ABSTRACT

T4 polynucleotide kinase–phosphatase (Pnkp) exemplifies a family of enzymes with 5′-kinase and 3′-phosphatase activities that function in nucleic acid repair. The polynucleotide 3′-phosphatase reaction is executed by the Pnkp C-terminal domain, which belongs to the DxDxT acylphosphatase superfamily. The 3′-phosphatase reaction entails formation and hydrolysis of a covalent enzyme-(Asp165)-phosphate intermediate, driven by general acid–base catalyst Asp167. We report that Pnkp also has RNA 2′-phosphatase activity that requires Asp165 and Asp167. The physiological substrate for Pnkp phosphatase is an RNA 2′,3′-cyclic phosphate end (RNA > p), but the pathway of cyclic phosphate removal and its enzymic requirements are undefined. Here we find that Pnkp reactivity with RNA > p requires Asp165, but not Asp167. Whereas wild-type Pnkp transforms RNA > p to RNAOH, mutant D167N converts RNA > p to RNA 3′-phosphate, which it sequesters in the phosphatase active site. In support of the intermediacy of an RNA phosphomonoester, the reaction of mutant S211A with RNA > p results in transient accumulation of RNAp en route to RNAOH. Our results suggest that healing of 2′,3′-cyclic phosphate ends is a four-step processive reaction: RNA > p + Pnkp → RNA-(3′-phosphoaspartyl)-Pnkp → RNAOH + phosphoaspartyl-Pnkp → Pi + Pnkp.

INTRODUCTION

T4 polynucleotide kinase–phosphatase (Pnkp) exemplifies a family of enzymes that heal broken termini in RNA or DNA by converting 3′-PO4/5′-OH ends into 3′-OH/5′-PO4 ends, which are then sealed by RNA or DNA ligases. During T4 infection, Pnkp thwarts the accumulation of RNAp in vivo, which is a proton from the 5′-OH; and the Lys15 and Arg126 side chains and a divalent cation that stabilize the transition state of the ATP γ phosphor.
By contrast, the removal of a 3′-phosphate by the Pnkp phosphatase module is a more complex two-step “ping-pong” reaction entailing the formation and subsequent hydrolysis of a covalent enzyme-(aspartyl-O6)-phosphate intermediate (Figure 1A). As with other members of the acylphosphatase superfamily (20), the first aspartate of the DxDXT signature motif (Asp165 in Pnkp) is the site of phosphoryl transfer to the enzyme. The chemistry is driven by (i) general acid–base catalysis by Asp167, which donates a proton to the ribose O3′ leaving group in the first step and accepts a proton from the water nucleophile in the second step; and (ii) transition-state stabilization by contacts to the phosphate oxygens from side chains Lys258 and Ser211 and an enzyme-bound magnesium ion (Figure 2) (15,17). The constituents of the octahedral Mg2+ coordination complex are: Asp165, Asp278, the Asp167 main-chain carbonyl, a phosphate oxygen, and two water molecules (Figure 2). The atomic contacts of these essential moieties of the T4 Pnkp phosphatase module are confidently inferred from the crystal structure of the homologous protein serine phosphatase Fcp1, captured as a covalent aspartyl–BeF3− adduct (a mimetic of the aspartyl-phosphate intermediate) with Mg2+ in the active site (Figure 2) (21). Mg2+ occupies a similar position in T4 Pnkp (15), but there are as yet no crystal structures of T4 Pnkp with a polynucleotide 3′-phosphate or phosphomimetic in the phosphatase active site.

Whereas the mechanistic model of the 3′-phosphomonooesterase reaction of Pnkp is on firm ground, it is fair to say that virtually nothing is known about the pathway by which RNA 2′,3′-cyclic phosphate ends are healed. That is because biochemical studies of the phosphatase reaction have used either 3′-phosphorylated oligodeoxyribonucleotides or deoxynucleoside 3′-monophosphates as the substrates (5–7,15–17). Recombinant T4 Pnkp has an apparent turnover number of 24 s−1 for the hydrolysis of 3′-dTMP under steady-state conditions (17). By contrast, T4 Pnkp is unable to hydrolyze ribonucleoside 3′-monophosphates in vitro (5,6). This is paradoxical given that 3′-dNMPs are effective substrates and that Pnkp is adept at hydrolyzing the 3′-phosphate of oligoribonucleotides (6). To our knowledge, there have been no studies published regarding the enzymic moieties required for RNA 3′-phosphate hydrolysis by Pnkp and no insights to the (presumably) multistep pathway by which Pnkp removes a 2′,3′-cyclic phosphate. Here we address both issues by studying the reaction of Pnkp and several phosphatase active site mutants with otherwise identical RNA oligonucleotides with 3′-phosphate versus 2′,3′-cyclic phosphate termini.

**MATERIALS AND METHODS**

**T4 Pnkp**

pET–Pnkp plasmids encoding wild-type or mutant Pnkp proteins fused to an N-terminal His10 tag (15,17) were introduced into *Escherichia coli* BL21(DE3). Recombinant protein production was induced by adjusting exponentially growing cultures (1000 ml) to 0.3 mM IPTG and incubating them at 17°C for 15 h with continuous shaking. The wild-type and mutant His10-Pnkp proteins were purified from soluble bacterial lysates by Ni-agarose chromatography, as described previously (16). Protein concentrations were determined by using the BioRad dye reagent with bovine serum albumin as the standard.

**RNA substrates**

The 20-mer RNA3p oligonucleotide labeled with 32P at the penultimate phosphate was prepared by T4 Rnl1-mediated addition of [5′-32P]PpCp to a 19-mer synthetic oligoribonucleotide, as described (22). The RNA3p was treated with *E. coli* RNA 3′-terminal phosphatase cyclase and ATP to generate a 2′,3′-cyclic phosphate derivative, RNAp (22). The RNA3p and RNAp substrates were gel-purified before use in Pnkp phosphatase assays. A 2′-phosphate terminated derivative, RNAp, was produced by reacting RNAp with purified *Arabidopsis thaliana* tRNA ligase (9) as follows: a reaction mixture (100 μl) containing 50 mM Tris–HCl (pH 8.0), 2 mM DTT, 10 mM EDTA, 1 μM RNAp and 50 μM AtRNL was incubated for 30 min at 37°C. The RNA2p product was recovered by phenol–chloroform extraction and precipitation with ethanol; the conversion of RNAp to RNA2p was verified by digesting the 20-mers with RNase T1 and analysing the 32P-labeled terminal fragments by urea-PAGE.

**Phosphatase assay**

Reaction mixtures containing 100 mM Tris-acetate (pH 6.0), 10 mM MgCl2, 2 mM DTT, 50 mM 32P-labeled RNAp or RNAp substrate and wild-type or mutant Pnkp (specified as fmol or pmol of the enzyme monomer) were incubated at 22°C. The reactions were quenched by adding an equal volume of 100 mM EDTA. The products were digested for 30 min at 37°C with RNase T1 (1000 U; Fermentas). The samples were supplemented with 0.5 volume of 90% formamide, 0.01% bromophenol blue/xylene cyanol, 50 mM EDTA and then analysed by electrophoresis (at 50 W constant power) through a 40-cm 20% polyacrylamide gel containing 8 M urea in 45 mM Tris borate, 1.2 mM EDTA. The 32P-labeled RNAs were visualized by autoradiography of the gel and, where specified, quantified by scanning the gel with a Fuji Film BAS-2500 imager.

**RESULTS**

**RNA 3′-phosphate and 2′,3′-cyclic phosphate removal activities of Pnkp**

A goal of the present study was to assess and compare the pathways of RNA end healing by T4 Pnkp at 3′-phosphate versus 2′,3′-cyclic phosphate termini. The substrates used were otherwise identical 20-mer RNAs with 3′-phosphate (p) or 2′,3′-cyclic phosphate (>p) ends and a single radiolabel between the 3′-terminal and penultimate nucleosides (Figure 3). The RNA3p and RNAp substrates (0.5 pmol) were incubated for 20 min at 22°C...
Figure 1. Pathways of RNA 3’ end-healing by T4 Pnkp. (A) Two-step chemical mechanism of 3’-phosphate removal through formation (Step 1) and hydrolysis (Step 2) of a covalent phosphoaspartyl-enzyme intermediate. (B) Two hypothetical pathways for removal of a 2’,3’-cyclic phosphate are shown. A common first step entails attack of the aspartate nucleophile on the RNA 5’-p end to form (Step 1) and then hydrolyze (Step 2) a covalent RNA-(phosphoaspartyl)-enzyme intermediate. In the Step 1 reaction shown, the ribose O2’ is the leaving group and the RNA 3’-phosphate is attached to the enzyme. Two variants of the Step 2 hydrolysis reaction (Steps 2a or 2b) are illustrated. In Step 2a, the enzymic aspartate is the leaving group and RNA3’p is the product. In Step 2b, the ribose O3’ is the leaving group and a phosphoaspartyl-enzyme remains.
with wild-type Pnkp. To enable resolution of the reactants and products with different 3’ ends, the mixtures were digested with RNase T1 before analysis by denaturing PAGE (Figure 3). RNase T1 incised the substrates 3’ of the most distal guanosine to yield the 32P-labeled tetranucleotides \( \text{HOCUUpC}_2 \text{p} \) or \( \text{HOCUUpC} > \text{p} \) (Figure 3, lanes 0).

Reaction of increasing concentrations of Pnkp with the RNA substrates depleted the \( \text{HOCUUpC}_2 \text{p} \) T1 fragment and generated a more slowly migrating T1 fragment corresponding to the dephosphorylated product, \( \text{HOCUUpC}_{\text{OH}} \) (Figure 3B). The same \( \text{HOCUUpC}_{\text{OH}} \) product was generated when Pnkp reacted with RNA > p (Figure 3A). Whereas both dephosphorylation reactions proceeded to completion at saturating enzyme, the extent of RNA\text{OH} formation at limiting enzyme was higher for RNA3p than RNA > p (Figure 3). Quantification of the enzyme titration data from three separate experiments indicated that the Pnkp specific activity was ~3-fold higher with RNA3p (Figure 4A) versus RNA > p (Figure 4B).

It was notable that we detected no accumulation of a phosphorylated T1 fragment \( \text{HOCUUpC}_p \) (which migrates faster than \( \text{HOCUUpC} > p \)) during the reaction of sub-saturating levels of input Pnkp with the RNA > p substrate (Figure 3A). Nor did we detect a phosphorylated species when assaying the time course of the reaction of wild-type Pnkp with the RNA > p substrate (not shown). These results suggested that (i) either the pathway of 2’,3’-cyclic phosphate removal does not involve the formation of a free RNAp intermediate; or (ii) an RNAp intermediate is formed, but is rapidly hydrolyzed to RNA\text{OH}, making it difficult to detect.

**The Asp165 nucleophile is required for removal of a 2’,3’-cyclic phosphate**

Previous studies showed that mutation of the Asp165 nucleophile to asparagine abolished the 3’-phosphatase activity of T4 Pnkp with a 3’-dTMP mononucleotide substrate (17). Here we queried the impact of the D165N change on the removal of the terminal phosphates of the 20-mer RNA3p and RNA > p strands. Whereas 0.16 pmol of wild-type Pnkp sufficed for near-quantitative conversion of RNA3p to RNA\text{OH} (Figure 3B), we detected no product formation by up to 40 pmol of the D165N mutant (Figure 4A and data not shown). This result is consistent with a mechanism of 3’-phosphate hydrolysis through a covalent aspartyl-phosphate intermediate (Figure 1A). The key question is whether the same mechanism of covalent catalysis applies when Pnkp acts at a 2’,3’-cyclic phosphate, that is, does the initial step entail a nucleophilic attack by Asp165 on the cyclic phosphate to form a covalent RNA-(phosphoryl)-Asp165 adduct? [Note: if the ribose O2’ is the leaving group, then the initial step in cyclic phosphate removal would generate an RNA-(3’-phosphoryl)-Asp165 adduct, as depicted in Figure 1B. In the event that the ribose O3’ is the leaving group (analogous to Step 1 in Figure 1A), then an RNA-(2’-phosphoryl)-Asp165 adduct would be formed.] If a mechanism of covalent catalysis applies, then the D165N mutation should abolish the reactivity of Pnkp at a 2’,3’-cyclic phosphate end. However, one can envision an alternative mechanism whereby water is the relevant nucleophile in the initial attack on RNA > p. A hydrolysis mechanism could directly generate an RNA 3’-phosphate end in a single step that would then enter the phosphomonoesterase reaction pathway in Figure 1A. If hydrolysis is the initial event, then we might find that the D165N mutant is capable of converting RNA > p to RNAp, which ought to accumulate because Asp165 is clearly required for downstream reactions at a monophosphate end (Figure 4A). We observed that D165N was unreactive with the RNA > p substrate at up to 40 pmol of input enzyme (Figure 4B and data not shown). A simple interpretation of this result is that covalent catalysis by Asp165 is the initial step in 2’,3’-cyclic phosphate removal.
could account for our inability to detect RNAp during the 2',3'-cyclic phosphate removal reaction. The alternative pathway (Step 2A) is that Asp165 is the leaving group in the hydrolysis of an RNA-phosphoaspartyl-Pnkp, which generates RNAp and Pnkp apoenzyme as the products. In this case, RNAp might instantly enter the phosphomonoesterase reaction pathway (Figure 1A) to be converted to RNAOH. To discriminate among these pathway options, we sought to expose the existence of an otherwise fleeting RNAp intermediate, by mutating the phosphatase active site in the hope of identifying lesions that selectively impact 3'-phosphate removal while sparing the initial Steps 1 and 2A of the hypothetical pathway of 2',3'-cyclic phosphate removal.

Our initial attention focused on the Asp167 general acid–base catalyst, mutation of which to alanine or asparagine ablates 3'-phosphatase activity with a 3'-TMP mononucleotide substrate (17). Here we found that the D167N and D167A mutants were inert in dephosphorylating the RNA2p substrate at up to 40 pmol of input enzyme (Figures 3B, 4A, and data not shown). By contrast, D167N and D167A were reactive with the RNA2p substrate, which they converted to RNAp as the sole product detected by PAGE (Figure 3A and data not shown). The extent of RNAp formation was proportional to the input D167N and D167A proteins (Figure 4B), with 82% of the RNA2p being converted to RNAp by 4 pmol of either mutant enzyme. The specific activity of D167N as a cyclic phosphodiesterase acting on RNA2p was ∼7% of the specific activity of wild-type Pnkp in converting RNAp to RNAOH (Figure 4B). Thus, eliminating the proton donating/accepting ability of Asp167 selectively ablated the Pnkp 3'-phosphomonoesterase, while preserving an appreciable 2',3'-cyclic phosphodiesterase activity.

The kinetic profile of the reaction of D167N (500 nM) with RNA2p (50 nM) is shown in Figure 4C. The reaction attained an end point in 10 min with an 85% yield of RNAp product. Because increasing the D167N concentration to 1000 nM had no effect on the rate of RNAp formation or the reaction end point (not shown), we surmise that the reaction in enzyme excess is not limited by the initial rate of D167N binding to RNA2p. Nonetheless, the kinetic data for RNAp formation did not fit to a single exponential (not shown), but instead fit very well to a biphasic kinetic model (Figure 4C) in which, as calculated by non-linear regression in Prism, the rapid phase proceeded with a rate constant of 0.14 ± 0.02 s⁻¹ (and accounted for 65% of the RNAp end-product) and the slow phase proceeded with a rate constant of 0.0065 ± 0.0017 s⁻¹. We infer that the rapid phase reflects the rate of the chemical step(s) of the 2',3'-cyclic phosphodiesterase reaction of D167N with RNA2p bound in the phosphatase active site. The slow phase is readily explained by the propensity of a fraction of the input RNA2p strands (which have 5’-OH termini) to bind to the phosphoacceptor site of the Pnkp kinase domain (14), where no 5’ reaction will occur in the absence of an NTP donor and where the 2',3’>p end is inaccessible to the phosphatase domain.

**Mutating the Asp167 general acid–base allows capture of an RNAp intermediate in the pathway of 2',3'-cyclic phosphate removal**

Invocation of a covalent RNA-phosphoaspartyl-Pnkp adduct in the removal of the 2',3'-cyclic phosphate mandates a second step in the reaction pathway in which the adduct is hydrolyzed. The proposed attack by water could, in principle, occur in either of two orientations, as illustrated in Figure 1B. In the event that the ribose oxygen is the leaving group (Step 2B), the hydrolysis reaction yields RNAOH and phosphoaspartyl-Pnkp. (The latter can be readily hydrolyzed to restore the Pnkp apoenzyme; Figure 1A). Such a pathway through Step 2B
The consequence is that the slow phase is limited by the rate of dissociation of the RNAp from the kinase domain and its redistribution to the D167N phosphatase domain.

**D167N sequesters the RNAp product of the cyclic phosphodiesterase reaction**

We performed an order-of-addition experiment to test the susceptibility of the RNAp product generated by D167N to subsequent processing by wild-type Pnkp (per the scheme in Figure 5A). Control experiments verified that 4 pmol of D167N and 0.5 pmol of wild-type Pnkp sufficed for efficient conversion of the input RNAp strand to RNAp and RNAOH products, respectively (Figure 5A, lanes 2 and 3). The initially surprising finding was that simply adding wild-type Pnkp to the reaction mixture after a 20-min incubation of D167N with RNAp failed to convert any of the RNAp product to RNAOH (Figure 5A, lane 4), signifying that the RNAp end was either unreactive or shielded from reaction with wild-type Pnkp. This issue was resolved by heating the reaction mixture after the 20-min incubation with D167N and before supplementation with wild-type Pnkp. This maneuver rendered the RNAp species completely accessible to hydrolysis by wild-type Pnkp to yield RNAOH (Figure 5A, lane 5). These results suggested that the RNAp product of the D167N cyclic phosphodiesterase reaction remains bound in the phosphatase active site.

We examined this issue by asking whether pre-incubation of an RNA 3’-phosphate-terminated substrate with increasing amounts of D167N would protect the RNAp from subsequent hydrolysis during a 20-min reaction with 0.5 pmol of wild-type Pnkp (per the scheme in Figure 5B). We observed a progressive decline in the accessibility of the RNAp substrate as the level of D167N was increased, with virtually complete protection from hydrolysis being achieved at 4 pmol of input D167N. This experiment attests to the apparent stability of the D167N•RNAp complex and suggests that a pathway of 2’,3’-cyclic phosphate removal through an RNAp intermediate might be processive.

**Pnkp mutant S211A transiently accumulates RNAp during 2’,3’-cyclic phosphate removal**

Trapping of RNAp as a product of the D167N reaction supports the intermediacy of RNAp in cyclic phosphate removal, but the result does not rule out the possibility that RNAp formation is an “off-pathway” event prompted by the loss of the acid–base catalyst. The case for intermediacy would be fortified by identifying another change in the active site (one not affecting general acid–base catalysis) that promotes accumulation of RNAp by selectively slowing the phosphomonoesterase reaction. We considered that this might be achieved in light of the differences in the electrostatics of the presumptive pentacoordinate transition states of the 2’,3’-cyclic phosphodiesterase reaction (–2 charge) and the phosphomonoesterase reaction (–3 charge) in an associative phosphoryl transfer mechanism. If this difference pertains in the active site of the enzyme, then the 3’-phosphatase reaction might be more acutely reliant than the 2’,3’-cyclic phosphodiesterase on the Ser211 hydroxyl, which donates a hydrogen bond to one of the non-bridging phosphate oxygens of the aspartyl-phosphate adduct (Figure 2). Initial enzyme titration experiments with the S211A mutant Pnkp showed that its specific activity with the RNAp substrate was similar to that of wild-type Pnkp (Figure 4B), whereas S211A activity with the RNA3p substrate was 3-fold less than that of wild-type Pnkp (Figure 4A). Moreover, we readily
Reaction mixtures (10 μl) containing 100 mM Tris-acetate (pH 6.0), 10 mM MgCl₂, 2 mM DTT, 0.5 pmol (50 nM) 32P-labeled RNAp, and increasing amounts of Pnkp-D167N as specified were pre-incubated for 20 min at 22°C. The reactions in lanes 1, 2, and 3 were quenched with 50 mM EDTA. The three remaining Pnkp-D167N reaction mixtures were treated as follows. Wild-type Pnkp (0.5 pmol) with RNAp was reacted for 20 min at 22°C and then quenched with EDTA. The D167N reaction mixture in lane 6 was heated at 95°C for 2 min, after which the wild-type Pnkp-supplemented D167N reaction mixtures were incubated for 20 min at 22°C and then quenched with EDTA. The D167N reaction mixture in lane 6 was heated at 95°C for 2 min, incubated for an additional 20 min at 22°C without wild-type Pnkp supplementation, and then quenched with EDTA. The RNAs of all six reaction mixtures were then digested with RNase T1 and analysed by urea-PAGE (lanes 1–6). An RNase T1 digest of the 32P-labeled RNAp-20-mer was analysed in parallel in lane 7. An autoradiogram of the gel is shown. The phosphorylation states of the terminal RNA T1 fragments are indicated at left. (B) A schematic of the experiment is shown above the results. Reaction mixtures (10 μl) containing 100 mM Tris-acetate (pH 6.0), 10 mM MgCl₂, 2 mM DTT, 0.5 pmol (50 nM) 32P-labeled RNAp, and increasing amounts of Pnkp-D167N as specified were pre-incubated for 20 min at 22°C. The reaction mixtures were then supplemented with 0.5 pmol of wild-type Pnkp and incubated for 20 min at 22°C. The reactions were then quenched with EDTA. The RNAs were digested with RNase T1 and analysed by urea-PAGE. The extent of RNAOH formation is plotted as a function of the amount of Pnkp-D167N included during the pre-incubation phase. Each datum is the average of three independent experiments (±SEM).

Figure 5. D167N sequesters the RNAp product of the cyclic phosphodiesterase reaction. (A) A schematic of the experiment is shown above the results. Six reaction mixtures (10 μl) containing 100 mM Tris-acetate (pH 6.0), 10 mM MgCl₂, 2 mM DTT, 0.5 pmol (50 nM) 32P-labeled RNAp, and either no enzyme (lane 1), 0.5 pmol (50 nM) wild-type Pnkp (lane 3), or 4 pmol (400 nM) Pnkp-D167N (lanes 2, 4, 5 and 6) were incubated for 20 min at 22°C. The reactions in lanes 1, 2, and 3 were quenched with 50 mM EDTA. The three remaining Pnkp-D167N reaction mixtures were treated as follows. Wild-type Pnkp (0.5 pmol) was either added directly (lane 4) or after heating the reaction mixture at 95°C for 2 min (lane 5), after which the wild-type Pnkp-supplemented D167N reaction mixtures were incubated for 20 min at 22°C and then quenched with EDTA. The D167N reaction mixture in lane 6 was heated at 95°C for 2 min, incubated for an additional 20 min at 22°C without wild-type Pnkp supplementation, and then quenched with EDTA. The RNAs of all six reaction mixtures were then digested with RNase T1 and analysed by urea-PAGE (lanes 1–6). An RNase T1 digest of the 32P-labeled RNAp-20-mer was analysed in parallel in lane 7. An autoradiogram of the gel is shown. The phosphorylation states of the terminal RNA T1 fragments are indicated at left. (B) A schematic of the experiment is shown above the results. Reaction mixtures (10 μl) containing 100 mM Tris-acetate (pH 6.0), 10 mM MgCl₂, 2 mM DTT, 0.5 pmol 32P-labeled RNAp, and increasing amounts of Pnkp-D167N as specified were pre-incubated for 20 min at 22°C. The reaction mixtures were then supplemented with 0.5 pmol of wild-type Pnkp and incubated for 20 min at 22°C. The reactions were then quenched with EDTA. The RNAs were digested with RNase T1 and analysed by urea-PAGE. The extent of RNAOH formation is plotted as a function of the amount of Pnkp-D167N included during the pre-incubation phase. Each datum is the average of three independent experiments (±SEM).

RNA 2'-phosphatase activity of Pnkp
The PAGE system used for analysis of the T1 fragments does not discriminate whether the RNAp product formed by the D167N cyclic phosphodiesterase reaction has a 3'-phosphate or 2'-phosphate terminus. Given that wild-type Pnkp is competent to hydrolyze an RNA 3'-phosphate, and that the RNAp formed in the cyclic phosphodiesterase reaction is also hydrolyzed by wild-type Pnkp, it is tempting to assume that the CPDase reaction product is itself a 3'-phosphomonoester. However, as far as we know, there is no report in the literature concerning the capacity of T4 Pnkp to remove both the aspartate nucleophile and the aspartate general acid–base. Enzyme titration showed that Pnkp had similar detected RNAp when RNA p was reacted for 20 min with limiting levels of S211A enzyme (not shown).

The kinetic profile of the reaction of excess S211A (500 nM) with RNA > p (50 nM) is presented in Figure 6A and quantified in Figure 6B. The reaction was notable for the early accumulation of RNAp, which comprised 25% and 48% of the total radiolabeled material at 5 and 10s, respectively, at which times RNAOH comprised only 8% and 34% of the total. The abundance of RNAp declined steadily thereafter, concomitant with the accumulation of RNAOH to an extent of 93% of the total labeled RNA at 5 min (Figure 6B). These results are consistent with a precursor–product relationship between RNAp and RNAOH. By plotting the data in Figure 6B as the sum of the RNAp and RNAOH species as a function of reaction time, we derived a rate constant of 0.14 ± 0.01 s⁻¹ for the 2',3'-cyclic phosphodiesterase reaction of the S211A enzyme.

The reaction of excess S211A with the RNA3p substrate displayed biphasic kinetics with an end point of 91% RNAOH formation (Figure 6C). Non-linear regression curve fitting of the data in Prism highlighted a fast phase with an apparent rate constant of 0.092 ± 0.019 s⁻¹ that accounted for 57% of the reaction product. (The rate of the slow phase was 0.012 ± 0.0026 s⁻¹). Taken together, the kinetic data for the two substrates indicate that the rate of the S211A 2',3'-cyclic phosphodiesterase reaction is ~1.5-fold faster than that of the 3'-phosphatase step, thereby accounting for the accumulation of the RNAp intermediate during the reaction of S211A with RNA > p.
The extent of RNAOH formation (expressed as the percent of total labeled RNA) is plotted as a function of time. Each datum in the graph is the average of three independent experiments (±SEM). (Figure 7B).

**DISCUSSION**

The present study advances our understanding of the mechanism by which the T4 Pnkp phosphatase enzyme heals RNA ends. We show that Pnkp is adept at healing RNA 3′-phosphomonoesters and RNA 2′-phosphomonooesters to yield the respective hydroxyl termini in a reaction pathway that relies on both the Asp165 nucleophile and the Asp167 general acid–base.
The ability of either the ribose O3' or O2' to be the leaving group during the attack of Asp165 on the terminal phosphate suggests some flexibility in the active site, whereby (i) the terminal ribose can bind in alternative conformations that place either the O2' or O3' close to the Asp167 general acid; or (ii) the terminal ribose binds in a fixed orientation and the Asp167 general acid has some range of motion to access either a 2'-0-bridging or a 3'-0-bridging phosphate oxygen.

It is well-established that T4 Pnkp is an RNA repair enzyme in vivo, dedicated to the healing and sealing of broken tRNAs with 2'-,3'-cyclic phosphate and 5'-OH ends (1,2,9). Our studies of the Pnkp-D167N and Pnkp-S211A enzymes reveal that removal of a 2'-,3'-cyclic phosphate occurs through sequential cyclic phosphodiesterase and phosphomonoesterase reactions. Pnkp-D167N activity arrests after conversion of RNA:p to RNA30:p, which remains sequestered in the phosphatase active site. The D167N CPDase reaction outcome (a 3'-PO4) suggests that the terminal 2'-,3'-cyclic phosphate binds in a specific orientation that places the O2' leaving group apical to the Asp165 nucleophile. Apparently, the O2' leaving group can be expelled when Asp167 is not available as a proton donor, albeit slowly. The S211A mutation, affecting the coordination of the scissile phosphate (Figure 2), results in transient accumulation of high levels of an RNAp intermediate that is subsequently hydrolyzed to RNAOH. It is our assumption that cyclic phosphate removal by wild-type Pnkp also proceeds through an RNAp intermediate, but we have been unable to confirm this because the rate of 3' end healing by wild-type Pnkp under conditions of enzyme excess is too fast to measure by manual methods.

It is worthwhile to compare and contrast the properties of T4 Pnkp phosphatase (a Mg2+-dependent
aciphosphatase) with those of three other types of RNA repair enzymes that act on 2',3'-cyclic phosphate ends (Table 1). Clostridium thermocellum (Cth) Pnkp exemplifies a family of RNA end-healing and sealing enzymes found in diverse bacteria from many phyla. CthPnkp is composed of three catalytic modules: an N-terminal polynucleotide 5'-kinase; a central 2',3'-phosphatase; and a C-terminal ligase (29). The CthPnkp phosphatase domain, which belongs to the binuclear metallophosphoesterase superfamily, catalyzes the release of P\(\text{I}\) from 2'-PO\(\text{A}\), 3'-PO\(\text{A}\) or 2',3'-cyclic phosphate ribonucleotides and requires either Mn\(^{2+}\) or Ni\(^{2+}\) as the metal cofactor (29–33). As with T4 Pnkp, CthPnkp converts a 2',3'-cyclic phosphate to 3'-OH, 2'-OH end-product through sequential diesterase and monoesterase reactions. However, unlike T4 Pnkp, which generates an exclusive 3'-PO\(\text{A}\) product of its CPDase reaction (as shown for “diesterase-only” mutant D167N), a diesterase-only mutant of CthPnkp opens a 2',3'-cyclic phosphodiester to form an exclusive 2'-PO\(\text{A}\) product (32,33). The chemical mechanism of the CthPnkp phosphoesterase is through attack of a metal-bound water on the scissile phosphate, that is, the reaction does not involve a covalent phosphoenzyme intermediate.

Yeast and plant tRNA ligases are composed of an N-terminal ligase domain, a central polynucleotide kinase domain and a C-terminal 2',3'-cyclic phosphodiesterase domain (9,25,26). The CPDase domain of yeast/plant tRNA ligase belongs to the 2H phosphoesterase domain (9,25,26). The CPDase domain of yeast/plant tRNA ligase (9,25,26) opens a 2',3'-cyclic phosphodiester to form an exclusive 2'-PO\(\text{A}\) product (24). And unlike T4 Pnkp, the plant tRNA ligase CPDase reaction is independent of a metal cofactor (B. Remus and S. Shuman; unpublished data) and does not involve a covalent intermediate.

The recently elucidated pathway of 2',3'-cyclic phosphate/5'-OH end joining by E. coli RtcB involves an initial manganese-dependent 2',3'-cyclic phosphodiesterase reaction that converts RNA >p to RNAp and a subsequent manganese-dependent 3'-PO\(\text{A}\)/5'-OH ligation reaction that requires GTP and proceeds through covalently activated RtcB-(histidinyl-N)-GMP and RNA(3)ppG intermediates (22,27,28). Available evidence suggests that the RtcB CPDase and ligase reactions are executed at a single active site. Whereas T4 Pnkp and RtcB both generate 3'-PO\(\text{A}\), 2'-OH ends through their CPDase activities, RtcB, unlike T4 Pnkp, does not further hydrolyze the 3'-PO\(\text{A}\) end.

It is apparent that nature has evolved many different chemical and structural solutions to the problem of 3' end healing in RNA repair pathways (Table 1). In the cases of T4 Pnkp, CthPnkp and yeast/plant tRNA ligase, the phosphoesterase components are members of widely distributed enzyme superfamilies that have been harnessed to RNA repair, by the acquisition of substrate specificity determinants and by fusion of the phosphatase domain to one or more other catalytic components of the repair pathway. By contrast, RtcB exemplifies a novel enzyme family and a stand-alone catalytic unit. Too little is known at present about the RtcB family to speculate whether the many bacterial, archaeal and eukaryal RtcB homologs are devoted to RNA repair/splicing or whether their unique chemistry might be applied to other biological purposes. However, we are willing to speculate that the four flavors of cyclic phosphate end-healing enzymes in Table 1 are not the end of the story and that new enzymes and strategies for repairing broken ends await discovery.

**FUNDING**

Funding for open access charge: NIH [GM63611].

**Conflict of interest statement.** None declared.

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