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Bacterial Toxin RelE: A Highly Efficient Ribonuclease with Exquisite Substrate Specificity Using Atypical Catalytic Residues

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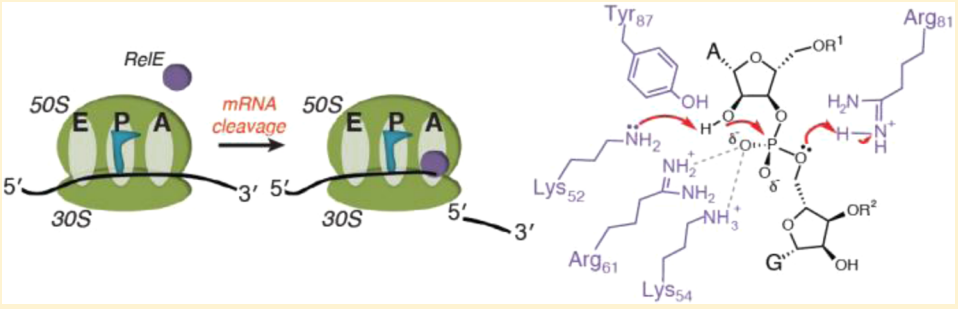
Supporting Information



ABSTRACT: The toxin RelE is a ribosome-dependent endoribonuclease implicated in diverse cellular processes, including persistence. During amino acid starvation, RelE inhibits translation by cleaving ribosomal A-site mRNA. Although RelE is structurally similar to other microbial endoribonucleases, the active-site amino acid composition differs substantially and lacks obvious candidates for general acid−base functionality. Highly conserved RelE residues (Lys52, Lys54, Arg61, Arg81, and Tyr87) surround the mRNA scissile phosphate, and specific 16S rRNA contacts further contribute to substrate positioning. We used a single-turnover kinetic assay to evaluate the catalytic importance of individual residues in the RelE active site. Within the context of the ribosome, RelE rapidly cleaves A-site mRNA at a rate similar to those of traditional ribonucleases. Single-turnover rate constants decreased between 10^2- and 10^6-fold for the RelE active-site mutants of Lys52, Lys54, Arg61, and Arg81. RelE may principally promote catalysis via transition-state charge stabilization and leaving-group protonation, in addition to achieving in-line substrate positioning in cooperation with the ribosome. This kinetic analysis complements structural information to provide a foundation for understanding the molecular mechanism of this atypical endoribonuclease.

Prokaryotic lifestyle changes are initiated in response to environmental cues by pathways that sense physiological differences and initiate downstream events. Toxin−antitoxin (TA) modules have recently emerged as central players in the regulation of diverse cellular processes that modulate bacterial activities, including the general stress response, biofilm formation, and persistence.[1−3] TA systems are abundant genetic modules that encode a toxin protein that interferes with normal cell physiology and a cognate antitoxin that inactivates the toxin under normal environmental conditions.[1,3,4] In type II TA systems, the antitoxins and toxins are proteins that tightly associate in an inactive complex that autoregulates operon transcription.[1,3] The antitoxins are proteolytically degraded upon environmental stresses, including nutrient starvation, which liberates free toxin and relieves TA operon downregulation.[5,6]

A major class of type II toxins act as mRNA interferases, which cleave mRNA.[7,8] One of the most well-characterized type II toxins is the endoribonuclease RelE, which cleaves translating mRNA in the ribosomal A-site upon amino acid starvation.[5,9−13] RelE is normally in a tightly associated complex with the antitoxin RelB, which is nucleolytically inactive. Upon amino acid starvation, Lon protease degrades RelB to liberate the active RelE protein.[5,6] RelE demonstrates strict ribosome dependence and cleaves translating mRNA between the second and third nucleotides of ribosomal A-site codons, preferentially upstream of purines.[10,13−15] The relaxed codon sequence specificity may allow RelE to globally modulate translation rates and facilitate the fast adaptation of cells to environmental changes.

The limited similarity of RelE toxins to standard ribonucleases (RNases) has hindered efforts to understand the mechanism of RelE-mediated phosphodiester bond cleavage.[4,8,16,17] RelE is most similar to the broad class of metal-independent RNases that yield a 2′,3′-cyclic phosphate and 5′-hydroxyl upon phosphodiester bond breakage.[18] Phosphodiester bond cleavage by these RNases typically proceeds via general acid−base chemistry mediated by a glutamate-histidine pair that readily promotes catalysis under physiological pH conditions.[18] These active-site residues are replaced with basic residues in RelE, yet structural studies of *Escherichia coli* and archaeal RelE proteins revealed that despite

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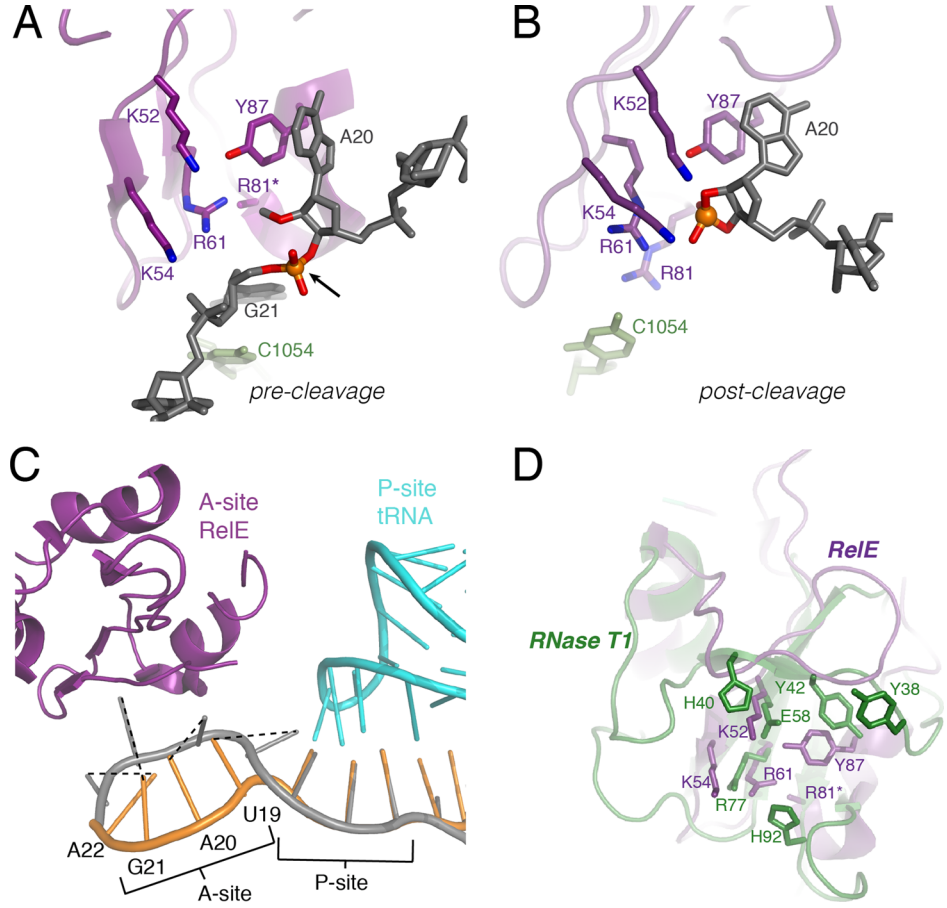

Figure 1. Structural insights into the RelE cleavage mechanism. Active sites of (A) precleavage and (B) postcleavage cocrystal structures with RelE (purple) with a modified mRNA substrate (2′-*O*-methyl at nucleotide 20) or cyclic phosphate product (gray) and nearby ribosomal 16S rRNA (light green). Heteroatoms that may be important for catalysis are colored blue (nitrogen) and red (oxygen). The arrow denotes the scissile phosphate, shown as an orange sphere. In the precleavage structure, R81(*) is mutated to an alanine (PDB entries 3KIQ and 3KIU). (C) RelE induces a conformational change in the A-site mRNA. Overlay of mRNA in the 70S ribosomal A-site and P-site when RelE is bound (gray) or a cognate tRNA (not shown) is bound (orange) in the A-site. Dashed lines denote the corresponding mRNA nucleobases between each state (PDB entry 2J00). (D) Structure of *E. coli* RelE from panel A (purple) superimposed on the structure of RNase T1 (green, PDB entry 1RGA).

the lack of sequence similarity, RelE shares a microbial RNase fold with these endoribonucleases.[17,19,20]

The cocrystal structures of *E. coli* RelE with the ribosome-bound mRNA substrate in the pre- and postcleavage states provided the first opportunity to examine the RelE active site with substrate and with product (Figure 1A–C).[12] The mRNA is sequestered over 7 Å from its normal A-site path into the highly positively charged RelE active site and is additionally stabilized by contacts with the 16S rRNA (Figure 1C).[12] The distorted mRNA configuration exposes the scissile phosphate and aligns the 2′-hydroxyl for an in-line nucleophilic attack. These structures also confirmed that although the RelE active-site residues overlay well with other RNase active sites, the side chain identities differ (Figure 1D and Figure S1 of the Supporting Information).[12,21,22] The most conserved residues in RelE (Arg61, Arg81, Tyr87, Lys52, and Lys54) are within hydrogen bonding distance of the scissile phosphate, its 5′- and 3′-nucleotides, or the mRNA 2′-hydroxyl (Figure 1A,B).[12]

Even with the RelE structures, the fundamental question of how the largely basic side chains that constitute the RelE active site promote phosphodiester bond cleavage remains. On the basis of the cocrystal structure, Neubauer et al. conducted preliminary tests of several active-site mutants but observed only modest effects on mRNA cleavage.[12] Despite these small effects, Neubauer et al. proposed a general acid–base mechanism in which Arg81 and Ty87 act as the general acid–base pair, Arg61 provides transition-state charge stabilization, and Lys52 and Lys54 may contribute to phosphate charge stabilization and substrate binding.[12] However, the biochemical results were not consistent with the structural predictions or this proposed mechanism. In other RNases, the measured mutational rate effects for catalytic side chains can be on the order of 10^3–10^5-fold.[23−26]

Here we report the kinetic analysis of the ribosome-dependent mRNA cleavage by *E. coli* toxin RelE using a single-turnover cleavage assay to directly monitor RelE cleavage and substrate association. This kinetic analysis served as the framework to examine how specific RelE active-site residues contribute to catalysis and substrate binding. These results provide biochemical data to complement the structural information regarding RelE function within the ribosome. The detailed enzymatic analysis of RelE also has applicability to other noncanonical endoribonucleases.

EXPERIMENTAL PROCEDURES

RelE and RelB Overexpression and Purification. The *relBE* locus from *E. coli* K-12 MG1655 with an N-terminal six-His tag was cloned into pET22-b between the NdeI and

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BamHI sites under T7 RNA polymerase control. Internal deletion mutants in *E. coli relb* were constructed with site-directed mutagenesis to disrupt the antitoxin's strong interactions with RelE and aid in RelE purification. The following RelB mutants were used: Δ3 (deletion of Ala19−Glu21), Δ6 (deletion of Ala19−Gly24), and Δ9 (deletion of Ala19−Pro27). Wild-type RelE was overexpressed and purified using the Δ9-His_{6x}-RelB:RelE construct. RelE mutants were generated via site-directed mutagenesis and overexpressed in the background of the Δ9 (K52A, K54A, Y87A, K52A/Y87F), Δ6 (R61A), or Δ3 (Y87F) RelB strain. The RelBE complexes were all expressed in *E. coli* BL21(DE3) and purified as follows. A 5 mL overnight culture was diluted into 600 mL of LB with 100 μg/mL ampicillin and grown to OD_{600} of 0.8 at 37 °C before being induced with 1 mM IPTG. After 3 h, cells were harvested via centrifugation and the pellet was resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 5 mM 2-mercaptoethanol, and 0.2 mg/mL lysozyme)[11] and lysed by sonication at 4 °C. Lysate was cleared by centrifugation at 4 °C and incubated with nickel-NTA agarose resin (Qiagen) (1 h, 4 °C). Resin was washed with lysis buffer with 35 mM imidazole before RelE was selectively eluted by denaturation in 100 mM NaH_2PO_4, 10 mM Tris-HCl, 9.8 M urea, and 1 mM 2-mercaptoethanol (pH 8.0).[11] RelE proteins were purified to apparent homogeneity as monitored by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (PAGE) with Coomassie Brilliant Blue staining. Purified protein was dialyzed into 50 mM Bicine (pH 8.4) and 8 M urea before refolding via dialysis in 50 mM Tris-HCl (pH 7.5), 70 mM NH_4Cl, 300 mM KCl, 7 mM $MgCl_2$, 1 M urea, and 1 mM dithiothreitol. Refolded protein was concentrated under argon using a 5000 molecular weight cutoff ultrafiltration membrane and filtered (0.22 μm) before being stored in 50 mM Tris-HCl (pH 7.5), 70 mM NH_4Cl, 30 mM KCl, 7 mM $MgCl_2$, 1 mM dithiothreitol, and 20% glycerol. His_{6x}-RelB was expressed in the context of the *relBE* locus with the *relE* start codon (ATG) mutated to isoleucine (ATT). The protein was overexpressed and purified as described for the RelBE complex; however, RelB was eluted from the Ni-NTA resin using 250 mM imidazole under native conditions. Purified RelB was also stored in 50 mM Tris-HCl (pH 7.5), 70 mM NH_4Cl, 30 mM KCl, 7 mM $MgCl_2$, 1 mM dithiothreitol, and 20% glycerol.

Circular Dichroism Spectroscopy. Circular dichroism spectra were recorded using an Applied Photophysics Chirascan spectrometer and a 2 mm path length quartz cuvette. Samples were prepared at 10 μM as described previously.[11] Scans were baseline-corrected to buffer alone, and each spectrum represents the average from at least three scans (from 260 to 202 nm) with a 0.5 nm step size recorded at 4 and 20 °C.

mRNA and Ribosome Complex Preparation. The *E. coli* 70S ribosomes were prepared as described previously.[27] The deprotected mRNA oligonucleotide (5′-GGCAAGGAGGUAAAAAUGUAGAAAAACAAU-3′) were 5′-^{32}P-labeled as described previously.[28] Ribosome complexes (RC) (400 nM $tRNA^{fmet}$, 100 nM mRNA oligonucleotide, a trace of 5′-^{32}P-labeled mRNA oligonucleotide, and 300 nM 70S ribosomes) were formed at 37 °C in reaction buffer [50 mM Tris-HCl (pH 7.5), 70 mM NH_4Cl, 30 mM KCl, 7 mM $MgCl_2$, and 1 mM dithiothreitol] for 30 min before dilution with reaction buffer to a final mRNA oligonucleotide concentration of 40 nM. Ribosome complexes containing the phosphorothioate mRNA substrates were prepared in the same manner. The mRNA oligonucleotides containing phosphorothioate substitutions between the second and third nucleotide of the A-site codon ($UA_{ps}G$) were constructed using a splinted T4 RNA ligase reaction with a 12-nucleotide oligonucleotide harboring the substitution and a 5′-phosphate (5′-P-UA_{ps}GAAAAACAAU-3′) ligated to an 18-nucleotide RNA (5′-GGCAAGGAGGUAAA-AAUG-3′) using a 22-mer nucleotide DNA splint.[29] Prior to ligation, the R_P and S_P diastereomers were separated using reversed-phase high-performance liquid chromatography on a C18 column.[30]

Single-Turnover Kinetic Measurements. Single-turnover kinetic measurements were taken for wild-type RelE using a rapid-quench-flow apparatus (KinTek Corp.). Reactions were initiated by adding an equal volume of RelE (final concentrations of 0.1−20 μM) to the RC (final RNA concentration of 20 nM) at 20 °C and quenching with excess chemical quench [80% formamide, 50 mM Tris-MES (pH 6.5), 65 mM EDTA, 0.2 mg/mL bromophenol blue, and 0.2 mg/mL xylene cyanol]. Cleavage rate constants for Y87F, K52A, and K54A were measured using the same quench-flow apparatus methods. The remaining RelE mutants were measured using manual benchtop quench methods, which were similarly initiated upon addition of an equal volume of RelE to RC at 20 °C, and reactions were stopped with excess chemical quench. Single-turnover reactions in the absence of the ribosomes were performed using the benchtop reaction procedures. Wild-type RelE (final concentration of 10 μM) alone, or premixed with a 6-fold excess of RelB, was added to mRNA substrate (final mRNA oligonucleotide concentration of 20 nM and a trace of 5′-^{32}P-labeled mRNA oligonucleotide) in reaction buffer at 20 °C, and reactions were stopped with excess chemical quench. Reactions in the absence of ribosomes were monitored up to 1.5 h after being initiated. For all reactions, the extent of A-site mRNA cleavage was determined by separating substrate and product RNAs by 15% denaturing PAGE and visualization with a STORM phosphorimager (Molecular Dynamics). Individual band intensities were quantified with ImageQuant (GE Healthcare), and the fraction cleaved was quantified as the fraction product oligonucleotide of the total.

Individual time courses for cleaved product accumulation for reactions were fit to double-exponential eq 1 using Prism 6:

$$\text{fraction cleaved} = A_{\text{fast}}(1 - e^{-k_{\text{fast}}t}) + A_{\text{slow}}(1 - e^{-k_{\text{slow}}t}) + Y_0 \quad (1)$$

where A_{fast} and A_{slow} are the amplitudes of the two phases, k_{fast} and k_{slow} are the rate constants of the phase (inverse seconds), t is the reaction time (seconds), and Y_0 is the initial fraction reacted. Because of the slow progress of the reactions of R61A and K52A/Y87F, it was not possible to measure the complete reaction and these time courses were best fit to single-exponential eq 2:

$$\text{fraction cleaved} = A(1 - e^{-kt}) + Y_0 \quad (2)$$

where A is the amplitude, k is the rate constant of the phase (inverse seconds), t is the reaction time (seconds), and Y_0 is the initial fraction reacted. The observed rates for the fast and slow phases were plotted as a function of RelE enzyme concentration (micromolar). The data points from each replicate were fit independently by hyperbolic eq 3:

$$k_{\text{obs}} = ([E]k_2)/([E] + K_{1/2}) \quad (3)$$

where k_{obs} is the observed rate constant, [E] is RelE enzyme concentration (micromolar), k_2 is the cleavage rate constant at

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a saturating enzyme concentration (inverse seconds), and $K_{1/2}$ is the apparent dissociation constant (micromolar). The mean and standard error from at least three independent determinations are reported.

Equilibrium Pulse−Chase with RelB. To confirm that RelB quenches the single-turnover RelE cleavage reactions, RC or RelE was incubated with a 6-fold molar excess of RelB antitoxin over RelE prior to the initiation of reactions. For RelB rapid quench-flow experiments, RelE and RC were reacted for the programmed reaction time in addition to the travel time through the exit line (t_1). Unquenched reaction mixtures were collected into a tube with 75 μL of chemical quench or RelB (6-fold molar excess) followed by addition of 150 μL of chemical quench after 10 reaction half-lives, t_2. For benchtop RelB quench experiments, RelE and RC were mixed for reaction time t_1 followed by the addition of either excess chemical quench or a 6-fold excess of RelB. After the quench delay time, t_2, excess chemical quench was added to the reaction mixture. Reactions were analyzed as described above.

RESULTS

We set out to characterize the kinetics of wild-type RelE cleavage activity and a series of active-site mutants. Estimates of wild-type multiple-turnover activities for different A-site substrates have been measured, but the kinetic characterization of RelE catalysis has been limited.[10,12,16,17] To provide a framework for understanding how RelE functions at both microscopic and global levels, we have used a single-turnover assay to directly measure mRNA cleavage rate constants. We then used this kinetic framework to examine the effects of RelE active-site modifications on mRNA cleavage.

mRNA Cleavage by RelE. Efficient overexpression of RelE protein for kinetic assays was achieved by co-expression with N-terminally His-tagged RelB antitoxin.[11,16] RelE protein was purified from RelB under strong denaturing conditions, and circular dichroism far-UV analysis of RelE was consistent with refolding (Figure S2 of the Supporting Information).[11]

We developed a single-turnover kinetic assay to directly monitor RelE cleavage of mRNA. Cleavage activity was measured with RelE enzyme in excess of the ribosomal complex (RC) substrate comprised of 70S *E. coli* ribosomes, an mRNA containing an A-site UAG codon, and a P-site tRNA. The addition of excess RelE enzyme to RC resulted in selective A-site codon cleavage between the second and third nucleotides, as described previously.[10,12] No cleavage attributable to RelE was detected in the absence of ribosomes (data not shown). For the ribosome−RelE reaction, time courses for accumulation of cleaved mRNA product reached end points of approximately 85% and were best described by a double exponential (Figure 2A). In most reactions, the second rate did not significantly contribute to the total reaction amplitude, but on the basis of the systematic residuals resulting from single-exponential fits, a double-exponential function was selected. The two-state kinetic behavior was consistently observed in all time courses for wild-type and mutant RelE reactions. It may result from different substrate or enzyme populations. In all cases, the relative magnitude of the effects is similar for the fast and slow rate constants, but for the sake of clarity, the fast rate constant is used for mechanistic interpretations. The reaction rate was dependent on the enzyme concentration at low RelE concentrations (0.1−3 μM) but reached a maximum at higher concentrations, with rate constant k_2 equaling 380 s^{-1} (Figure 2A,B). The values for k_2 and $K_{1/2}$ were obtained from fitting the hyperbolic dependence of k_{obs} versus RelE enzyme concentration (Table 1, Figure 2, and Table S1 of the Supporting Information).

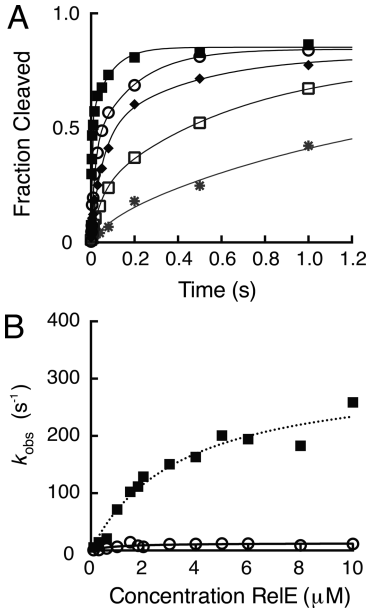

Figure 2. Wild-type RelE rapidly cleaves ribosomal mRNA. (A) Measurement of single-turnover rates for wild-type RelE. Reaction mixtures contained 20 nM RC and increasing concentrations of wild-type RelE enzyme: 0.1 (∗), 0.3 (□), 0.6 (◆), 1 (○), or 10 μM (■). The fractions of mRNA substrate cleaved during the first 1.2 s of each reaction are shown, but reactions were measured for at least 3 s and were fit as described in Experimental Procedures. (B) Dependence of cleavage rates on wild-type RelE enzyme concentration. The rates, k_{obs}, for the fast (■) and slow phases (○) for a single replicate are plotted as a function of RelE concentration. The rate constants (k_2) and dissociation constants (K_d) were extracted from hyperbolic fits of each replicate, and the mean and SEM for each constant are listed in Table 1 and Table S1 of the Supporting Information.

Table 1. RelE Single-Turnover Cleavage Rate and Dissociation Constants

RelE	$k_{2,fast}$ (s^{-1})[a]	x-fold change[b]	$K_{d,fast}$ (μM)[a]
wild-type	380 ± 25		3.1 ± 0.4
K52A	0.18 ± 0.01	2.1×10^3	0.48 ± 0.13
K54A	0.14 ± 0.01	2.7×10^3	1.5 ± 0.3
R61A	0.00014 ± 0.00001	2.7×10^6	0.88 ± 0.07
R81A	0.0064 ± 0.0001	6.0×10^4	0.81 ± 0.13
Y87F	3.0 ± 0.1	1.3×10^2	1.1 ± 0.3
Y87A	0.0021 ± 0.0002	1.8×10^5	1.8 ± 0.3
K52A/Y87F	0.00037 ± 0.00006	1.0×10^6	1.0 ± 0.4

[a]Means ± SEM from at least three independent determinations. [b]Calculated as the change in the cleavage rate constant for each mutant relative to that of the wild type.

To determine whether reactions were in rapid equilibrium, we used a pulse−chase approach that exploited the nanomolar affinity interaction between RelE and antitoxin RelB.[6,19] Direct protein−protein interaction with RelB prevents RelE from entering the ribosomal A-site and cleaving the mRNA substrate.[19] Preincubation of either RelE or RC with a 6-fold molar excess of antitoxin RelB resulted in negligible levels of RNA cleavage. For the pulse−chase experiment, RelE and RC were allowed to react for time t_1, before being pulsed with

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Table 2. Thio Effects on Wild-Type and Y87A RelE Cleavage Rate Constants[a]

	O	S (R_P)		S (S_P)	
RelE	k_{fast} (s^{-1})[b]	k_{fast} (s^{-1})[b]	thio effect[c]	k_{fast} (s^{-1})[b]	thio effect[c]
wild-type	380 ± 25	9.7 ± 0.1	39-fold	0.42 ± 0.18	900-fold
Y87A	0.0021 ± 0.0002	0.00027 ± 0.00006	8-fold	0.00037 ± 0.00014	6-fold

[a]O, unsubstituted mRNA; S (R_P) or S (S_P), sulfur substitution at the nonbridging oxygen between mRNA nucleotides 20 and 21. [b]Means ± SEM from at least three independent determinations of the rate constant at a saturating enzyme concentration. [c]Calculated as the change in cleavage rate for sulfur-substituted mRNA substrates relative to that of the unsubstituted substrate.

excess RelB. Any free RelE or RelE that rapidly dissociated from RC should be irreversibly sequestered into a complex with RelB. This complex formation would prevent any additional accumulation of product before the chemical quench was added at time t_2. In the single-turnover reaction, RelB quenched the RelE cleavage reaction to an extent equal to that of the chemical quench, indicating that the rate of RelE dissociation (k_{-1}) is faster than the rate of cleavage (k_2) for RC-bound RelE. Therefore, the RelE reaction is in rapid equilibrium, and $K_{1/2}$ is equivalent to the dissociation constant, where K_d = 3.1 ± 0.4 μM for the wild-type RelE−RC enzyme−substrate interaction.

We next examined RelE cleavage of an RNA harboring a phosphorothioate substitution at the scissile phosphate. This substrate was previously found to be cleaved to almost the same extent as the unmodified RNA under conditions with a 5-fold excess of RelE enzyme; however, the reaction rate was not measured.[12] If the chemical step is rate-limiting in our assay, the intrinsic difference in chemical reactivity between the oxygen- and sulfur-containing substrates should be reflected in a modest decrease in the rate constant of approximately 4−10-fold.[31] These thio effects would be masked under conditions where reaction steps other than chemistry, such as a conformational change in the mRNA, were rate-limiting. RelE cleaved the phosphorothioate diastereomers to end points comparable to the parent mRNA substrate, but both were less efficiently cleaved (Table 2). The 39- and 900-fold differences in rate constants for R_P and S_P substrates, respectively, provide evidence that the chemical step is at least partially rate-limiting in this RelE assay. Thus, the single-turnover assay provides a framework in which RelE mutants can be considered for their effects on substrate binding and chemical catalysis. However, the magnitudes of the thio effects are larger than expected for a classical general acid−base mechanism and may be indicative of transition-state interactions between RelE and the nonbridging phosphate oxygens.[32−34]

Kinetic Characterization of RelE Active-Site Mutants. Using the single-turnover assay developed for wild-type RelE, we investigated the contributions of the active-site residues (Lys52, Lys54, Arg61, Arg81, and Tyr87) to the RelE cleavage mechanism. In addition to being highly conserved, these residues are positioned to directly participate in catalysis and were proposed to be involved in the reaction mechanism (Figure 1).[12] Single alanine or phenylalanine substitutions were introduced for these five residues to examine their contributions to RelE catalysis. Additionally, a double mutant, K52A/Y87F, was tested to investigate interactions, such as a hydrogen bonding network, between these two residues that are in the proximity of each other and the 2′-hydroxyl. All RelE active-site mutants were purified following the same scheme used for the wild-type protein. Circular dichroism analysis for each mutant was similar to that of wild-type RelE and consistent with refolding (Figure S2 of the Supporting Information).[11]

All seven mutants were active in the single-turnover kinetic assay and cleaved the RNA between the second and third nucleotides of the A-site UAG codon. Mutant cleavage reactions exhibited kinetic behavior similar to that of the wild type, and all rates reached a plateau at high RelE concentrations (Figure 3 and Figure S3 of the Supporting Information). The cleavage rate constant effects for these mutants span four orders of magnitude, emphasizing that not all RelE active-site residues contribute equally to RelE phosphodiesterase activity (Table 1 and Table S1 of the Supporting Information). Using the RelB pulse−chase experiment described for wild-type RelE, reactions were confirmed to be in rapid equilibrium, and therefore, all $K_{1/2}$ values report on the dissociation constant (K_d) for each RelE−RC interaction (Table 1, Figure 3, and Table S1 of the Supporting Information). None of the mutations tested significantly impacted the binding affinity of RelE for the ribosome-bound mRNA substrate (Table 1 and Table S1 of the Supporting Information).

Arg61 and Arg81, two of the most highly conserved residues in RelE, were implicated in catalysis by translation inhibition and cellular toxicity studies.[16,17,19] Furthermore, Arg61 and Arg81 assume positions comparable to those of the catalytic residues responsible for stabilizing the scissile phosphate and for 5′-leaving-group protonation in other RNases.[12,18,21,22] Alanine substitution reduced RelE cleavage rate constants by 2.7 × 10^6-fold for R61A and 6.0 × 10^4-fold for R81A. These effects are at least 1 order of magnitude stronger than those of most other single-alanine mutants tested.

The structurally conservative Y87F mutation yielded the most catalytically active mutant among those tested with just a 130-fold effect on the reaction rate constant. However, replacement of Tyr87 with alanine severely inhibited cleavage activity, decreased 1.8 × 10^5-fold relative to that of the wild type. As with the other mutants tested, each had a K_d similar to that of the wild type (Table 1). The Y87A substitution should abolish the stacking interactions with nucleotide 2 and was expected to reduce the rate of efficient sequestration of mRNA into the RelE active site (Figure 1). To confirm that chemistry remained rate-limiting for this mutant, the Y87A cleavage rate for the phosphorothioate substrates was measured. Thio effects were measured for each substrate (5-fold for R_P and 6-fold for S_P), suggesting that chemistry remains at least partially rate-limiting for this mutant (Table 2). However, these effects were substantially smaller than those observed for the wild type, and the apparent stereospecificity of the wild type was not observed with the Y87A mutant. The magnitude of these effects is closer to what it is expected for the intrinsic thio effect and may suggest that the reaction mechanism differs in the Y87A mutant.

The terminal amino groups from Lys52 and Lys54 are near Tyr87 and within hydrogen bonding distance of the RNA 2′-hydroxyl and phosphate. We found that under single-turnover conditions the K52A and K54A mutations each decreased the

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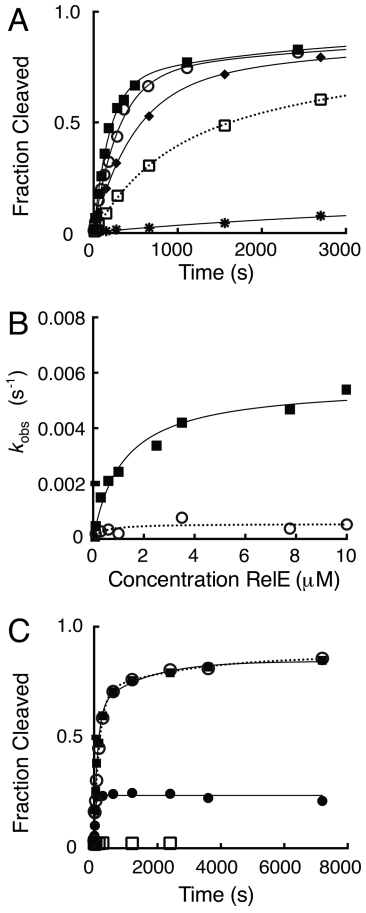

Figure 3. Analysis of single-turnover reaction kinetics of mutant RelE proteins. (A) Measurement of single-turnover rates for R81A RelE. Reaction mixtures contained 20 nM RC and increasing concentrations of R81A RelE enzyme: 0.1 (∗), 0.3 (□), 0.6 (◆), 2.5 (○), or 10 μM (■). The first 3000 s of the time course is shown, and full time courses were fit as described for the wild type. (B) Dependence of cleavage rates on R81A RelE enzyme concentration. The rates, k_{obs}, for the fast (■) and slow phases (○) for a single replicate are plotted as a function of RelE concentration. The rate constants (k_2) and dissociation constants (K_d) extracted from hyperbolic fits of each replicate and the mean and SEM for each constant are listed in Table 1 and Table S1 of the Supporting Information. (C) Antitoxin RelB pulse−chase quench experiments with R81A RelE. Reaction mixtures contained 20 nM RC and 10 μM R81A RelE; reactions were quenched either chemically (○) or enzymatically with a 6-fold excess of RelB (■), or the RC was premixed with RelB prior to the addition of RelE (□). Product formation for the pulse−chase reaction with antitoxin RelB is plotted for the total time (reaction time, t_1, plus quench delay time or chase, t_2) where RelB was added after t_1 of 78 s (●).

RelE rate constant by 2 × 10^3-fold (Table 1). The proximity of Lys52 to the conserved Tyr87 and to the 2′-hydroxyl may be critical for deprotonation at the 2′-hydroxyl. To investigate any interactions between these critically positioned residues, in particular if Tyr87 contributes to the positioning and activation of Lys52, the effect of the Lys52 mutation was measured in the context of the Y87F mutant. A modest synergistic effect for this secondary mutation renders RelE minimally active, with a 1.0 × 10^6-fold decrease in the rate from that of the wild type, suggesting distinct mechanistic contributions by each residue.

DISCUSSION

RelE is a specific endoribonuclease that cleaves mRNA only in the context of the ribosome. Because of its nonstandard active-site residues, RelE uses an alternative strategy from more traditional endoribonucleases for efficient substrate recognition and catalysis. Features of the RelE active site that are conserved, yet catalytically unconventional, provide substrate positioning, general acid−base function, and charge stabilization during the reaction. The unusual RelE active-site composition may lead to a requirement for substrate positioning for the achievement of catalytic activation. The obligate ribosome dependence may derive from the ability of the ribosomal A-site to provide scaffolding that promotes productive binding interactions or the formation of the appropriate microenvironment that activates RelE catalytic side chains.[12,35]

RelE does not efficiently cleave free mRNA, but in the context of the ribosome, RelE rapidly cleaves ribosomal mRNA in the A-site (Figure 2).[10] The single-turnover catalytic rate for RelE observed in this study (k_2 = 380 s^{-1}) approaches that of the prototypical RNase A, which cuts the free oligonucleotide with a k_{chem} of ∼1000 s^{-1},[36] yet under these conditions, there is negligible RelE-mediated cleavage of mRNA in the absence of ribosomes (data not shown) and the ribosome is estimated to enhance the RelE reaction by at least 10^8-fold. This specificity might result from nonproductive substrate binding to free mRNA because the global ribosomal contacts may drive productive RelE−mRNA association. In RNase T1, the downstream mRNA is engaged by residues, Tyr38, His92, and Phe100, but for RelE, the ribosome must provide contacts to hold the nucleotides in place and maintain backbone distortion.[12,18,21] This distortion promotes favorable substrate geometry for the intramolecular in-line nucleophilic attack by the mRNA 2′-hydroxyl.[18] However, no rRNA or ribosomal proteins directly contact the mRNA nucleophile or leaving group, which are instead surrounded by RelE basic side chains.

The biochemical evaluation of RelE active-site side chains by Neubauer et al. led to the proposal of a general acid−base mechanism that invoked Arg81 and Tyr87 as the general acid−base pair, and Arg61 as transition-state charge stabilization, with Lys52 and Lys54 providing additional transition-state charge stabilization (Figure 4A).[12] However, the biochemical data presented with the structure were not consistent with this proposal. It is likely that significant catalytic contributions from each active-site side chain were overlooked because the extent of mRNA cleavage was measured after a single 15 min time point with a small excess of substrate.[12] Given a cleavage rate constant of 380 s^{-1}, 15 min corresponded to approximately 500000 single-turnover reaction half-lives for wild-type RelE. Under such conditions, only the most debilitating RelE mutations would show even the slightest effect, and the magnitude of the effects could not be defined.

To discern differences between wild-type and mutant RelE that are mechanistically important, we used a single-turnover assay to isolate RelE catalysis. By direct measurement of mRNA cleavage rate constants, mutational effects can be ascribed to each RelE active-site residue. In addition to requiring in-line positioning, phosphodiester bond cleavage is promoted by the combination of nucleophile deprotonation, leaving-group protonation, and stabilization of the transition state.[18,37] In solution, although a dianionic intermediate is expected during base-catalyzed reactions, monoanionic or neutral intermediates are possible for acid-catalyzed reactions, whereby nonbridging

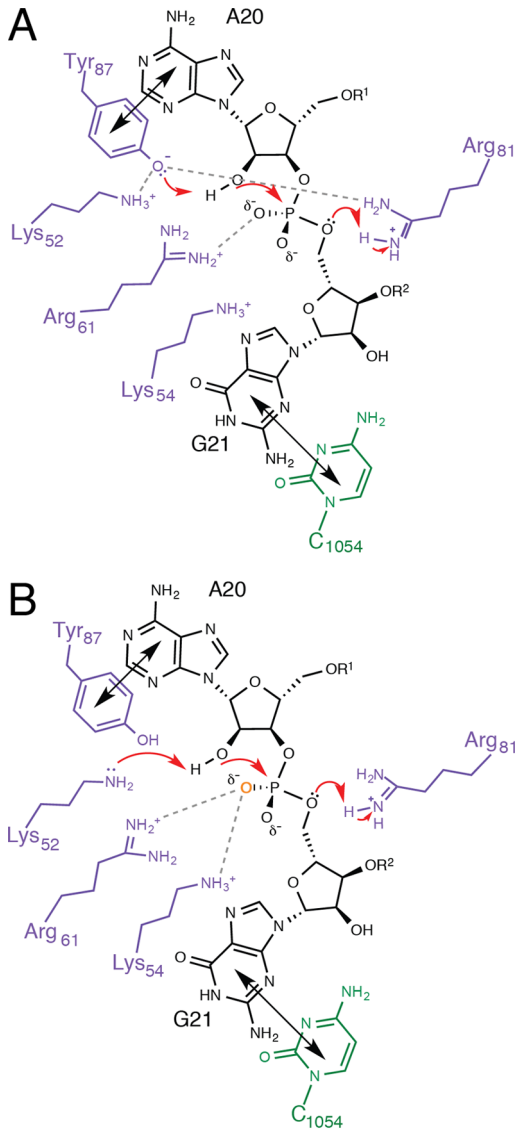

Figure 4. Mechanism of phosphodiester bond cleavage by RelE. (A) Previously proposed mechanism for RelE catalysis with RelE (purple), mRNA (black), and 16S rRNA C1054 (green).[12] Tyr87 and C1054 stack with the second and third nucleotides, respectively (shown with black double arrows), to position the substrate for nucleophilic attack. The positively charged environment shifts the pK_a of Tyr87, which could act a general base; Arg61 stabilizes the transition state, and Arg81 protonates the leaving group, acting as a general acid. Dashed lines indicate proposed interactions. (B) Revised model for RelE catalysis, depicted as in panel A. Here, Lys52 is activated as the general base by the positively charged microenvironment; Arg61 and Lys54 stabilize the transition state, and Arg81 acts as a general acid. The large phosphorothioate effects suggest strong interactions between RelE and the pro-S_p nonbridging oxygen (orange), possibly from Arg61 and the neighboring Lys54.

phosphate oxygens are protonated.[38,39] These different protonation states of the phosphorane intermediate are also possible in the context of enzymatic reactions. The reaction mechanism and catalytic functions have not been readily assigned for the noncanonical RelE reaction. Together with the cocrystal structures, the measured mutational effects provide a foundation for considering catalytic contributions of individual residues and future mechanistic investigations of RelE.

While the single-turnover effects do not explicitly implicate a particular RelE residue for a specific catalytic role, they do demonstrate that individual active-site residues make significant mechanistic contributions. The interpretation of these effects in the context of the available structural information has led to a revised mechanistic proposal for RelE-mediated cleavage (Figure 4B). In the revised mechanism, Lys52 is activated by the positively charged microenvironment to act as a general base, Arg61 and Lys54 contact and stabilize the negatively charged phosphate, and Arg81 acts a general acid. Well-conserved positively charged residues Arg61 and Arg81 are situated near the phosphate and 5′-oxygen leaving group, and each significantly affects the chemical rate constant. As such, their proposed roles in transition-state charge stabilization and leaving-group protonation, respectively, appear to be indispensible to RelE.[12] The positive charges and Tyr87 engage the RNA substrate in the distorted conformation required for the intramolecular in-line nucleophilic attack at the phosphate.[12] Large effects (10^3) upon mutation of either lysine adjacent to the 2′-oxygen are indicative that both lysines contribute catalytically. A pK_a shift of one amine, possibly Lys52, in the context of a highly positively charged microenvironment may allow efficient proton abstraction at the 2′-hydroxyl. Direct contacts to the phosphate oxygens, in particular the pro-S_P oxygen, from Lys54 and Arg61, may polarize the phosphate bonds and further promote catalysis. Each RelE active-site substitution negatively impacted chemistry but did not perturb the substrate binding affinity, indicating that these residues stabilize the transition state but not the ground state.

The large catalytic effect measured for R61A (10^6-fold) is consistent with its proposed role in transition-state stabilization. The universal conservation of Arg61 and the analogous role in charge stabilization attributed to structurally equivalent residues in RNases, Arg77 in RNase T1 and Arg71 in RNase Sa2, further reinforce the importance of this arginine in promoting nucleophilic attack by stabilizing transition-state charge buildup on the scissile phosphate (Figure 1 and Figure S1 of the Supporting Information).[12,21,22] Furthermore, the guanidinium group of RelE Arg61 is located at the juncture between other RelE side chains (Lys54, Arg81, and Tyr87) and the 2′-hydroxyl and nonbridging phosphate oxygens on the substrate (Figure 1).[12] From this position, Arg61 may coordinate active-site organization and substrate positioning, further accounting for the considerable effect on the rate upon mutation (Figure 4B).

The other active-site arginine in RelE, Arg81, was evoked as a general acid, and the significant rate effect for its mutation reinforces the assignment of a critical catalytic function (Table 1). Leaving-group protonation in other protein phosphodiesterase enzymes is often achieved by histidine because of its near-neutral pK_a, but there is precedent for arginine-mediated general acid activity in enzymes such as X-family DNA polymerases and fumarate reductase.[18,40−42] The positive charge of both this arginine and Arg61 may help lower the pK_a of neighboring active-site residues and aid general base activity. In phosphodiesterase reactions, leaving-group protonation may be the rate-limiting step.[37,43−45] Evidence from recent kinetic isotope effect measurements and computational calculations for enzyme and solution base-catalyzed reactions suggests that RNA transphosphorylation proceeds via a late transition state with rate-limiting expulsion of the 5′-oxygen leaving group.[38,39] Therefore, general acid catalysis is particularly important in RNase active sites for both protein

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and RNA enzymes. In some instances, stabilization of the developing charge at the 5′-oxygen may be more critical than optimal general base activity.[43]

A primary challenge in understanding RelE-mediated phosphodiesterase activity involves nucleophile activation. Deprotonation of the 2′-hydroxyl in RNases is often initiated by glutamate or histidine side chains (Figure S1 of the Supporting Information),[18] yet there are examples of alternative general base side chains (lysine, arginine, and tyrosine) in diverse enzymes such as oxidative decarboxylases, dehydrogenases, and epimerases.[40,46−48]

In RelE, Tyr87 has generated speculation with regard to its role as a general base, but our results are inconsistent with it functioning in this role. The loss of a catalytically essential group in an RNase active site can abolish all measurable activity, as seen in Barnase upon mutation of general base Glu73.[23] However, in RelE, the phenyl ring at position 87 appears to be nearly sufficient for the maintenance of catalytic efficiency, with only a 130-fold effect (Table 1). Tyr87 structurally aligns with tyrosines in RNase active sites, such as Tyr42 in T1 and Tyr103 in Barnase (Figure 1 and Figure S1 of the Supporting Information).[12,21,22] Substitution of Tyr103 in Barnase with phenylalanine yielded results similar to those for Tyr87 in RelE, with almost no difference in cleavage or binding.[24] In T1, mutation to alanine weakened binding but not activity.[49] Conserved tyrosine and other aromatic residues in RNase active sites are primarily thought to help position the nucleotide adjacent to the cut site and possibly improve specificity.[18,21,22,24]

The large catalytic effects and structural proximity to the 2′-hydroxyl suggest that Lys52 and/or Lys54 may play a role as the general base. Lys52 structurally coincides with the glutamate general base in RNase active sites (Figure 1D and Figure S1 of the Supporting Information).[12,21,22] Across bacterial RelE proteins, Lys52 and Lys54 are well-conserved, but they are replaced by the analogous residue arginine in many archaeal examples.[4,12,50] A study examining archaeal RelE translational inhibition activity identified residue Arg58 (Lys52 in *E. coli* RelE) as being functionally important, and the single-turnover cleavage rate constant decreased by 3 orders of magnitude upon mutation of either lysine (Table 1).[17] The solution pK_a of each active-site side chain is far from neutrality, but shifts of up to 5 pK_a units have been measured for lysine and arginine in other contexts.[40,51,52] The RelE microenvironment created by the positively charged residues could sufficiently shift the pK_a of a lysine side chain to enhance its general base functionality as seen in other enzymes.[40,51,52] Therefore, RelE may be inactivated with an increasing pH as a result of deprotonation of active-site residues critical for maintaining this microenvironment.

On the basis of the identical rate effects, we cannot exclude the possibility that Lys54 acts as general base in RelE. However, Lys54 may be better situated to interact with Lys52 and help to lower its pK_a while also offering phosphate stabilization as proposed.[12] Each lysine was proposed to function in additional charge stabilization at the phosphate, similar to RNase Sa2.[12,22] In RNase Sa2, two arginine residues polarize the phosphate−oxygen bonds and prime the phosphate for intramolecular nucleophilic attack (Figure S1 of the Supporting Information).[22] The large thio effects for wild-type RelE may result from the weaker propensity to hydrogen bond with or protonate a sulfur atom and therefore reflect strong polarization of the phosphate oxygen bonds in the transition state or formation of a triester as proposed for RNase T1.[32−34] Specifically, the pro-S_P oxygen may be engaged in interactions with RelE (Figure 4B). This nonbridging oxygen can be protonated to form a phosphate triester, similar to the acid-catalyzed solution reaction that proceeds via a monoanionic intermediate.[38] This proton can be donated from various sources, including the general acid and 2′-hydroxyl, resulting in a phosphate triester that is 10^3−10^5-fold more reactive than the diester substrate.[32−34] The higher reactivity of a triester substrate could weaken the requirement for a robust general base in RelE. Alternatively, the positive side chains could merely neutralize the charged phosphate. Lys54 of RelE, in particular, is situated like Lys27 in Barnase and His40 in RNase T1, which are critical residues for electrostatic catalysis in their respective enzymes (Figure 1D and Figure S1 of the Supporting Information).[12,21,23,53,54]

Although the RelE active-site positive charges would be expected to aid ground-state binding of the negatively charged RNA substrate, their loss is detrimental to only the transition state, suggesting this interaction may be largely facilitated by global interactions between RelE and the ribosomal substrate. There is a charge redistribution expected for phosphoryl transfer reaction transition states that proceed via a negatively charged pentacoordinate intermediate, which RelE may preferentially stabilize over the ground state.[37,43,55] Furthermore, RelE side chains could contribute to the stabilization of the catalytically competent distorted mRNA conformation. The differential stabilization of the highly negatively charged or distorted phosphorane intermediate may be responsible for the observed chemical rate effects in the absence of binding effects.

Conserved and catalytically important features of the RelE active site include residues that offer substrate positioning, general acid functionality, and charge stabilization.[4,12,16] RelE, which is a highly efficient and specific nuclease, may represent a shift in the RNase general acid−base catalytic paradigm. The strategy used by RelE relies heavily on catalytic side chains that may require activation through a positive charge network and promote catalysis predominantly by leaving-group protonation, charge stabilization, and possibly triester formation. This may render RelE largely dependent on optimal substrate geometry and overall active-site organization, which are provided only in the context of the ribosomal A-site thereby preventing indiscriminate nuclease activity. Unlike the numerous RNases such as RNase A, T1, and Barnase that are poised for RNA cleavage with a variety of free RNA substrates, RelE can efficiently act only in the context of its ribosomal "cofactor".[10,18]

A number of other nucleases have unusual active sites that are similar to that of RelE. MqsR is another bacterial toxin that may require the ribosome for mRNA cleavage, and Colicin E5 targets the anticodon loop of specific tRNA molecules.[56−58] Both nucleases cleave very specific RNA structures where it can be expected that substrate recognition and positioning are critical for function. Each active site is similarly composed of basic lysine and arginine side chains in addition to glutamine (both MqsR and E5) and tyrosine (MqsR).[56,58] Because all three enzymes act on highly structured RNA substrates and share active-site composition features, these nucleases may use a mechanistic strategy similar to that of RelE.

These and other examples of noncanonical nuclease active sites similar to the widespread RelE toxin suggest that it may represent an alternative solution to nuclease activity that provides efficient catalysis, while ensuring strict substrate

specificity. RelE relies on features of the ribosome-bound substrate, to bind and orient the substrate. The efficiency of RelE-mediated cleavage and ribosome dependence allow RelE to specifically modulate translation rates and regulate cellular responses to environmental changes.

■ ASSOCIATED CONTENT

Supporting Information

Additional figures and tables for kinetic experiments, circular dichroism analysis, and structural discussions of ribonuclease active-site organization. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

RNase, ribonuclease; RC, ribosomal complex substrate; PAGE, polyacrylamide gel electrophoresis; PDB, Protein Data Bank; SEM, standard error of the mean.

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