ME\textsubscript{1}, a type I ribosome-inactivating protein (RIP), belongs to a family of enzymes long believed to possess rRNA N-glycosidase activity directed solely at the universally conserved residue A4324 in the sarcin/ricin loop of large eukaryotic and prokaryotic rRNAs. We have investigated the effect of modifying the structure of non-ribosomal RNA substrates on their interaction with ME\textsubscript{1} and other RIPs. ME\textsubscript{1} was shown to depurate a variety of partially denatured nucleic acids, randomly removing adenine residues from single-stranded regions and, to a lesser extent, guanine residues from wobble basepairs in hairpin stems. A defined sequence motif was not required for recognition of non-paired adenosines and cleavage of the N-glycosidic bond. Substrate recognition and ME\textsubscript{1} activity appeared to depend on the physical availability of nucleotides, and denaturation of nucleic acid substrates increased their interaction with ME\textsubscript{1}. Pretreatment of mRNA at 75 °C rather than 60 °C, for example, lowered the apparent \( K_{d} \) from 87.1 to 73.9 nM, making it more vulnerable to depurination by RIPs. Exposure to ME\textsubscript{1}, \textit{in vitro} completely abolished the infectivity of partially denatured RNA transcripts of the potato spindle tuber viroid, suggesting that RIPs may target invading nucleic acids before they reach host ribosomes \textit{in vivo}. Our data suggest that the extensive folding of many potential substrates interferes with their ability to interact with RIPs, thereby blocking their inactivation by ME\textsubscript{1} (or other RIPs).

Ribosome-inactivating proteins (RIPs)\textsuperscript{1} are cytotoxic enzymes that have been identified in plants, fungi, and bacteria and act as glycosidases that specifically cleave nucleotide N-glycosidic bonds (1). It has been proposed that RIPs inhibit protein synthesis by virtue of their enzymatic activity, selectively removing a specific adenine residue from the highly conserved and surface-exposed \( \alpha \)-sarcin/ricin loop in the large rRNA (1–3). This enzymatic cleavage prevents the binding of the EF-2-GTP complex to the ribosome, with the subsequent arrest of protein synthesis and eventually cell death (4).

The universally conserved adenine residue A4324 of the eukaryotic 28 S rRNA was long considered the only enzymatic substrate of RIPs, but several lines of evidence have recently identified a variety of alternative substrates. For instance, it has been shown that several RIPs can release adenine from multiple sites in rRNA (5). Furthermore, saporin-L1 can release adenine residues from a variety of nucleic acid substrates, including poly(A), mRNA, tRNA, and DNA (6, 7). More than 50 other RIPs are active on DNA (8). Certain RIPs also display enzymatic activity toward RNA transcripts derived from various plant and animal viruses including the human immunodeficiency virus (6, 9–16). However, enzymatic activity on these non-ribosomal substrates requires a high protein:substrate ratio, and the biological relevance of these observations is unclear.

Several studies using the ricin A-chain (RTA) have suggested that RIP catalytic activity requires a specific substrate structure such as a tetraloop with the sequence \( 5'\text{GAGA}^4 \) (17–19). The stem of this structure possesses tilted Watson-Crick base pairing in the stem with an unusual G1:A4 base pairing in the loop region (19, 20). The depurination site adenine (A2) occupies an exposed position outside the solvent-accessible loop, whereas the other nucleotide bases are buried within the phosphodiester backbone by hydrogen bonding and base stacking. In contrast, pokeweed antiviral protein (PAP) does not exhibit an absolute requirement for the tetraloop structure to exhibit enzymatic activity (18). Recent reports propose that PAP recognizes and binds to the cap structure of mRNAs, specifically depurinating downstream adenine residues (21, 22). Based on these results, PAP may bind to capped viral RNA, subsequently depurinating viral RNAs rather than host ribosomes during the infection process (21). However, this hypothesis does not explain the activity of PAP with substrates such as rRNA or DNA that lack a cap structure (8).

Possible explanations for this remarkable array of enzymatic activities include inherent differences among RIPs, the diversity of RNA substrates, or, as explored in this paper, differences in experimental conditions. Temperature, pH, and ionic composition of the assay buffer change not only the catalytic efficiency \( k_{cat}/K_{m} \) of RIPs but also their target sites (7, 23, 24). Experimental conditions also determine the catalytic activities of different RIPs (8). Efficient catalysis may require that
substrate(s) assume a particular structural conformation induced by specific experimental conditions. These observations prompted us to ask whether the structural changes of substrates induced by experimental treatments such as heating could affect the enzymatic activity of RIPs.

RNAs are highly flexible molecules whose structures are influenced by such factors as the vectorial nature of transcription and translation, trans-acting factors, the presence of RNA-binding proteins or RNA chaperones, and the cellular environment, including ion homeostasis (25). Thus far, staining of RNA secondary structure has been carried out almost exclusively in vitro, and it remains to be proven whether conclusions from these studies also apply in vivo. For example, a recent study of telomerase RNA has shown that RNA structures can be different in vitro and in vivo, as exemplified by the in vitro formation of the phylogenetically conserved pseudoknot in the 5′-part of the telomerase RNA, which was not observed in vivo (26). Importantly, changes in RNA structure in vivo can cause loss of the ability to interact with other molecules (27). Experimental variables such as temperature or ionic concentration often trap RNAs in inactive conformations that interfere with their interactions with other molecules (28, 29).

In the present study, we have systematically perturbed the conformation of various nucleic acids to further understand the substrate specificity of RIPs. Our results demonstrate that the enzymatic activity of ME1, an RIP from Mirabilis expansa, on non-ribosomal substrates is highly structure-dependent. Extensive structural folding caused by experimental conditions was found to interfere with the interaction of ME1, and non-ribosomal substrates. Enzymatic specificity and kinetics vary with the conformation of non-ribosomal substrates, and a specific motif was not found to be necessary for substrate recognition by ME1.

**EXPERIMENTAL PROCEDURES**

**Proteins—** ME1 was purified from storage roots of *M. expansa* using immunoaffinity chromatography. A polyclonal antibody raised previously in our laboratory against reverse-phase high pressure liquid chromatography-purified ME1 (30) was coupled to CNBr-activated Sepharose at pH 8.0. Total root proteins of *M. expansa* were equilibrated with 75 mM Tris-HCl, pH 8.0, to facilitate protein-ligand interaction, and immunobound proteins were then eluted with 100 mM glycine-HCl, pH 2.7, containing 0.5 M NaCl. ME1 was further separated using cation-exchange high performance liquid chromatography (POROS HS; Applied Biosystems), resulting in >99% pure protein as judged by SDS-PAGE with silver staining and sequence analysis. Purified RTA and saporin-56 were purchased from Sigma. Their purities were checked by SDS-PAGE with silver staining, and protein concentration was determined as described by Bradford (31) using a protein assay kit (Bio-Rad) and bovine serum albumin as a standard.

**DNA and Oligonucleotide—** The open reading frame region of *M. expansa* DNA and Oligonucleotide—pap-h open reading frame were amplified from the corresponding pGEM®-T Easy plasmid by PCR using gene-specific primers 5′E30 and 3′E28. After electrophoresis in a 1.5% (w/v) agarose gel and staining in 0.5 mg/ml ethidium bromide, the DNA fragments were excised from the gel and recovered using the Quantum Prep gel slice kit (Bio-Rad). The isolated DNA was double-stranded and amplified by PCR from hairy roots of pokeweed (*Phytolacca americana*) using RNA polymerase. A polyclonal antibody raised previously in our laboratory against reverse-phase high pressure liquid chromatography-purified ME1 (31) using a protein assay kit (Bio-Rad) and bovine serum albumin as a standard.

**Temperature-gradient Gel Electrophoresis—** Temperature-gradient gel electrophoresis of 32P-labeled PSTVd RNAs was carried out using a commercially available apparatus (Qiagen). The horizontal 5% polyacrylamide, 0.17% bisacrylamide gel and buffer reservoirs contained 45°C Tris-borate-EDTA, 5 mM NaCl. Following preincubation at 26°C (20 min, 30°C), PSTVd RNA transcripts were diluted with 165 µl of loading buffer (18 mM NaCl, 17 mM sodium cacodylate, 3 mM sodium citrate, 0.2 mM EDTA, pH 7.0) and 25 µl of loading dye (50% glycerol, 1× Tris-borate-EDTA, 2% bromphenol blue-xylene cyanol) and applied to the single 12-cm sample slot. Sixty min later, the current flow was stopped for 30 min while a 25–65°C temperature gradient was generated across the gel. The current was reapplied, the voltage was increased to 350 V, and 1 h 45 min later, the gel was fixed in 10% (v/v) ethanol, 1% (v/v) acetic acid and dried before overnight autoradiography.

**Primer Extension—** pap-h RNA transcripts treated with ME1, as
described above were purified by extraction with phenol:chloroform and chloroform:isoamyl alcohol followed by ethanol precipitation. RNAs were resuspended in 5 μl of 2× primer extension buffer (100 mM Tris-HCl, pH 8.3, 100 mM KCl, 20 mM MgCl₂, 20 mM dithiothreitol, 1 mM spermidine, 2 mM dNTPs) and annealed for 20 min at 58 °C with an oligonucleotide primer (5'-CTTGGCTGTAGTTGCTTAGG-3') complementary to a sequence located 200 bases downstream from the 5'-end of pap-h mRNA. Primer extension by reverse transcriptase was carried out for 30 min at 42 °C in a final reaction volume of 20 μl with the addition of 40 mM sodium phosphate buffer. RNA templates were destroyed by incubation with RNase A before the addition of formamide loading buffer (20 μl) to terminate the reaction. Following electrophoresis in 6% polyacrylamide gels containing 7 M urea, extension products were visualized by silver staining using the Silver Sequence™ DNA sequencing system (Promega). The position of the 5'-end was verified by comparison with a DNA sequencing ladder from a pGEM®-T Easy plasmid encoding pap-h that was prepared with the same primer used for primer extension.

In-line Probing of RNA—Values for the half-maximal apparent dissociation constant (apparent Kₐ) of each construct were determined by conducting in-line probing of RNA, wherein the concentration of the ME₁, was varied between 0 and 100 μM. Composite plots of the fraction cleavage at the site(s) of depurination, and small nucleotide fragment(s) are released. As a result, the amount of intact nucleic acid quantitatively decreases.

**RESULTS**

**Immunopurification of ME₁.**—To obtain ME₁ with a high degree of purity in a single step, we used an affinity chromatography approach involving a polyclonal anti-ME₁ antibody. This antibody, which was raised previously in our laboratory (30), was used as a ligand to generate an affinity matrix via coupling to CNBr-activated Sepharose. Total root proteins of *M. expansa* were applied to the column, and immunobound proteins were eluted with pH linear gradients as outlined under “Experimental Procedures.” As shown in Fig. 1A and B, the single protein peak produced by the elution process contained two protein bands. These bands corresponded to ME₁ (30 kDa; Ref. 35) and ME₂ (27 kDa; Ref. 30), two RIPs previously found to be present in *M. expansa* roots. ME₁ was further purified by cation-exchange chromatography, and its RNA N-glycosidase activity was confirmed with ribosomes prepared from *S. cerevisiae* as described previously (36). As shown in Fig. 1C, the immunopurified ME₁ depurinated the 28 S rRNA and released the diagnostic 367-nucleotide fragment upon treatment with aniline, thereby demonstrating that immunopurified ME₁ is an enzymatically active RIP.

**Activity of ME₁ on Partially Denatured Non-ribosomal Substrates**—As the first step in understanding the enzymatic activity of RIPs on nucleic acids, we examined the reaction catalyzed by ME₁ on such partially denatured non-ribosomal substrates as DNA, rRNA isolated from yeast ribosomes, and ssDNA encoding PAP-H (RIP identified in the hairy roots of pokeweed; Ref. 36). Before incubation with ME₁, each nucleic acid substrate was preheated to temperatures ranging from 30 to 90 °C to induce structural modifications. Non-covalent interactions such as hydrogen bonding and base stacking that stabilize double-stranded DNA (dsDNA) can be disrupted by simply raising the temperature. When a depurinated nucleic acid is treated with aniline, the phosphodiester backbone undergoes cleavage at the site(s) of depurination, and small nucleotide fragment(s) are released. As a result, the amount of intact nucleic acid quantitatively decreases.

As shown in Fig. 2A, the fact that dsDNA levels were not affected by preincubation at 30 °C followed by incubation with ME₁ and aniline treatment indicates that ME₁ neither depurinates nor degrades dsDNA. Increasing the preincubation temperature to 45 or even 75 °C resulted in the release of single-stranded DNA (ssDNA), but ME₁ activity remained undetectable. In contrast, the quantitative decrease in intact ssDNA template visible in the electrophoretic profiles of dsDNA preincubated at 90 °C showed that ME₁ readily depuri-
Nucleic acids were subsequently fractionated on 6% (w/v) polyacrylamide gels containing 7 M urea and stained with ethidium bromide. Importantly, the enzymatic activity of ME1 was both rapid and spontaneous. The fact that ssDNA depurination was completed in 30 s (Fig. 2B) allowed us to assess ME1-nucleic acid interaction after nucleic acid substrates were able to refold at 37 °C (see Supplemental Fig. 1). Throughout this study, aniline treatment of partially denatured nucleic acids alone did not result in cleavage (results not shown).

We next examined the catalytic action of ME1 on two partially denatured RNA substrates. As shown in Fig. 3A (lanes d and e), ME1 was not active on deproteinized rRNAs stored at 0 °C. Data presented in Fig. 3B show, however, that preincubation of rRNAs at relatively low temperatures (i.e. 45 and 60 °C) followed by exposure to ME1 resulted in the depurination of 26 S rRNA. Increasing the preincubation temperature to 75 °C resulted in depurination of both 26 S and 18 S rRNA. These results were similar to those obtained with ssDNA substrates; i.e. exposure to as little as 100 pg of ME1 resulted in the rapid (≥30 s) depurination of rRNA (data provided as Supplemental Fig. 2). Interestingly, the electrophoretic pattern showed that the ME1-depurinated rRNAs released fragments of many different sizes after aniline treatment. Because rRNA templates were stable up to 90 °C under the buffer conditions used (Fig. 3A, lanes f–i), the fragment patterns observed in Fig. 3B indicated that ME1 can depurinate rRNAs at multiple sites. As shown in Fig. 3C, heat-treated pap-h mRNA was also susceptible to ME1. Taken together, these results indicate that (i) onset of ME1 enzymatic activity is closely related to the secondary structure of potential single-stranded nucleic acid substrates and that (ii) ME1 cleavage of physically available N-glycosidic bonds is random.

Activity of ME1 on a Single-stranded RNA with Multiple Adenines—To further examine the enzymatic activity and specificity of ME1 on partially denatured mRNA substrates, pap-h mRNA synthesized in vitro was incubated with ME1 and subjected to primer extension analysis using a primer annealing 200 bases downstream from its 5’ terminus. Inspection of the electrophoretic profiles shown in Fig. 4A reveals that pap-h mRNA fragmentation was highly temperature-dependent. Preincubation of pap-h mRNA at temperatures >45 °C led to modification/cleavage at several sites. ME1 activity was greater on mRNA pretreated at 75 °C than on those preincubated at lower temperatures. Several sites (e.g. A11, A19, A25, A45, A64, A68, and A108) were susceptible to ME1 at all temperatures. None of the ME1-targeted sites, however, showed any sequence homology, indicating that ME1 recognition and catalysis do not require a defined sequence motif.

As shown in Fig. 4B, the results of primer extension were largely congruent with the computationally predicted secondary structures of pap-h mRNA at various temperatures; i.e. depurination occurred most frequently at unpaired adenine residues in potential single-stranded regions and bulge loops. Contrary to previous reports (17), only three of the adenine residues susceptible to depurination (A45, A121, and A123) are predicted to be located in hairpin loops. Although these results would suggest that ME1 acts mostly on adenine residues, two guanine residues (G117 and G133) were also modified when the mRNA was preincubated at 60 °C. Repeated experiments confirmed this same site-specific guanine removal, indicating that although ME1 preferentially targets adenine residues, guanine residues are also potential targets. It is possible that susceptible guanine residues are involved in a specific tertiary structure(s).

To further understand the interaction between ME1 and potential mRNA substrates, enzymatic activity was analyzed by monitoring the extent of base removal at several ME1-susceptible sites within pap-h mRNA. ME1 activity was assessed by comparing the relative band densities at four sites (i.e. A11, A64, A68, and A103) that were consistently susceptible to ME1 in mRNA preincubated at different temperatures. As shown in Fig. 4C, the breakdown of mRNA structure led to increased ME1 activity at all four positions.

The enzymatic specificity of ME1 was further examined by determining the apparent dissociation constant (apparent KD) over a range of ME1 concentrations. When trace amounts of pap-h mRNA pretreated at 60 °C were incubated with 0–100 µM ME1, half-maximal cleavage was observed in the presence of ~87.1 nM ME1. The probing of mRNA pretreated at 75 °C, in contrast, yielded an apparent KD of 73.9 nM. The results presented in Fig. 5A indicate that ME1 activity increases with the degree of substrate denaturation. However, it is worth noting that the KD values for individual nucleotide-ME1 interactions might differ from the overall apparent KD values. As shown in Fig. 5B, the KD value of individual nucleotides varied, and ME1 was more active at some sites present in RNA pretreated at 60 °C than at these sites in RNA pretreated at 75 °C. For
example, the apparent $K_D$ values for positions A103 and A108 were lower for mRNAs pretreated at 75 °C than for those pretreated at 60 °C. Just the opposite behavior was observed for positions A11, A64, and A68.

ME$_1$ Depurinates a Variety of Uncapped mRNAs—To determine whether ME$_1$ activity on single-stranded RNA is a general phenomenon, RNA transcripts derived from three defense-related fungal and plant open reading frames (i.e. B26, Tom PR-O’b, and At2g14610) were partially denatured by heating and incubated with ME$_1$. These open reading frames encode an acidic α-elicitin from Phytophthora cryptogea (B26; Ref. 37), a β-1,3-glucanase from L. esculentum (Tom PR-O’b; Ref. 38), and pathogenesis-related protein-1 (PR-1) from Arabidopsis thaliana (At2g14610; GenBank™/EBI accession number AY117187). As shown in Fig. 6A, heat treatment rendered all three RNAs susceptible to ME$_1$ at temperatures up to 60 °C. However, at temperatures higher than 75 °C, RNA transcripts became resistant to ME$_1$ activity, indicating that the cap structure is not absolutely required for mRNA depurination (Fig. 6B, b and c). In all cases, RIP activity appeared to be tightly regulated by mRNA conformation.

Activity of ME$_1$ on a Single-stranded Plant Pathogenic RNA—To examine the possible biological effects of ME$_1$ activity on plant pathogenic RNAs, we tested the ability of ME$_1$ to depurate PSTVd. PSTVd is a small (359 nucleotides), covalently closed circular RNA molecule whose ability to replicate in cultivated potato results in a disease known as spindle tuber.

The temperature-dependent conversion of its rod-like structure to an open circular form occurs via a series of discrete, well characterized structural transitions (39, 40) whose possible role in modulating the biological properties of PSTVd remains poorly understood. This denaturation process is illustrated in Fig. 7A where PSTVd RNA transcripts synthesized in vitro were subjected to temperature-gradient gel electrophoresis under low ionic strength conditions. Over a temperature range of 30–60 °C, the transition of PSTVd from a highly base-paired, rapidly migrating
structure to a more open, slowly migrating structure is clearly visible. Comparing these results with those from the depurination analysis shown in Fig. 7

Comparing these results with those from the depurination analysis shown in Fig. 7

**ME**₁ can be seen to act on PSTVd transcripts preincubated at temperatures 45°C, biochemical evidence that ME₁ can act directly on a pathogenic nucleic acid.

**Finally, to examine the biological effects of ME₁ action, the ME₁-treated PSTVd RNAs were inoculated onto the cotyledons of young tomato (L. esculentum) seedlings. Five weeks postinoculation, the epinasty and stunting symptoms typical of PSTVd infection began to appear in the foliage of plants inoculated with PSTVd RNAs incubated in the absence of ME₁ (Fig. 7C), and the inoculated plants were subsequently tested for the presence of PSTVd by dot-blot hybridization. As shown in Table I, incubation of PSTVd RNA transcripts pretreated at temperatures ≥75°C with ME₁ completely abolished infectivity.**

**ME₁ Preferentially Targets a Non-ribosomal Nucleic Acid Substrate**—To further explore the affinity of ME₁ for various potential substrates in vitro, we examined (i) the ability of ME₁ to act on ribosomes isolated from *M. expansa* leaf tissue and (ii) the ability of such ribosomes to compete against an SSO substrate. As shown in Fig. 8A, ME₁ depurinated *M. expansa* 28S ribosomal rRNA at a specific site, releasing a diagnostic fragment upon aniline treatment. However, ME₁ activity on *M. expansa* ribosomes did not exhibit the kinetics typical of an RIP. Although this fragment was detectable after incubation with as little as 0.1 nM ME₁, enzymatic activity did not increase with concentration (Fig. 8B). Relative intensities of the diagnostic fragment remained the same at ME₁ concentrations as high as 1 μM; also, depurination was detectable only after relatively long incubation times (>15 min), and the amount of fragment released remained the same up to 1 h (Fig. 8C). At least trace amounts of intact 28S rRNA were visible in all assays.
To determine whether or not *M. expansa* ribosomes could be the primary target for ME1 action, we first investigated its ability to compete against 23SSO-A12. 23SSO-A12 contains only one adenine residue, and the activity was measured using a slight modification of a fluorometric method described previously by Zamboni et al. (33). Using a fixed preincubation temperature and reaction time (45 °C/20 min), assays containing 10 nM 23SSO-A12 yielded an apparent $K_D$ value of 105.9 nM (Fig. 8D). We subsequently examined the activity of ME1 toward *M. expansa* ribosomes in the presence of increasing concentrations of 23SSO-A12 preincubated at 45 °C. The reactions were carried out for 20 min, and data presented in Fig. 8E showed that the presence of 23SSO-A12 prevented the release of the diagnostic fragments from *M. expansa* ribosomes. Detectable amounts of the diagnostic rRNA fragments did appear after extended (>40 min) incubation (data not shown). Taken together, these results indicated that ribosomes are unlikely to be the primary target of RIP activity.

**DISCUSSION**

Previous studies have shown that RIPs can act on non-ribosomal substrates *in vitro*, but the biological significance of these observations remained unclear. To determine whether or not ME1-substrate interaction could explain the exquisite specificity of this RIP for biological substrates, we have used as substrates large, nearly full-length nucleic acids rather than the small synthetic oligonucleotides used by others (e.g., see Refs. 41 and 42). Our results clarify the role of secondary/
tertiary structure in regulating the ability of ME1 to depurinate a wide range of non-ribosomal nucleic acids. RNA structure was unambiguously shown to play a key role in regulating the catalytic activity of this type 1 RIP.

**RNA Structure Modulates the Enzymatic Activity of ME1**

RNA folding is stabilized by a combination of hydrogen bonds, metal ions, and tertiary interactions (43). However, the most stable structure of an RNA molecule is not always optimal for RNA-protein interaction (25). Under *in vitro* conditions, the free energy of folding is considered small enough to facilitate RNA-protein interaction (44, 45). We have shown that partial denaturation of potential RNA substrates *in vitro* enhances the enzymatic activity of ME1, possibly by optimizing RNA-RIP interaction. These results indicate that depending on the precise structure of a potential substrate, RIP activity observed *in vitro* could be quite different from that occurring *in vivo*.

Comparison of catalytic activities on *pap-h* mRNA pretreated to 60 or 75 °C indicated that ME1-RNA interaction is controlled by the structure of the RNA. This observation could explain the previously reported nonspecific depurination activity of RIPS under acidic pH conditions and/or in the absence of cofactors such as Mg²⁺ (8, 24, 46, 47). Our assays contained only minimal concentrations of Mg²⁺ (1 mM) to stabilize RNA substrates upon heat treatment. Lowering either the pH or the concentration of Mg²⁺ causes the *Tm* of RNA molecules to decrease (48, 49). In addition, Mg²⁺ is not required for proper RNA folding, but its presence enhances the stability of those structure(s) that do form (50, 51). Therefore, the presence of additional Mg²⁺ as reported in other studies may have inhibited RNA-RIP interaction instead of acting as a cofactor to increase enzymatic activity. Taken together, all of these results suggest that RIPS are able to actively depurinate adenosines by interacting with as yet undefined structural motifs.

At this time, we do not know whether the secondary or tertiary structure of an RNA molecule plays the most important role in its interaction with ME1. However, changes in RNA tertiary structure may explain the activity of certain RIPS on guanine, a nucleotide considered to be a minor substrate site on both ribosome and non-ribosomal substrates (1, 21, 24). Thus far, only ricin and PAP have been shown to have deguanylation activity (52), and recent experiments using highly purified RIPS have ruled out deguanylation activity for gelonin, momordin, PAP-S, and saporin-S6 (53). Our results indicate that ME1 can only remove guanine residues from *pap-h* mRNA pretreated at 60 °C. As shown in Fig. 4B, computer predictions suggest that all three ME1-susceptible guanine residues may be located in G:U wobble base pairs next to internal loops. Because G117 and G133 are still predicted to be paired at

**Table 1. Effect of ME1 treatment on PSTVd infectivity**

| Pretreatment | Inoculum concentration | Infectivity*  |
|--------------|------------------------|---------------|
| °C           | ng/ml                  |               |
| 30           | 100                    | 6:10          |
|              | 10                     | 2:10          |
| 45           | 100                    | 8:10          |
|              | 10                     | 3:5           |
| 60           | 100                    | 3:5           |
|              | 10                     | 0:5           |
| 75           | 100                    | 2:5           |
|              | 10                     | 0:5           |
|              | 100 (+ ME1)            | 0:5           |

*Infectivity is expressed as number of plants infected:number of plants inoculated.

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75 °C, ME1 activity appears to be regulated by more than just the secondary structure of a potential substrate.

Substrate Recognition and Enzymatic Activity by ME1 Do Not Require Specific Sequence or Structural Motifs—Cleavage of small synthetic RNA substrates by RIPs requires specific sequence and structural motifs. Using such substrates, a GAGA tetraloop closed by CG base pairs has been identified and proposed as the identity element required for RIP recognition and catalysis (17, 23, 41, 54–56). The second adenine residue in this motif was identified as the sole site of RIP activity, and similar size loop structures with different sequences were not recognized by RIPs (19). In addition to the adenosine at the depurination site, the 3' flanking guanosine (G3) has also been proposed to play a critical role in RIP recognition (57).

pap-h mRNA contains only two potential GAGA recognition sites, one starting at G451 and the second at G698, which are predicted to be located in hairpin loops at 37 °C. Using a combination of primer extension assays and computational structure prediction, we have shown that ME1 activity occurs not only at these sites but also at multiple adenine and guanine residues located in single-stranded regions. Recognition of an adenine residue by ME1 did not require the presence of an adjacent guanine residue, and the sequences surrounding A45, A121, and A123 lack any obvious sequence homology. The fact that denaturation leads to an increase of ME1 activity is also consistent with the lack of a requirement for a specific motif for RNA recognition and catalytic activity.

We have reported recently that ME1 inhibits the translation of uncapped luciferase mRNA in a rabbit reticulocyte translation system (35). The present study provides direct evidence that the cap analog m7GpppG is not necessary for ME1 recognition. Additional experiments using RTA and saporin-S6 suggest that many RIPs can recognize and depurinate RNAs lacking a specific binding motif. Because we did not test the activity of PAP on uncapped luciferase transcripts, we cannot rule out the possibility that this RIP requires a cap structure for depurination activity (21, 22). RIP binding to the cap of mRNA may enhance the stability of the RIP-mRNA interaction, but the primary determinant of ME1, RTA, and saporin-S6 enzymatic activity is the conformation of the mRNA.

ME1 Can Directly Inactivate Pathogenic Nucleic Acids—An increasing body of evidence indicates that RIPs possess antimicrobial activity that is effective against a broad array of animal and plant viruses (for review, see Ref. 58). The widely accepted mechanism for antiviral action identifies host ribosomes as the target of RIP activity. Viral infection is thought to alter the structure of the host cell, allowing RIPs to gain access to the ribosomes and leading to arrest of protein synthesis and cell death, thereby blocking viral replication and spread (59). Somewhat surprisingly, transgenic plants expressing PAP do
not exhibit a hypersensitive response or other symptoms of spontaneous cell death in response to viral infection, although they are resistant to a wide range of viruses (60, 61).

Our results suggest that the enzymatic activity of ME1 primarily targets pathogenic non-ribosomal substrates rather than the host ribosomes. Because viral genomes must form single-stranded templates that are largely free of coat protein at some stage during the infection process, deproteinization of virus-related nucleic acids could provide direct protection against infection. The presence of high concentrations of RIPs within the cell wall (30, 36, 59) could facilitate RIP-viral nucleic acid contact; alternatively, RIPs could enter the cytoplasm together with the virions via mechanical damage to the cell wall and plasma membrane and act on viral RNA during co-translational disassembly (62, 63). Because viroids lack the protein capsid that protects almost all conventional viruses, one might expect them to be particularly sensitive to RIPs.

We have shown that ME1 can depurinate partially denatured PSTVd RNA, thereby leading to a dramatic decrease in infectivity. Detailed structural studies by Riesner and co-workers (64, 65) have characterized a series of rearrangements involving nucleotides within the conserved region of PSTVd that are required for the cleavage/ligation of multimeric replicative intermediates into mature circular progeny. One of these rearrangements results in the formation of a loop E motif very similar to those found in rRNAs. Several years ago, Wassenegger et al. (66) reported that mechanical inucleolusation of tobacco (Nicotiana tabacum L.) plants with the PSTVd intermediate strain results in the appearance of a novel variant containing a single C−U substitution at position 259 within this motif. More recently, Zhu et al. (67) have shown that a U−A change at position 257 has similar effects on the ability of PSTVd to replicate in tobacco. Although the possible role of RIP(s) in restricting the host range of viroids remains to be determined, our results shed new light on the biological function of RIPs in plants, showing that these enzymes can depurinate a diverse array of nucleic acid substrates, possibly at specific stages of plant development and/or pathogen challenge.

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REFERENCES

1. Endo, Y., Mitsui, K., Motizuki, M., and Tsurugi, K. (1987) J. Biol. Chem. 262, 5908−5913.
2. Endo, Y., and Tsurugi, K. (1987) J. Biol. Chem. 262, 8128−8130.
3. Hartley, M. R., Legname, G., Osborn, R. W., Chen, Z., and Lord, J. M. (1991) FEBS Lett. 290, 65−68.
4. Osborn, R. W., and Hartley, M. R. (1990) Eur. J. Biochem. 193, 401−407.
5. Barbieri, L., Ferreras, J. M., Barreco, A., Ricci, P., and Stirpe, F. (1992) Biochem. J. 286, 1−4.
6. Barbieri, L., Gori, P., Vallbonesi, P., Castiglionetti, P., and Stirpe, F. (1994) Nature 372, 624.
7. Barbieri, L., Vallbonesi, P., Gori, P., Pession, A., and Stirpe, F. (1996) Biochim. Biophys. Acta 1319, 507−513.
8. Barbieri, L., Vallbonesi, P., Bonora, E., Gori, P., Bolognesi, A., and Stirpe, F. (1997) Nucleic Acids Res. 25, 518−522.
9. Umesky, M. A., Irvin, J. D., and Hardesty, B. (1977) Ann. N. Y. Acad. Sci. 284, 431−440.
10. Aron, G., and Irvin, J. D. (1980) Antimicrob. Agents Chemother. 17, 1032−1033.
11. McGrath, M. S., Hwang, K. M., Caldwell, S. E., Gaston, I., Luk, K. C., Wu, P., Ng, V. L., Crowe, S., Daniels, J., Marsh, J., Dehlert, T., Lekas, P. U., Ueunna, J. C., Yeung, H. W., and Lidon, J. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2844−2848.
12. Zaring, J. M., Moran, R. A., Haffar, O., Saas, J., Richmann, D. D., Spina, D. A., Myers, D. E., Kuechbel, V., Ledbetter, J. A., and Uckun, F. M. (1990) Nature 347, 92−95.
13. Taylor, S., Massiah, A., Lomonosoff, G., Roberts, L., Lord, J. M., and Hartley, M. (1984) Plant J. 3, 827−835.
14. Rajamohan, V., Venkatachalakumari, T. K., Irvin, J. D., and Uckun, F. M. (1999) Biochem. Biophys. Res. Commun. 260, 453−458.
15. Rajamohan, V., Venkatachalakumari, T. K., and Uckun, F. M. (1999) Biochem. Biophys. Res. Commun. 263, 419−424.
The N-Glycosidase Activity of the Ribosome-inactivating Protein ME1 Targets Single-stranded Regions of Nucleic Acids Independent of Sequence or Structural Motifs

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