Kinetic mechanism of nick sealing by T4 RNA ligase 2 and effects of 3'-OH base mispairs and damaged base lesions

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ABSTRACT

T4 RNA ligase 2 (Rnl2) repairs 3'-OH/5'-PO₄ nicks in duplex nucleic acids in which the broken 3'-OH strand is RNA. Ligation entails three chemical steps: reaction of Rnl2 with ATP to form a covalent Rnl2-(lysyl-N ζ)-AMP intermediate (step 1); transfer of AMP to the 5'-PO₄ of the nick to form an activated AppN- intermediate (step 2); and attack by the nick 3'-OH on the AppN- strand to form a 3'-5' phosphodiester (step 3). Here we used rapid mix-quench methods to analyze the kinetic mechanism and fidelity of single-turnover nick sealing by Rnl2-AMP. For substrates with correctly base-paired 3'-OH nick termini, k_{step2} was fast (9.5 to 17.9 sec⁻¹) and similar in magnitude to k_{step3} (7.9 to 32 sec⁻¹). Rnl2 fidelity was enforced mainly at the level of step 2 catalysis, whereby 3'-OH base mispairs and oxoguanine, oxoadenine, or abasic lesions opposite the nick 3'-OH elicited severe decrements in the rate of 5'-adenylation and relatively modest slowing of the rate of phosphodiester synthesis. The exception was the noncanonical A:oxoG base pair, which Rnl2 accepted as a correctly paired end for rapid sealing. These results underscore (1) how Rnl2 requires proper positioning of the 3'-terminal ribonucleoside at the nick for optimal 5'-adenylation and (2) the potential for nick-sealing ligases to embed mutations during the repair of oxidative damage.

Keywords: 8-oxoguanine; RNA repair; abasic lesions; transient state kinetics

INTRODUCTION

Bacteriophage T4 RNA ligase 2 (Rnl2) is the exemplar of a distinct clade of ATP-dependent RNA ligases that repair 3'-OH/5'-PO₄ nicks in duplex RNA (Ho and Shuman 2002; Nandakumar et al. 2004, 2006). Rnl2 is also adept at sealing hybrid RNA:DNA substrates in which the 3'-OH strand at the nick is RNA and the 5'-PO₄ strand and template strand are DNA (Nandakumar and Shuman 2004). The Rnl2 enzyme family includes the RNA-editing ligases (RELs) of the protozoan parasites *Trypanosoma* and *Leishmania* (Blanc et al. 1999; Schnauffer et al. 2001; Palazzo et al. 2003; Deng et al. 2004). RELs are essential for kinetoplastid mRNA editing, which involves RNA-guided mRNA incision, RNA templated gap filling by UMP additions to form a duplex nick, and then nick sealing by the ligase. Thus, the RELs are promising drug targets for treatment of trypanosomiasis and leishmaniasis (Amaro et al. 2008; Durrant et al. 2010; Moshiri et al. 2011).

The ligation reaction of Rnl2 entails three nucleotidyl transfer steps akin to those of DNA ligases. In the first step, Rnl2 reacts with ATP in the absence of nucleic acid to form a covalent enzyme-(lysyl-N ζ)-AMP intermediate and re-

lease pyrophosphate. In step 2, Rnl2-AMP binds to the nicked duplex nucleic acid and transfers the adenylate to the 5'-PO₄ terminus to form an adenylated nicked intermediate. In step 3, Rnl2 directs the attack of the nick 3'-OH on the 5'-phosphoanhydride linkage, resulting in a repaired 3'-5' phosphodiester and release of AMP.

T4 Rnl2 is the best-studied RNA ligase, and its properties reflect how the RELs are likely to function during mRNA editing (Nandakumar et al. 2006). T4 Rnl2 and RELs consist of two conserved domains. The structure of their N-terminal adenylyltransferase domains and the architecture of their AMP binding sites are nearly identical and are similar to the adenylyltransferase domains of DNA ligases (Deng et al. 2004; Ho et al. 2004). The adenylyltransferase domain of T4 Rnl2 is competent per se for catalysis of step 1 and step 3, but it is inactive in overall nick sealing and defective in binding to a nicked duplex substrate (Ho et al. 2004; Nandakumar and Shuman 2004, 2005). The Rnl2 C-terminal domain is required for step 2 of the ligation pathway. Crystal structures of Rnl2 have been captured at discrete steps along

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the reaction pathway: the covalent Rnl2–AMP intermediate; Rnl2 bound to an adenylylated nicked duplex, in the state immediately following step 2; and Rnl2 at an adenylylated nick in a state poised for step 3 (Nandakumar et al. 2006). These structures disclosed a distinctive fold for the Rnl2 C-terminal domain, illuminated the stereochemistry of the nucleotidyl transfer steps, and revealed how remodeling of active-site contacts and conformational changes at the nick propel the ligation reaction forward. Extensive mutational analysis of Rnl2 has defined the amino acid functional groups required for each of the three chemical steps of strand joining (Ho and Shuman 2002; Yin et al. 2003; Nandakumar et al. 2004, 2006; Nandakumar and Shuman 2005).

The unique properties of Rnl2 have inspired novel practical applications. The ability of the Rnl2 N-terminal domain to ligate a pre-adenylylated polynucleotide to an RNA 3'-OH terminus has been harnessed for cloning and sequencing small RNAs (Pfeffer et al. 2005; Viollet et al. 2011). Full-length Rnl2 provides for highly efficient synthesis of RNAs containing site-specific modifications or labels by templated joining of multiple oligonucleotides. Indeed, Rnl2 is the best reagent available for templated joining of RNA strands (Nandakumar and Shuman 2004; Bullard and Bowater 2006).

Here, our goal was to explore the kinetic mechanism of Rnl2, by implementing rapid mix-quench methods to analyze the transient-state kinetics of nick sealing by pre-formed Rnl2–adenylate. After deriving rate constants for the 5'-adenylation (step 2) and phosphodiester synthesis (step 3) reactions, we proceeded to gauge the effects of base mispairs and base damage at the nick 3'-OH terminus on the kinetics of nick sealing. Our studies provide new insights to ligase fidelity and the potential for ligase-mediated mutagenesis during the repair of 3'-OH RNA nicks.

RESULTS AND DISCUSSION

Kinetic mechanism of nick sealing by Rnl2

We and others have applied rapid mix-quench methods to study the transient state kinetics of DNA nick sealing by ATP-dependent DNA ligases (Lohman et al. 2011; Samai and Shuman 2011a,b, 2012; Taylor et al. 2011). Here we extended this approach to T4 Rnl2. The substrates we used were singly nicked duplexes composed of an 18-mer RNA_{OH} strand and an 18-mer pDNA strand (labeled with ³²P at the nick 5'-PO₄) annealed to a 36-mer DNA template strand (Fig. 1A). We varied the identity of the N:X base pair at the nick 3'-OH end, as U:A, A:T, G:C, C:G, or C:I. The nicked duplexes (100 nM) were reacted with a 20-fold molar excess of Rnl2–AMP (2 μM) in the presence of 10 mM Mg²⁺ but in the absence of ATP. Omission of ATP restricts the system to one round of catalysis of 5'-adenylation (step 2) and phosphodiester formation (step 3). The reactions were quenched with EDTA and formamide, and the products were analyzed by urea-PAGE. An exemplary product analysis

FIGURE 1. Kinetics of single-turnover nick sealing by Rnl2 at correctly paired 3'-OH ends. (A) The singly nicked duplex substrate for sealing by Rnl2–AMP is shown with the 5' ³²P-label at the nick denoted by •. The 3'-OH strand is all RNA. The 3' N:X base pair at the nick (highlighted in the shaded box) is variable. A product analysis by denaturing PAGE of a sealing reaction of Rnl2 at a 3' C:I nick (where I is inosine) is shown. The times at which the reactions were quenched are specified above the lanes. The positions of the radiolabeled 18-mer 5'-PO₄ strand (pNick), the 5'-adenylylated intermediate (AppNick), and the sealed product are indicated on the left. (B) The distributions of radiolabeled AppNick intermediate and sealed product during the reaction of Rnl2 at a 3' C:I nick are plotted as a function of time. Each datum in the graph is the average of three separate experiments. The curve fits to the kinetic scheme are shown. (C) The step 2 and step 3 rate constants for sealing nicks with the indicated 3' N:X pairs.

for the 3' C:I nicked duplex is shown in Figure 1A. The use of a Kintek rapid mix-quench apparatus accessed the reaction on a millisecond timescale, which was necessary to observe the formation and intermediacy of the AppDNA species en route to the sealed 36-mer RNApDNA product (Fig. 1A). The intermediate and sealed products were quantified and plotted as a function of reaction time (see Fig. 1B for the 3' C:I substrate), with each datum being the average of three independent

experiments. The kinetic profiles were fitted in MATLAB to a unidirectional scheme of 5'-PO₄ adenylation (step 2: Rnl2-AMP • pNick → Rnl2 • AppNick) and subsequent phosphodiester synthesis (step 3: Rnl2 • AppNick → Rnl2 • AMP • RNApDNA), with allowance for reversible branching of Rnl2 • AppNick to an “out-of-pathway” state, as described in our prior studies of *Chlorella* virus DNA ligase (Samai and Shuman 2011a). The observed step 2 and step 3 rate constants (k_{step2} and k_{step3}) for the series of correctly paired 3' N:X nicks are shown in Figure 1C. (Control experiments with a 3' U:A nicked duplex verified that the regime of 20-fold Rnl2-AMP excess sufficed to attain pseudo-first-order kinetics [i.e., the rate of the reaction was not responsive to a twofold increase or a twofold decrease in ligase concentration], signifying that initial binding of Rnl2-AMP to the nick was not rate-limiting.)

The salient theme from the kinetic analysis is that rates of step 2 and step 3 are rapid and similar in magnitude for the nicks with a correctly paired 3'-OH end. When the base pair at the nick 3'-OH terminus was U:A (mimicking the 3'-OH U:A nick terminus after the last uridylylate addition across from A in the guide RNA during mRNA editing), we derived rate constants of $k_{\text{step2}} = 14.3 \text{ sec}^{-1}$ and $k_{\text{step3}} = 15.6 \text{ sec}^{-1}$. The rates for the 3' A:T substrate were similar ($k_{\text{step2}} = 17.9 \text{ sec}^{-1}$ and $k_{\text{step3}} = 18.8 \text{ sec}^{-1}$), as were those for the 3' G:C nick ($k_{\text{step2}} = 9.5 \text{ sec}^{-1}$ and $k_{\text{step3}} = 14.8 \text{ sec}^{-1}$) and the 3' C:I nick ($k_{\text{step2}} = 9.9 \text{ sec}^{-1}$ and $k_{\text{step3}} = 7.9 \text{ sec}^{-1}$). Only in the case of the 3' C:G nick ($k_{\text{step2}} = 12.6 \text{ sec}^{-1}$ and $k_{\text{step3}} = 32 \text{ sec}^{-1}$) was the rate of step 3 more than twofold faster than step 2. These results indicate that Rnl2 has little specificity for the 3'-OH nucleobase or base pair when sealing a 3'-OH/5'-PO₄ nick.

It was instructive to compare the results for Rnl2 with the kinetic parameters for single-turnover sealing of DNA nicks by ATP-dependent DNA ligases. The observed step 2 and step 3 rate constants for *Chlorella* virus DNA ligase (ChVLig) were 2.4 sec^{-1} and 25 sec^{-1} , respectively (Samai and Shuman 2011b, 2012). Thus, for ChVLig, the attack of the nick 3'-OH on AppDNA is an order of magnitude faster than the formation of AppDNA. Similar single-turnover kinetic studies were reported for human DNA ligase I ($k_{\text{step2}} = 2.6 \text{ sec}^{-1}$; $k_{\text{step3}} = 12 \text{ sec}^{-1}$) (Taylor et al. 2011) and T4 DNA ligase ($k_{\text{step2}} = 5.3 \text{ sec}^{-1}$; $k_{\text{step3}} = 38 \text{ sec}^{-1}$) (Lohman et al. 2011). It would appear that diverse ATP-dependent DNA ligases have similar kinetic properties with respect to steps 2 and 3, whereby step 2 is rate limiting. In contrast, Rnl2 catalysis of step 2 is faster than that of DNA ligases and is not disproportionate to the step 3 rate.

Rnl2 fidelity: effect of 3'-base mispairs on the kinetics of nick sealing

“Fidelity” refers to the capacity of a ligase to discriminate in the sealing of substrates containing paired versus mispaired bases flanking the nick. Different DNA ligases display distinct patterns of fidelity, with respect to how well the ligase discrim-

inates and the hierarchy of which mispairs are or are not accepted by the ligase (Tomkinson et al. 1992; Husain et al. 1995; Shuman 1995; Luo et al. 1996; Sriskanda and Shuman 1998; Bhagwat et al. 1999; Tong et al. 2000; Nakatani et al. 2002; Lamarche et al. 2005; Wang et al. 2007a). The consistent theme is that DNA ligase fidelity is greatest on the 3'-OH side of the nick. High-fidelity DNA ligases have been used to develop detection/amplification methods for molecular diagnostics, including the detection of single-nucleotide polymorphisms and disease-associated mutations (Barany 1991; Li et al. 2005). Ligase-based detection entails the annealing of specific 3'-OH and 5'-PO₄ polynucleotide probes complementary to adjacent sequences in the gene of interest in a biological sample so that they form a duplex nick. For the method to work perfectly, the ligase must link the two adjacent probes only when the nucleotides at the nick are correctly base-paired. Because Rnl2 is naturally adept at ligating nicks templated by RNA, it could be a useful reagent for detecting RNA sequence variations based on similar principles. The caveat is that the fidelity of Rnl2 is uncharted territory. What is known is that the RNA sealing activity of Rnl2 is reduced drastically when the duplex substrate contains either a 1-nucleotide (nt) gap or unpaired 1-nt “flaps” at the 3'-OH or 5'-PO₄ end instead of a correctly paired nick (Nandakumar et al. 2004).

Here we evaluated the fidelity of Rnl2, focusing on the effects of 3'-OH base mispairs on the transient state kinetics of nick sealing. First, we gauged the effects of all 3' N:purine mispairs, including those to inosine. (The inosine experiments are pertinent to whether Rnl2 can provide a ligase-detection assay for sites of A-to-I editing in biological RNA samples.) An exemplary kinetic profile for single-turnover sealing of a 3' A:A mispaired nick is shown in Figure 2. Whereas the A:A mispair did not affect the yield of ligated product, it did slow the reaction, such that the endpoint was attained at 30 sec. The step 2 and step 3 rate constants for sealing the 3' A:A mispair (0.22 sec^{-1} and 1.9 sec^{-1}) were 65-fold and eightfold slower than the respective step 2 and 3 rates for a correctly paired 3' U:A nick (Fig. 2). Thus, the 3' A:A mispair exerted a more profound impact on the 5'-adenylation reaction than on the subsequent step of phosphodiester synthesis. A similar selective effect on step 2 rate versus step 3 was seen for the 3' C:A ($k_{\text{step2}} = 0.34 \text{ sec}^{-1}$; $k_{\text{step3}} = 4.7 \text{ sec}^{-1}$) and 3' G:A ($k_{\text{step2}} = 0.43 \text{ sec}^{-1}$; $k_{\text{step3}} = 2.0 \text{ sec}^{-1}$) mispairs.

The component rates of sealing of the 3' U:G mispaired nick ($k_{\text{step2}} = 0.42 \text{ sec}^{-1}$; $k_{\text{step3}} = 7.0 \text{ sec}^{-1}$) were 30-fold and fivefold slower than the rates for the correctly paired 3' C:G nick (Fig. 2), again highlighting selective impact of a 3'-perturbation on adenylation of the 5'-PO₄. The selectivity was progressively less for the 3' A:G mispair ($k_{\text{step2}} = 0.71 \text{ sec}^{-1}$; $k_{\text{step3}} = 4.1 \text{ sec}^{-1}$) and the 3' G:G mispair ($k_{\text{step2}} = 0.53 \text{ sec}^{-1}$; $k_{\text{step3}} = 1.4 \text{ sec}^{-1}$), where the step 2 defects were of the same magnitude, but the step 3 defects were greater (Fig. 2). The 3' inosine mispairs U:I ($k_{\text{step2}} = 0.86 \text{ sec}^{-1}$; $k_{\text{step3}} = 3.2 \text{ sec}^{-1}$), A:I ($k_{\text{step2}} = 1.5 \text{ sec}^{-1}$; $k_{\text{step3}} = 4.5 \text{ sec}^{-1}$) and G:I

FIGURE 2. Effects of 3'-OH N:purine mispairs on the kinetics of nick sealing. (Top panel) The distributions of radiolabeled AppNick intermediate and sealed product during the reaction of Rnl2 at a 3' A:A mispaired nick are plotted versus time. Each datum is the average of three separate experiments. The curve fits are shown. (Bottom panel) The step 2 and step 3 rate constants for sealing nicks with the indicated 3'-OH N:purine configurations.

($k_{\text{step2}} = 0.60 \text{ sec}^{-1}$; $k_{\text{step3}} = 2.8 \text{ sec}^{-1}$) slowed step 2 by 12-fold, sevenfold, and 17-fold compared with the correctly paired 3' C:I nick. The step 3 rates were slowed by only two-fold to threefold.

We proceeded to analyze the effects of 3' N:pyrimidine mispairs. The kinetic profile for single-turnover sealing of a 3' C:C mispaired nick is shown in Figure 3. The C:C mispair did not affect the yield of ligated product, but it depressed the reaction rate, such that the endpoint was attained at 80 sec. The step 2 and 3 rate constants for sealing the 3' C:C mispair (0.054 sec^{-1} and 3.0 sec^{-1}) were 175-fold and fivefold slower than the rates for a correctly paired 3' G:C nick (Fig. 3). The 3'-OH C:C nick was the most deleterious of all mispairs with respect to step 2 catalysis. The five other 3' N:C and N:T mispairs slowed step 2 by factors ranging from sixfold to 55-fold compared with the correct G:C or A:T pairs. All six 3' N:C and N:T mispairs exerted relatively modest effects on step 3 rate (2.5-fold to six-fold decrements compared with G:C or A:T).

Effects of damaged base lesions on the kinetics of nick sealing by Rnl2

Oxidative damage to purine nucleobases in nucleic acids generates 8-oxoguanine (oxoG) and 8-oxoadenine (oxoA). The

effects and metabolism of oxoG in DNA have been studied intensively in light of its mutagenic properties, which ensue from the ability of oxoG in the *syn* nucleoside conformation to mispair with adenine (see Fig. 4). This pairing prompts DNA polymerases to embed A:oxoG mispairs, which can ultimately lead to G $\rightarrow$ T transversions if not corrected. RNA is also sensitive to oxidative base damage. Indeed, the frequency of oxoG lesions in total RNA is reported to be higher than in total DNA, and several studies correlate elevated RNA oxoG levels with human neurodegenerative diseases (for review, see Wurtmann and Wolin 2009). Kinetoplastid mRNA editing occurs in the oxidative milieu of the mitochondrion, where the guide-RNA purine-tract template for U insertion might acquire 8-oxopurine lesions that could affect the efficiency and fidelity of RNA gap-filling and, in turn, influence the efficiency and outcome of the final ligation step executed by Rnl2-like RELs. Were polynucleotide ligases to seal mispairs at 8-oxopurines in the template strand, this would be promutagenic at the DNA level and might result in misediting of kinetoplastid mRNAs. There have been reports of oxopurine effects on T4 and human DNA ligases (Hashimoto et al. 2004; Zhao et al. 2007), but no studies of oxidative lesion effects on RNA joining.

To fill this knowledge gap, we determined the transient-state kinetics of T4 Rnl2 in sealing nicked duplexes with oxoG and oxoA bases in the template strand opposite the 3'-OH ribonucleotide at the nick. By testing all N:oxoG and

FIGURE 3. Effects of 3'-OH N:pyrimidine mispairs on the kinetics of nick sealing. (Top panel) The distributions of radiolabeled AppNick intermediate and sealed product during the reaction of Rnl2 at a 3' C:C mispaired nick are plotted versus time. Each datum is the average of three separate kinetic experiments. The curve fits to the kinetic scheme are shown. (Bottom panel) The step 2 and step 3 rate constants for sealing nicks with the indicated 3'-OH N:pyrimidine configurations.

FIGURE 4. Sealing of nicks with 3' N:oxopurine lesions. (Top panel) The distributions of radiolabeled AppNick intermediate and sealed product during the reaction of Rnl2 at a 3' A:oxoG nick are plotted versus time. Each datum is the average of three separate kinetic experiments. The curve fits are shown. (Inset) The chemical structure of the A:oxoG pair with oxoG in the *syn* conformation; (dashed lines) hydrogen-bonding contacts. (Bottom panel) The step 2 and step 3 rate constants for sealing nicks with the indicated 3'-OH N:oxoG and N:oxoA configurations.

N:oxoA pairs and then comparing the rate constants for sealing these damaged substrates to the rates for the corresponding undamaged N:G and N:A nicks, we aimed to answer the question of whether certain damaged mispair configurations are more or less prone to “unfaithful” sealing by Rnl2. The kinetic profile for sealing of the 3' A:oxoG substrate is shown in Figure 4. The apparent step 2 and step 3 rate constants of 14.4 sec^{-1} and 17.8 sec^{-1} were typical of a correctly paired nick and were much faster than the rates of sealing the undamaged 3' A:G nick (0.71 sec^{-1} and 4.1 sec^{-1}). Thus, Rnl2 accepts the 3' A:oxoG mispair as a properly paired nick. Indeed, Rnl2 seals the A:oxoG nick as well as when the template oxoG is correctly paired with C ($k_{\text{step2}} = 13.5 \text{ sec}^{-1}$; $k_{\text{step3}} = 15.3 \text{ sec}^{-1}$). In contrast, the U:oxoG ($k_{\text{step2}} = 0.28 \text{ sec}^{-1}$; $k_{\text{step3}} = 2.9 \text{ sec}^{-1}$) and G:oxoG ($k_{\text{step2}} = 0.43 \text{ sec}^{-1}$; $k_{\text{step3}} = 2.1 \text{ sec}^{-1}$) termini slowed the rates of step 2 and step 3 catalysis (Fig. 4) to a degree similar to that seen for the undamaged U:G and G:G mispairs (Fig. 2). We infer that (1) an 8-oxo atom in guanine is not deleterious per se, and (2) the consequence of oxoG in the template strand across from the nick 3'-OH is to enable the embedding of rAMP in lieu of rCMP at the repair junction.

The rates of sealing of the 3' N:oxoA substrates are shown in Figure 4. The paired U:oxoA nick was an effective substrate for Rnl2 ($k_{\text{step2}} = 8.4 \text{ sec}^{-1}$; $k_{\text{step3}} = 12.3 \text{ sec}^{-1}$). The A:oxoA ($k_{\text{step2}} = 0.88 \text{ sec}^{-1}$; $k_{\text{step3}} = 2.4 \text{ sec}^{-1}$) and G:oxoA ($k_{\text{step2}} = 0.90 \text{ sec}^{-1}$; $k_{\text{step3}} = 2.3 \text{ sec}^{-1}$) mispairs were sealed at the

same slowed rates as each other, and they displayed rate decrements in the range of those seen for the undamaged A:A and G:A mispairs. The C:oxoA mispair was the most deleterious configuration ($k_{\text{step2}} = 0.27 \text{ sec}^{-1}$; $k_{\text{step3}} = 0.99 \text{ sec}^{-1}$), especially with respect to the step 3 rate. Thus, Rnl2 does not recognize any but U as the proper partner for pairing with oxoA in the template strand across from the nick 3'-OH.

Effects of abasic sites on the kinetics of nick sealing

Abasic sites formed during base excision repair are among the most common DNA lesions and represent a challenge to genome integrity, e.g., by causing polymerases to stall, slip, or misincorporate at an abasic site in the template strand. How ligases contend with abasic sites is an open question. Here we endeavored to answer it for Rnl2 via single-turnover kinetic analysis of the sealing of nicked duplexes with THF (tetrahydrofuran) abasic sites in the template strand opposite the 3'-OH ribonucleotide at the nick. All N:abasic configurations were tested. We envisioned that comparison of the step 2 and step 3 rate constants for N:abasic nicks with those of the correctly paired N:X substrates, and to N:X mispairs, would reveal, and potentially distinguish, the effects of having no base-pairing to template at the nick versus the effects of mispairing.

The kinetic profile for Rnl2 sealing of the 3' A:abasic substrate is shown in Figure 5, along with the rate parameters for all four 3' N:abasic nicks. The template abasic lesion was deleterious no matter the 3'-OH nucleotide across from it, especially with respect to the rate of step 2 catalysis. The order of preference by Rnl2 for the N base opposite the abasic template was $A > G > C > U$, with k_{step2} values spanning a 10-fold range from 0.87 sec^{-1} for A:abasic to 0.08 sec^{-1} for

FIGURE 5. Sealing of nicks with 3' N:abasic lesions. (Top panel) The distributions of radiolabeled AppNick intermediate and sealed product during the reaction of Rnl2 at a 3' A:abasic nick are plotted versus time. Each datum is the average of three separate kinetic experiments. The curve fits are shown. (Bottom panel) The step 2 and step 3 rate constants for sealing nicks with the indicated 3'-OH N:abasic configurations are shown.

U:abasic nicks (Fig. 5). The step 2 and step 3 rates for the A:abasic substrate were similar to those for the A:G, A:I, and A:C nicks, signifying that absence of a base across from the 3'-OH A had much the same effect as any of these several A:N mispairs. The effects of the G:abasic lesion ($k_{\text{step2}} = 0.29 \text{ sec}^{-1}$; $k_{\text{step3}} = 2.3 \text{ sec}^{-1}$) were similar to those of G:A, G:G, and G:I mispairs. In contrast, the U:abasic nick had a more detrimental effect on the step 2 rate (0.08 sec^{-1}) than did any of the U:N mispairs: U:G (0.42 sec^{-1}), U:I (0.86 sec^{-1}), U:C (0.43 sec^{-1}), and U:T (0.32 sec^{-1}). The step 2 defect of the C:abasic nick (0.15 sec^{-1}) was in the mid-range of those seen for C:N mispairs ($0.054\text{--}0.34 \text{ sec}^{-1}$).

Conclusions and implications

Here we elucidated the kinetic mechanism of single-turnover nick sealing by T4 Rnl2-AMP and determined the effects of 3'-OH mispairs and base damage lesions on the rates of nick 5'-adenylation and phosphodiester synthesis. The pertinent findings with respect to the sealing of perfectly paired nicks were that the rates of step 2 catalysis were rapid ($9.5\text{--}17.9 \text{ sec}^{-1}$) and similar in magnitude to the step 3 rates ($7.9\text{--}32 \text{ sec}^{-1}$). Rnl2 was kinetically sensitive to all 3'-OH base mispairs. The consistent and instructive results were that fidelity was manifest most strongly at the level of step 2 catalysis, whereby base mispairs elicited more severe decrements in the rate of 5'-adenylation than phosphodiester synthesis. We surmise that Rnl2 is acutely dependent on proper positioning of the 3'-terminal ribonucleoside to attain optimal rates of 5'-adenylation and that the mispair effects on step 2 rate reflect the degree to which the nick is structurally perturbed. This view is consistent with prior studies showing that (1) the majority of the atomic contacts of Rnl2 with the nicked duplex substrate are located on the 3'-OH side of the nick (Nandakumar et al. 2006); and (2) replacing the terminal 3'-OH at the nick with a 3'-H slows step 2 by a factor of 1000, even though the 3'-OH is not chemically transformed during the step 2 reaction (Nandakumar and Shuman 2005). Step 3 catalysis is also affected by modifications at the terminal sugar; to wit, replacing the 2'-OH at the nick with 2'-H slows step 3 by a factor of 25–35, with little effect on step 2 (Nandakumar and Shuman 2005).

Our evaluation of the effects of oxopurines and abasic sites in the template strand opposite the nick 3'-OH underscored the importance of a correctly positioned 3'-OH ribonucleotide for nick sealing by Rnl2. This was most evident in the “gain-of function” elicited by a noncanonical 3' A:oxoG pair (Fig. 4) that Rnl2 readily accepts for rapid sealing.

Rnl2 displayed a hierarchy of mispair effects on the rates of nick sealing under single-turnover conditions, whereby C:C and A:A were the most detrimental. Thus, Rnl2 could, in principle, be a suitable reagent for RNA mutation detection. However, because the yield of ligated product is not affected by 3'-OH mispairs with the template strand, successful implementation would require optimization of reaction times

and conditions, for example, by performing the ligations under steady-state conditions (at substoichiometric Rnl2 concentrations in the presence of ATP) and exploring how variations in reaction components might enhance ligase fidelity (Bhagwat et al. 1999).

Despite the wealth of information concerning the structure and biochemical properties of T4 Rnl2 and its value as a molecular biology reagent, we remain ignorant of its biological role during T4 infection. It is noteworthy that phage T4 encodes two RNA ligases with quite different substrate specificities. RNA ligase 1 (Rnl1) was discovered and purified in 1972 based on its ability to seal single-stranded RNA ends (Silber et al. 1972). Rnl1 quickly became an essential reagent for RNA 3'-radiolabeling (England and Uhlenbeck 1978), notwithstanding that its biological function remained obscure until 1987, when it was shown that Rnl1 collaborates with T4 polynucleotide kinase to repair a break in the anticodon loop of tRNA^{Lys} inflicted by the *Escherichia coli* antiviral ribotoxin PrrC (Amitsur et al. 1987). It was subsequently demonstrated that T4 Rnl1 has high inherent specificity for sealing breaks in the tRNA anticodon loop (Wang et al. 2007b). In contrast, T4 Rnl2 is most adept at sealing nicked duplex substrates in which the 3'-OH strand is RNA and, as shown here, the nick 3'-OH end is correctly paired. Given the strong structural and biochemical conservation of Rnl2 and RELs, it is reasonable to invoke a role for Rnl2 in the repair of breaks in RNA duplexes, generated perhaps as part of an RNA-damaging host response to virus infection (analogous to tRNA restriction by PrrC). The damage might be targeted to a pre-existing stable duplex segment of a bacterial RNA or entail guide-RNA mediated incision of a single-stranded segment of a viral RNA, akin to guided RNA slicing by Argonaute proteins or CRISPR RNA-guided targeting of foreign nucleic acids, including RNA targets (Hale et al. 2009, 2012). We are attracted to the idea that Rnl2 enzymes encoded by T4 and other DNA viruses are purposed to antagonize RNA-targeting CRISPR systems that provide acquired host immunity to infection. Also, the ability of Rnl2 to efficiently and rapidly seal RNA nicks when the template strand is DNA might come into play in repairing RNA damage inflicted by bacterial Argonautes that use DNA guides to direct endonucleolytic cleavage of RNA targets (Yuan et al. 2005; Wang et al. 2008).

MATERIALS AND METHODS

T4 Rnl2-AMP

His₁₀Rnl2 was produced in *E. coli* BL21(DE3) and purified by Ni-agarose affinity chromatography (Nandakumar et al. 2004). The protein concentration was determined with the Bio-Rad dye reagent using bovine serum albumin as the standard. The recombinant Rnl2 preparation comprises a mixture of ligase apoenzyme and enzyme-adenylate intermediate. The concentration of Rnl2-AMP was determined by measuring ATP-independent sealing of a singly nicked 36-bp substrate (with a 3'-OH C:G pair at the nick) as a function of

input Rnl2, whereby there is a 1:1 correspondence between molar yield of ligated 36-mer and the molar amount of catalytically active Rnl2-AMP in the reaction mixture. From the slope of the titration curve in the linear range of Rnl2 dependence, we determined that 45% of the enzyme was pre-adenylylated and active in nick sealing.

Nicked duplex substrates

RNA oligonucleotides were purchased from Dharmacon and deprotected as instructed by the vendor. Unmodified DNA oligonucleotides and DNAs containing single inosine, 8-oxoguanine, 8-oxoadenine, or THF abasic nucleoside modifications were purchased from Eurofins MWG Operon. The 18-mer DNA strand was 5' ³²P-labeled using T4 polynucleotide kinase and [γ -³²P]ATP and then purified by electrophoresis through a nondenaturing 18% polyacrylamide gel. To form the nicked substrates, mixtures of 18-mer 5'-PO₄ strand, 18-mer 3'-OH RNA strand, and 36-mer complementary template strand were annealed at a molar ratio of 1:5:2 in 200 mM NaCl, 10 mM Tris-HCl (pH 6.8), and 1 mM EDTA by serial incubation for 10 min at 65°C, 15 min at 37°C, and 30 min at 22°C. The substrates were stored at -20°C and thawed on ice immediately prior to use.

Kinetics of single-turnover nick sealing

A Kintek RQF3 rapid chemical quench apparatus was used to assay the reaction of a ³²P-labeled nicked duplex with a 20-fold molar excess of pre-formed Rnl2-AMP at 22°C in the absence of added ATP. The rapid kinetic measurements were initiated by mixing two solutions (20 μ L each) of 50 mM Tris acetate (pH 6.5), 5 mM DTT, 10 mM MgCl₂, and 40 mM NaCl, containing 0.2 μ M nicked duplex substrate and 4 μ M Rnl2-AMP, respectively. The reactions were quenched by rapid mixing with 110 μ L of 90% formamide, 40 mM EDTA. The products were analyzed by urea-PAGE, and the distribution of ³²P-labeled nucleic acids (as sealed 36-mer RnapDNA product, 18-mer AppDNA intermediate, and residual 18-mer pDNA substrate) was quantified by scanning the gel with a Fujix BAS2500 imager. Each experiment was repeated in triplicate. The distributions of AppDNA intermediate and sealed product were plotted as a function of reaction time. As noted previously for *Chlorella* virus DNA ligase (Samai and Shuman 2011a), our initial modeling of the data in Prism to a simple sequential reaction pathway described by two rate constants—for 5'-adenylylation (step 2) and phosphodiester synthesis (step 3)—yielded poor fits to the experimental kinetic profiles for the 5'-adenylylated intermediate, the level of which typically declined more slowly than could be simulated by the simple sequential pathway. To better model the experimental profiles for AppDNA and sealed product, we used the kinetic scheme described for *Chlorella* virus DNA ligase (Samai and Shuman 2011a), which includes a reversible transition of the step 2 product from an active “in pathway” state to an inactive “out of pathway” state. Simulations and curve fitting to the experimental data to this model were performed in MATLAB by inputting an initial k_{step2} value (obtained by plotting the sum of AppDNA plus sealed RnapDNA as a function of time and fitting the data to a single exponential in Prism) and stipulating that all other rates be greater than zero. Exemplary curve fits are shown in Figures 1–5. The step 2 and step 3 rate constants are compiled in the figures; the error values represent one standard deviation.

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