

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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ME₁, 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 ME₁ and other RIPs. ME₁ was shown to depurinate a variety of partially denatured nucleic acids, randomly removing adenine residues from single-stranded regions and, to a lesser extent, guanine residues from wobble base-pairs 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 ME₁ activity appeared to depend on the physical availability of nucleotides, and denaturation of nucleic acid substrates increased their interaction with ME₁. 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 ME₁ *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 *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 ME₁ (or other RIPs).

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 α -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 ¹GAGA⁴ (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 the

Ribosome-inactivating proteins (RIPs)¹ are cytotoxic enzymes that have been identified in plants, fungi, and bacteria

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¹ The abbreviations used are: RIP, ribosome-inactivating protein; RTA, ricin A-chain; PAP, pokeweed antiviral protein; PSTVd, potato spindle tuber viroid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; SSO, small synthetic oligodeoxynucleotide; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.

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, studies of RNA secondary structure have 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 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-S6 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 *pap-h* was amplified by PCR from hairy roots of pokeweed (*Phytolacca americana*) using gene-specific primers 5E.30 (5'-ATGCATGTTTCATCTGATCAATCATAAAAGT-3') and 3E.28 (5'-ATCAGAATCCCTGAAATAGATTACCAAG-3') designed from its cDNA sequence (GenBank™/EBI accession number AY071928). The PCR product was then subcloned into pGEM®-T Easy vector (Promega). pGEM®-T Easy recombinant plasmids encoding defense-related genes *B26*, *Tom RP-O'b*, and *At2g14610* were gifts of Dr. Christopher B. Lawrence (Department of Bioagricultural Sciences and Pest Management, Colorado State University, Fort Collins, CO). The small oligonucleotide 23SSO-A12 (5'-GGGCCGGCGGUACCGCCGGCGCC-3') was synthesized by the Macromolecular Resources Facility (Department of Biochemistry, Colorado State University, Fort Collins, CO).

Isolation of Ribosomes—To isolate ribosomes, yeast cells (10 g, *Saccharomyces cerevisiae* strain YPH500) and leaves of *M. expansa* (50 g) were ground in liquid N₂ with a mortar and pestle, and 100 ml of extraction buffer (200 mM KCl, 25 mM MgCl₂, 25 mM EGTA, 200 mM sucrose, and 25 mM β-mercaptoethanol in 200 mM Tris-HCl, pH 9.0) was added. After centrifugation at 10,000 × *g* for 20 min at 4 °C, the resulting supernatant was layered onto a sucrose cushion (1 M sucrose for yeast ribosomes and 2 M sucrose for *M. expansa* ribosomes, 20 mM

KCl and 5 mM MgCl₂ in 25 mM Tris-HCl, pH 7.6) in Ti-70 tubes (Beckman Instruments) and centrifuged at 200,000 × *g* for 4 h at 4 °C (L-70 Ultracentrifuge, Beckman Instruments). The pellets were resuspended in 25 mM Tris-HCl buffer (pH 7.6) with 25 mM KCl and 5 mM MgCl₂. A further preparation of protein-free rRNAs was extracted from ribosomes with phenol:chloroform (1:1, v/v) and precipitated with ethanol for 2 h at -80 °C.

Preparation of Substrates—DNA fragments encoding the *pap-h* open reading frame were amplified from the corresponding pGEM®-T Easy plasmids by PCR using gene-specific primers 5E.30 and 3E.28. After electrophoresis in a 1.5% (w/v) agarose gel and staining in 0.5 mg·ml⁻¹ ethidium bromide, the DNA fragments were excised from the gel and recovered using the Quantum Prep gel slice kit (Bio-Rad).

pap-h, *B26*, *Tom RP-O'b*, and *At2g14610* mRNAs were transcribed from the corresponding pGEM®-T Easy plasmid DNAs using T7 RNA polymerase (Promega). Template DNAs were linearized by SpeI digestion and purified by phenol:chloroform extraction and ethanol precipitation. Transcription reactions containing 40 mM Tris-HCl, pH 7.5, 9 mM MgCl₂, 10 mM NaCl, 2 mM spermidine, 10 mM dithiothreitol, 20 units of RNasin, the four nucleoside triphosphates at 1.5 mM each, 50 nM DNA templates, and 3 units/μl T7 polymerase were incubated at 37 °C for 1 h. Potato spindle tuber viroid (PSTVd) RNA transcripts were synthesized using SP6 RNA polymerase, plasmid pST64-B5 (32) as a template, and four nucleoside triphosphates with or without supplemental 5'-[α-³²P]UTP (3000 Ci/mmol; Amersham Biosciences). Transcription reactions were subsequently extracted with phenol and chloroform, and the nucleic acids were precipitated with ethanol for 2 h at -80 °C before purification by electrophoresis on 6% (w/v) polyacrylamide gels containing 1× Tris-borate-EDTA buffer, 5 M urea and elution in 500 mM ammonium acetate, 0.1% (w/v) SDS, 0.1 mM EDTA. RNA transcripts were then recovered by ethanol precipitation.

Heat Treatment and Depurination Analysis—Partial denaturation of DNA and RNA substrates was carried out for 30 s at various temperatures (30, 45, 60, 75, and 90 °C). Each reaction (15 μl) contained ~150 nM either DNA in incubation buffer PS-1 (10 mM Tricine-KOH, pH 8.7, 2.5 mM KCl, 3 mM MgCl₂, 3.75 μg/ml bovine serum albumin, 0.005% (v/v) Tween 20) or RNA transcripts in buffer PS-2 (1.25 mM Tris-HCl, pH 7.4, 2.5 mM KCl, 1.25 mM MgCl₂, 0.01 mM EDTA). Reactions were quenched by placing the tubes on ice. Following addition of 85 μl of RIP incubation buffer PS-3 (10 mM Tris-HCl, pH 7.2, 40 mM KCl) containing 1 ng of RIP, the reaction mixtures were incubated at 37 °C for 10 min. Nucleic acids incubated in the absence of RIPs served as negative controls. Following incubation, RIPs were removed by phenol:chloroform (1:1, v/v) extraction, and the aqueous phase containing the nucleic acids was divided in half. One aliquot was incubated for 30 min on ice with 1 M aniline acetate (pH 4.5) before precipitation with ethanol; the second aliquot was precipitated directly. Both aniline-treated and untreated nucleic acids were fractionated by electrophoresis in 6% (w/v) polyacrylamide gels containing 7 M urea and stained with ethidium bromide.

Adenine release from a small synthetic oligonucleotide (SSO) substrate (*i.e.* 23SSO-A12) was measured as described by Zamboni *et al.* (33). Following preincubation at 45 °C for 30 s in buffer PS-2, the SSO (10 nM) was diluted into buffer PS-3 and incubated at 37 °C for 20 min in the absence and presence of RIP. One volume of cold ethanol was added, and after 10 min at -80 °C, the ethanol-soluble fractions were recovered by centrifugation. Free adenine was converted into its etheno derivative by diluting a 0.2-ml portion of the ethanol-soluble fractions to 1 ml with H₂O, adding 0.4 ml of 0.1 M sodium acetate buffer, pH 5.1, containing 0.14 M chloroacetaldehyde, and then heating the samples in a water bath at 80 °C for 40 min. Fluorescence was measured in an Aminco-Bowman spectrophotofluorometer. Excitation and emission wavelengths were set at 280 and 400 nm, respectively.

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 0.2× Tris-borate-EDTA, 5 mM NaCl. Following pre-electrophoresis at 26 °C (200 V, 30 min), PSTVd transcripts (~40,000 cpm) were diluted with 165 μl of loading buffer (18 mM NaCl, 17 mM sodium cacodylate, 3 mM cacodylic acid, 0.2 mM EDTA, pH 7.0) and 25 μl of loading dye (50% glycerol, 1× Tris-borate-EDTA, 2% bromophenol 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 established across the gel. Upon resumption of electrophoresis, 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* mRNA transcripts treated with ME₁ as

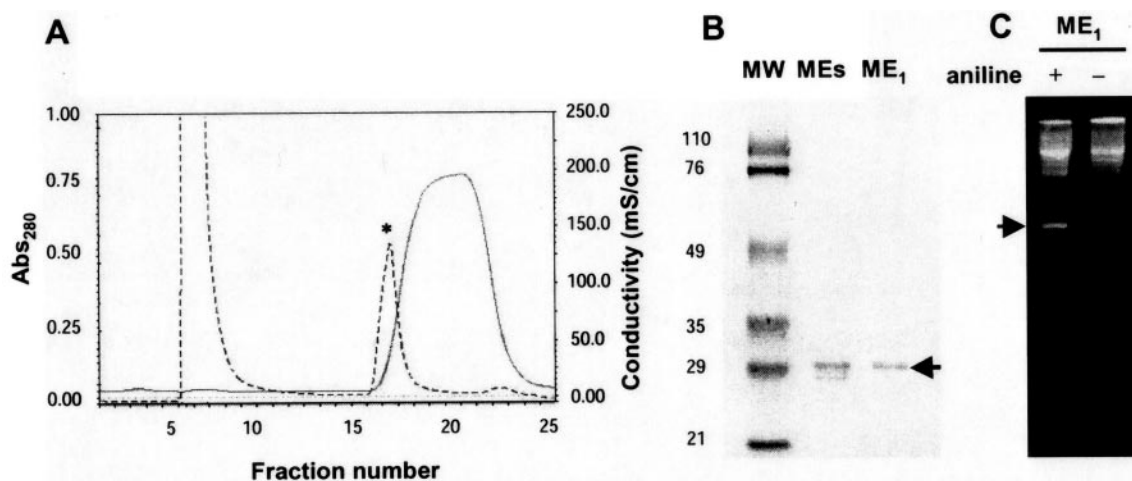


FIG. 1. **Immunoaffinity chromatography of ME₁.** A, fractionation of total root protein from *M. expansa* on a Sepharose polyclonal anti-ME₁ antibody column. Proteins were equilibrated with 75 mM Tris-HCl, pH 8.0, and loaded onto the column. The affinity column was attached to a fast protein liquid chromatography system, and the chromatogram was monitored by a Bio-Rad BioLogic Duo-Flow system. The *dashed line* indicates A₂₈₀, and the *solid line* indicates conductivity; the peak of immunobound proteins eluted with 100 mM glycine-HCl, pH 2.7, containing 0.5 M NaCl is indicated by an *asterisk*. B, SDS-PAGE analysis of fractions resulting from affinity (MEs) followed by cation-exchange (ME₁) chromatography. Approximately 5 μ g of protein was loaded/lane and visualized by silver staining. The *arrow* indicates the position of purified ME₁. MW, molecular weight markers. C, enzymatic activity of purified ME₁ *in vitro*. Ribosomes isolated from *S. cerevisiae* were incubated with purified ME₁. Following phenol:chloroform extraction and ethanol precipitation, rRNAs were treated with aniline, fractionated on a 4.5% (w/v) urea-polyacrylamide gel, and stained with ethidium bromide. The *arrow* indicates the diagnostic 367-nucleotide product of 28 S rRNA cleavage.

described above were purified by extraction with phenol:chloroform and chloroform:isoamyl alcohol followed by ethanol precipitation. RNAs were resuspended in 5 μ l of 2 \times 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'-CTTTGGCTTGATTGCGTAGAG-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 μ l 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 μ l) 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 K_D) of each construct were determined by conducting in-line probing of RNA, wherein the concentration of the ME₁ was varied between 0 and 100 μ M. Composite plots of the fraction of RNA cleaved at specific sites *versus* the logarithm of the concentration of ME₁ were generated to provide an estimate of the apparent K_D . Fraction-cleaved values were normalized relative to the highest and lowest cleavage values measured for each site. The graphs were generated and analyzed using the Gel Doc 2000 gel documentation system and quantification software (Bio-Rad).

PSTVd Infectivity Assays—PSTVd transcripts incubated in the presence or absence of ME₁ after partial denaturation were diluted with 20 mM NaPO₄, pH 7.0, to a final concentration of either 10 or 100 μ g/ μ l. Aliquots (10 μ l) were then placed on the carborundum-dusted cotyledons of 7-day-old tomato (*Lycopersicon esculentum* Mill cv. Rutgers) seedlings and gently rubbed with the side of a sterile micropipette tip. Control plants were inoculated with buffer alone. Inoculated seedlings were maintained for 4–6 weeks in a greenhouse under conditions suitable for viroid replication and periodically tested for the presence of PSTVd progeny by dot-blot hybridization using full-length, digoxigenin-labeled RNA probes specific for (+)PSTVd (34).

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. 1, A 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 mRNA 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-

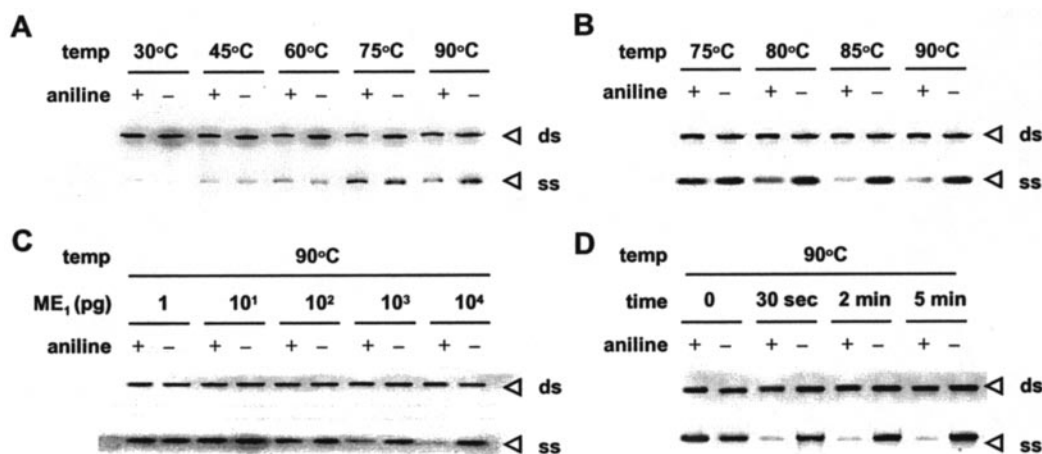


FIG. 2. **Enzymatic activity of ME₁ on heat-denatured ssDNA substrates.** A and B, the *N*-glycosidase activity of ME₁ on DNA substrates pretreated at 30–90 °C. C and D, concentration- (1 pg–10 ng) and time-dependent (0–5 min) *N*-glycosidase activity of ME₁ toward ssDNAs (ss) produced by preincubation at 90 °C. DNA substrates were heated before incubation with ME₁ as described under “Experimental Procedures.” Nucleic acids were subsequently fractionated on 6% (w/v) polyacrylamide gels containing 7 M urea and stained with ethidium bromide. Preincubation temperature (*temp*), ME₁ concentration (*pg*), reaction time, and the presence (+) or absence (–) of aniline treatment are shown above each lane.

rates ssDNA. Data presented in Fig. 2B showed that ssDNAs produced at >80 °C were susceptible to ME₁, and incubation of ssDNA released by pretreatment at 90 °C with as little 100 pg of ME₁ resulted in detectable depurination (Fig. 2C). Most importantly, the enzymatic activity of ME₁ was both rapid and spontaneous. The fact that ssDNA depurination was completed in ≤30 s (Fig. 2D) allowed us to assess ME₁-nucleic acid interaction before 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 indicate 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

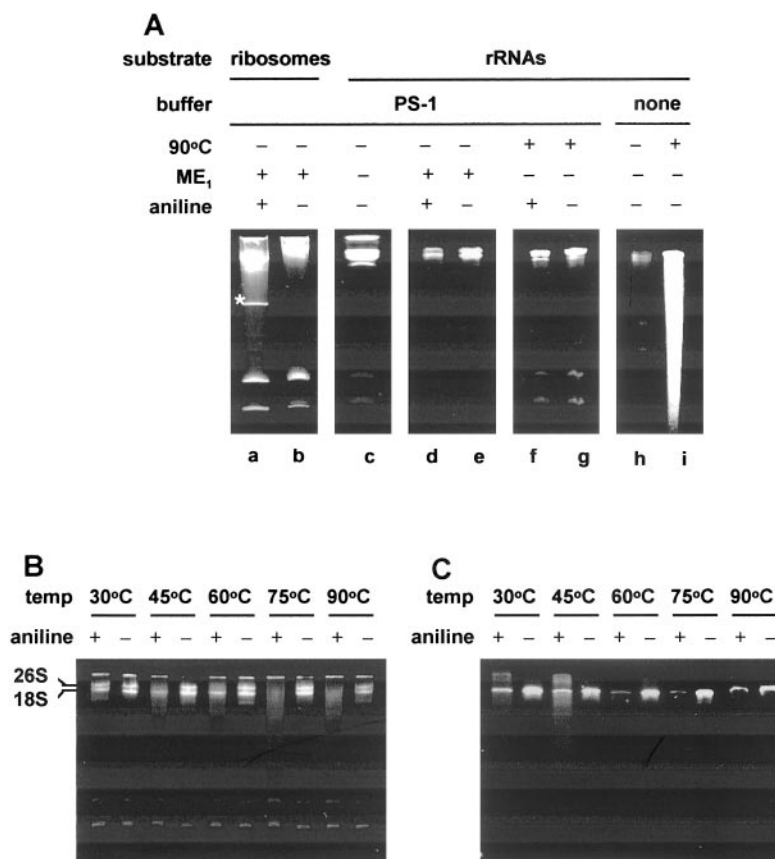
mRNA pretreated at 75 °C than on those preincubated at lower temperatures. Several sites (*e.g.* A11, A19, A25, A45, A64, A68, and A108) were susceptible to ME₁ at all temperatures. None of the ME₁-targeted sites, however, showed any sequence homology, indicating that ME₁ recognition and catalysis do 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 K_D) 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 K_D 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 K_D values for individual nucleotide-ME₁ interactions might differ from the overall apparent K_D values. As shown in Fig. 5B, the K_D 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

FIG. 3. Enzymatic activity of ME₁ on heat-treated deproteinized rRNA and mRNA substrates. Shown is the N-glycosidase activity of ME₁ on intact ribosomes (A, lanes a and b), deproteinized rRNA (A, lanes c–i, and B), and *pap-h* mRNA (C). Nucleic acids were heated before incubation with ME₁ as described under “Experimental Procedures.” Nucleic acids were subsequently fractionated on 6% (w/v) polyacrylamide gels containing 7 M urea and stained with ethidium bromide. Preincubation temperature (*temp*) and the presence (+) or absence (–) of aniline treatment are shown above each lane. The asterisk in lane a of A indicates the presence of the diagnostic 367-nucleotide product of 26 S rRNA cleavage.



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.

ME₁ Depurinates a Variety of Uncapped mRNAs—To determine whether ME₁ 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 ME₁. These open reading frames encode an acidic α -elicitor 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 ME₁. RNAs pretreated at 0 °C were unaffected by incubation with ME₁ (data not shown), but depurination at selective sites could be seen at temperatures as low as 30 °C. Interestingly, the enzymatic efficiency of ME₁ appeared to be mRNA-dependent.

ME₁ depurinated multiple sites on all three transcripts but was less active on *B26* RNA. Preincubation of *Tom PR-O'b* and *At2g14610* transcripts at high temperatures resulted in extensive depurination, and intact RNA was undetectable after aniline treatment (Fig. 6A, compare *b* and *c*). In contrast, intact *B26* transcripts were visible at all temperatures tested, even though the appearance of a “smear” of fragments after aniline treatment indicated that ME₁ acts at multiple sites (Fig. 6A, *a*). These results provided clear evidence that ME₁ can act in a structure-dependent fashion at multiple sites within a single-stranded RNA substrate.

Hudak *et al.* (21, 22) have proposed recently that recognition of luciferase mRNA by PAP requires the presence of a cap structure. Recognition is followed by depurination of downstream adenine residues. To determine whether or not a cap

structure is required for recognition of luciferase mRNA, uncapped luciferase transcripts were pretreated under various conditions and then incubated with ME₁. As expected (see Refs. 21 and 22), depurination was not detectable with uncapped luciferase mRNAs stored at 0 °C (results not shown). However, uncapped luciferase mRNAs that had been pretreated at higher temperatures were clearly susceptible to ME₁. As shown in *a* of Fig. 6B, multiple sites in uncapped luciferase transcripts became susceptible to ME₁ after preincubation at 45 °C, releasing a number of small fragments after aniline treatment. Note also that the catalytic efficiency of ME₁ increased with the extent of substrate denaturation.

RIP activity on uncapped luciferase mRNA was not limited to ME₁. Parallel experiments involving several other well known RIPs such as RTA and saproin-S6 yielded RNA fragmentation patterns similar to those produced by ME₁, 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 ME₁ on a Single-stranded Plant Pathogenic RNA—To examine the possible biological effects of ME₁ activity on plant pathogenic RNAs, we tested the ability of ME₁ to depurinate 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 native 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

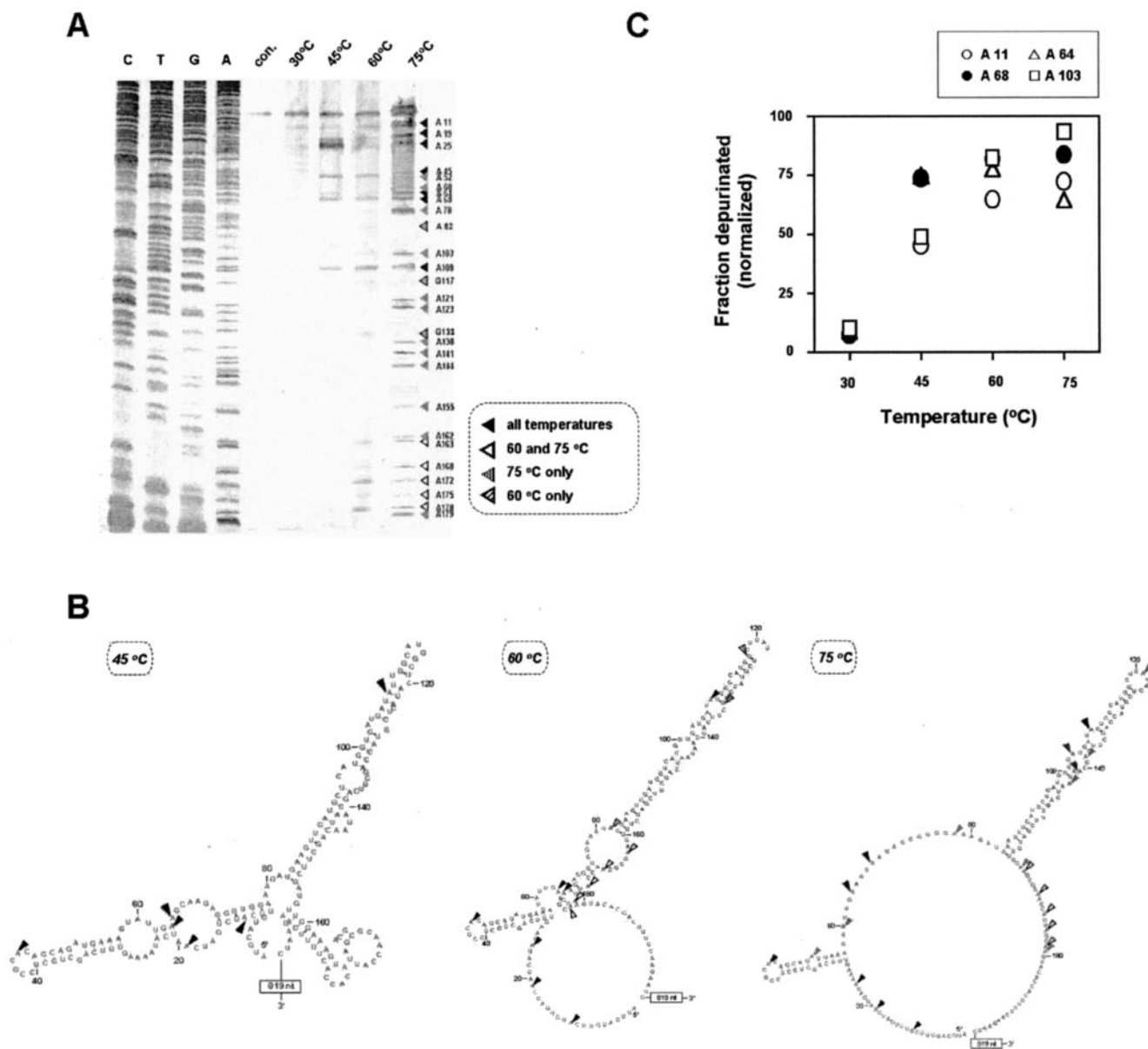


FIG. 4. Identification of ME₁-susceptible sites in *pap-h* mRNA. A, primer extension analysis of the 5'-portion of *pap-h* mRNA transcribed *in vitro* and pretreated at various temperatures before incubation with ME₁. A primer complementary to sequences located ~200 nucleotides from the 5' terminus of *pap-h* mRNA was extended with reverse transcriptase. Extension products corresponding to various cleavage sites are numbered and identified by arrowheads. con., control. B, possible secondary structures of the 5'-region of *pap-h* mRNA as predicted by a combination of computer modeling and ME₁ probing (68, 69). Coding details as in A (see box). nt, nucleotide(s). C, the effect of pretreatment temperature on the accessibility of specific adenosine residues in *pap-h* mRNA to modification by ME₁.

structure to a more open, slowly migrating structure is clearly visible. Comparing these results with those from the depurination analysis shown in Fig. 7B, ME₁ 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* 28 S 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 μ M; 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 28 S rRNA were visible in all assays.

FIG. 5. Affinity of ME₁ for partially denatured *pap-h* mRNA. A, relationship between ME₁ concentration and the extent of RNA cleavage at selected sites in *pap-h* mRNA pretreated at 60 °C (left) and 75 °C (right). Dotted arrows reflect the estimated concentration of ME₁ needed to attain half-maximal cleavage of RNA (apparent K_D). B, logarithm of the apparent K_D values for individual positions at the same temperatures.

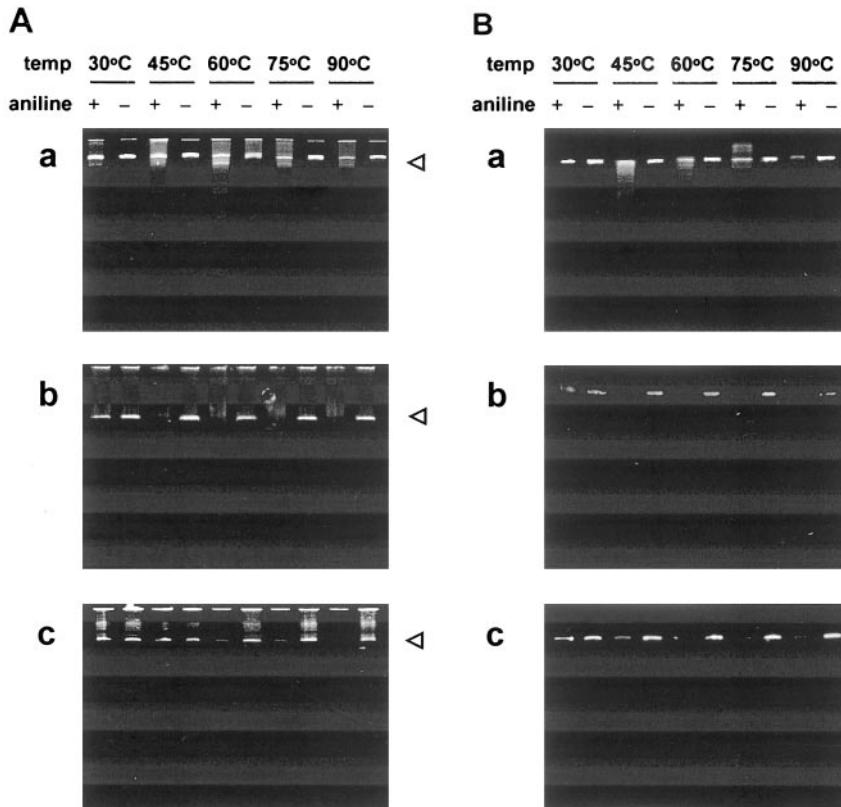
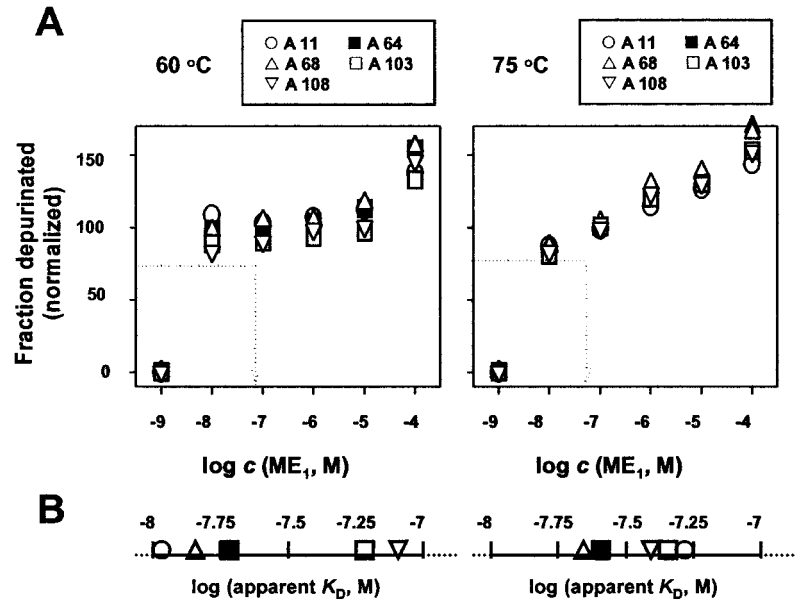


FIG. 6. Effect of partial denaturation on the recognition of uncapped mRNAs by ME₁. A, N-glycosidase activity of ME₁ on (a) acidic α -elicitin (B26) from *P. cryptogea*, (b) β -1,3-glucanase (Tom RP-O'b) from *L. esculentum*, and (c) pathogenesis-related protein-1 (PR-1; *At2g14610*) from *A. thaliana*. The unfilled arrowheads indicate the position of intact mRNA transcripts synthesized *in vitro*. *temp.*, temperature. B, N-glycosidase activity of (a) ME₁, (b) RTA, and (c) saporin-S6 on uncapped luciferase mRNAs after partial denaturation. mRNA templates were preincubated at the indicated temperatures, incubated with RIPs, treated with aniline, and fractionated on a 7 M urea, 6% (w/v) polyacrylamide gel.

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. Detect-

able 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/

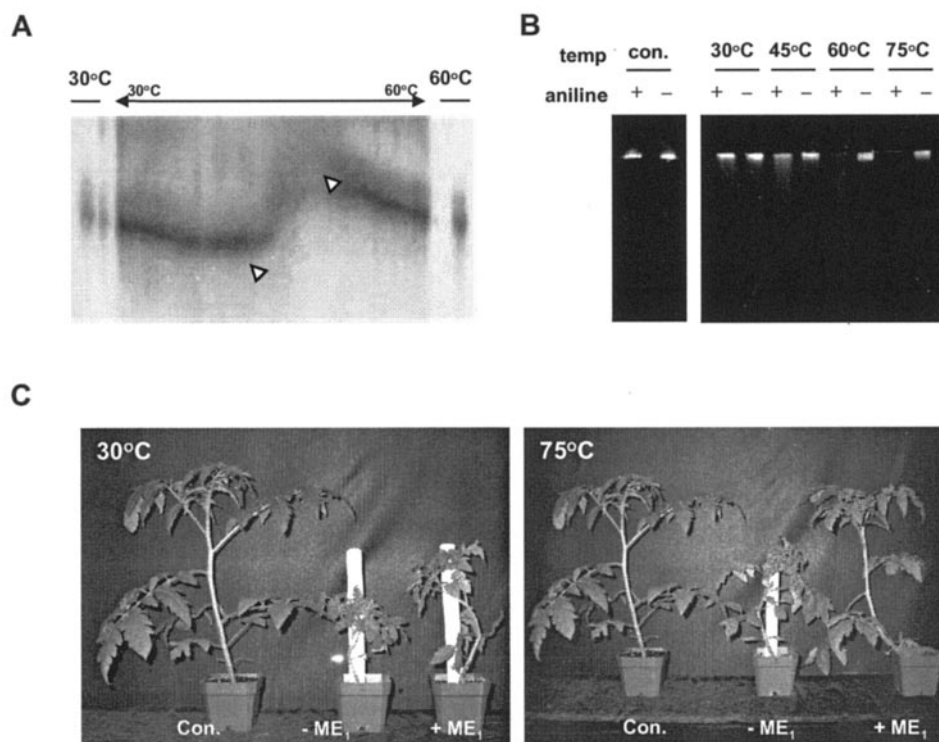


FIG. 7. **Direct action of ME₁ on a single-stranded plant pathogenic RNA.** A, thermal denaturation of linear PSTVd under low ionic strength conditions. Temperature-gradient gel electrophoresis analysis of linear ³²P-labeled PSTVd RNA was carried out at temperatures ranging from 30 to 60 °C. B, depurination of PSTVd by ME₁. RNA transcripts synthesized *in vitro* were partially denatured, incubated with ME₁, treated with aniline, and fractionated on a 7 M urea, 6% (w/v) polyacrylamide gel. *temp*, temperature; *con.*, control. C, infectivity of ME₁-treated PSTVd RNAs. Cotyledons of 1-week-old tomato seedlings were dusted with carborundum and inoculated with aliquots (10 μl) of either untreated or ME₁-treated PSTVd RNA transcripts serially diluted in 20 mM sodium phosphate, pH 7.0. Inoculated seedlings were maintained in a greenhouse and photographed 5 weeks postinoculation.

TABLE I
Effect of ME₁ treatment on PSTVd infectivity

Pretreatment	Inoculum concentration	Infectivity ^a
°C	ng/ml	
30	100	6:10
	10	2:10
	100 (+ ME ₁)	8:10
45	100	3:5
	10	1:5
	100 (+ ME ₁)	1:5
60	100	3:5
	10	0:5
	100 (+ ME ₁)	1:5
75	100	2:5
	10	0:5
	100 (+ ME ₁)	0:5

^a Infectivity is expressed as number of plants infected: number of plants inoculated.

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 pre-treated 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 *T_m* 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 pre-treated 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

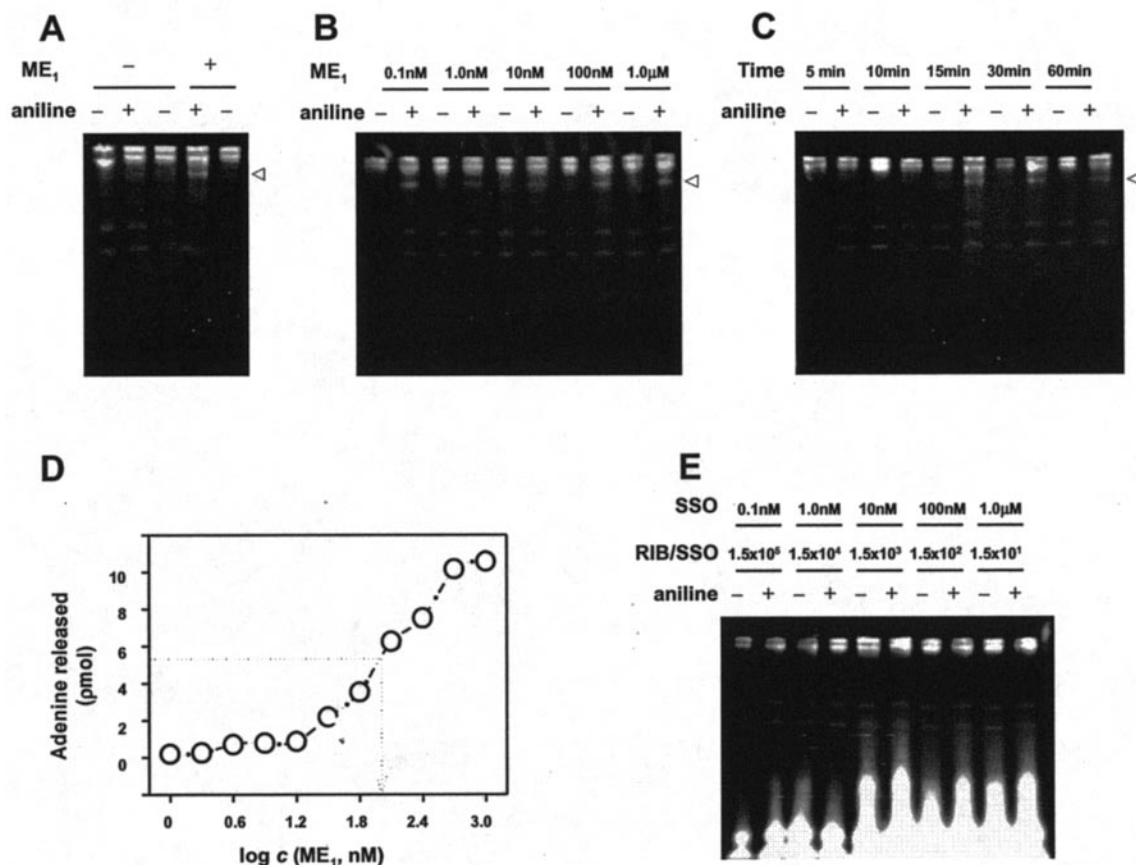


FIG. 8. Relative affinity of ME₁ for ribosomal and non-ribosomal substrates. A–C, *N*-glycosidase activity of ME₁ on *M. expansa* ribosomes. The left lane in A is protein-free rRNA. The presence (+) or absence (–) of ME₁, ME₁ concentrations, and reaction times (min) are indicated above each lane in A, B, and C, respectively. The presence (+) or absence (–) of aniline is denoted. The unfilled arrowheads indicate the predicted product of 28 S rRNA cleavage. D, relationship between ME₁ concentration and the extent of small oligonucleotide cleavage. The SSO was pretreated at 45 °C, and adenine release from SSO was assayed by fluorometry as described under “Experimental Procedures.” Dotted arrows indicate the estimated concentration of ME₁ needed to attain half-maximal cleavage of RNA (apparent *K_D*). E, inhibition of ME₁ activity on *M. expansa* ribosomes by SSO. Pretreated SSO was added to tubes containing ME₁ and *M. expansa* ribosomes, and resulting mixtures were incubated at 37 °C for 20 min. rRNAs were then extracted, treated with aniline, and fractionated on 4.5% (w/v) urea-polyacrylamide gels. SSO concentrations, the concentration ratio of substrates (ribosome:SSO (*RIB/SSO*)), and the presence (+) or absence (–) of aniline are indicated above each lane.

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, depurination 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 central 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 inoculation 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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