Distinctive kinetics and substrate specificities of plant and fungal tRNA ligases

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ABSTRACT
Plant and fungal tRNA ligases are trifunctional enzymes that repair RNA breaks with 2',3'-cyclic-PO4 and 5'-OH ends. They are composed of cyclic phosphodiesterase (CPDase) and polynucleotide kinase domains that heal the broken ends to generate the 3'-OH, 2'-PO4, and 5'-PO4 required for sealing by a ligase domain. Here, we use short HORNA>p substrates to determine, in a one-pot assay format under single-turnover conditions, the order and rates of the CPDase, kinase and ligase steps. The observed reaction sequence for the plant tRNA ligase AtRNL, independent of RNA length, is that the CPDase engages first, converting HORNA>p to HORNA2, which is then phosphorylated to pRNA2,p by the kinase. Whereas the rates of the AtRNL CPDase and kinase reactions are insensitive to RNA length, the rate of the ligase reaction is slowed by a factor of 16 in the transition from 10-mer RNA to 8-mer and further by eightfold in the transition from 8-mer RNA to 6-mer. We report that a single ribonucleoside-2',3'-cyclic-PO4 moiety enables AtRNL to efficiently splice an otherwise all-DNA strand. Our characterization of a fungal tRNA ligase (KlaTrl1) highlights important functional distinctions vis à vis the plant homolog. We find that (1) the KlaTrl1 kinase is 300-fold faster than the AtRNL kinase; and (2) the KlaTrl1 kinase is highly specific for GTP or dGTP as the phosphate donor. Our findings recommend tRNA ligase as a tool to map ribonucleotides embedded in DNA and as a target for antifungal drug discovery.

Keywords: 2',3' cyclic phosphodiesterase; RNA ligase; RNA repair; polynucleotide kinase

INTRODUCTION
Plant and fungal tRNA ligases, exemplified by Arabidopsis thaliana AtRNL and Saccharomyces cerevisiae Trl1 (SceTrl1), are essential agents of informational and stress response pathways involving the repair of programmed tRNA and mRNA breaks with 2',3'-cyclic phosphate and 5'-OH ends. Plant and fungal tRNA ligases perform three distinct RNA repair reactions: (1) The 2',3'-cyclic phosphate (>p) end is hydrolyzed to a 3'-OH,2'-PO4 by a cyclic phosphodiesterase (CPDase); (2) the 5'-OH end is phosphorylated by an NTP-dependent polynucleotide kinase; and (3) the 3'-OH,2'-PO4 and 5'-PO4 ends are sealed by an ATP-dependent RNA ligase to form an unconventional 2'-PO4, 3'-5' phosphodiester at the splice junction (Konarska et al. 1982; Greer et al. 1983; Schwartz et al. 1983; Pick et al. 1986; Pick and Hurwitz 1986; Englert and Beier 2005). AtRNL and SceTrl1 are composed of three separable catalytic domains: a C-terminal CPDase module belonging to the "2H" phosphoesterase superfamily; a central kinase module of the P-loop phosphotransferase superfamily; and an N-terminal ligase domain that belongs to the covalent nucleotidytransferase superfamily (Fig. 1A; Apostol et al. 1991; Sawaya et al. 2003; Wang and Shuman 2005; Wang et al. 2006).

The CPDase and kinase reactions are collectively referred to as "end-healing" (Schwer et al. 2004). Healing provides the proper termini for end sealing, which is dependent on the 2'-PO4 moiety. The sealing phase of tRNA splicing requires ATP and proceeds via three adenylate transfer steps. First, the ligase domain reacts with ATP to form a covalent ligase-(lysyl-ηN)-AMP intermediate and displace pyrophosphate. Adenylation occurs at a conserved lysine (Lys152 in AtRNL) (Fig. 1A) within a signature KxG motif. Second, ligase transfers AMP to the 5'-PO4 RNA terminus to form an RNA-adenylate intermediate (AppRNA). Third, ligase directs the attack of the 3'-OH on AppRNA to form the splice junction and displace AMP.

Despite the importance of tRNA splicing in plant and fungal physiology, little was known about the kinetics of the RNA repair reactions catalyzed by tRNA ligases or how the specificity for sealing 2'-PO4 ends is enforced. Focusing initially on AtRNL, we aimed to close this knowledge gap, by

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determining the rates of end-healing and sealing under single-turnover conditions. We exploited linear 20-mer RNA substrates with different terminal structures and a collection of AtRNL mutants to dissect individual steps or subsets of steps along the RNA repair pathway (Remus and Shuman 2013). The linear RNAs used for those studies mimic an excised tRNA intron, which is efficiently sealed by AtRNL to form a circular RNA product (Englert and Beier 2005; Nandakumar et al. 2008).

We found that the isolated CPDase reaction was faster than the kinase and ligase reactions, suggesting that the 2′,3′-p // 3′-OH break is converted to a 3′-OH,2′-PO4 // 5′-OH prior to its conversion by the kinase to a 3′-OH,2′-PO4 // 5′-PO4 that can be joined by the ligase module. The rates of sealing of the half-healed H10RNA2′p and fully healed pRNA2′p (0.056 sec⁻¹) pair intermediates were similar to one another and to the rate of the complete H10RNA>p repair pathway (0.048 sec⁻¹), implying that the kinase and ligase reactions are jointly rate limiting. Whereas the ligase is strictly dependent on ATP as the nucleotide substrate for end sealing, the kinase readily used ATP, GTP, CTP, UTP, or dATP as the phosphate donor (Remus and Shuman 2013). Thus, the AtRNL kinase is not fastidious with respect to the nucleobase or the pentose sugar of the NTP.

We gained the first mechanistic insights into the 2′-PO4 specificity of ligation by showing that AtRNL readily seals a pre-adenylated AppRNAOH substrate with a 3′-OH,2′-OH end. The similar rates of AppRNA2′p and AppRNAOH sealing indicated that the 2′-PO4 moiety makes little contribution to the phosphodiester synthesis step (step 3) of the ligase reaction. Rather, 2′-PO4 specificity of AtRNL is enforced at the level of RNA 5′ adenyllylation (step 2), whereby the conversion of pRNAOH to AppRNAOH and ligated RNA in the presence of ATP was several hundredfold slower than the sealing of pRNA2′p under the same reaction conditions. We proposed that the strategy employed by AtRNL for discriminating 2′-PO4 vs. 2′-OH ends provides a quality control checkpoint to ensure that only purposeful RNA breaks are sealed and to avoid nonspecific capping of 5′-PO4 ends (Remus and Shuman 2013).

Here, we extend the analysis of plant tRNA ligase to address the following questions: (1) What is the minimal RNA substrate for end-healing and sealing? (2) How do decrements in RNA chain length affect the kinetics of the healing and sealing steps? and (3) Can AtRNL splice a 5′-OH DNA substrate with a single ribonucleoside-2′,3′-cyclic-PO4 end?

We also analyze the kinetics and substrate requirements of an exemplary fungal tRNA ligase, Kluyveromyces lactis Trl1 (KlaTrl1). Fungal Trl1 enzymes are potential therapeu- tic targets, because their structures and biochemical mechanisms are unique compared to the RtcB-type tRNA repair systems elaborated by metazoans, archaea, and many bacteria (Popow et al. 2011; Tanaka and Shuman 2011; Tanaka et al. 2011; Chakravarty et al. 2012; Englert et al. 2012; Das et al. 2013a; Desai et al. 2013). Indeed, mammalian proteomes have no discernable homologs of the sealing domain of fungal tRNA ligase. These features highlight the sealing components of tRNA ligases as untapped targets for the discovery of new anti-fungals. Even more attractive is the prospect of inhibiting multiple enzymatic steps in the fungal tRNA splicing pathway, which would reduce the risk of the emergence of resistance. For this scenario to be plausible, the case must be made that the CPDase or kinase modules of fungal Trl1 enzymes have “drugable” properties.

In the present study, we were especially interested in the nucleotide substrate specificity of the KlaTrl1 kinase module, in light of prior studies indicating that SceTrl1 preferentially uses GTP as the phosphate donor for its kinase reaction in vitro and in vivo (Westaway et al. 1993; Sawaya et al. 2003). We show that KlaTrl1 is highly selective for GTP or dGTP as the phosphate donor, and we exploit nucleotide analogs.
to elucidate the atomic determinants of nucleobase specificity. Kinetic analysis reveals that (1) the fungal kinase is much faster than the homologous plant kinase, and (2) the ligation steps are rate limiting for the joining of linear RNAs by \( Kla \text{-Trl1} \). Thus, fungal and plant tRNA ligases have distinctive substrate specificities and kinetic properties when executing the same chemical reactions.

**RESULTS**

**One-pot assay for monitoring the AtRNL end-healing and sealing reactions**

Previously, we employed a \( 3' \) \( \text{\textsuperscript{32}P} \)-labeled 20-mer \( \text{HORNA} \text{p} \) substrate to monitor the progress of the AtRNL splicing reaction. A technical limitation to this approach was the inability to separate by urea-PAGE the input 20-mer \( \text{HORNA} \text{p} \) substrate from the \( \text{HORNA} \text{p} \) product of the CPDase reaction; consequently, the measurement of CPDase activity entailed an additional (and cumbersome) step in which the reactants and products were digested with RNase T1 to yield \( 3' \) \( \text{\textsuperscript{32}P} \)-labeled tetra-nucleotides with 2',3'-cyclic-PO\(_4\) or 2'-PO\(_4\) ends that could be resolved by PAGE (Remus and Shuman 2013). Here, in order to assay the healing and sealing pathway of AtRNL in a one-pot format, we used a 10-mer \( \text{HORNA} \text{p} \) substrate with a single radiolabel between the \( 3' \)-terminal and penultimate nucleosides (Fig. 1A). The 10-mer \( \text{HORNA} \text{p} \) substrate (20 nM) was reacted for 5 min with a 50-fold molar excess of recombinant wild-type AtRNL in the presence of 10 mM Mg\(^{2+}\) and 0.1 mM ATP. To limit the reaction to just the CPDase step, we either omitted Mg\(^{2+}\) and/or ATP, or replaced wild-type AtRNL with mutated versions K700A-S701A or D726A that are devoid of kinase activity (Remus and Shuman 2013). To limit the reaction to just the kinase step, we used the CPDase-defective T1001A mutant (Remus and Shuman 2013). To allow end-healing but preclude the ligation reaction, we used the K152A mutant. The reaction products were analyzed by urea-PAGE and visualized by autoradiography (Fig. 1A).

In the reactions limited to the CPDase, the \( \text{HORNA} \text{p} \) substrate was hydrolyzed to form a more rapidly migrating \( \text{HORNA} \text{p} \) species (Fig. 1A). When the ligation reaction was prevented by the K152A mutation, the \( \text{HORNA} \text{p} \) substrate was converted into a fully healed pRNA\(_p\) species that migrated ahead of the \( \text{HORNA} \text{p} \) CPDase reaction product (Fig. 1A). The CPDase-defective T1001A mutant performed the \( 5' \) phosphorylation step, leading to the formation of a pRNA\(_p\) species that could not be ligated. In contrast, wild-type AtRNL converted the 10-mer \( \text{HORNA} \text{p} \) substrate into a circular product, migrating more rapidly than the healed pRNA\(_p\) species, via intramolecular end joining (Fig. 1A). Thus, we can resolve the healed intermediates and sealed products of the AtRNL splicing pathway by using a short RNA substrate.

**Order and kinetic profile of the AtRNL reactions under single-turnover conditions**

The temporal progress of the reaction of 1 \( \mu \text{M} \) AtRNL with 20 nM 10-mer \( \text{HORNA} \text{p} \) substrate is shown in Figure 1C. The substrate was sequentially converted into \( \text{HORNA} \text{p} \) and pRNA\(_p\) intermediates en route to the ligated circle product. The distribution of labeled RNAs is plotted in Figure 1B, with each datum being the average of three separate kinetic experiments \( \pm \text{SEM} \). The data were fit by nonlinear regression in Prism to a sequential three-step mechanism depicted in Figure 1B, whereby the reaction order is first CPDase, then kinase, then ligase. The single-turnover rate constants were \( 0.10 \pm 0.005 \text{ sec}^{-1} \) for the CPDase, \( 0.049 \pm 0.0015 \text{ sec}^{-1} \) for the kinase; and \( 0.035 \pm 0.0011 \text{ sec}^{-1} \) for the ligase (Fig. 1B). The rate of the AtRNL ligation step with the 10-mer substrate \( \text{HORNA} \text{p} \) (0.035 sec\(^{-1}\)) was similar to the rate determined previously with a 20-mer \( \text{HORNA} \text{p} \) (0.056 sec\(^{-1}\)) (Remus and Shuman 2013).

**Effects of RNA size decrements on the kinetics of end-healing and sealing**

It was of interest to determine the effects of shortening the \( \text{HORNA} \text{p} \) substrate on the outcomes and kinetics of the...
component steps of the AtRNL splicing pathway, with the aim of identifying minimized substrates for each enzymatic module. We began by shortening the 10-mer substrate to an 8-mer, removing the 5′ dinucleotide but otherwise maintaining the nucleotide sequence (Fig. 2A). Reactions of wild-type AtRNL and kinase-defective, ligase-defective, or CPDase-defective AtRNL mutants with the 3′-32P-labeled 8-mer HORNA>p substrate (depicted at the bottom of the panel with the 32P-label denoted by •) yielded half-healed HORNA2>p or pRNA2>p intermediates or a fully healed pRNA2>p that were resolvable by urea-PAGE. An autoradiogram of the gel is shown in panel B. The levels of the healed pRNA2>p and pRNA2>p intermediates and the ligated circle product were quantified by scanning the gel and are plotted as a function of time in panel C. Each datum is the average of three separate time-course experiments ± SEM. The data were fit by nonlinear regression in Prism to a sequential three-step reaction pathway shown at right in panel B. The apparent rate constants for the CPDase, kinase, and ligase reactions are indicated.

FIGURE 2. Reaction of AtRNL with an 8-mer HORNA>p substrate. (A)Wild-type AtRNL and the indicated mutants were reacted with a 3′-32P-labeled 8-mer HORNA>p substrate (depicted at the bottom of the panel with the 32P-label denoted by •). Reaction mixtures (20 µL) containing 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM DTT, 10 mM MgCl2, 100 µM ATP, 20 nM 8-mer HORNA>p, and 1 µM AtRNL (wild-type or mutant as specified) were incubated for 5 min at 22°C. The reactions were quenched with formamide, EDTA, and the products were analyzed by urea-PAGE. An autoradiograph of the gel is shown. The position and identities of the radiolabeled product were quantified by scanning the gel and are plotted as a function of time in panel C. The time 0 sample was withdrawn and quenched prior to adding AtRNL. The products were resolved by urea-PAGE. An autoradiogram of the gel is shown in panel B. The levels of the healed 6-mer HORNA2>p and pRNA2>p intermediates and the ligated circle product were quantified by scanning the gel and are plotted as a function of time in panel C.

A single ribonucleoside 2′,3′-cyclic-PO4 enables AtRNL to splice DNA
AtRNL and fungal Trl1 enzymes are essential components of RNA repair pathways in vivo. Their 2′-PO4 requirement for
end joining mandates that the reactive 3'-OH terminus be a ribonucleoside. To our knowledge, the extent of the RNA requirement for productive and kinetically vigorous splicing has not been determined. A simple way to address this issue is to query whether any or all of the nucleotides of a spliceable polynucleotide substrate for AtRNL (exclusive of the 3'-nucleotide) can be replaced by deoxynucleotides. This approach has been fruitful in delineating the minimal RNA content required for other RNA repair enzymes, including T4 RNA ligase 2 (Nandakumar and Shuman 2004), the RNA terminal 2'-O-methyltransferase Hen1 (Jain and Shuman 2011), the RNA 3'-terminal cyclase RtcA (Das and Shuman 2013), and the RNA 3'-PO4/5'-OH ligase RtcB (Das et al. 2013a).

Here, we tested whether AtRNL could splice a HOD17R>p substrate, consisting of 17 deoxynucleotides and a single 32P-labeled 3'-terminal ribonucleoside-2',3'-cyclic-PO4 (Fig. 4). The substrate was prepared by templated enzymatic ligation of a cold HOD17ROH strand, 5'-d(CATATCCGTCGCCC T)(rC), to a 5' 32P-labeled 10-mer DNA strand, 5'-pATTCC GATAG, followed by digestion of the isolated D17RpD10 strand with RNase A to yield HOD17Rp, which was then converted to HOD17R>p by RtcA (Das and Shuman 2013).

The HOD17R>p substrate was reacted with AtRNL under single-turnover conditions, and the products were analyzed by urea-PAGE (Fig. 4, top panel). We detected the formation of a healed intermediate pD17R2>p at early time points en route to a more rapidly migrating circular end-product. (Note that the initial HOD17R2>p intermediate was not resolvable from the input substrate by the PAGE procedure.) A plot of the extent of ligation as a function of time is shown in Figure 4, the bottom panel. The data were fit by nonlinear regression to a single exponential with a rate constant of 0.027 ± 0.02 sec⁻¹ for the composite splicing reaction. This value for an all-DNA substrate with a single ribonucleoside-2',3'>p compares favorably with the rate constant of 0.048 sec⁻¹ for the composite splicing reaction of an all-RNA 20-mer HORNA>p substrate (Remus and Shuman 2013). We conclude that the terminal ribonucleoside is the sole RNA

FIGURE 3. Reaction of AtRNL with a 6-mer HORNA>p substrate. A reaction mixture (250 µL) containing 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM DTT, 0.1 mM ATP, 20 nM 6-mer HORNA>p substrate (depicted at the bottom of panel A with the 32P-label denoted by ●), and 1 µM AtRNL was incubated at 22°C. Aliquots (20 µL) were removed at the times specified and quenched immediately with formamide, EDTA. The time 0 sample was withdrawn and quenched prior to adding AtRNL. The products were resolved by urea-PAGE. An autoradiogram of the gel is shown in panel A. The levels of the healed HORNA2>p and pRNA2>p intermediates and the ligated dimer product were quantified by scanning the gel and are plotted as a function of time in panel B. Each datum is the average of three separate time-course experiments ±SEM. The data were fit by nonlinear regression in Prism to a sequential three-step reaction pathway shown at right in panel B. The apparent rate constants for the CPD, kinase, and ligase reactions are indicated.

FIGURE 4. AtRNL efficiently splices a 5'-OH DNA with a ribonucleoside-2',3'-cyclic-PO4 terminus. A reaction mixture (160 µL) containing 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM DTT, 2 mM MnCl₂, 100 µM ATP, 1 µM AtRNL, and 20 nM 3'-32P-labeled 18-mer HOD17Rp substrate (shown with the 3' ribonucleoside shaded and the 32P-label denoted by ●) was incubated at 22°C. Aliquots (20 µL) were withdrawn at the times specified and quenched immediately with an equal volume of 90% formamide, 30 mM EDTA. The time 0 sample was withdrawn and quenched prior to adding AtRNL. The products were analyzed by urea-PAGE. An autoradiograph of the gel is shown in the top panel. The position of the linear substrate (HOD17Rp) is indicated on the left; the positions of the healed intermediate (pD17R2>p) and the ligated circle product are indicated on the right. The kinetic profile of the intramolecular end joining reaction is plotted in the bottom panel. Each datum in the graph is the average of three separate experiments ±SEM. A nonlinear regression curve fit of the data to a single exponential is shown.
specificity determinant for polynucleotide ligation by plant tRNA ligase. The implications of this finding for practical applications of tRNA ligases will be discussed.

Distinctive end-healing properties of *K. lactis* tRNA ligase

An earlier comparison of the specific activities of the isolated healing (kinase-CPDase) and sealing (ligase) domains of ScTrl1 and AtRNL showed that the plant sealing module was more active in vitro than the yeast homolog, whereas the yeast healing domain was more active than the plant homolog, when each was paired with an excess of its own partner domain (Nandakumar et al. 2008). Moreover, a 1:1 cross-species mixture of the yeast healing and plant sealing domains displayed 25-fold higher specific activity in tRNA splicing activity than a reciprocal 1:1 combination of the plant healing and yeast sealing domains. These initial results hinted at the existence of distinctive properties of one or both enzymatic activities of the yeast kinase-CPDase module. One reported difference is that the AtRNL kinase uses any common NTP as a phosphate donor, whereas the *Sce* Trl1 kinase prefers GTP (Westaway et al. 1993; Remus and Shuman 2013). Indeed, Greer and colleagues discovered that the *Sce* Trl1 kinase was 100-fold more efficient with GTP vs. ATP, as reflected in the yield of pRNA product as a function of NTP concentration (Westaway et al. 1993).

Here, we aimed to analyze the kinetics and substrate specificity of end-healing by *Kluveromyces lactis* tRNA ligase. KlaTrl1 is an 813-aa polypeptide with 45% amino acid sequence identity to *Sce* Trl1. We produced the C-terminal kinase-CPDase domain, KlaTrl1-(385–813), in *E. coli* and purified the 49-kDa recombinant protein (Supplemental Fig. S1A). We reacted 1 µM KlaTrl1-(385–813) with 20 nM 3′32P-labeled 10-mer HORNA>p substrate and a divalent cation for 1 min, in the absence of added NTP or in the presence of 100 µM ATP, GTP, CTP, or UTP. The reactions were quenched with EDTA, and the products were analyzed by urea-PAGE. Absent an added NTP, the HORNA>p was hydrolyzed quantitatively by the CPDase to a HORNA2p species that migrated ahead of the input substrate (Fig. 5A). Inclusion of 100 µM GTP or UTP elicited the formation of the fully healed product, pRNA2p, in quantitative (97%) yield (Fig. 5A). CTP and ATP at 100 µM supported phosphorylation of 38% and 9% of the substrate, respectively (Fig. 5A,B). When 100 µM dNTPs were tested as phosphate donors, only dGTP was effective (Fig. 5A,C).

To better gauge the phosphate donor preferences of the *K. lactis* kinase, we back-titrated the nucleotide concentrations in 10-fold decrements and quantified the extents of phosphorylation, which are displayed in bar graph format in Figure 5, B and C. Lowering the GTP concentration by a factor of 100 or 1000, to 1 µM or 0.1 µM, had virtually no effect on the yields of phosphorylated RNA (96% and 92%, respectively) after a 1-min reaction in enzyme excess. In contrast, whereas UTP was an effective kinase substrate at the highest concentration tested (100 µM), back-titrations progressively diminished the extent of phosphorylation, to 25% at 1 µM UTP and 6% at 0.1 µM UTP (Fig. 5B). The intermediate yield of product formation observed with 100 µM CTP (38%) fell off quickly at 10 µM and 1 µM CTP, to 10% and 5%, respectively (Fig. 5B). Thus, GTP is the strongly preferred phosphate donor among the four canonical rNTPs. Among the dNTPs titrated for

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kinase activity, dGTP supported 96% phosphorylation at 1 µM concentration (Fig. 5C), signifying that the K. lactis kinase was not stringently specific for the pentose sugar, so long as guanine was the nucleobase. However, dUTP was not an effective substrate, even at 100 µM, supporting only 9% phosphorylation at 10 µM UTP (Fig. 5B). We surmise that the ribose sugar matters when the NTP nucleobase is suboptimal.

Purine NTP analogs illuminate nucleobase recognition by the K. lactis kinase

We gained insights into the nucleobase specificity of the yeast kinase by testing purine ribonucleoside triphosphate analogs as substrates for 5′,3′ RNA p phosphorylation. The NTPs were included at 1, 10, and 100 µM concentration, and the extents of phosphorylation in a 1-min reaction under single-turnover conditions were quantified (Fig. 6). Inosine triphosphate was the most effective substrate, supporting 95% phosphorylation at 1 µM ITP (equivalent to the yield at 1 µM GTP), signifying that the exocyclic 2-NH₂ moiety of GTP is not an important determinant of nucleobase recognition per se. In contrast, 2-aminopurine triphosphate was a much less effective substrate for the kinase reaction, with yields of 3%, 16%, and 52% phosphorylation at 1, 10, and 100 µM concentration, respectively. This result highlights the importance of the 6-oxo atom of guanine for phosphate donor function. This conclusion was fortified by the feeble donor activity of 6-chloropurine triphosphate, which supported 5% and 13% phosphorylation at 10 µM and 100 µM concentration, respectively. Because inosine and 6-chloropurine are isosteres differing only in the 6-oxo vs. 6-chloro atom, the several hundredfold difference in their efficacy as kinase substrates (on a concentration basis) provides a rough gauge of the contribution of the 6-oxo atom to nucleobase recognition, albeit specifically in the absence of a 2-NH₂ moiety on the purine ring. In the same vein, the finding that 2-aminopurine, which lacks a 6-oxo atom, was an approximately fourfold better donor NTP than 6-chloropurine (at 100 µM concentration) roughly gauges the contribution of the 2-NH₂ substituent to nucleobase recognition. Finally, we found that 6-O-methyl guanosine triphosphate was a very effective donor, sustaining 68% and 94% phosphorylation at 1 µM and 10 µM 6-OMeGTP, respectively. We infer that the 6-oxo atom is essential because it acts as a hydrogen-bond acceptor, presumably from an amino acid side chain hydrogen donor or a main chain amide donor on the kinase enzyme. This scenario would account for the unique ability of UTP to serve as an alternative phosphate donor, by virtue of its pyrimidine 4-oxo group accepting the putative hydrogen bond in lieu of the guanine 6-oxo.

Single-turnover kinetics of the K. lactis kinase reaction

The rate of 5′ phosphorylation of the 10-mer 16S RNA p by the K. lactis healing domain under conditions of enzyme excess was too fast to measure manually. Therefore, we employed a rapid mix–quench apparatus to access the reaction on a millisecond time scale. Equal volumes of enzyme and substrate solutions were mixed at 22°C to achieve final concentrations of 20 nM 16S RNA p, 1 µM kinase-CPDase, 10 mM Mg²⁺, and 100 µM GTP or UTP. The rapid mix reactions were quenched with EDTA, and the products were resolved by urea-PAGE. The kinetic profiles for the GTP and UTP reactions are shown in Figure 7, A and B, where each datum is the average of three experiments ±SEM. Nonlinear regression fitting of the data to a single exponential yielded rate constants of 14.3 ± 1.2 sec⁻¹ with GTP as the phosphate donor and 0.7 ± 0.03 sec⁻¹ with UTP. The salient points of this analysis are that (1) the chemistry of the single-turnover K. lactis kinase reaction was 20-fold faster with 100 µM GTP vs. 100 µM UTP as the phosphate donor, and (2) the rate of phosphorylation of the 10-mer 16S RNA p substrate by K. lactis kinase was 300-fold faster than the rate of the plant AtRNL kinase (0.049 sec⁻¹) (Fig. 1).
Kinetic profile of the composite *Kla*Trl1 3′ -RNA>5′ splicing reaction

Recombinant full-length *Kla*Trl1 (Supplemental Fig. S1) was used to study the kinetics and outcome of the ligation reaction under single-turnover conditions (1 µM *Kla*Trl1 and 20 nM 3′ -32P-labeled 20-mer HORNA>p substrate) in the presence of 100 µM GTP and 100 µM ATP. The temporal progress of the reaction is shown in Figure 8A. Whereas the end-healing reactions were complete within 10 sec (the earliest time point sampled), the sealing of the pRNA>p strand proceeded slowly over 10 min to yield a mixture of monomer circle and multimeric products, formed by intramolecular and intermolecular end joining, respectively (Fig. 8A). We also detected the formation of an adenylylated RNA species, AppRNA2′p, migrating slower than the pRNA>p strand, that appeared in parallel with ligated products and decayed between 5 and 30 min as the circles and multimers accumulated. The fraction of the total labeled RNA that was ligated is plotted as a function of time in Figure 8B; 96% of the input RNA was sealed at the reaction endpoint. Nonlinear regression fitting of the data to a single exponential function yielded an apparent rate constant of 0.0048 ± 0.00036 sec⁻¹ for the *Kla*Trl1 splicing reaction. This value is 10-fold slower than the rate of single-turnover splicing of the same 20-mer HORNA>p substrate by AtRNL (0.048 sec⁻¹) (Remus and Shuman 2013). It is also slower by a factor of 1000 than the rate of the *K. lactis* kinase reaction.

The temporal profile of the reaction of *Kla*Trl1 with the shorter 10-mer HORNA>p substrate is shown in Figure 9A. Again, the end-healing reactions were complete within 10 sec, but the ligation reaction with the pRNA>p strand occurred quite slowly, yielding a mixture of AppRNA2′p and sealed RNA circles that persisted up to 30 min. (No multimers were formed in this reaction.) The fraction ligated is plotted as a function of time in Figure 9B; 78% of the input RNA was circularized in 30 min. By fitting the data to a single exponential, we derived an apparent rate constant of 0.0048 ± 0.00036 sec⁻¹ for the splicing reaction on the 10-mer.

The experiments in Figures 8 and 9 show that the sealing phase of the *K. lactis* reaction is severely rate limiting, at least in part because the step of phosphodiester synthesis in the three-step ligation reaction is slowed or aborted. This is a point of distinction between AtRNL and *Kla*Trl1. During the course of 10-mer ligation by AtRNL, we observed no AppRNA2′p intermediate, signifying that the rate of phosphodiester synthesis (i.e., the attack of the 3′-OH on the AppRNA end) is much faster than the rate of adenylate transfer from ligation-AMP to the 5′-PO₄ terminus to form AppRNA. In contrast, the adenylylated RNA species accumulated to a peak extent of 33% of total RNA during the *Kla*Trl1 reaction with the 10-mer, signifying that the step of phosphodiester synthesis was debilitated. The persistence of AppRNA at 30
mechanism and specificity of plant tRNA ligase AtRNL and the present study fills gaps in our understanding of the DISCUSSION. Our findings demonstrate that the reaction mixture (200 µL) containing 50 mM Tris-HCl, pH 8.0, 2 mM DTT, 10 mM MgCl₂, 100 µM ATP, 100 µM GTP, 20 nM 3²-P-labeled 10-mer HORNA>p substrate (depicted at the bottom of panel A with the ²-P-label denoted by ♦), and 1 µM KlaTrl1 was incubated at 22°C. Aliquots (20 µL) were withdrawn at the times specified and quenched immediately. The products were analyzed by urea-PAGE. An autoradiogram of the gel is shown in panel A. The position and identities of the radiolabeled substrate and products are indicated on the left and right. The extent of ligation (circle/total RNA) and the extent of RNA adenylation ([AppRNAp + circle]/total RNA) are plotted as a function of time in panel B. Each datum is the average of three separate time-course experiments ±SEM. Nonlinear regression curve fits of the data to a single exponential are shown.

FIGURE 9. Reaction of KlaTrl1 with a 10-mer ²³RNA>p substrate. A reaction mixture (200 µL) containing 50 mM Tris-HCl, pH 8.0, 2 mM DTT, 10 mM MgCl₂, 100 µM ATP, 100 µM GTP, 20 nM 3²-P-labeled 10-mer HORNA>p substrate (depicted at the bottom of panel A with the ²-P-label denoted by ♦), and 1 µM KlaTrl1 was incubated at 22°C. Aliquots (20 µL) were withdrawn at the times specified and quenched immediately. The products were analyzed by urea-PAGE. An autoradiogram of the gel is shown in panel A. The position and identities of the radiolabeled substrate and products are indicated on the left and right. The extent of ligation (circle/total RNA) and the extent of RNA adenylation ([AppRNAp + circle]/total RNA) are plotted as a function of time in panel B. Each datum is the average of three separate time-course experiments ±SEM. Nonlinear regression curve fits of the data to a single exponential are shown.

min suggested that a percentage of the sealing reactions were aborted subsequent to RNA adenylation, most likely because (1) the AppRNA₂⁻P intermediate dissociates from the KlaTrl1 ligase domain, (2) the apo-ligase reacts with ATP to form ligase–AMP, and (3) ligase–AMP is unable to rebind and seal the released AppRNA₂⁻P strand because the adenylate-binding pocket is occupied. This is the accepted explanation for trapping of adenylylated RNA and DNA species during abortive sealing by other polynucleotide ligases (Sriskanda and Shuman 1998; Ho and Shuman 2002). We derived a rate constant of 0.0043 ± 0.00015 sec⁻¹ for the RNA adenylation step of the KlaTrl1 ligase reaction on the 10-mer substrate by plotting the sum of the AppRNA₂⁻P and circular RNA species as a function of time and fitting the data to a single exponential (Fig. 9B).

DISCUSSION

The present study fills gaps in our understanding of the mechanism and specificity of plant tRNA ligase AtRNL and fungal tRNA ligase KlaTrl1. We exploited short ²³RNA>p substrates to determine, in a one-pot assay format, the order and rates of the CPDase, kinase, and ligase steps along the AtRNL splicing reaction pathway. Whereas the rate of the CPDase reaction was unaffected and the rate of the kinase reaction was affected only modestly by shortening the length of the RNA substrate, the rate of the sealing reaction was acutely sensitive, being slowed by a factor of 16 in the transition from 10-mer RNA to 8-mer and by eightfold in the transition from 8-mer RNA to 6-mer. The shift from 8-mer to 6-mer also changed the sealing mode from intramolecular to intermolecular end joining.

These results provide new insights into the minimal substrates and conformational constraints on the catalytic domains of AtRNL. The observed reaction sequence, independent of RNA length, is that the CPDase engages first, converting ²³RNA>p to ²³RNA>²-P, which is then phosphorylated to pRNA>²-P by the kinase. It is unclear whether the product ²-P₉₉₇ terminus dissociates from the CPDase active site prior to engagement of the 5'-OH end by the kinase active site. (If the ²-P₉₉₇ stays bound to the CPDase during the kinase reaction, it would imply, in the case of the 6-mer substrate, that the kinase phosphoacceptor site is located within ~45 Å of the CPDase ²-P₉₇ site, assuming an interval of 6.5 Å between the phosphates of an RNA chain.)

The ligase module of AtRNL is thought to have independent and specific binding sites for the ²-P₉₉₇ and ²-P₉₇ RNA ends. A key feature of the AtRNL ligation reaction is that transfer of AMP from the ligase–AMP adduct to the ²-P₉₇ RNA end requires the ²-P₉₇ end by the kinase (Remus and Shuman 2013). The order of end capture by the ligase is not known, but there are several plausible scenarios, including one in which the half-healed ²³RNA>²-P species dissociates from the CPDase and the ²-P₉₇ terminus is quickly engaged by the ligase module, so that subsequent phosphorylation of the 5'-OH by the kinase occurs while the ²-P₉₇ is bound to the ligase, after which the ²-P₉₇ end dissociates from the kinase and is immediately captured by the ligase (thereby favoring intramolecular sealing). The fact that ²³RNA>p substrates as short as an 8-mer are sealed by AtRNL to yield exclusively circular products tells us that the active site of the ligase is sufficiently “open” to attain the proper orientation of the reactive RNA termini, either by having the 8-mer RNA adopt a minicircle conformation (with the ends lined up in series) or a fold-back hairpin conformation (with anti-parallel ends). The proper geometry for RNA 5' adenylation apparently cannot be achieved when the RNA is shortened to a 6-mer; consequently, the outcome shifts to intermolecular end joining.

We report that a single ribonucleoside-²-P₉₇ cyclic-PO₄ moiety enables AtRNL to efficiently splice an otherwise all-DNA strand. Evidently, the kinase and ligase domains are not specific for RNA vs. DNA 5' ends. We surmise that RNA specificity is enforced solely at the level of the ²-P₉₇ requirement for adenylylation of the 5'-PO₄ terminus by
the ligase module. The DNA sealing ability of AtRNL expands its utility as a reagent for nucleic acid capture and sequencing. As conceived initially, Schutz et al. (2010) used AtRNL to ligate an RNA oligonucleotide tag to the population of cellular RNAs that naturally have 2′,3′-cyclic-PO4 termini. After treating the ligated products with Tpt1 to remove the 2′-PO4 at the splice junction, they used an oligonucleotide complementary to the tag to prime synthesis of a cDNA library, which was then amplified by PCR and sequenced. This method can be used to identify RNA species and processing intermediates that are not accessible by tagging with conventional RNA ligases such as T4 Rnl1 and T4 Rnl2 (Schutz et al. 2010).

Based on our findings here, we envision a new application for AtRNL in the tagging and characterization of DNAs with 2′,3′-cyclic-PO4 ends. DNA breaks with 2′,3′-cyclic-PO4 ends are generated by topoisomerase IB transesterification at sites where single ribonucleotides are embedded in the genome (Sekiguchi and Shuman 1997). Embedded ribonucleotides are among the most abundant natural lesions in DNA, and their cleavage by topoisomerase IB causes a sharp increase in deletion mutations at the incision site (Kim et al. 2011; Reijns et al. 2012). The metabolism of 2′,3′-cyclic-PO4 DNA breaks is entirely uncharted. A method to tag, quantify, and map the location of such breaks on a genome-wide scale would be a key advance—and AtRNL is an enabling reagent to accomplish this goal. Equally promising is the use of AtRNL to quantify and map the location of embedded ribonucleotides on a genome-wide scale. This can be accomplished by treating genomic DNA with alkali, which will yield a mixture of 2′-PO4 and 3′-PO4 ends at all sites where ribonucleotides are embedded. AtRNL can then selectively ligate an oligonucleotide tag to the DNA 2′-PO4 ends. The specificity of AtRNL ensures that no ligation will occur to a DNA 3′ end.

Our characterization of K. lactis tRNA ligase highlights important functional distinctions vis à vis the plant homolog. First, KlaTrl1 ligase domain is less effective than its AtRNL counterpart in sealing the short tRNA >=p substrates used here for the kinetic analyses. This echoes earlier findings that, whereas AtRNL is as adept at splicing linear single-stranded RNAs as it is in joining the breaks in the anti-codon stem–loop of pre-tRNAs that are the physiological substrates for tRNA splicing, the yeast enzyme SecTrl1 exhibits a preference for broken stem–loop substrates and is less active in sealing short linear RNAs (Englert and Beier 2005; Nandakumar et al. 2008). Our observation that the KlaTrl1 ligation reaction on the 10-mer tends to abort after the formation of AppRNA2>=p suggests that the fungal ligase active site is constrained in its ability to hold on to both ends of the 10-mer RNA.

The second, and more salient, difference between the fungal and plant tRNA ligases pertains to the kinase module. We find that (1) the KlaTrl1 kinase reaction is 300-fold faster than the AtRNL kinase reaction measured under single-turnover conditions with the same RNA substrate, and (2) the KlaTrl1 kinase is highly specific for GTP or dGTP as the phosphate donor. The GTP specificity of KlaTrl1 resonates with earlier findings for SecTrl1 (Westaway et al. 1993).

The strong GTP preference exhibited by the polynucleotide kinase domains of fungal tRNA ligases is unique among all polynucleotide kinase (Pnk) enzymes characterized to date. The best studied of these, with respect to structure, mechanism, and NTP substrate specificity, are bacteriophage T4 Pnk (Galburt et al. 2002; Wang et al. 2002) and Clostridium thermocellum Pnk (CthPnk) (Wang et al. 2012; Das et al. 2013b,c), both of which are end-healing components of a multienzyme RNA repair pathway and both of which can use any NTP as the phosphate donor. Crystal structures of CthPnk bound to ATP, GTP, CTP, UTP, and dATP revealed an extensive network of ionic and hydrogen-bonding contacts to the α, β, and γ phosphates. In contrast, there are no enzymic contacts to the ribose and none with the nucleobase other than a π-cation interaction with an arginine side chain. This arginine is conserved in T4 Pnk, where it makes the same π-cation stack on the nucleobase of the donor (Galburt et al. 2002), and it is also present in the kinase domains of AtRNL and the fungal Trl1 enzymes (Wang et al. 2006). Whereas the arginine contributes to donor NTP affinity in CthPnk (Das et al. 2013b), it does not explain the GTP specificity of the fungal Trl1 kinase domain. Our hypothesis, based on our nucleotide analog experiments, is that the fungal kinases make clade-specific hydrogen-bonding interactions with the guanine 6-oxo atom that enforce GTP specificity. This raises the prospect that the fungal kinase donor site can be targeted selectively by a small molecule inhibitor that interacts with the guanine specificity determinant(s) of Trl1 but does not bind to the donor sites of the many other cellular P-loop phosphotransferases, which either have no NTP donor preference or selectively use ATP as substrates.

MATERIALS AND METHODS

AtRNL purification

Recombinant wild-type AtRNL and mutants K152A, K700A-S701A, D726A, and T1001A were produced in Escherichia coli as His10-Smt3 fusions and purified as described (Remus and Shuman 2013). The tagged proteins were isolated from soluble bacterial extracts by Ni-agarose chromatography, followed by treatment with Smt3-specific protease Ulp1 to cleave the tag. After depletion of the tag by adsorption to Ni-agarose, the AtRNL proteins were subjected to gelfiltration through a Superdex-200 column equilibrated in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.05% Triton X-100, and 10% glycerol. Peak AtRNL fractions were pooled and stored at −80°C. Protein concentrations were determined by using the Biorad dye reagent with BSA as the standard.

KlaTrl1 production and purification

The full-length KlaTRL1 open reading frame (GenBank accession CAG98435) and the segment encoding the C-terminal kinase-
Preparation of 3′ 32P-labeled DNA>p substrate

The 3′ 32P-labeled HOD17RpD10 strand was prepared as follows. A 10-mer deoxyribonucleotide HODATTCGAGATGcmp was 5′ 32P-labeled by using [γ32P]ATP and T4 polynucleotide kinase-phosphatase (Pnkp), then gel-purified. The labeled pD10 strand was mixed with an 18-mer CATATCCGTGTCCGCTT(rC)OH strand (D17R) and a complementary 36-mer DNA strand to form a nicked DNA duplex with a monoribonucleotide on the 3′-OH side of the nick and a 3′-P32-labeled deoxyribonucleotide on the 5′-PO4 side of the nick. The nick was ligated by reaction with a 10-fold molar excess of Chlorella virus DNA ligase, 10 mM MgCl2, and 1 mM ATP for 1 h at 22°C to yield an internally 32P-labeled HOD17RpD10 strand. The ligation product was heated for 10 min at 95°C in the presence of a 10-fold molar excess of an unlabeled 36-mer DNA that competes with the radiolabeled ligation product for reannealing to the bridging template DNA strand. This mixture was digested with 50 µg RNase A for 10 min at 37°C, thereby converting the HOD17RpD10 species to a 3′ 32P-labeled HOD17Rp strand, which was gel-purified. HOD17Rp was converted to HOD17R>p by reaction with RNA 3′-terminal cyclase and ATP, then recovered by phenol: chloroform extraction and precipitation with ethanol.

Rapid kinetic assay of KlaTrl1 kinase activity

A Kintek RQF3 rapid chemical quench apparatus was used to assay the reaction of 32P-labeled DNA>p with a 50-fold molar excess of KlaTrl1-(385–813) at 22°C in the range of 0.05- to 1-sec reaction times. The reaction mixtures (40 µL) contained 50 mM Tris-HCl, pH 8.0, 2 mM DTT, 10 mM MgCl2, 100 µM GTP or UTP, 20 nM 32P-labeled 32P-labeled RNA>p substrate, and 1 µM AtRNL. The rapid kinetic measurements were initiated by mixing two buffer solutions (20 µL each). One solution contained 50 mM Tris-HCl, pH 8.0, 2 mM DTT, 10 mM MgCl2, 100 µM GTP or UTP, and 40 nM 32P-labeled RNA>p. The other solution contained 50 mM Tris-HCl, pH 8.0, 2 mM DTT, 10 mM MgCl2, 100 µM GTP or UTP, and 2 µM KlaTrl1-(385–813). The reactions were quenched by rapid mixing with 110 µL of 90% formamide, 50 mM EDTA. The samples were analyzed by urea-PAGE. The 32P-labeled RNAs were quantified by scanning the gel with a Fujix BAS2500 imager.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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