A distinct RNA recognition mechanism governs Np₄ decapping by RppH

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Abstract

Dinucleoside tetraphosphates, often described as alarmones because their cellular concentrations increase in response to stress, have recently been shown to function in bacteria as precursors to dinucleoside tetraphosphate (Np₄) RNA caps. Removal of this cap is critical for initiating 5’ end-dependent degradation of those RNAs, potentially affecting bacterial adaptability to stress; however, the predominant Np₄ decapping enzyme in prokaryotes, RppH, is activated by the very conditions of ribosidase stress that enable Np₄-capped RNAs to accumulate to high levels. Here, we show that, in Escherichia coli cells experiencing such stress, the RNA pyrophosphohydrolase RppH assumes a leading role in decapping those transcripts, preferring them as substrates over their triphosphorylated and diphosphorylated counterparts. Unexpectedly, this enzyme recognizes Np₄-capped 5’ ends by a mechanism distinct from the one it uses to recognize other 5’ termini, resulting in a one-nucleotide shift in substrate specificity. The unique manner in which capped substrates of this kind bind to the active site of RppH positions the δ-phosphate, rather than the β-phosphate, for hydrolytic attack, generating triphosphorylated RNA as the primary product of decapping. Consequently, a second RppH-catalyzed depredation step is required to produce the monophosphorylated 5’ terminus needed to stimulate rapid RNA decay. The unconventional manner in which RppH recognizes Np₄-capped 5’ ends and its differential impact on the rates at which such termini are deprotected as a prelude to RNA degradation could have major consequences for reprogramming gene expression during disulfide stress.

Significance

Dinucleoside tetraphosphate alarmones function in bacteria as precursors to 5’-terminal dinucleoside tetraphosphate (Np₄) caps, becoming incorporated at high levels into RNA during stress and thereby influencing transcript lifetimes. However, little is known about how these noncanonical caps are removed as a prelude to RNA degradation. Here, we report that the RNA pyrophosphohydrolase RppH assumes a leading role in decapping those transcripts under conditions of disulfide stress and that it recognizes Np₄-capped 5’ ends by an unexpected mechanism, generating a triphosphorylated RNA intermediate that must undergo further depredation by RppH to trigger degradation. These findings help to explain the uneven distribution of Np₄ caps on bacterial transcripts and have important implications for how gene expression is reprogrammed in response to stress.

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Research in our lab has demonstrated that disulfide stress is a major contributor to gene expression changes in bacteria. We report here that the RNA pyrophosphohydrolase RppH, which is activated by disulfide stress, prefers to decap Np₄-capped RNAs over their triphosphorylated counterparts. This preference is due to the unique way in which RppH recognizes Np₄-capped 5’ ends, resulting in a one-nucleotide shift in substrate specificity. The authors propose that this mechanism could have major implications for how gene expression is reprogrammed in response to disulfide stress.
RNA degradation. Dinucleoside tetraphosphates (Np4As) were first described over 50 y ago as byproducts of transfer RNA aminoacylation and are present in all realms of life (22–24), yet their biological function as cap precursors was only recently recognized. Increases in their abundance are correlated with many interesting bacterial phenotypes including several related to pathogenesis (25–28). Np4As are often characterized as alarmones because their cellular concentration rises dramatically in response to certain stresses, especially disulfide stress induced by cysteine cross-linkers like cadmium and diamide (29, 30), which arrest growth. The same stress conditions greatly increase the cellular concentration of Np4-capped RNAs, raising their abundance to such an extent that they come to represent 4 to 76% of most primary transcripts (20). As a result of molecular symmetry (Np4A = Ap4N) and the mechanism of cap acquisition, both the cap nucleotide and the first transcribed nucleotide of Np4-capped RNAs can be A, G, C, or U.

Cap removal is a critical first step in the degradation of Np4-capped transcripts via the 5′ end-dependent pathway. Both in vitro and in E. coli, Np4-capped RNA can be deprotected by either of two enzymes, ApaH or RppH (20, 31), which also function as Np4A hydrolases (32–34). Of the two, ApaH appears to be the principal Np4-decapping enzyme in unstressed E. coli cells. Its inactivation by cysteine cross-linkers enables Np4-capped transcripts to accumulate to a high cellular concentration during disulfide stress, a phenomenon recapitulated in unstressed apaH deletion mutants, which lack this enzyme altogether (20). By contrast, although RppH contributes to deprotecting Np4-capped RNA in E. coli under ordinary growth conditions, deleting the rppH gene is not sufficient to cause Np4-capped RNAs to rise to measurable levels. Despite the importance of ApaH and RppH for governing the lifetimes of Np4-capped transcripts, nothing is known about their substrate specificity as decapping enzymes, which could have a major impact on gene expression during stress.

Because ApaH is inactivated in disulfide-stressed cells (20), we have now examined the importance of RppH for deprotecting Np4-capped transcripts under those conditions and found that it remains active. Biochemical and crystallographic studies have revealed unexpected differences in the recognition of capped versus triphosphorylated RNAs by this enzyme, resulting in distinct substrate preferences and reaction products.

Fig. 1. Effect of disulfide stress on RppH activity in E. coli. (A) Structure of the 5′ end of Ap4-capped yeiP mRNA. Only the cap and the first transcribed nucleotide of this A-initiated RNA are shown. The α-, β-, γ-, and δ-phosphates of the cap are labeled. (B) Rate of loss of capped yeiP mRNA after arresting transcription in E. coli mutants lacking ApaH and/or RppH. (Top) Northern blots. Cultures of the indicated strains (ΔapaH or ΔapaH ΔrppH) growing in MOPS-glucose medium either were treated with cadmium chloride (cadmium stressed; Right) or were not (unstressed; Left), and equal amounts of total RNA extracted at time intervals after inhibiting transcription were cleaved site-specifically with a deoxyribozyme targeting yeiP (SI Appendix, Table S3) and analyzed by boronate gel electrophoresis and blotting with a radiolabeled yeiP probe. (Bottom) Graphs. The amount of capped yeiP mRNA that remained was plotted semilogarithmically as a function of time, and best-fit lines were calculated by linear regression. First-order rate constants for the disappearance of capped yeiP mRNA were determined from the slopes of these lines. Representative experiments are shown.
These studies suggest an explanation for the strikingly uneven distribution of Np4 caps on cellular transcripts and provide key insights into the mechanism by which Np4-capped transcripts are degraded when such caps are abundant.

**Results**

**Effect of Disulfide Stress on RppH Activity in E. coli.** Two *E. coli* pyrophosphohydrolases, ApaH and RppH, have each been shown to remove Np4 caps from RNA 5' ends, both in vitro and in vivo (20, 31). In *E. coli*, ApaH appears to be the predominant source of Np4 decapping activity under ordinary growth conditions, where it is so efficient that Np4-capped RNAs are virtually undetectable. Np4-capped transcripts are able to accumulate to significant levels under conditions that inactivate ApaH, such as disulfide stress, or in mutant strains (ΔapaH) that lack this enzyme altogether (20). To determine whether RppH remains active during disulfide stress, we induced this condition in isogenic *E. coli* mutants lacking either ApaH alone or both ApaH and RppH, blocked further RNA synthesis, and measured the rate at which Np4-capped transcripts were lost. In principle, any difference between the rates in these two strains can be attributed to the action of RppH.

As a model RNA, we chose the *E. coli* yeiP transcript, which encodes a paralog of the translation elongation factor EF-P and is highly capped in wild-type *E. coli* cells experiencing disulfide stress and in unstressed ΔapaH cells (20). We first determined the rate of yeiP decapping by RppH in the absence of stress. Log-phase cultures of ΔapaH and ΔapaH ΔrppH cells were treated with rifampicin to arrest transcription, and total RNA was harvested at time intervals thereafter. Capped and uncapped cellular transcripts were then separated by electrophoresis through a polyacrylamide matrix modified with boronate side chains that selectively retard the migration of capped RNAs by transiently forming a covalent adduct with the vicinal diol of the cap nucleoside (35). Distinct bands representing capped and uncapped yeiP mRNA were subsequently detected by Northern blotting (Fig. 1, B, Left). By subtracting the rate of disappearance of the capped yeiP transcript in the ΔapaH ΔrppH strain from the rate in the isogenic ΔapaH strain, we calculate that the first-order rate constant for yeiP decapping by RppH is 0.115 ± 0.008 min⁻¹ in unstressed *E. coli* cells (SI Appendix, Table S1).

We then repeated these measurements in the same *E. coli* strains after treatment with cadmium chloride to induce disulfide stress (Fig. 1, B, Right). Under these conditions, the rate constant for yeiP decapping by RppH was calculated to be 0.031 ± 0.004 min⁻¹ (SI Appendix, Table S1). Thus, RppH is 27 ± 4% as active during cadmium stress as it is in unstressed cells. We conclude that RppH retains the ability to deprotect Np4-capped transcripts in *E. coli* under disulfide stress conditions that completely inactivate ApaH. The presence of yeiP decay intermediates resulting from 5’ monophosphate-dependent cleavage by RNase E (36) in ΔapaH but not ΔapaH ΔrppH cells (Fig. 1B) shows that RNase E also remains active under these stress conditions.

**Requirements for Unpaired Nucleotides at the 5’ End.** Because RppH not only contributes to the deprotection of Np4-capped RNA under normal growth conditions but also appears to be the primary decapping enzyme during disulfide stress, knowing the substrate preferences of this enzyme is crucial for understanding the diverse rates at which capped transcripts are degraded in *E. coli*. To examine those preferences, we employed a set of structurally unambiguous RNA substrates similar to those that had previously been used to investigate the specificity of RppH when removing pyrophosphate from triphosphorylated RNA (11, 14, 37). Synthesized by in vitro transcription in the

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The presence of a specific NpA (synonymous with ApN due to molecular symmetry), each substrate comprised an Np-capped 5′-terminal RNA segment that was not base paired followed by two 5′-terminal stem-loops, one of which bore a fluorescein tag in its apical loop (Fig. 2A and SI Appendix, Fig. S1A). Another Np-capped RNA containing a third stem-loop (ApA8XL; SI Appendix, Fig. S1B) was included in every reaction as an internal standard. To compare rates of decapping, each RNA substrate was combined with ApA8XL and RppH, and reaction samples were quenched with ethylenediaminetetraacetate (EDTA) at various times thereafter. The substrates and reaction products were then separated by boronate gel electrophoresis and visualized by fluorescence.

The first capped substrate tested was ApG3, which contained three unpaired 5′-terminal nucleotides, the first of which was G (Fig. 2A). RppH was able to efficiently decap both ApG3 and ApA8XL, whereas catalytically inactive RppH-E57A was unable to remove the cap from either substrate, as expected (Fig. 2B). Because a 5′-terminal stem-loop is known to prevent RppH-catalyzed pyrophosphate removal from triphosphorylated RNA (3, 14), we next examined whether such a structure also inhibits Np cap removal by this enzyme. A substrate with no unpaired nucleotides at the 5′ end, ApG0, was completely resistant to RppH, while ApA8XL in the same reaction mixture was readily decapped (Fig. 2C). To determine the number of unpaired 5′-terminal nucleotides required for decapping by RppH, we tested three more Ap-capped substrates containing one, two, or four unpaired nucleotides there (ApG1, ApG2, and ApG4, respectively). RppH reacted slowly with ApG1, substantially faster with ApG2, and even more rapidly with ApG4, whose reactivity resembled that of ApG3 (Fig. 2D). The internal standard ApA8XL was efficiently decapped in every reaction (SI Appendix, Fig. S2A). We conclude that RppH requires at least one unpaired nucleotide at the 5′ end for Np cap removal and prefers two or more. These requirements differ from those observed for pyrophosphate removal from triphosphorylated RNA, where RppH was found to require at least two unpaired 5′-terminal nucleotides and to prefer three or more (14).

**Effect of Nucleotide Identity on Decapping.** The requirement for at least one unpaired nucleotide at the 5′ end of capped substrates raised the possibility that RppH might be sensitive to the identity of that nucleotide. We therefore compared the reactivity of four Ap-capped substrates (ApA4, ApG4, ApC4, and ApU4; SI Appendix, Fig. S1C) that differed in sequence only at the first of four unpaired 5′-terminal nucleotides (i.e., at the first transcribed nucleotide). The substrate was combined with Ap4A8XL and RppH, and reaction samples were quenched with ethylenediaminetetraacetate (EDTA) at various times thereafter. The substrates and reaction products were then separated by boronate gel electrophoresis and visualized by fluorescence.

Because ApA4, ApG4, ApC4, and ApU4 have been observed in E. coli (20), we also investigated whether RppH discriminates between different types of caps (ApA8, ApG8, ApC8, and ApU8; SI Appendix, Fig. S1D). No significant disparities were observed in its reactivity with substrates that differed only in the identity of the cap nucleobase (Fig. 3B and SI Appendix, Fig. S2C).

**Influence of the 5′-Terminal Transcribed Nucleotide in E. coli.** To determine whether the 5′-terminal nucleotide preference of RppH observed in vitro is also evident in vivo, we examined the influence of the first nucleotide of efp mRNA on its rate of decapping by RppH in E. coli. This transcript, which encodes the translation elongation factor EF-P, naturally begins with C at the 5′ end, as does its capped counterpart in Δefp cells (20). Plasmids encoding wild-type efp mRNA or either of two efp point mutants in which the 5′-terminal pyrimidine had been replaced with a purine (efp-C1A or efp-C1G) were introduced into an isogenic pair of Δefp and Δefp ΔrppH strains lacking the chromosomal copy of the efp gene. Log-phase cultures growing without stress were then treated with rifampicin, and the rate constant for RppH-mediated decapping of efp mRNA was calculated from the difference in the rate of disappearance of the Np-capped transcript in the two strains, as determined by boronate gel electrophoresis and Northern blotting.

The three efp transcripts were Np-capped to varying degrees in Δefp cells (Fig. 4A), and their rates of deprotection by RppH also differed substantially. Both of the 5′-terminal purine substitution mutants were decapped much faster than the wild-type, C-initiated transcript (Fig. 4 B–D). The rate constant for decapping by RppH in E. coli was 0.099 ± 0.009 min⁻¹ for efp-C1A and 0.089 ± 0.012 min⁻¹ for efp-C1G versus only 0.034 ± 0.005 min⁻¹ for wild-type efp, a nearly threefold acceleration (Fig. 4E and SI Appendix, Table S1). The influence of the first transcribed nucleotide on the rate of efp decapping by RppH in vivo, where the enzyme forms a heteromeric complex with the diaminopimelate epimerase DapF (17–19), was in full agreement with the 5′-terminal sequence preference observed for decapping by monomeric RppH in vitro, evidence that DapF does not alter the substrate specificity of decapping by RppH.
of RppH as a decapping enzyme differ from those observed for its reaction with triphosphorylated and diphosphorylated RNAs (4, 14); in particular, cap removal requires one fewer unpaired nucleotide at the 5′ end and prefers a purine as the first transcribed nucleotide rather than as the second transcribed nucleotide. These findings suggest that the positioning of capped RNA bound to RppH may be shifted by one nucleotide relative to the positioning of triphosphorylated and diphosphorylated RNA substrates.

To visualize the interaction of RppH with the 5′ end of NP4-capped RNA, we determined the X-ray crystal structure of this enzyme bound to Ap4A at 1.6 Å resolution (SI Appendix, Table S2). The structure was determined in the precleavage state with a catalytically competent enzyme, whose activity was inhibited by adding fluoride ions that replace the reactive water molecule in the catalytic center. In the structure, the tetraphosphate moiety of Ap4A splay out on a positively charged surface of the protein (Fig. 5A), extending from the catalytic center where three Mg2+ ions are present to a semiopen cleft where one of the adenines (Ade1) is sandwiched between R27 and Q95 above and L83, V137, and F139 below (Fig. 5B). The side chain of R27 forms a cation-π interaction with the nucleobase of Ade1 but does not hold it tightly in the cleft, as evidenced by its partial electron density map (Fig. 5C). The other adenosine (Ade0) is disordered in the structure. The tetraphosphate bridge connecting the two nucleosides accepts 10 hydrogen bonds from seven amino-acid residues that line the binding path, including three each formed by the γ- and δ-phosphates and two each formed by the α- and β-phosphates (Fig. 5F). In addition, the δ-phosphate coordinates all three Mg2+ ions, and the γ-phosphate contacts MgI (SI Appendix, Fig. S3). Together, these interactions position the substrate optimally for catalysis.

The structure readily explains the catalytic mechanism for asymmetrical Ap4A cleavage. The electron density peak positioned between Mg2 and Mg3 and assigned to a fluoride ion corresponded to a water molecule in the noninhibited structure (16). This nucleophile is located 2.9 Å from the phosphorus atom of the δ-phosphate at an angle (175°) that is ideal for in-line attack to sever the δ-phosphate from the γ-phosphate (Fig. 5B). Cleavage there by water would yield AMP and ATP as products, as previously reported (34).

Comparison of this structure to that of RppH bound to the 5′-triphosphorylated RNA oligonucleotide pppAGU (16) revealed striking similarities, including the absence of any appreciable change in protein conformation, as well as important differences. Specifically, Ade1 of Ap4A replaces the second nucleoside (Gua2AGU) of pppAGU in the cleft, the α-phosphate of Ap4A replaces the phosphodiester linkage between Gua2AGU and the preceding nucleoside, and the γ- and δ-phosphates of Ap4A replace the α- and β-phosphates of the RNA ligand (Fig. 5D and E). As a result, the 5′-terminal nucleoside of pppAGU (Ade1AGU) is replaced nonspecifically by the β-phosphate of Ap4A. This phosphate forms two new hydrogen bonds with RppH residues R27 and Q95 that could not be formed by the ribose of Ade1AGU. Thus, the four phosphates of Ap4A maximize the number of interactions with RppH and, together with Ade1, effectively replace the two 5′-terminal nucleotides of the RNA ligand, thereby positioning the δ-phosphate of Ap4A in the catalytic center, where the β-phosphate of pppAGU would have been.

**Products of Decapping by RppH.** Because Ap4A is symmetrical, its complex with RppH does not alone reveal the bound orientation of NP4-capped RNA. However, the preference of RppH for NP4-capped substrates that begin with a purine suggests that the first transcribed nucleotide of capped RNA replaces Ade1 of Ap4A in the cleft, where R27 could engage in a stronger cation-π interaction with a purine than with a pyrimidine (Fig. 5B). Likewise, the
indifference of RppH to the identity of the cap nucleoside suggests that the cap replaces Ade0, whose disordered structure in the complex of Ap4A with RppH implies a lack of stable base-specific interactions with the protein. Binding Np4-capped RNA in this manner would position its δ-phosphate for hydrolytic attack in the catalytic center. If so, the deprotection of such substrates by RppH should release the cap nucleoside as a monophosphate, thereby generating triphosphorylated RNA susceptible to rapid degradation by RNase E.

To test this hypothesis, we examined the RppH reaction products of the Np4-capped RNA tetramer Ap4GUAA. We chose a substrate of this length to ensure that the substrate and each of the possible reaction products would have distinct chro-
motographic mobilities on phosphoethyleneimine (PEI)-cellulose. As determined by thin-layer chromatography and ultraviolet (UV) shadowing, RppH released the cap of Ap4GUAA primarily as AMP while generating an initial RNA product that comigrated with triphosphorylated GUAA (Fig. 6A). Little (<20%) of the cap was released as ATP. To rule out the possibility that much of the cap released as ATP was quickly converted to AMP, we tested the reactivity of ATP with RppH under the same conditions and found it to be largely inert (Fig. 6B), consistent with prior observations (38). Moreover, what little ATP did react was converted primarily to ADP rather than AMP. As expected, Ap4GUAA did not react with catalytically inactive RppH-E57A.

To ensure that the positioning of Ap4GUAA on the surface of RppH was not biased by its nucleotide sequence, which might favor binding of the 5′-terminal purine in the enzyme cleft, we tested another capped substrate, Ap4GAUA, in which the first two transcribed nucleotides were both purines. Despite the presence of a purine at both of these positions, this substrate also yielded AMP as the principal mononucleotide product (Fig. 6 C and D). Together, these findings suggest that RppH deprotects all Np4-capped transcripts by releasing the cap primarily as a nucleoside monophosphate, thereby generating triphosphorylated RNA as the initial reaction product regardless of the 5′-terminal RNA sequence.

Finally, the reactivity of Ap4GUAA and Ap4GAUA with RppH was compared to that of their triphosphorylated and diphosphorylated counterparts by monitoring the reaction of each as a function of time. The Ap4-capped substrates were the most reactive of all, irrespective of the RNA sequence (SI Appendix, Figs. S1 and S2).


**Discussion**

It was long believed that only eukaryotic RNAs are capped at the 5'-end. However, the recent discovery of 5'-caps on bacterial transcripts and their impact on RNA degradation has transformed thinking about the 5'-terminal regulatory events that govern RNA function in bacteria (10, 20, 39). The abundant types of bacterial caps yet reported are the Np4 caps observed in *E. coli* during disulfide stress, when the Np4A hydrolyse and decapping enzyme ApaH becomes inactivated (20). Our findings now show that, under these conditions, the enzyme binds the second nucleotide of these uncapped substrates in a cleft, where that nucleoside is held in place by cation-π and hydrophobic interactions on either side that favor a purine there (14, 16). By doing so, RppH positions the β-phosphate for hydrolytic attack promoted by magnesium ions in the catalytic center, generating monophosphorylated RNA and either pyrophosphate or orthophosphate, respectively, as reaction products. By analogy, one might have expected an Np4-capped substrate to be bound in a similar manner. However, the distinct substrate preferences of RppH when catalyzing the deprotection of Np4-capped rather than uncapped substrates suggest otherwise. In particular, instead of requiring at least two unpaired 5'-terminal nucleotides, the second of which should ideally be a purine (14), RppH recognizes only one unpaired nucleotide at the 5'-end of Np4-capped substrates and prefers for that 5'-terminal nucleotide to be a purine. This one-nucleotide shift in specificity suggests that RppH binds the first transcribed nucleotide rather than the second transcribed nucleotide of Np4-capped RNA in the cleft to form a complex that resembles RppH bound to Ap4A. As a result, the δ-phosphate, instead of the β-phosphate, is positioned for hydrolytic attack. Corroborating this conclusion, the reaction of Ap4A-capped RNAs with RppH releases the cap primarily as AMP rather than ATP, even when the capped substrate is designed to increase the potential for the second nucleotide to bind in the cleft. A third possible mode of interaction, in which the cap nucleotide binds in the cleft, is ruled out by the indifference of the enzyme to the identity of that nucleotide and by the release of the cap principally as AMP.

What explains the distinct binding modes of Np4-capped and uncapped substrates? For one thing, triphosphorylated and diphosphorylated RNAs would be unreactive if the 5'-terminal nucleotide, rather than the second nucleotide, were to bind in the cleft, as their phosphate chains would then be too short to reach the nucleophilic water molecule in the catalytic center. By contrast, although Np4-capped RNAs could, in principle, bind productively in any of three ways, their decapping by RppH appears to rely primarily on only one of these binding modes, in which the first transcribed nucleotide docks in the cleft. The structure of RppH bound to Ap4A and the influence of the number of unpaired 5'-terminal nucleotides on reactivity suggest likely explanations for this preference. When bound to RppH, an adenylyl nucleotide and the γ- and δ-phosphates of Ap4A (a structural analog of Np4-capped RNA) isostERICALLY replace the second nucleotide and the α- and β-phosphates of triphosphorylated RNA and make similar contacts with the enzyme and magnesium ions (Fig. 5D). The key difference between these enzyme-substrate complexes is the β-phosphate of Ap4A, which replaces the first nucleoside of triphosphorylated RNA and is uniquely able to accept hydrogen bonds from the positively charged side chain of R27 to a negatively charged nonbridging oxygen and from the side chain of Q95 to a bridging oxygen. These energetically favorable interactions would be lost if the second transcribed nucleotide of Np4-capped RNA were to bind in the cleft. These interactions likely also explain the greater reactivity of Np4-capped RNA as an RppH substrate compared to both triphosphorylated (31) and diphosphorylated RNA. The failure of this enzyme in vitro and in vivo and discovered that it binds Np4-capped 5'-ends in an unexpected manner to generate RNA products whose 5'-phosphorylation state (primarily triphosphorylated) is unlike that of any product yet described for any decapping enzyme. Np4-capped transcripts are the most reactive RNA substrates of RppH yet identified, exceeding the reactivity of their triphosphorylated (31) and even their diphosphorylated counterparts. These insights are key to understanding how bacterial mRNAs are degraded and their lifetimes are regulated during stress.

Previously determined structures of RppH bound to triphosphorylated and diphosphorylated RNA have revealed that the enzyme binds the second nucleotide of these uncapped substrates in a cleft, where that nucleoside is held in place by cation-π and hydrophobic interactions on either side that favor a purine there (14, 16). By doing so, RppH positions the β-phosphate for hydrolytic attack promoted by magnesium ions in the catalytic center, generating monophosphorylated RNA and either pyrophosphate or orthophosphate, respectively, as reaction products. By analogy, one might have expected an Np4-capped substrate to be bound in a similar manner. However, the distinct substrate preferences of RppH when catalyzing the deprotection of Np4-capped rather than uncapped substrates suggest otherwise. In particular, instead of requiring at least two unpaired 5'-terminal nucleotides, the second of which should ideally be a purine (14), RppH recognizes only one unpaired nucleotide at the 5'-end of Np4-capped substrates and prefers for that 5'-terminal nucleotide to be a purine. This one-nucleotide shift in specificity suggests that RppH binds the first transcribed nucleotide rather than the second transcribed nucleotide of Np4-capped RNA in the cleft to form a complex that resembles RppH bound to Ap4A. As a result, the δ-phosphate, instead of the β-phosphate, is positioned for hydrolytic attack. Corroborating this conclusion, the reaction of Ap4A-capped RNAs with RppH releases the cap primarily as AMP rather than ATP, even when the capped substrate is designed to increase the potential for the second nucleotide to bind in the cleft. A third possible mode of interaction, in which the cap nucleotide binds in the cleft, is ruled out by the indifference of the enzyme to the identity of that nucleotide and by the release of the cap principally as AMP.

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Previously determined structures of RppH bound to triphosphorylated and diphosphorylated RNA have revealed that the enzyme binds the second nucleotide of these uncapped substrates in a cleft, where that nucleoside is held in place by cation-π and hydrophobic interactions on either side that favor a purine there (14, 16). By doing so, RppH positions the β-phosphate for hydrolytic attack promoted by magnesium ions in the catalytic center, generating monophosphorylated RNA and either pyrophosphate or orthophosphate, respectively, as reaction products. By analogy, one might have expected an Np4-capped substrate to be bound in a similar manner. However, the distinct substrate preferences of RppH when catalyzing the deprotection of Np4-capped rather than uncapped substrates suggest otherwise. In particular, instead of requiring at least two unpaired 5'-terminal nucleotides, the second of which should ideally be a purine (14), RppH recognizes only one unpaired nucleotide at the 5'-end of Np4-capped substrates and prefers for that 5'-terminal nucleotide to be a purine. This one-nucleotide shift in specificity suggests that RppH binds the first transcribed nucleotide rather than the second transcribed nucleotide of Np4-capped RNA in the cleft to form a complex that resembles RppH bound to Ap4A. As a result, the δ-phosphate, instead of the β-phosphate, is positioned for hydrolytic attack. Corroborating this conclusion, the reaction of Ap4A-capped RNAs with RppH releases the cap primarily as AMP rather than ATP, even when the capped substrate is designed to increase the potential for the second nucleotide to bind in the cleft. A third possible mode of interaction, in which the cap nucleotide binds in the cleft, is ruled out by the indifference of the enzyme to the identity of that nucleotide and by the release of the cap principally as AMP.

What explains the distinct binding modes of Np4-capped and uncapped substrates? For one thing, triphosphorylated and diphosphorylated RNAs would be unreactive if the 5'-terminal nucleotide, rather than the second nucleotide, were to bind in the cleft, as their phosphate chains would then be too short to reach the nucleophilic water molecule in the catalytic center. By contrast, although Np4-capped RNAs could, in principle, bind productively in any of three ways, their decapping by RppH appears to rely primarily on only one of these binding modes, in which the first transcribed nucleotide docks in the cleft. The structure of RppH bound to Ap4A and the influence of the number of unpaired 5'-terminal nucleotides on reactivity suggest likely explanations for this preference. When bound to RppH, an adenylyl nucleotide and the γ- and δ-phosphates of Ap4A (a structural analog of Np4-capped RNA) isostERICALLY replace the second nucleotide and the α- and β-phosphates of triphosphorylated RNA and make similar contacts with the enzyme and magnesium ions (Fig. 5D). The key difference between these enzyme-substrate complexes is the β-phosphate of Ap4A, which replaces the first nucleoside of triphosphorylated RNA and is uniquely able to accept hydrogen bonds from the positively charged side chain of R27 to a negatively charged nonbridging oxygen and from the side chain of Q95 to a bridging oxygen. These energetically favorable interactions would be lost if the second transcribed nucleotide of Np4-capped RNA were to bind in the cleft. These interactions likely also explain the greater reactivity of Np4-capped RNA as an RppH substrate compared to both triphosphorylated (31) and diphosphorylated RNA. The failure of
Np4-capped substrates to bind RppH in the opposite orientation with the cap in the cleft may be related to the substantially greater reactivity of substrates bearing additional unpaired nucleotides downstream of the nucleotide in the cleft, which implies favorable interactions of those nucleotides with the enzyme that would be sacrificed if the cap were to occupy the cleft.

The mechanism by which E. coli RppH recognizes Np4 caps is likely to be replicated in nearly all \( \alpha, \beta, \gamma, \) and \( \epsilon \)-proteobacteria, including many important pathogens, as the active-site residues of RppH that interact with such caps and with \( \text{Mg}^{2+} \) are highly conserved in those species (14). It remains to be determined whether nonhomologous RNA pyrophosphohydrolases from \( \delta \)-proteobacteria like Bdellovibrio bacteriovorus (8) and Firmicutes like Bacillus subtilis (9) can recognize Np4 caps.

Besides its crucial role in Np4 cap removal during stress, E. coli RppH has been shown to react in vitro with certain additional types of RNA caps and cap analogs but not with others. Our insights into the various modes of substrate binding by this enzyme now make it possible to explain those differences in reactivity and to predict the effect of RNA sequence and structure on the rate at which those caps are removed. For example, diadenosine polyphosphates bearing three to six bridging phosphates have been tested as substrates for RppH, and all but Ap4A are inactive (34). In the case of Ap4A, Ap5A, and Ap6A ATP is always one of the reaction products, a finding consistent with the structure reported here for Ap4A bound to RppH, in which adenine binding in the cleft positions the \( \delta \)-phosphate beside a nucleophile poised for attack. Lacking a \( \delta \)-phosphate, Ap4A would be too short to react. Similarly, the eukaryotic cap analog m\(^{G}\)p\(^{G}\)G is unreactive, whereas m\(^{G}\)p\(^{G}\)capped RNA reacts with RppH to produce m\(^{G}\)GDP and monophosphorylated RNA (40), presumably because the second transcribed nucleotide of this capped RNA can bind in the cleft and position the \( \beta \)-phosphate for hydrolytic attack. The same rationale undoubtedly explains why RppH reacts slowly with RNA bearing an NAD or NADH cap to yield nicotinamide mononucleotide and monophosphorylated RNA but does not react with NAD or NADH themselves (10, 12, 34, 41), which each contain only two bridging phosphates. Because transcripts that are m\(^{G}\)p\(^{G}\)-capped by RNA polymerase (21) are expected to resemble triphosphorylated RNA in their productive mode of binding, we predict that their efficient decapping by RppH requires at least two unpaired 5'-terminal nucleotides and prefers a purine at the second position. Therefore, the mechanism by which RppH recognizes Np4-capped RNAs and positions them in the active site is probably unique among both capped and uncapped transcripts.

The 5'-end-dependent degradation pathway is a key contributor to RNA turnover in E. coli (3). The rate at which transcripts are degraded in this manner depends both on whether or not they are capped and on the activity and specificity of each enzyme in the pathway. In unstressed wild-type cells, where the Np4A hydroxylase and decapping enzyme ApaH is active, Np4A levels are very low and Np4-capped transcripts are undetectable (20). Under these conditions, the RNA substrates available for 5'-terminal depletion are predominantly triphosphorylated and diphosphorylated, and RppH acts not as a decapping enzyme but as an RNA pyrophosphohydrolase that converts their 5' ends to monophosphates (3, 4). Uncapped RppH substrates of this kind are most reactive if they contain at least two, and preferably three or more, unpaired 5'-terminal nucleotides and bear a purine at the second position, characteristics that also enable ready cleavage of their monophosphorylated reaction products by the endonuclease RNase E (14, 15). What little Np4-capped RNA is made undergoes rapid decapping by ApaH to produce a diphosphorylated decay intermediate that must then react with RppH to generate the monophosphorylated 5' end favored by RNase E (20). During disulfide stress, ApaH inactivation allows the cellular concentration of Np4As and Np4-capped transcripts to increase markedly (20) and forces RppH to assume responsibility for Np4 cap removal. Therefore, the initial RNA product of decapping is primarily triphosphorylated under these conditions. As a result of the one-nucleotide shift in RppH specificity, decapping of these transcripts by this enzyme requires only one unpaired 5'-terminal nucleotide and prefers two or more while favoring RNAs that begin with a purine (Figs. 2–4). Nevertheless, to attain a 5' phosphorylation state vulnerable to rapid cleavage by RNase E, which remains active under these conditions, the triphosphorylated products of decapping must react a second time with RppH to convert their 5' ends to monophosphates, a reaction with distinct substrate preferences (14). As a consequence, the identities of both the first and second transcribed nucleotide appear to be critical for governing rates of RppH-mediated depletion of Np4-capped transcripts, whose susceptibility to 5'-end-dependent degradation during disulfide stress is expected to be maximized by the presence of two unpaired 5'-terminal purines followed by at least one more unpaired nucleotide of any kind.

A recent survey of 14 E. coli RNAs showed that the steady-state percentage of each that was Np4 capped when ApaH was inactivated by cadmium stress was 33 to 76% for A-initiated transcripts, 36 to 72% for C-initiated transcripts, and 26% for the lone U-initiated transcript examined yet was only 4 to 8% for G-initiated transcripts (20). Moreover, changing the first transcribed nucleotide of yeiP mRNA from A to G reduced the capped fraction of that transcript from 76 to 5%. Consistent with those observations, we now find in apaH cells that the steady-state percentage of Np4-capped efp mRNA is only slightly affected by changing the 5'-terminal transcribed nucleotide from C (25 ± 3%) to A (21 ± 1%) but falls to just 4 ± 1% when that nucleotide is changed to G (Fig. 44). The percentage of an RNA that is capped at steady state depends on the combined effects of multiple cellular processes, especially the ratio of the rates of cap acquisition and cap removal. Rates of cap acquisition should depend on the availability of cap precursors (Np4As = Ap4Ns) relative to the NTP with which they compete for 5'-terminal incorporation during transcription initiation by RNA polymerase (21). In cadmium-stressed E. coli, the molar ratio of Ap4G to GTP (0.26) is lower than the ratio of Ap4C to CTP (0.73) and Ap4U to UTP (0.68) and much lower than the ratio of Np4A to ATP (2.6, where N = A, G, C, or U) (42). Furthermore, our findings now show that RppH decaps G- and A-initiated transcripts three to five times faster than C- and U-initiated transcripts (Figs. 34 and 4E). The combination of a low cap-acquisition rate and a high decapping rate probably explains the small percentage of G-initiated transcripts that are Ap4-capped at steady state. Conversely, the much higher rate of cap acquisition by A-initiated RNAs, whose synthesis can begin with any Np4A (21), likely counterbalances their rapid decapping by RppH, allowing a substantial percentage of A-initiated transcripts to be Ap4-capped at steady state. Similarly, the ample fraction of C- and U-initiated transcripts that are Ap4-capped at steady state can be attributed to their intermediate rate of synthesis and slow rate of decapping.

The removal of Np4 caps appears to be crucial for governing E. coli mRNA lifetimes during disulfide stress. By controlling rates of RppH-dependent decapping and degradation, the substrate recognition mechanism described here may help cells to recover from this stress by increasing the production of key stress-response proteins and reducing the synthesis of other proteins of less immediate importance. The ability to reset levels of gene expression under these perilous conditions may be critical for survival.
Materials and Methods

Strains and Plasmids. Measurements of 5′ cap levels and decapping rates in E. coli were performed in isogenic derivatives of the K-12 strain BW25113 (43) bearing in-frame deletions of the apaH and rppH coding regions, either individually or in combination (20). The strains used to analyze efp mRNA contained plasmid p604 to facilitate detection of that mRNA by increasing its cellular concentration (36). The strains used to analyze eps, efp-C1A, and efp-C1G mRNA contained plasmid pEFp1, pEFp1-C1A, or pEFp1-C1G [derivatives of plasmid pBR322H (13) encoding efp mRNA or a variant thereof] and lacked the chromosomal efp gene, which was deleted by P1 transduction from the efp::kan strain of the Keio collection (44).

Rates of Decapping by RppH in E. coli. E. coli cells were grown to mid-log phase (an optical density of 0.3 at 650 nm) at 37 °C in MOPS-glucose medium (45) before arresting transcription with rifampicin (200 μM). For measurements made during cadmium stress, the mid-log phase cells were treated with CdCl2 (0.2 mM) for 90 min before adding rifampicin. Total RNA was extracted at time intervals after inhibiting transcription, and equal amounts of RNA (10 μg) were analyzed by electrophoresis on a borated gel to separate capped from uncapped RNA, and analyzed by Northern blotting (20). Band intensities were graphed semilogarithmically as a function of time, and first-order rate constants for the disappearance of capped efp or efp mRNA were obtained from the slope of the best-fit line as determined by linear regression. The rate constant for RppH-mediated decapping in E. coli was then calculated by subtracting the rate constant for the disappearance of the capped transcript in ΔapaH RppH cells from the corresponding rate constant in ΔapaH cells.

RNA Synthesis by In Vitro Transcription. To prepare double-stranded DNA templates for synthesizing ApU4G and related substrates by in vitro transcription, pairs of complementary oligodeoxynucleotides (200 pmol each; SI Appendix, Table S3) were annealed and then extended with the Klenow fragment of DNA polymerase I (five units/μL New England Biolabs) and deoxyuridine triphosphates (0.5 mM each) in a solution (20 μL) containing Tris HCl, pH 7.9 (10 mM), NaCl (50 mM), MgCl2 (10 mM), and dithiothreitol (10 mM) (37). The fully double-stranded products were phenol extracted, ethanol precipitated, and dissolved in water. Templates for the synthesis of ApU4Bx, ApA4, and the four NpA4B8 RNAs contained a T7 ±6 promoter; the other templates all contained a T7 +65 promoter (46). Capped RNAs were synthesized by in vitro transcription of the double-stranded DNA template (0.25 pmol/μL) for 8 h at 37 °C with T7 RNA polymerase (5 units/μL New England Biolabs) in the presence of ApA4, GpA4, CpA4, or UpA4 at a concentration of 1 μM. The resulting transcripts were purified by gel electrophoresis and elution of the band of interest.

ApU4GUA and ApA4GUA were synthesized by in vitro transcription of the double-stranded DNA template (2 pmol/μL; SI Appendix, Table S3) for 3 h at 37 °C with T7 RNA polymerase (0.1 μg/μL) in a solution containing Tris HCl, pH 8.0 (100 mM), MgCl2 (20 mM), spermidine (2 mM), dithiothreitol (40 mM), ApG (3 mM), ATP (6 mM), and TTP (3 mM) (47). Their triphosphorylated, diprophosphorylated, and monophosphorylated counterparts (ppU4GUA, ppGUA, ppA4GUA, and pGUA4) were prepared in the same manner, except that ApG was replaced by GTP, GDP, or GMP (3 mM), respectively. The products of transcription were purified by anion-exchange chromatography on a 1-mL MonoQ (5/50) column (GE Healthcare). RNA was eluted with a 25-mL 0 to 50% gradient of 2 M triethylammonium bicarbonate buffer, pH 8.5, at a flow rate of 1 mL/min, and the desired RNA fractions were frozen and lyophilized. The dried RNAs were dissolved in water, and their concentrations were determined spectrophotometrically. The identity of each RNA product was confirmed by mass spectrometry (Bruker UltraFLEX MALDI-TOF).

Affinity chromatography on TALON beads, and tagged at N-terminus with a hexahistidine tag, were produced in E. coli strain BL21(DE3) and purified by anion-exchange chromatography on a HiTrap SP column and gel filtration on Superdex 75 (GE Healthcare). For decapping reactions, RNA was diluted in a high-salt buffer containing HEPES, pH 7.0 (20 mM), MgCl2 (10 mM), NaCl (300 mM), dithiothreitol (1 mM), rNasin (10 units; Promega), and glycerol (10% vol/vol) in a volume of 34.1 μL. An unreacted sample (7 μL) was removed and mixed with loading buffer (7 μL; 95% formamide, 20 mM EDTA, pH 8, containing bromophenol blue). RppH was freshly diluted for each reaction into a high-salt buffer containing HEPES, pH 7.0 (20 mM), MgCl2 (10 mM), NaCl (300 mM), dithiothreitol (1 mM), and glycerol (10% vol/vol), and the diluted enzyme (7.9 μL) was added to the preincubated reaction mixture to achieve a final concentration of 30 μM. Reaction samples (8.5 μL) were quenched with loading buffer (8.5 μL) after 15, 30, and 60 s, and all four samples were subjected to electrophoresis on a denaturing gel containing 13.7% acrylamide: bisacrylamide (19:1), 0.3% 3-acylamidophenylboronic acid, 7 M urea, and 0.1 M Tris-acetate, pH 9.0 (35, 48). The fluorescein-labeled RNAs were then washed with a Typhoon Trio image (GE Healthcare) and quantified with ImageQuant TL software. The percentage of each RNA that was capped at each time point was calculated from the ratio of band intensities (capped versus uncapped) and then normalized to the percentage that was capped at time 0.

Structure Determination by X-Ray Crystallography. RppH was prepared in a solution of 20 mM sodium acetate, pH 5.0, 100 mM NaCl, and 1 mM dithiothreitol. Crystals of RppH, were grown in sitting drop format, typically against 0.4 M of reservoir solution at 20 °C for 4 d. Crystals were grown from a mixture comprising 1 μL of 1 mM protein and 2 μL of reservoir solution (0.4 M NaH2SO4, 10% [w/v] PEG3350, and 10% glycerol). To soak ApA4 into the crystals, they were transferred to 1 μL of soaking solution containing 0.1 mM sodium cacodylate, pH 7.0, 0.2 M NaCl, 0.1% [v/v] PEG3350, 10% glycerol, and 10% ApA4. After incubating the crystals for 20 min, they were transferred to a fresh 1-μL drop of the same solution and cross-linked by incubation over a 2-μL drop of 50% glutaraldehyde for 30 min. The crystals were transferred to a 2-μL drop of 50 mM MOPS, pH 7.0, 25 mM MgCl2, 25 mM NaF, 1 mM ApA4, 30% (v/v) pentaerythritol propoxylate S4/A POI/OH, and 15% PEG3350 for 20 min and then mounted and frozen in a cryostream without additional cryoprotector.

Diffraction data were collected at 100 K at the home Rigaku X-ray source and on beamline 24ID-C of the Advanced Photon Source (Argonne National Laboratory). Data were processed by using the X-ray Detector Software (XDS) suite (49). The crystal structure was solved by molecular replacement using the structure of E. coli RppH (Protein Data Bank [PDB] code 4S2W) as a search model and the Phaser-MR implementation in PHENIX (50). The models were rebuilt manually in COOT (51) and refined in PHENIX. The ligand, water molecules, and ions were added at the last stages of refinement based on the Fo-Fc and 2Fo-Fc electron density maps. Three density maps peak in the catalytic center were assigned to Mg2+ ions based on their octahedral coordination geometry and coordination distances in the range of 1.9 to 2.2 Å, which are characteristic of these cations.

Products of Decapping by RppH. To characterize the products of RppH-mediated decapping, either ApA4GUA, ppA4GUA, ApA4GUA, or ppA4GUA (6 nmol) was preincubated at 37 °C for 2 min in a buffer containing HEPES, pH 7.5 (20 mM), MgCl2 (10 mM), and dithiothreitol (1 μM), resulting in a total volume of 6 μL. For decapping reactions, RppH was added to the preincubated reaction mixture to achieve a final enzyme concentration of 14 μM, and the diluted enzyme (1 μL) was added to the prewarmed substrate, resulting in a final enzyme concentration of 1 μM. The resulting mixture was preincubated at 37 °C for 30 s, then cooled to 0 °C, and the reaction was quenched with the addition of 2 μL of 20% glycerol, 10 mM EDTA, and 50% formamide, followed by transferring the reaction mixture to a fresh 1-μL drop containing 10% glycerol. Following 2 min of incubation, the crystals were transferred to a fresh 1-μL drop of the same solution and cross-linked by incubation over a 2-μL drop of 50% glutaraldehyde for 30 min. The crystals were transferred to a 2-μL drop of 50 mM MOPS, pH 7.0, 25 mM MgCl2, 25 mM NaF, 1 mM ApA4, 30% (v/v) pentaerythritol propoxylate S4/A POI/OH, and 15% PEG3350 for 20 min and then mounted and frozen in a cryostream without additional cryoprotector.
of 3 μM. The reactions were quenched after 8 min by adding 0.7 μL EDTA (220 mM), and the products were analyzed by thin-layer chromatography on fluorescent PEI-cellulose plates (Millipore Sigma) developed with 0.4 M ammonium sulfate, pH 5.5. Spots visualized by UV shadowing were photographed and quantified by using ImageJ software. Comparisons of the reactivity of Ap4GUA, ppGpGUA, ApGpGUA, ppGpGUA, and ppGpGUA were performed identically, except that the reaction mixtures were scaled up 5.5-fold and samples (7 μL) were quenched after 0, 2, 4, and 8 min. The percent of reaction at each time point was calculated from the molar ratio of substrate to RNA product(s), with correction for the difference in the extinction coefficients of the capped and uncapped RNAs.

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Data Availability. The atomic coordinates for the complex of RppH with Ap4A have been deposited in the PDB under accession no. 7SP3. All other data are included in the figures and S Appendix.

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