and its variants were ≥ 20 mg per liter of culture, which are comparable to those from previous studies (Lee and Raines, 2003).

Crystal Preparation. Crystals of wild-type ONC•d(AUGA) complex were grown by the hanging-drop method from a solution containing 10 mg/ml protein and 3.0 mM nucleic acid in water mixed with an equal amount of reservoir solution containing 25% (v/v) polyethylene glycol (PEG) 3350 in 0.10 M 4-(2-hydroxyethyl)piperazine-1- ethanesulfonic acid (HEPES) pH 7.5 at 20 °C. Crystals were soaked in reservoir solutions supplemented with increasing amounts of ethylene glycol up to 20% (v/v) and were flash-frozen in a stream of cryogenic nitrogen gas. Crystals of T89N/E91A ONC were grown by the hanging-drop method from a solution containing 21.4 mg/ml protein in water mixed with an equal amount of reservoir solution containing 30.6% (w/v) polyethylene glycol monomethyl ether (MEPEG) 2K and 50 mM 5'-AMP in 90 mM bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane (BIS-TRIS) pH 6.5 at 20 °C. The crystals were soaked in a reservoir solution supplemented with 5% (v/v) ethylene glycol and were flash-frozen in a stream of cryogenic nitrogen supplemented with 5% (v/v) ethylene glycol and were flash-frozen in a stream of cryogenic nitrogen supplemented with 5% (v/v) ethylene glycol

X-ray Data Collection. X-ray diffraction data for the wild-type ONC•d(AUGA) and the T89N/E91A ONC•5'-AMP complex were collected with a Bruker AXS Proteum R CCD detector and Microstar rotating-anode generator using copper K_{α} radiation. All data were processed and scaled with the programs SAINT and SADABS from the Proteum software suite (Bruker, Madison, WI).

Structure Refinement. Structures of the wild-type ONC•d(AUGA) and the T89N/E91A ONC•5'-AMP complex were solved by molecular replacement with apo-

ONC (PDB entry 10NC) as a model using the program MOLREP (Vagin and Teplyakov, 1997). The structures were completed using alternate cycles of manual building in COOT (Emsley and Cowtan, 2004) and refinement in REFMAC5 (Murshudov et al., 1997). The stereochemical quality of the final models were assessed using MolProbity (Lovell et al., 2003). The final coordinates were deposited in the RCSB Protein Data Bank (Berman et al., 2000) with accession numbers 215S and 2GMK for the wild-type ONC•d(AUGA) and T89N/E91A ONC•5'-AMP complexes, respectively.

Assays of Catalytic Activity. Ribonucleolytic activity of wild-type ONC and its variants was measured with a hypersensitive assay based on the cessation of fluorescence quenching as described in Chapter Two. To assess the *B*₂-subsite specificity of ONC, two fluorogenic substrates that contain distinct nucleobase sequences were used. Preference for the interaction with the guanine nucleobase was measured by using 6-FAM-dArUdGdA-6-TAMRA, and the interaction with the adenine base was measured by using 6-FAM-dArUdAdA-6-TAMRA. In order to confirm the previous study that showed that the major *in vivo* cleavage sequence was the guanosine–guanosine bond in the variable loop or D-arm in tRNA (Suhasini and Sirdeshmukh, 2006), we assessed the catalytic activity of wild-type ONC toward two novel ONC substrates, which contain a single cleavable guanosine–guanosine phosphodiester bond (6-FAM-dUrGdGdA-6-TAMRA and 6-FAM-dArGdGdA-6-TAMRA).

Inhibition of Catalytic Activity. Inhibition of ribonucleolytic activity was measured using 6-FAM-dArUdGdA-TAMRA as a substrate. Inhibition by 5'-AMP or 5'-GMP was assessed at 25 °C in 2.0 mL of 50 mM imidazole–HCl buffer, pH 6.0, containing NaCl

(0-0.25 M), 6-FAM-dArUdAdA-6-TAMRA (60 nM), and ONC (1-5 nM), with a minor modification from previous studies (Kelemen et al., 1999; Park et al., 2001). We assumed that the nucleobase moiety of 5'-AMP and 5'-GMP bind exclusively at the B_2 subsite, because the narrow B_1 subsite is not likely to accommodate the purine bases. This assumption is supported by our finding that ONC does not cleave our novel substrates containing a guanosine–guanosine bond (vide infra). Fluorescence (F) was measured with 493 and 515 nm as the excitation and emission wavelengths, respectively. The value of $\Delta F/\Delta t$ was measured for 3 min after the addition of ONC. Next, an aliquot of inhibitor (1) dissolved in the assay buffer was added, and $\Delta F/\Delta t$ was measured in the presence of the inhibitor for 3 min. The concentration of inhibitor in the assay was doubled repeatedly at 3-min intervals. Excess wild-type RNase A was then added to the mixture to ensure that less than 10% of the substrate had been cleaved prior to completion of the inhibition assay. Apparent changes in ribonucleolytic activity due to dilution were corrected by comparing values to those from an assay in which aliquots of buffer were added. Values of K_i were determined by non-linear least squares regression analysis of data fitted to Equation 3.1 (Kelemen et al., 1999; Park et al., 2001) with the program DELTAGRAPH 5.5 (Red Rock Software, Salt Lake City, UT),

$$\Delta F / \Delta t = (\Delta F / \Delta t)_0 (K_i / (K_i + [I]))$$
(Eq. 3.1)

where $(\Delta F/\Delta t)_0$ was the ribonucleolytic activity prior to the addition of the inhibitor.

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Assays of Cytotoxic Activity. The effect of wild-type ONC, its variants, and RNase A on cell proliferation was determined as described in Chapter . Cytotoxicity data were analyzed with the programs SIGMAPLOT (SPSS Science, Chicago, IL) and DELTAGRAPH 5.5. Each data point represents the mean (\pm S.E.) of at least three experiments performed in triplicate. The IC₅₀ value of each variant was determined with the following equation (Haigis *et al.*, 2002):

$$S = \frac{IC_{50}}{(IC_{50} + [ribonuclease])} \times 100$$

(Eq. 3.2)

where S is the percent of total DNA synthesis during the 4-h pulse as compared to that of a PBS control.

3.4 Results

Structure of Wild-type ONC•d(AUGA) Complex. The structure of the complex was refined to an *R*-factor of 0.178 using data from 32.49 to 1.90 Å. The RMSD from target geometries is 0.013 Å for bond lengths and 1.415 degrees for bond angles. The average *B*-factor was 16.60 Å². The electron density was continuous for the main chain and the side chain atoms. The overall dimensions of the complex were

129.1 Å \times 26.1 Å \times 32.5 Å. The nucleic acid bound to the enzyme in a productive mode, and this structure was used for further kinetic analyses

Structure of T89N/E91A ONC•5'-AMP Complex. The structure of the T89N/E91A ONC•5'-AMP complex was refined to an *R*-factor of 0.165 using data from 40.93 to 1.65 Å. The RMSD from target geometries is 0.010 Å for bond lengths and 1.718 degrees for bond angles. The average *B*-factor was 19.35 Å². The electron density was continuous for the main-chain and the side-chain atoms. The nucleic acid bound to the enzyme in a nonproductive mode, and the structure was not used for further kinetic analyses.

Residues near the Guanine moiety of d(AUGA). In the structure of crystalline wildtype ONC•d(AUGA) complex, Glu91 forms two H-bonds with the guanine moiety of d(AUGA) (Figure 3.1D). In addition, Thr89 is located in close proximity to the guanine base. We hypothesized that the two residues constitute the B_2 subsite of ONC (B_2 indicates the nucleobase 3' to the cleaved bond; see Figure 2.3). To test whether Thr89 and Glu91 constitute the B_2 subsite and to study the molecular basis for the guanine preference, we adopted the site-directed mutagenesis approach. We replaced the two residues with those that would differ in net charge and H-bond forming capability (Table 3.2), and tested whether these variants showed an altered nucleobase preference.

Variants with an amplified preference for guanine. Insertion of a positive charge at position 89 increased the guanine preference of wild-type enzyme by 100-fold (Table 3.2). T89R and T89K ONC displayed a 1000-fold and 500-fold preference for a guanine base over an adenine one, respectively. Furthermore, the absolute catalytic activity for the cleavage of a guanine-containing substrate (UpG) by T89R ONC was enhanced three fold-from that of the wild-type enzyme. Variants with a decreased preference for guanine. Elimination of the negative charge at position 89 not only lowered the guanine preference of the enzyme, but also improved the absolute catalytic activity for the cleavage of an adenine-containing substrate (UpA, Table 3.2). The E91A, E91Q, and E91N variants of ONC cleaved UpG 3- to 9-fold more quickly than UpA, indicative of significantly lower guanine preferences than that of the wild-type enzyme. The most significant change in preference was observed with E91K ONC, which had a slight preference for UpA over UpG (1.5 fold). All these variants above had 3- to 10-fold greater UpA-cleaving activity than wild-type enzyme.

Replacing Thr89 with an asparagine residue increased the catalytic activity of ONC for UpA cleavage by 3-fold. The guanine preference of this variant also decreased by 20-fold. An aspartate or glutamine residue at this position likewise decreased the guanine preference. T89D and T89Q ONC had a 10-fold and a 40-fold preference for the guanine base, respectively. Their absolute catalytic activity for UpA, however, did not improve as described with the T89N variant. Combining the T89N and E91A mutation augmented their individual effect. T89N/E91A ONC had the greatest catalytic activity toward UpA among all of the variants, with a greater than 11-fold improvement from the wild-type enzyme. Moreover, T89N/E91A ONC had a complete shift in nucleobase preference, favoring the adenine base over the guanine base by 2.5-fold.

Inhibition of Catalysis. Cleavage of 6-FAM–dArUdGdA–6-TAMRA by wild-type ONC and its variants was inhibited by the nucleotides 5'-GMP and 5'AMP (Table 3.3). Wild-type ONC was inhibited by 5'-GMP and 5'AMP with K_i values of

 $(6.7 \pm 0.5) \times 10^2 \,\mu$ M and $(3.7 \pm 0.3) \times 10^3 \,\mu$ M, respectively. The K_i values of T89K and T89R ONC, variants that manifested an increased preference for the guanine base, did not differ markedly from that of wild-type ONC. On the contrary, variants with a decreased guanine preference showed a relatively large changes in K_i values. Inhibition by 5'-AMP was promoted 3-fold and 6-fold in T89N ONC and T89N/E91A ONC, respectively. 5'-GMP inhibited E91A ONC twice poorer than the wild-type enzyme.

Rational Design of ONC Variants with Enhanced Catalytic Activity. Previous structural investigations have unveiled little flexibility in the structure of ONC with respect to other ribonucleases (Gorbatyuk *et al.*, 2004; Merlino *et al.*, 2005). Model-free analysis of the ¹⁵N-T₁, ¹⁵N-T₂, and ¹⁵N-¹H NOE relaxation data for the backbone amide, and molecular dynamics simulations revealed rigidity in the V-shaped β -sheet. The latter study also pointed out that ONC has a wider active-site cleft than does RNase A. It was hypothesized that the wide active-site cleft coupled with little flexibility might hinder facile interaction with the substrate. The structure of the wild-type ONC•d(AUGA) complex supports this hypothesis. The RMSD of the backbone C^{α} of ONC in the free and complexed form is just 0.4 Å (Shindyalov and Bourne, 1998), suggesting little conformational change upon substrate binding.

Global Approach. To resuscitate the low catalytic activity of ONC, we focused on increasing the flexibility of the β -sheet region. We envisioned that the increased flexibility would lead to an enhanced "global" breathing motion of the enzyme, which could help substrate binding. Interestingly, Merlino and coworkers (2005) have implicated the short loops of ONC as causing rigidity in the β -sheet region. Amphibian

ribonucleases are grouped into two subfamilies (Rosenberg *et al.*, 2001). The members of the subfamily with two short loops connecting α2-helix and β2-strand, and β4- and β5strands (ONC, RC-RNase 2, RC-RNase 4, and RC-RNase 6) show low catalytic activity (Liao *et al.*, 2000; Hsu *et al.*, 2003; Leu *et al.*, 2003). The second subfamily, represented by RC-RNase and RC-RNase L1, contain loops with additional amino acid residues (IVGG and ITP, Figure 3.1A), and exhibit relatively high catalytic activity. The difference in catalytic activity between the two subfamilies reaches up to five orders of magnitude. We reasoned that installing the longer loops from RC-RNase onto ONC could enhance its catalytic activity. Accordingly, two ONC variants (IVGG/ITP and L27I/F28Y/IVGG/ITP ONC) were designed and tested for their ribonucleolytic activity. The L27I/F28Y mutation was added to mimic the loop structure of RC-RNase more closely. Both variants did not, however, improve in catalytic activity.

Local Approach. The most conspicuous feature of the wild-type ONC•d(AUGA) complex was the absence of significant electron density for the two flanking adenosine moieties in the nucleic acid (Figure 3.1B). This lack of electron density suggests a negligible interaction with the enzyme. We asked whether the incorporation of residues that would promote "local" interaction with substrate could revive the catalytic activity of ONC. In RNase A, residues denoted as "P subsites" participate in substrate binding (*cf.*: Figure 2.3). These P-subsite residues are cationic lysine or arginine residues that can promote binding with the anionic phosphoryl groups in the nucleic acid backbone through favorable Coulombic interaction (Fontecilla-Camps *et al.*, 1994). Removal of any residues in the P subsites leads to a loss in catalytic activity and substrate binding

(Fisher *et al.*, 1998; Fisher *et al.*, 1998). In the ONC•d(AUGA) complex structure, no residues other than the active-site lysine residues (Lys9 and Lys31; P_1 subsite, Figure 3.1D) were interacting with phosphoryl groups in the nucleic acid. We hypothesized that installing a novel P subsite in ONC would increase its catalytic activity by enhancing substrate binding. A detailed structural comparison between the RNase A•d(ATAAG) complex (PDB entry 1RCN) and the ONC complex pointed towards Thr5 in ONC as being a promising location for a novel P subsite (as a P_2 subsite, data not shown). Consequently, we created the T5K and T5R variants and measured their catalytic activity. In this regard the T5R substitution was better than the T5K substitution, conferring a twofold increase in catalytic activity.

Val37 in RC-RNase is involved in nucleobase recognition (B_1 subsite)(Leu *et al.*, 2003). The corresponding residue in ONC is Lys33. K33V ONC was designed to test whether this substitution could increase nucleobase recognition in ONC. The catalytic activity of K33V ONC did not, however, differ from that of wild-type ONC.

Catalytic Activity toward Guanosine–Guanosine Bond In vitro. Excess wild-type ONC (200 μ M) did not catalyze the cleavage of 6-FAM-dUrGdGdA-6-TAMRA or 6-FAM-dArGdGdA-6-TAMRA with a rate measurable by our assay. We used RNase T1 as a positive control. RNase T1 cleaves RNA at the 3'-side of guanosine residues (Steyaert, 1997). One unit³ of RNase T1 catalyzed the cleavage of our novel substrates to completion within a minute (data not shown).

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³ One unit of RNase T1 activity corresponds to an increase of 0.0004 A_{260} units in 1 min in 1 mL at room temperature using GpA as substrate.

Cytotoxicity of ONC variants. All ONC variants with altered nucleobase preference that are tested for cytotoxicity exhibited lower toxicity than the wild-type enzyme towards K-562 leukemic cell line (Table 3.2). Likewise, ONC variants designed to possess an improved catalytic activity displayed a lowered cytotoxicity that wild-type ONC (IC₅₀ value from 0.4 μ M to 4.0 μ M).

3.5 Discussion

Molecular Basis for the B_2 -*subsite Specificity of ONC.* Catalysis of UpG cleavage by wild-type ONC is 10^2 -fold more efficient than is the cleavage of UpA (Table 3.1). We have demonstrated that this specificity can be altered by modifying residues involved in B_2 -nucleobase recognition. In the structure of the wild-type ONC•d(AUGA) complex, we noticed that $O_{\delta 1}$ and $O_{\delta 2}$ of Glu91 form two hydrogen bonds with N_1 and N_2 of the guanine moiety (Figure 3.1D). In addition, Thr89 is located proximal to the guanine moiety. The distances between $O_{\delta 1}$ and $C_{\delta 2}$ of Thr89, and O_{δ} of the guanine moiety are 3.57 Å and 4.25 Å, respectively (Figure 3.1D). Similar interactions were observed in the RC-RNase•d(ACGA) complex (Leu *et al.*, 2003). Specifically, Lys95 and Glu97 from RC-RNase establish an extensive network of hydrogen bonds with the nucleobase. Replacement of these residues with alanine resulted in a noticeable change in nucleobase preference (Leu *et al.*, 2003). To understand the molecular basis underlying the nucleobase specificity of ONC, we took a more systematic approach—we replaced Thr89 and Glu91 with residues that vary in their ability to form hydrogen bonds and in their net charge.

Each of our Glu91 variants exhibited a lower guanine-preference. The lysine substitution gave the largest effect, reducing the guanine preference to three fold from 100 fold displayed by the wild-type enzyme. It is worth noting that this specificity change is a contribution from a decrease in the catalytic activity toward the UpG substrate and an increase toward the UpA substrate. Surprisingly, a single variant, E91K ONC, gained 9fold in catalytic activity. All the substitutions made at position 91 (alanine, glutamine, asparagine, and lysine) necessarily led to the loss of the two Glu91- guanine hydrogen bonds observed in the crystalline complex (Figure 4.1D), which explains the observed decrease in catalytic activity for the cleavage of the UpG substrate. The contribution of hydrogen bonds in the enzyme-substrate interface to catalysis had been well established (Carlow et al., 1998; Loverix et al., 2000; Yin et al., 2001; Kicska et al., 2002; Kim et al., 2006). On the other hand, the dramatic enhancement in the catalytic activity for cleavage of the UpA substrate was not anticipated. It is difficult to explain such an improvement by a consideration of hydrogen bonds, as the E91A variant, devoid of such hydrogen bonds, gains activity similar to that of other variants. In addition, the fact that E91K ONC with +2Z showed the greatest change in nucleobase preference implicated that the importance of charge. To understand this specificity at the atomic level, we performed electrostatic potential calculations of guanine and adenine with the program SPARTAN (Wavefunction, Irvine, CA). Figure 3.3 illustrates the results of that calculation, wherein electron-rich regions of the nucleobase are colored red, neutral regions green, and electron-poor regions blue. The results are in agreement with previous calculations (Basu *et al.*, 2004), showing that guanine is electron poor on N_1 and N_2 .

Thus, the interaction of this region with the side chain of Glu91 would be promoted by favorable Coulombic interaction. In contrast, the electrostatic potential of adenine differs significantly from that of guanine. The corresponding region in adenine is electron-rich, and the presence of the negatively charged Glu91 would result in unfavorable interactions. Thus, the 100-fold preference (k_{cat}/K_M) for the guanine base that wild-type ONC exhibits can be explained, at least in part, by electrostatic interactions between Glu91 and the nucleobases. We conclude that Glu91 is a major contributor for the B_2 -nucleobase specificity of ONC.

The structure of the ONC•d(AUGA) complex predicts that O_6 of the guanine base would be located close to the positive charge added at position 89. In the RC-RNase•d(ACGA) complex, a lysine residue at the corresponding position makes a hydrogen bond with O_6 of guanine (Leu *et al.*, 2003). Our data demonstrate that a residue bearing a positive charge at this position (lysine or arginine) strengthens the guanine preference of ONC, while a negative charge (aspartate) attenuates that preference. Because O_6 on guanine is electron-rich, and N_6 on adenine is electron-poor (Figure 3.3), this result is yet again in agreement with the substrate specificity of ONC originating from its electrostatic interaction with the nucleobases. It is not clear, however, why T89N ONC has an increased catalytic activity for the UpA substrate, because this mutation does not involve change in net charge. We have also shown that the increase in catalytic activity toward the UpA substrate by the single substitutions can be enhanced further by their combination. T89N/E91A ONC, a variant that would possess two compatible interfaces for adenine, displays an 11-fold enhanced catalytic activity toward the UpA substrate. Furthermore, this double variant now clearly prefers adenine to guanine (2.5-fold).

We used the inhibitory effect of 5'-GMP or 5'-AMP on the cleavage of 6-FAM– dArUdGdA–6-TAMRA to assess the effect of substitutions on ground-state binding (Table 3.2). In this analysis, we assume that the mono-nucleotides bind at the B_2 and P_1 subsites, and that this binding competitively inhibits the cleavage of the substrate. The inhibitory effect of 5'-AMP is somewhat promoted in T89K ONC and T89R ONC, indicating that binding of adenine at the B_2 subsite is slightly stronger in these variants, despite the adverse Coulombic effect of adding a positive charge. The binding of guanine became only slightly stronger after the substitutions. Thus, the major ramification of the extra positive charge at position 89 is the destabilization of transition-state binding of adenine as shown in the considerably decreased k_{cat}/K_M values (Table 3.1). The T89N substitution lowers the ground-state energy of the enzyme-adenine complex as the threefold lower K_i value indicates. The E91A substitution destabilizes the ground-state binding of guanine.

Guanine and adenine are part of nucleotides with a broad range of distinct biological functions. Discrimination between these two nucleobases, similar in shape and size, is critical for many enzymes (*e.g.*, kinases, ATPases, and GTPases). These proteins usually exhibit a strong preference for their cognate nucleotide over the other. Statistical analysis of protein–nucleotide structures in the RCSB Protein Data Bank has suggested that a hydrogen-bonding network and Coulombic interactions (Nobeli *et al.*, 2001; Basu *et al.*, 2004) are the two major determinants for selectivity. The nature of hydrogen bond in

proteins is largely electrostatic (Jeffrey, 1997), and a correlation between hydrogen bonds and the electrostatic potential distribution in the protein–ligand interface was indeed found in those analyses. In agreement with these findings, our study suggests both hydrogen bonds and Coulombic interactions with Glu91 are utilized by ONC for its nucleobase specificity. The physiological implication of the specificity is unknown.

Rational Design of ONC with Enhanced Catalytic Activity. When ONC was implanted with extra amino acid residues (IVGG and ITP) designed to affect the "global" breathing motion, the catalytic activity did not improve (Table 3.2). The result implies that the factors causing the low catalytic activity of ONC reside outside the loops. The greater catalytic activity and flexibility attained by the M23L mutation (Notomista *et al.*, 2000; Gorbatyuk *et al.*, 2004; Merlino *et al.*, 2005) supports this notion. Met23 is located in a hydrophobic cavity created by the side chains of Ile22, Phe28, Lys31, Phe36, Cys68, and Tyr77. A bulky leucine residue at this position is thought to alter the position of the residues, especially that of Lys31, which is critical for catalysis. In this regard, it would be interesting to identify those amino acid residues responsible for rigidity in the β -sheet region by thorough sequence and structural comparison of amphibian ribonucleases. The "local" modifications (T5K, T5R, and K33V mutations) did not result in a sizeable improvement in catalytic activity. It is possible that the envisioned local interactions were not realized due to the low flexibility of ONC.

In vivo Cleavage Sites for ONC. Previously, it has been shown that cellular tRNA is a predominant target for ONC (Saxena *et al.*, 2002). It is still a matter of debate whether cellular tRNA is the sole substrate for ONC in the cell (Ardelt *et al.*, 2003). Members of

the RNase A superfamily usually cleave single-stranded RNA that is not base-paired, and how extensively base-paired tRNA becomes a target for ONC has been unknown. Moreover, calculations on mammalian mitochondria and E. coli predict that most of their tRNA exist as a ternary complex with EF-Tu and GTP in equilibrium (Cai et al., 2000), which will limit its accessibility to ONC even further. Elongation factor (EF) Tu promotes the binding of aminoacyl-tRNA to the acceptor site of the ribosome. A recent report by Suhasini and Sirdeshimukh (2006) shed some light on this issue. Through an extensive sequence analysis on the cleavage site in tRNA, the authors demonstrated that ONC preferentially targets the variable loop or the D-arm in t-RNA. These regions of tRNA lack base pairing and are thus candidates for cleavage by ONC. In our ONC•d(AUGA) structure, ONC makes minimal contacts with substrate (only one P and two B subsites), implying that a compact single-stranded region in RNA is sufficient for an ONC substrate. In addition, the structure of the EF-Tu•GMP•tRNA^{Phe} complex (PDB entry 1TTT) confirms that the variable loop and the D-arm regions are not blocked by EF-Tu. Remarkably, the authors found that the predominant cleavage site is a guanosineguanosine phosphodiester bond. It is well known that ONC prefers to cleave a phosphodiester linkage 3' to pyrimidine bases. In our complex structure, the narrow B_1 subsite composed of Lys33, Thr35, and Phe100 makes a close contact with the uracil base. Hence, binding of the relatively large guanine base at this subsite is not likely. To determine whether ONC catalyzes the cleavage of the guanosine–guanosine bond in vitro, we developed two novel substrates (6-FAM-dUrGdGdA-6-TAMRA and 6-FAM-dArGdGdA-6-TAMRA). The inability of ONC to cleave either substrates lead us

to conclude that the B_2 subsite of ONC is too constricted to accommodate a large guanine nucleobase in an unstructured RNA molecule. In addition, it is evident that additional structural elements in ONC or the tRNA enable targeting of a guanosine–guanosine bond *in vivo*.

3.6 Conclusions

We have determined the crystalline structure of a productive ONC•DNA complex and a nonproductive ONC•mono-nucleotides complex at 1.9 Å and 1.7 Å resolution, respectively. Inspired by the structural data, we have identified the molecular basis for the nucleobase specificity of ONC. Our findings reveal that Glu91 is a major contributor to the nucleobase specificity, and ONC takes advantage of a hydrogen bond network and Coulombic interactions for its specificity. We have demonstrated that amino acid substitutions can shift this specificity from guanine to adenine. We propose that the factors responsible for the low catalytic activity of ONC reside outside of its two short loops. Finally, we propose involvement of unidentified structural elements in the *in vivo* targeting of ONC.

Acknowledgements. We are grateful to Drs. G. N. Phillips Jr., E. Bae, and C. A. Bingman for the solution of the ONC complex structures. We thank J. Binder for help with calculations for the electrostatic potential of nucleobases. We thank Dr. E. L. Myers and T. J. Rutkoski for contributive discussions.

	Wild-type ONC•d(AUGA)	T89N/E91A ONC•5'-AMP
Space group	P21212	P2 ₁ 2 ₁ 2 ₁
Unit cell parameters (Å)	a=129.2, b=26.1, c=32.5	a=29.0, b=52.1, c=66.1
Data collection statistics		
Resolution range (Å)	64.62 - 1.90 (1.95 - 1.90)	66.14 - 1.65 (1.70 - 1.65)
Number of reflections (measured/unique)	91217/9270	336231/12645
Completeness (%)	99.7 (95.9)	99.9 (99.6)
R _{merge} ^b	0.125 (0.517)	0.054 (0.663)
Redundancy	9.8 (3.3)	26.6 (5.1)
Mean σ/I	12.30 (2.52)	40.66 (3.32)
Refinement statistics		
Resolution range (Å)	32.49 - 1.90	40.93 - 1.65
Number of reflections (total/test)	9229/441	12597/612
$R_{\mathrm{cryst}}^{c}/R_{\mathrm{free}}^{d}$	0.178/0.240	0.165/0.217
RMSD bonds (Å)	0.013	0.010
RMSD angles (deg)	1.415	1.718
Average B factor $(Å^2)$	16.60	19.35
Number of water molecules	155	196
Ramachandran favored (%)	100.0	98.1
Ramachandran allowed (%)	0.0	1.9

Table 3.1: Summary of Data Collection and Refinement Statistics^a

^a Values in parentheses are for the highest-resolution shell

^b $R_{\text{merge}} = \sum_{h} \sum_{i} |I_i(h) - \langle I(h) \rangle | / \sum_{h} \sum_{i} I_i(h)$, where $I_i(h)$ is the intensity of an individual measurement of the reflection and $\langle I(h) \rangle$ is the mean intensity of the reflection.

^c $R_{\text{cryst}} = \sum_{h} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{h} |F_{\text{obs}}|$, where F_{obs} and F_{calc} are the observed and calculated structure factor amplitudes, respectively.

 d R_{free} was calculated as R_{cryst} using 5.0% of the randomly selected unique reflections that were omitted from structure refinement.

ONC	$k_{\rm cat}/K_{\rm M}~({\rm M}^{-1}{\rm s}^{-1})$		m/z °		IC ₅₀
UNC -	UpG ^a	UpA ^b	expected	observed	$(\mu M)^d$
Wild-type	$(4.2 \pm 0.5) \times 10^4$	$(3.8 \pm 0.2) \times 10^2$	11820	11820	0.2
T5K	$(5.8\pm0.1)\times10^4$	N.D.	11846	11852	0.4
T5R	$(8.3\pm0.5)\times10^4$	N.D.	11874	11881	1.2
T89A	$(1.5\pm0.1)\times10^4$	$(4.7\pm0.4)\times10^2$	11790	11793	0.4
T89K	$(4.1 \pm 0.3) \times 10^4$	$(7.0 \pm 0.4) \times 10^{1}$	11820	11824	1.0
T89R	$(1.4 \pm 0.1) \times 10^5$	$(1.1\pm0.1)\times10^2$	11875	11868	0.8
T89D	$(1.2 \pm 0.1) \times 10^3$	$(1.1 \pm 0.1) \times 10^2$	11834	11840	2.0
T89Q	$(1.4 \pm 0.1) \times 10^3$	$(3.5 \pm 0.2) \times 10^{1}$	11847	11852	N.D.
T89N	$(2.6 \pm 0.2) \times 10^3$	$(1.2 \pm 0.1) \times 10^3$	11833	11826	0.7
E91A	$(3.9 \pm 0.3) \times 10^3$	$(1.6 \pm 0.1) \times 10^3$	11762	11750	1.5
E91Q	$(9.4 \pm 0.3) \times 10^3$	$(1.1\pm0.1)\times10^3$	11819	11827	0.6
E91N	$(4.2 \pm 0.3) \times 10^3$	$(1.6 \pm 0.3) \times 10^3$	11811	11805	N.D.
E91K	$(2.4 \pm 0.1) \times 10^3$	$(3.8 \pm 0.3) \times 10^3$	11819	11828	N.D.
T89N/E91A	$(1.7 \pm 0.3) \times 10^3$	$(4.4 \pm 0.2) \times 10^3$	11775	11780	2.5
T89K/E91A	$(7.5 \pm 0.3) \times 10^3$	$(5.6 \pm 0.2) \times 10^2$	11788	11793	1.7
K33V	$(4.4\pm0.3)\times10^4$	$(3.7 \pm 0.3) \times 10^2$	11791	11795	N.D.
K33V/T89K	$(7.1 \pm 0.7) \times 10^4$	$(1.6 \pm 0.2) \times 10^2$	11818	11823	N.D.
IVGGITP	$(1.9 \pm 0.2) \times 10^4$	$(5.3 \pm 0.4) \times 10^2$	12447	12454	4.0
L27I/F28Y IVGGITP	$(3.4\pm0.2)\times10^4$	$(6.0 \pm 0.2) \times 10^2$	12463	12470	4.0

Table 3.2: Values of k_{cat}/K_M , m/z, and IC₅₀ for ONC and its Variants

^{*a,b*}Values of k_{cat}/K_M are for the cleavage of 6-carboxyfluorescein–dArUdGdA–6-

carboxytetramethylrhodamine or 6-carboxyfluorescein-dArUdAdA-6-

carboxytetramethylrhodamine in 0.10 M MES–NaOH buffer (pH 6.0) containing NaCl (0.10 M) at 23 ± 2 °C.

Values of m/z were determined by MALDI-TOF mass spectrometry.

^{*d*}Values of IC₅₀ (\pm S.E.) are for incorporation of [*methyl*-³H]thymidine into the DNA of K-562 cells exposed to an onconase, and were calculated with Equation 3.2.

ONC	$K_{\rm i} (\mu {\rm M})^a$		
UNC	5'-GMP	5'-AMP	
Wild-type	$(6.7 \pm 0.5) \times 10^2$	$(3.7\pm0.3)\times10^3$	
Т89К	$(5.3 \pm 0.2) \times 10^2$	$(2.0 \pm 0.2) \times 10^3$	
T89R	$(4.3 \pm 0.6) \times 10^2$	$(2.4 \pm 0.4) \times 10^3$	
T89N	$(1.0 \pm 0.1) \times 10^3$	$(1.2 \pm 0.1) \times 10^3$	
E91A	$(1.3 \pm 0.3) \times 10^3$	$(3.2 \pm 0.4) \times 10^3$	
T89N/E91A	$(9.9 \pm 0.7) \times 10^2$	$(5.9 \pm 0.2) \times 10^2$	

Table 3.3: Values of K_i for ONC and its Variants

^{*a*}The inhibition of ribonucleolytic activity for the cleavage of 6-carboxyfluorescein–dArUdGdA–6-carboxytetramethylrhodamine was assessed in 0.10 M MES–NaOH buffer (pH 6.0) containing NaCl (0.10 M) at 23 ± 2 °C.

Amino acid sequence and structure of Onconase[®]. (A) Amino acid Figure 3.1 sequence alignment of ONC and RC-RNase. The secondary structure of ONC is labeled as h (α -helix), s (β -strand), or t (turn). Residues conserved between the two ribonucleases are in gray boxes. < E denotes a pyroglutamate residue. Key active-site residues are in black boxes. Cysteine residues that form intramolecular disulfide bonds are in yellow boxes. Residues that participate in the nucleobase recognition at the B_2 subsite in ONC are in green. Extra amino acid residues in the loops of RC-RNase are in red. (B) Ribbon diagram of the three-dimensional structure of wild-type ONC•d(AUGA) complex (PDB entry 2I5S). The two flanking adenosine nucleotides are not included in the diagram because of their low electron density. The two arrowheads indicate the two loops that were subjected to elongation. (C) Ribbon diagram of the three-dimensional structure of T89N/E91A ONC•5'AMP complex (PDB entry 2GMK). (D) Active site and the B_2 subsite of wild-type ONC. Glu91 forms two hydrogen bonds with the guanine moiety of DNA. Thr89 is located close to the carbonyl oxygen of guanine. Images were created with the program MOLSCRIPT and rendered with the program RASTER3D (Merritt and Murphy, 1994).



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Figure 3.2 Structure and electron density diagram of (A) 9-methylguanine and (B) 9methyladenine. Electron density of the nucleobases was calculated with the program SPARTAN. Electron-rich regions are colored red; electronpoor regions are blue; neutral regions are green.



Chapter Four

Cytotoxicity of Bovine Seminal Ribonuclease:

Monomer versus Dimer

Portions of this chapter were published as:

Lee, J. E. and Raines, R. T. (2005) Cytotoxicity of bovine seminal ribonuclease: Monomer *versus* dimer. *Biochemistry* 44, 15760-15767.

4.1 Abstract

Bovine seminal ribonuclease (BS-RNase) is a homolog of bovine pancreatic ribonuclease (RNase A). Unlike RNase A, BS-RNase has notable toxicity for human tumor cells. Wild-type BS-RNase is a homodimer linked by two intermolecular disulfide bonds. This quaternary structure endows BS-RNase with resistance to inhibition by the cytosolic ribonuclease inhibitor protein (RI), which binds tightly to RNase A and monomeric BS-RNase. Here, we report on the creation and analysis of monomeric variants of BS-RNase that evade RI but retain full enzymatic activity. The cytotoxic activity of these monomeric variants exceeds that of the wild-type dimer by up to 30-fold, indicating that the dimeric structure of BS-RNase is not required for cytotoxicity. Dimers of these monomeric variants are more cytotoxic than wild-type BS-RNase, suggesting that the cytotoxicity of the wild-type enzyme is limited by RI inhibition following dissociation of the dimer in the reducing environment of the cytosol. Finally, the cytotoxic activity of these dimers is less than that of the constituent monomers, indicating that their quaternary structure is a liability. These data provide new insight into structurefunction relationships of BS-RNase. Moreover, BS-RNase monomers described herein are more toxic to human tumor cells than is any known variant or homolog of RNase A including Onconase[®], an amphibian homolog in Phase III clinical trials for the treatment of unresectable malignant mesothelioma.

4.2 Introduction

Several homologs of RNase A are endowed with natural cytotoxic activity (Youle and D'Alessio, 1997; Leland and Raines, 2001; Matousek, 2001; Makarov and Ilinskaya, 2003). Bovine seminal ribonuclease (BS-RNase) has been shown to kill tumor cells *in vitro* and *in vivo* (Matoušek, 1973; Vescia *et al.*, 1980). Onconase[®] (ONC), a frog homolog of RNase A, is in Phase III clinical trials for the treatment of unresectable malignant mesothelioma, an asbestos-related lung cancer (Mikulski *et al.*, 2002; Saxena *et al.*, 2003). The ability of these ribonucleases to enter cells and cleave cellular RNA leads to apoptosis (Saxena *et al.*, 2002; Haigis and Raines, 2003).

BS-RNase has 83% sequence identity with RNase A (Figure 4.1A), but possesses a distinct quaternary structure. Unlike RNase A (Figure 4.1B), BS-RNase exists as a homodimer crosslinked by disulfide bonds between Cys31 of one subunit and Cys32 of the other (Figure 4.1C). BS-RNase exists as a mixture of two distinct forms, M=M and M×M. In the M=M form, the two subunits are linked only by the two intersubunit disulfide bonds; whereas in the M×M form, the two subunits share additional noncovalent interactions established by the swapping of their N-terminal α -helices.² At equilibrium, the M×M form exists in a slight molar excess (Piccoli *et al.*, 1992; Kim and Raines, 1995).

Ribonuclease inhibitor (RI) is a 50-kDa protein present in the cytosol of mammalian cells (Hofsteenge, 1997; Shapiro, 2001; Dickson *et al.*, 2005). The binding between RI and RNase A is among the tightest of known protein–protein interactions, having an equilibrium dissociation constant (K_d) near 10⁻¹⁴ M (Lee and Vallee, 1989; Vicentini *et*

al., 1990; Shapiro and Vallee, 1991). This high affinity has likely evolved to protect cells from rogue ribonucleases (Haigis *et al.*, 2003). Hence, for a ribonuclease to be cytotoxic, it must evade RI. Monomers of BS-RNase do not evade RI and are not cytotoxic (Murthy and Sirdeshmukh, 1992; Kim and Raines, 1995). In contrast, dimeric BS-RNase is resistant to RI (Murthy *et al.*, 1996) and is cytotoxic (Vescia *et al.*, 1980; Kim *et al.*, 1995; Kim *et al.*, 1995).

The two intersubunit disulfide bonds of BS-RNase are unlikely to be stable in the reducing environment of the cytosol. As a consequence, it is possible that internalized BS-RNase dissociates into monomers, which are subject to inhibition by RI. Indeed, the M=M form is known to dissociate more rapidly than does the M×M form (Cafaro *et al.*, 1995; Kim and Raines, 1995), which benefits from the noncovalent interactions derived from domain swapping. Moreover, the M×M form is more cytotoxic than is the M=M form, suggesting that RI does mediate the cytotoxicity of BS-RNase.

ONC provides the benchmark for the cytotoxic activity of ribonucleases. Its extraordinary cytotoxicity is believed to originate, in large part, from its effective RIevasion (estimated $K_d \ge 10^{-6}$ M (Boix *et al.*, 1996)). BS-RNase is less cytotoxic than ONC, even though BS-RNase has greater ribonucleolytic activity than does ONC (Matousek *et al.*, 2003). Inhibition of BS-RNase by RI after reductive dissociation could be responsible for the lower cytotoxic activity of BS-RNase.

Here, we probe the effect of quaternary structure on the cytotoxicity of BS-RNase. Specifically, we ask if monomers of BS-RNase could be endowed with potent cytotoxic activity. Guided by the structure of the RI·RNase A complex, we create

monomeric variants of BS-RNase that have markedly decreased affinity for RI but retain full enzymatic activity. We then use these variants to demonstrate that the dimeric form of BS-RNase is not only unnecessary for cytotoxicity, but can actually be *detrimental* to cytotoxic activity.

4.3 Experimental Procedures

Materials. The fluorogenic ribonuclease substrate, 6-carboxyfluorescein– dArUdAdA– 6-carboxytetramethylrhodamine (6-FAM–dArUdAdA–6-TAMRA), was from Integrated DNA Technology (Coralville, IA). [*methyl-*³H]Thymidine was from PerkinElmer Life Sciences (Boston, MA). Phosphate-buffered saline (PBS) contained (in 1.00 liter) KCl (0.20 g), KH₂PO₄ (0.20 g), NaCl (8.0 g), and Na₂HPO₄·7H₂O (2.16 g), and had pH 7.4. All other chemicals and reagents were of commercial grade or better, and were used without further purification.

K-562 cells, which derive from a continuous human chronic myelogenous leukemia line, were from the American Type Culture Collection (Manassas, VA). Cell culture medium and supplements were from Invitrogen (Carlsbad, CA).

Instrumentation. Protein molecular mass was determined by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry using a Voyager-DE-PRO Biospectrometry Workstation (Applied Biosystems, Foster City, CA) and a 3,5dimethoxy-4-hydroxycinnamic acid (sinapinic acid) matrix (Sigma Chemical). Fluorescence measurements were performed with a QuantaMaster 1 Photon Counting Fluorometer equipped with sample stirring (Photon Technology International, South

Brunswick, NJ). Radioactivity was quantitated with a Microbeta TriLux liquid scintillation and luminescence counter (PerkinElmer, Wellesley, MA).

Production of Wild-Type BS-RNase and its Variants. Wild-type BS-RNase was produced in *Escherichia coli* strain BL21(DE3) under the direction of pSR1, which is a pET-17b-based vector that contains a synthetic gene for BS-RNase, as described previously (Kim and Raines, 1993; Leland et al., 1998). DNA encoding variants of BS-RNase was made from pSR1 with the QuikChange[®] Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA). Oxidative folding of wild-type BS-RNase and its dimeric variants was performed as described previously (Smith et al., 1978; D'Alessio et al., 2001). Briefly, protein produced as inclusion bodies was solubilized by adding 0.020 M Tris-HCl buffer (pH 8.0) containing guanidine-HCl (7.0 M) and EDTA (0.01 M). DTT was added to a final concentration of 0.1 M, and the resulting mixture was stirred for 2 h at room temperature. The solution was dialyzed overnight against 20 mM acetic acid, and the insoluble material was removed by centrifugation. The protein was folded oxidatively in the presence of glutathione (0.6 mM oxidized, 3.0 mM reduced) in 0.10 M Tris-HCl buffer (pH 8.4) containing L-arginine (0.5 M). After 18 h, dimerization was achieved by reduction of mixed disulfides between glutathione and Cys31 and Cys32 by the addition of a 10-fold molar excess of DTT, followed by air-oxidation overnight. Oxidative folding of monomeric variants of BS-RNase was carried out in the same manner as for the dimeric enzyme but without the dimerization process.

Production of Human RI. Human RI was produced according to methods described previously with minor changes (Leland *et al.*, 1998; Klink *et al.*, 2001). Briefly, DNA

encoding human RI was cloned into a pET-22b(+) vector between the *NdeI* and *SalI* sites with a Met(-1) sequence. The resulting vector was used to transform cells of *E. coli* strain BL21(DE3). Transformed cells were grown to mid-log phase at 37 °C; expression of the hRI cDNA was induced by the addition of IPTG (to 0.5 mM), and the induced cells were grown overnight at 20 °C. Cells were then lysed with a French pressure cell, and insoluble material was removed by centrifugation. RI was purified using RNase A-affinity chromatography followed by anion-exchange chromatography. Purified RI was stored in PBS containing DTT (5 mM) to prevent oxidation. The molar concentration of active RI was determined by titration against a known concentration of RNase A (Neumann and Hofsteenge, 1994), using 6-FAM–dArUdAdA–6-TAMRA as a substrate (*vide infra*).

Assays of Ribonucleolytic Activity. Ribonucleolytic activity was measured with a hypersensitive assay based on the cessation of fluorescence quenching (Kelemen *et al.*, 1999). Briefly, the increase of fluorescence at 515 nm was measured upon adding enzyme to PBS containing 6-FAM–dArUdAdA–6-TAMRA (50 nM) at 23 ± 2 °C. Values for k_{cat}/K_{M} were calculated with the equation 2.1.

Assays of Cytotoxic Activity. The effect of wild-type BS-RNase, its variants, ONC, and RNase A on cell proliferation was determined as described in Chapter Two. The IC_{50} value of the wild-type BS-RNase and its variants were calculated as described in Chapter Three.

Assays of Binding to Human RI. The ability of monomeric variants of BS-RNase to bind to human RI was assessed by a competition assay reported previously with a minor

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modification (Abel *et al.*, 2002). The assay was done in PBS containing DTT (5 mM) to prevent oxidation by air. First, the initial fluorescence (excitation: 491 nm; emission: 511 nm) of the fluorescein-labeled G88R RNase A (final concentration: 50 nM, K_d for human RI-G88R RNase A complex: 0.54 nM) was measured. Next, human RI was added (to 50 nM), and the decrease of the fluorescence evoked by RI-binding was measured after a 15-min equilibration. Finally, varying concentrations of a BS-RNase were added to compete with the fluorescein-labeled enzyme, and the increased fluorescence due to the dissociation of fluorescein-labeled enzyme from RI was measured after each addition and equilibration. The concentration of the remaining human RI–fluorescein-labeled G88R RNase A was calculated by comparing the fluorescence to the initial fluorescence. Values of K_d were obtained by a nonlinear least-squares analysis of the binding isotherm with the program DELTAGRAPH 5.5 (Red Rock Software, Salt Lake City, UT).

4.4 Results

Rational Design of RI-Evading BS-RNase Variants. A recent analysis of the RI-RNase A complex revealed several regions of high shape complementarity between RI and RNase A (Rutkoski *et al.*, 2005), three of which were of interest to us here (Figure 4.1D). Gly88 and Asn67 of RNase A are proximal to Trp259 and Val405, respectively; Asp38 and Arg39 of RNase A form favorable Coulombic interactions with Arg453 and Glu397 of RI. Without any detailed structural information of the complex with monomeric BS-RNase, we reasoned that RNase A can serve as a good model for

monomeric BS-RNase because of its high sequence identity (83%) and similar threedimensional structure (Sica *et al.*, 2003).

Our design strategy was as follows. First, we prepared C31A/C32A/G88R BS-RNase. Replacing the two cysteine residues with alanine excises two sulfur atoms and thereby prevents dimer formation. As in G88R RNase A (Leland et al., 1998), the bulky arginine residue at position 88 should create a steric clash with RI residues (Figure 4.1D). Substitutions in this region are known to diminish the affinity of RNase A (Leland et al., 1998; Suzuki et al., 1999; Rutkoski et al., 2005; Rutkoski et al., 2005), BS-RNase (Antignani et al., 2001), and RNase 1 (which is a human homolog (Leland et al., 2001)) for RI. Next, we attempted to disrupt a favorable Coulombic interaction between RI and C31A/C32A/G88R BS-RNase by swapping residues 38 and 39. In RNase A, swapping Asp38 and Arg39 converts two attractive interactions into two repulsive ones (Rutkoski et al., 2005). The corresponding residues in BS-RNase are Gly38 and Lys39. Accordingly, two distinct sets of substitutions were introduced in this region. We employed the strategy validated with D38R/R39D RNase A (Rutkoski et al., 2005), incorporating arginine and aspartate residues at the corresponding positions in BS-RNase to create C31A/C32A/G38R/K39D/G88R BS-RNase. In addition, we swapped the endogenous BS-RNase residues, Gly38 and Lys39, to create

C31A/C32A/G38K/K39G/G88R BS-RNase. Then, we replaced Asn67 with an arginine residue in the C31A/C32A/G38K/K39G/G88R variant. This substitution is known to generate an additional steric clash in the RNase A·RI (Rutkoski *et al.*, 2005). Finally, we generated dimers of these variants by reinstalling Cys31 and Cys32. It is noteworthy that

none of the altered residues are known to contribute to the catalysis of RNA cleavage by BS-RNase.

Protein Production. Wild-type BS-RNase and its variants were produced in *E. coli* to yield ≥ 20 mg of purified enzyme per liter of culture. Purified enzymes appeared as a single band after electrophoresis in a polyacrylamide gel in the presence of sodium dodecyl sulfate (data not shown), and each had the expected mass to within 0.065% according to MALDI–TOF mass spectrometry (Table 4.1). Wild-type BS-RNase and its dimeric variants ran in the gel as a dimer in the absence of a reducing agent, and as a monomer if boiled in reducing sample buffer (data not shown). This result indicates that the quaternary structure of the dimers is maintained by disulfide bonds.

Human RI was produced in *E. coli* to yield 5 mg of purified protein per liter of culture.

Ribonucleolytic Activity. Each monomeric variant of BS-RNase had ribonucleolytic activity within 30% of that of C31A/C32A BS-RNase (Table 4.1). Each dimeric variant had ribonucleolytic activity within 20% of that of wild-type BS-RNase. Thus, none of the substitutions had an adverse affect on the ability of BS-RNase to catalyze RNA cleavage. The dimeric enzymes had approximately twofold greater ribonucleolytic activity on a molar basis than did the monomeric enzymes, reflecting their two independent active sites (Mazzarella, 1993; Sica *et al.*, 2004).

Affinity for Human RI. The C31A/C32A/G88R substitution decreased the affinity of monomeric BS-RNase for RI by 250-fold. The C31A/C32A/G38K/K39G/G88R, C31A/C32A/G38R/K39D/G88R, and C31A/C32A/G38K/K39G/N76R/G88R

substitutions each decreased the affinity for RI by 10⁴-fold. Our competition assay does not provide an accurate measure of the affinity of the dimeric BS-RNases (too low) and C31A/C32A BS-RNase (too high) for RI (Abel *et al.*, 2002). The dimeric BS-RNases are likely to form a complex with $K_d > 2 \mu M$,⁴ (Abel *et al.*, 2002) as reported previously for wild-type BS-RNase (Antignani *et al.*, 2001). Similarly, C31A/C32A BS-RNase is likely to form a complex with K_d near 9.3 ± 3.2 pM, as reported for C31K/C32S BS-RNase

(Antignani et al., 2001).

Cytotoxic Activity. Each monomeric variant of BS-RNase was toxic to cultured K-562 cells (Figure 4.2). C31A/C32A/G88R BS-RNase had an IC₅₀ value of $0.11 \pm 0.02 \mu$ M (Table 4.1). C31A/C32A/G38R/K39D/G88R BS-RNase had an IC₅₀ value of $0.07 \pm 0.01 \mu$ M. The C31A/C32A/G38K/K39G/G88R and C31A/C32A/G38K/K39G/N76R/G88R variants were more cytotoxic, having IC₅₀ values of 0.046 ± 0.009 and $0.048 \pm 0.01 \mu$ M, respectively. In contrast, C31A/C32A BS-RNase had an IC₅₀ value of >50 μ M. ONC had an IC₅₀ value of $0.2 \pm 0.1 \mu$ M (Figure 4.2), which is similar to values reported previously (Leland *et al.*, 2000; Dickson *et al.*, 2003; Haigis *et al.*, 2003).

⁴ Values of K_i reported by Antignani and coworkers (2001) were determined by assaying the inhibition of ribonucleolytic activity by RI. Such values are equivalent to values of K_d for the RI-RNase A complex determined by the competition assay used herein.
All three dimeric BS-RNase variants were more cytotoxic than wild-type BS-RNase, which had an IC₅₀ value of $1.3 \pm 0.1 \mu$ M. G88R BS-RNase and G38R/K39D/G88R BS-RNase had four-fold greater cytotoxic activity than did the wildtype enzyme, and G38K/K39G/G88R BS-RNase had 15-fold greater cytotoxic activity.

4.5 Discussion

The widely accepted mechanism for BS-RNase–mediated cytotoxicity requires that the enzyme exhibit ribonucleolytic activity in the cytosol. In support of this mechanism, catalytically inactive BS-RNase is not cytotoxic (Kim *et al.*, 1995). In addition, the dimeric structure of the enzyme enables evasion of cytosolic RI (Cafaro *et al.*, 1995; Kim and Raines, 1995; Kim *et al.*, 1995). Monomers of wild-type BS-RNase derived from reduction of the two intersubunit disulfide bonds are inhibited by RI (Murthy and Sirdeshmukh, 1992; Murthy *et al.*, 1996) and are not cytotoxic (Kim and Raines, 1995). Thus, we reasoned that monomers of BS-RNase that resist RI as well as dimers composed of such monomers could provide new insight into the structure and function of BS-RNase.

RI Affinity of Monomeric BS-RNase Variants. In previous work, we designed semisynthetic monomers of BS-RNase that evade RI (Matousek *et al.*, 1997). Specifically, we modified the sulfhydryl group of Cys31 in C32S BS-RNase or Cys32 in C31S BS-RNase with a carboxymethyl group. The resulting semisynthetic enzymes were shown to evade RI in a qualitative assay, and to be toxic for spermatogenic layers in

mice. The semisynthetic enzymes were not, however, toxic to K-562 or other mammalian cells. Here, we have extended this approach.

In RNase A, the G88R substitution decreases the affinity for RI by nearly 10⁴-fold (Leland *et al.*, 1998). In BS-RNase, the same substitution decreases the affinity for RI by 250-fold (Table 4.1). Thus, changing Gly88 in an RNase A homolog provides a reliable means to diminish affinity for RI without decreasing ribonucleolytic activity.

Perturbing the presumed Coulombic interactions between RI and residues 38 and 39 of BS-RNase further enhances RI evasion. Both C31A/C32A/G38K/K39G/G88R BS-RNase and C31A/C32A/G38R/K39D/G88R BS-RNase have 50-fold less affinity for RI than does C31A/C32A/G88R BS-RNase (Table 4.1). Swapping residues 38 and 39 of RNase A had a similar effect on the affinity of that enzyme for RI (Rutkoski *et al.*, 2005). Thus, substitutions to residues 38 and 39 in an RNase A homolog can be used to diminish affinity for RI without decreasing ribonucleolytic activity. In accord with our experimental results, a recent computational analysis by Camacho and coworkers designated Arg39 of RNase A as a secondary "anchor residue" in the RI RNase A complex (Rajamani *et al.*, 2004).

Asn67 of RNase A is in close contact with Val405 of RI in the RI–RNase A complex (Figure 4.1D) and was identified by Camacho and coworkers as the primary "anchor residue" in the RI·RNase A complex (Rajamani *et al.*, 2004). Replacing Asn67 with an arginine was found to decrease the affinity of RNase A for RI (Rutkoski *et al.*, 2005). We tried to enhance the ability of C31A/C32A/G38K/K39G/G88R BS-RNase to evade RI by

installing an arginine residue at position 67. Surprisingly, this substitution had a negligible effect on the affinity for RI.

Cytotoxicity of Monomeric BS-RNase Variants. Monomeric BS-RNase variants that evade RI are cytotoxic (Figure 4.2A; Table 4.1). C31A/C32A/G88R BS-RNase has $>10^3$ fold greater cytotoxic activity than does C31A/C32A BS-RNase. The addition of a cationic residue (arginine) could enhance the affinity of the enzyme for the anionic surface of a mammalian cell (Futami *et al.*, 2001; Ilinskaya *et al.*, 2002), and thus be partly responsible for the enhanced cytotoxicity of G88R BS-RNase. The even greater cytotoxic activity of C31A/C32A/G38R/K39D/G88R BS-RNase, which has the same molecular charge as C31A/C32A BS-RNase, demonstrates that evasion of RI does indeed play a key role in the process. Likewise, C31A/C32A/G38K/K39G/G88R BS-RNase is a more potent cytotoxin than is C31A/C32A/G88R BS-RNase, even though the two enzymes have the same molecular charge. Thus, the substitution of three residues converts a nontoxic enzyme into a cytotoxin that is not only potent, but more potent than ONC (Figure 4.2A), which provides the benchmark for the cytotoxic activity of ribonucleases (Matousek *et al.*, 2003). Indeed, C31A/C32A/G38K/K39G/G88R BS-RNase is the most cytotoxic variant or homolog of RNase A reported to date.

Cytotoxicity of Dimeric BS-RNase Variants. The cytotoxic activity of wild-type (that is, dimeric) BS-RNase is greater if the enzyme is composed of monomers that evade RI (Figure 4.2B; Table 4.1). Interestingly, the same trends in cytotoxic activity observed with monomeric BS-RNases are also evident with their dimeric counterparts. For variants of the same molecular charge, constituent monomers with a higher K_d value always give

rise to a dimer with greater cytotoxic activity. For example, G38R/K39D/G88R BS-RNase is more cytotoxic than is the wild-type enzyme, and G38K/K39G/G88R BS-RNase is more cytotoxic than is the G88R variant. In other words, the cytotoxic activity of a BS-RNase variant correlates with the ability of its constituent monomers to evade RI. BS-RNase is known to dissociate into monomers in a buffer that mimics the reducing environment of the cytosol (Kim *et al.*, 1995). Our data indicate that a significant fraction of BS-RNase dissociates in the cytosol, and enable us to elaborate the mechanism of BS-RNase cytotoxicity (Figure 4.3)(Bracale *et al.*, 2002).

Recently, D'Alessio and coworkers reported that G88R/S89E BS-RNase is not significantly more cytotoxic than wild-type BS-RNase, even though this variant can evade RI after monomerization ($K_d = 2.5 \pm 0.5$ nM (Antignani *et al.*, 2001)). The apparent discrepancy with our work can be explained by the greater cytotoxic activity of G88R BS-RNase arising largely from its acquisition of a positive charge, which could enhance cellular uptake of the enzyme (*vide supra*). The S89E substitution in G88R/S89E BS-RNase negates the benefit of the additional positive charge. We conclude that the evasion of RI accrued from the G88R substitution alone is not sufficient to endow dimeric BS-RNase with enhanced cytotoxicity.

Molecular Charge and Cytotoxicity. Some ribonucleases are highly cationic proteins (Table 4.2). This cationic character is thought to facilitate cellular uptake of the enzyme. Moreover, increasing this cationic character by chemical modification (Futami *et al.*, 2001), site-directed mutagenesis (Ilinskaya *et al.*, 2002), or addition of an Arg₉ tag (Fuchs and Raines, 2005) enhances its cytotoxicity. G38K/K39G/G88R BS-RNase and

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C31A/C32A/G38K/K39G/G88R BS-RNase are both more cationic and more cytotoxic than G38R/K39D/G88R BS-RNase and C31A/C32A/G38R/K39D/G88R BS-RNase, respectively, but do not differ significantly in molecular size, ribonucleolytic activity, or affinity for RI (Figure 4.2; Table 1). It is noteworthy that in creating a monomeric BS-RNase variant, D'Alessio and coworkers increased the cationicity of the enzyme by replacing Cys31 with a lysine residue (Antignani *et al.*, 2001). The resulting variant, C31K/C32S BS-RNase, exhibited significant cytotoxic activity (IC₅₀ = 80 ± 7 µg/mL or $5.9 \pm 0.5 \mu$ M) even though it had high affinity for RI ($K_d = 9.3 \pm 3.2 p$ M).

Monomer versus Dimer. Monomeric BS-RNase variants that evade RI display greater cytotoxic activity than do their dimeric BS-RNase counterparts (Figure 4.2; Table 1). This finding was unexpected, as the ability of a dimeric BS-RNase to evade RI should always exceed that of its constituent monomers. Other factors that contribute to the cytotoxicity of a ribonuclease, such as efficient internalization or translocation (Figure 4.3), must be responsible for this discrepancy. Regardless, our data demonstrate that BS-RNase monomers that evade RI can be supremely potent cytotoxins.

4.6 Conclusions

We have investigated the significance of the dimeric structure of BS-RNase in its cytotoxicity. In so doing, we created monomeric BS-RNases with greater cytotoxic activity than ONC, an amphibian ribonuclease in Phase III trials as a cancer chemotherapeutic, or any dimeric BS-RNase. Thus, the dimeric structure of BS-RNase provides a means to evade RI but is not necessary for cytotoxicity and can actually

decrease cytotoxic activity. Finally, our data indicate that the BS-RNase dimer is severed in the cytosol, where RI limits its cytotoxicity.

Acknowledgments. We are grateful to B. D. Smith for providing a production system for human RI, and to Drs. J. C. Mitchell, B. G. Miller, and E. A. Kersteen, and T. J. Rutkoski and R. J. Johnson for contributive discussions.

BS-RNase	∆Z/molecule	$\frac{k_{\rm cat}/K_{\rm M}}{(10^7 {\rm M}^{-1} {\rm s}^{-1})^{a}}$	K (nM) ^b		<i>m</i> / <i>z</i> ^g	
			Nd (IIIVI)	IC ₅₀ (μινι)	expected	observed
Wild-type	0	3.5 ± 0.3	$>2 \times 10^{3 d}$	1.3 ± 0.1	27,464	27,472
G88R	+2	3.5 ± 0.1	ND ^e	0.28 ± 0.06	27,662	27,674
G38K/K39G/G88R	+2	3.4 ± 0.2	ND ^e	0.091 ± 0.02	27,662	27,671
G38R/K39D/G88R	0	4.1 ± 1.0	ND ^e	0.26 ± 0.04	27,834	27,850
C31A/C32A	0	1.9 ± 0.2	$(9.3 \pm 3.2) \times 10^{-3 f}$	>50	13,660	13,668
C31A/C32A/G88R	+1	2.3 ± 0.1	2.3 ± 0.6	0.11 ± 0.02	13,769	13,762
C31A/C32A/G38K/ K39G/G88R	+1	1.7 ± 0.03	$(1.0 \pm 0.4) \times 10^2$	0.046 ± 0.009	13,769	13,763
C31A/C32A/G38R/ K39D/G88R	0	1.4 ± 0.1	$(1.1 \pm 0.4) \times 10^2$	0.07 ± 0.01	13,855	13,864
C31A/C32A/G38K/ K39G/N67R/G88R	+2	2.1 ± 0.2	$(9.3 \pm 1.1) \times 10^{1}$	0.048 ± 0.01	13,811	13,819

 Table 1: Properties of Wild-Type BS-RNase and its Dimeric and Monomeric Variants

^{*a*} Values of k_{cat}/K_M (± S.E.) are for catalysis of 6-FAM–dArUdAdA–6-TAMRA cleavage at 23 ± 2 °C in PBS (pH 7.4). ^{*b*} Values of K_d (± S.E.) are for the complex with human RI at (23 ± 2) °C. ^{*c*} Values of IC₅₀ (± S.E.) are for incorporation of [*methyl*-³H]thymidine into the DNA of K-562 cells exposed to a BS-RNase, and were calculated with Equation 3.2. The IC₅₀ value for ONC is $0.2 \pm 0.1 \mu M$.

^d From Antignani and coworkers (2001).

^e ND, not determined.

^f For C31K/C32S BS-RNase (Antignani *et al.*, 2001).

^g Values of m/z were determined by MALDI-TOF mass spectrometry.

Table 4.2 : Molecular Charges of RNase A and its Monomeric Homo
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Ribonuclease	Arg	Lys	Asp	Glu	Z	Z/Residue
RNase A (cow)	4	10	5	5	+4	+3.2%
RNase 1 (human)	10	8	6	6	+6	+4.7%
Onconase®	3	12	6	3	+5 ^a	+4.8%
BS-RNase, monomer	4	14	4	5	+9	+7.3%

^{*a*} *N*-Terminus is uncharged because residue 1 is a pyroglutamate.

Figure 4.1 Amino acid sequences and three-dimensional structures of RNase A and BS-RNase. (A) Amino acid sequence alignment of RNase A and BS-RNase. The secondary structure of RNase A is identified with h (ahelix), s (β -strand), or t (turn). Residues conserved between the two ribonucleases are in gray boxes. Key active-site residues are in black boxes. The two cysteine residues that participate in the intersubunit linkage of BS-RNase are in a yellow box. Residues near regions of high shape complementarity in the porcine RI·RNase A complex are in red. (B) Three-dimensional structure of RNase A (PDB entry 7RSA (Wlodawer et al., 1988)). (C) Three-dimensional structure of BS-RNase in its M×M form (PDB entry 1BSR (Mazzarella, 1993)). (D) Threedimensional structure of the porcine RI·RNase A complex (PDB entry 1DFJ (Kobe and Deisenhofer, 1995)) highlighting regions disrupted herein. Images were created with the program MOLSCRIPT and rendered with the program RASTER3D (Merritt and Murphy, 1994).



Figure 4.2 Effect of BS-RNase, its variants, RNase A, and ONC on the proliferation of cultured K-562 cells. Cell proliferation was determined by incorporation of [*methyl-*³H]thymidine into cellular DNA after a 44-h incubation with a ribonuclease. Each value is the mean (± S.E.) of ≥3 independent experiments with triplicate samples, and is expressed as a percentage of the PBS control. Enzyme concentration is expressed in terms of µM active site to account for the two active sites in dimeric BS-RNases. (A) Monomeric BS-RNases: △, C31A/C32A BS-RNase; ◇, C31A/C32A/G88R BS-RNase; □, C31A/C32A/G38K/K39G/G88R; ○, C31A/C32A/G38K/K39G/N76R/G88R BS-RNase; ▼,
C31A/C32A/G38K/K39G/N76R/G88R BS-RNase; ▼,
C31A/C32A/G38K/K39G/N76R/G88R BS-RNase; ◇, G88R BS-RNase; □, G38K/K39G/G88R BS-RNase; ○, G38R/K39D/G88R BS-RNase;
□, G38K/K39G/G88R BS-RNase; ○, G38R/K39D/G88R BS-RNase;
□, RNase A; ●, ONC.



Figure 4.3

Putative mechanism for the cytotoxic activity of wild-type BS-RNase (Bracale *et al.*, 2002; Haigis and Raines, 2003). (A) BS-RNase binds to the cell surface and is internalized by dynamin-independent endocytosis. (B) BS-RNase reaches the cytosol by translocation from an endosome or the Golgi apparatus. (C) Intact BS-RNase degrades cellular RNA, leading to apoptosis. (D) Dissociated BS-RNase is inhibited by cytosolic RI (this work).





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