Dual Mechanisms whereby a Broken RNA End Assists the Catalysis of Its Repair by T4 RNA Ligase 2*

Jayakrishnan Nandakumar and Stewart Shuman†
From the Molecular Biology Program, Sloan-Kettering Institute, New York, New York 10021

T4 RNA ligase 2 (Rnl2) efficiently seals 3′-OH/5′-PO4 RNA nicks via three nucleotidyl transfer steps. Here we show that the terminal 3′-OH at the nick accelerates the second step of the ligase pathway (adenylylation of the 5′-PO4 strand) by a factor of 1000, even though the 3′-OH is not chemically transformed during the reaction. Also, the terminal 2′-OH at the nick accelerates the third step (attack of the 3′-OH on the 5′-adenylated strand to form a phosphodiester) by a factor of 25–35, even though the 2′-OH is not chemically reactive. His-37 of Rnl2 is uniquely required for step 3, providing a −106 rate acceleration. Biochemical epistasis experiments show that His-37 and the RNA 2′-OH act independently. We conclude that the broken RNA end promotes catalysis of its own repair by Rnl2 via two mechanisms, one of which (enhancement of step 3 by the 2′-OH) is specific to RNA ligation. Substrate-assisted catalysis provides a potential biochemical checkpoint during nucleic acid repair.

DNA ligases highlight a common fold and a conserved AMP-binding pocket composed of five peptide motifs (I, III, IIIa, IV, and V) (reviewed in Ref. 11). The motif I peptide, KXD/H/G, contains the lysine to which the AMP becomes covalently attached in step 1 of the ligase pathway. Studies of exemplary DNA ligases have highlighted a requirement for covalent adenylylation of the enzyme to recognize the 5′-PO4 nick structure (12, 13); this requirement has been attributed to a direct role of the adenylylate ribose sugar in coordinating the 5′-PO4 at the nick (14). T4 Rnl2 must also be adenylated at Lys-35 of the 35KHXG motif to stably bind to a nicked duplex ligand (10).

T4 Rnl2 is incapable of sealing nicked duplex DNA (9, 10). The RNA specificity of Rnl2 arises from a requirement for at least two ribonucleotides immediately flanking the 3′-OH of the nick (10). The rest of the nicked duplex can be replaced by DNA. The two key ribonucleotides play distinct roles in the ligation reaction, as surmised from the effects of sugar modifications on the ability of Rnl2-AMP to seal 3′-OH/5′-PO4 nicks under single-turnover conditions. Whereas the penultimate 2′-OH is important for nick recognition, the terminal 2′-OH at the nick is important for the attack of the 3′-OH on the 5′-adenylated strand to form a phosphodiester, but seemingly dispensable for nick recognition and adenylylation of the 5′-PO4 strand (10). Because the 2′-OH is not a chemical reactant in the sealing step, these results provided initial evidence for substrate-assisted catalysis during ligation by Rnl2. What role does the enzyme play in the sealing step? Preliminary studies indicated that the H37A mutation of motif I (35KHXG) selectively impaired the sealing step of the reaction of Rnl2-AMP with an 18-mer single-stranded 5′-PO4 RNA substrate, resulting in accumulation of the AppRNA intermediate (1, 6).

Here, we use chemically modified nicked substrates, mutant enzymes, and transient-state kinetic analysis to address the following mechanistic questions: What are the quantitative contributions of His-37, the ribose hydroxyls at the nick 3′ terminus, and the divalent cation during the catalysis of the 5′-adenyllylation and/or phosphodiester synthesis steps of the ligase reaction? What are the contributions of Lys-35 and the C-terminal domain of Rnl2 to the step 3 reaction? Is the requirement for a ribonucleotide on the 3′-OH side of the nick inherent to the N-terminal catalytic domain, or is it imposed by the C-terminal domain? Do the functional groups implicated in step 2 and step 3 catalysis act in concert to facilitate a single rate-limiting event or in parallel to facilitate different component substeps of the reactions?

EXPERIMENTAL PROCEDURES

Recombinant Rnl2 Proteins—Full-length wild-type Rnl2 and mutants R266A and D292A, and the N-terminal domain Rnl2-(1–249) were produced in Escherichia coli BL21(DE3) as His6-tagged fusions and purified from soluble bacterial extracts by nickel-agarose chromatography as described previously (2, 9). Gene fragments encoding N-terminal domain mutants Rnl2-(1–249)-K35A and Rnl2-(1–249)-H37A were amplified by PCR from plasmid templates containing the full-length K35A

* This work was supported in part by Grant GM63611 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† American Cancer Society Research Professor. To whom correspondence should be addressed. Fax: 212-717-3623; E-mail: s-shuman@ski.mskcc.org.

1 The abbreviations used are: AppRNA, RNA-adenylate; AppDNA, DNA-adenylate; pDNA, 5-PO4 DNA.

This paper is available online at http://www.jbc.org

23484
mixtures contained 250 nM wild-type Rnl2 (containing a stop codon in lieu of the Ala-250 codon and a BamH restriction site immediately 3' of the new stop codon. The PCR products were digested with BamH and NdeI and then inserted into pET16b (Novagen). The inserts of the mutant pET-RNL2 (1–249)-Ala plasmids were sequenced with BamHI and NdeI and then inserted into pET16b (Novagen). The distribution of ligated 24-mer product and the AppDNA intermediate, expressed as the fraction of the total 32P-labeled material, was quantified by scanning the gel with a Fujix BAS2500 imager.

**Ligation at a Preadenylated Nick**—Reaction mixtures (80 μl) containing 50 mM Tris acetate (pH 6.5), 10 mM MgCl2, 250 nM Rnl2 (corresponding to 125 nM Rnl2-AMP), and 50 nM 5'-adenylated nicked duplex substrate were incubated at 22 °C. Aliquots (10 μl) were withdrawn at the times specified and quenched immediately with formamide/EDTA. The products were resolved by denaturing PAGE, as described above. The accumulation of the ligated product, expressed as the fraction of the total 32P-labeled material, was quantified by scanning the gel.

### RESULTS

The RNA 2'-OH and His-37 Promote Phosphodiester Synthesis by Distinct Mechanisms—We showed previously that Rnl2 requires only two terminal ribonucleotides on the 3'-OH strand for optimal nick sealing activity and that the penultimate ribonucleotide can be replaced by a 2'-OCH3 nucleotide with no ill effect (10). Here, we prepared singly nicked 24-bp substrates in which the 12-mer 5'-OH strand and the 24-mer template strand were all DNA, and the 12-mer 3'-OH strand consisted of 10 deoxyribonucleotides at the 5'-end, a 2'-OCH3 nucleotide at the penultimate position flanking the 3'-OH end, and a terminal 3'-OH nucleoside containing either a 2'-OH or a 2'-H (Fig. 1). We examined the kinetics of single-turnover nick ligation of these substrates by Rnl2-AMP under conditions of enzyme excess, in the absence of exogenous ATP. The omission of ATP ensures that sealing will be limited to a single round of catalysis by preadenylated Rnl2-AMP, which comprised 50% of the protein in the purified Rnl2 preparation. The sealing of the 2'-OH substrate by wild-type Rnl2 was rapid and efficient; 98% of the input 32P-labeled 12-mer DNA strand was converted to a ligated 24-mer product within 1 min, and no DNA-adenylate intermediate was detected (not shown). The reaction attained 82% of the end point value after 5 s, which was the earliest time analyzed. From this datum, we calculated an apparent rate constant of 0.37 s⁻¹ for the composite step 2 + 3 reaction.

The H37A mutation of Rnl2 slowed step 3 of the single-turnover ligation reaction at a 2'-OH nick, resulting in the transient accumulation of high levels of the DNA-adenylate intermediate (Fig. 1B). DNA-adenylate comprised 78% of the total labeled DNA after 15 s, at which time only 5% of the substrate was sealed. Because the extent of consumption of the input 12-mer substrate DNA strand (the sum of DNA-adenylate and ligated

---

**FIG. 1.** Effects of a 3'-terminal deoxyribonucleotide and the H37A mutation on the kinetics of single-turnover ligation. Reaction mixtures contained 250 nM wild-type Rnl2 (containing a 3'-terminal deoxyribonucleotide (B) or deoxyribonucleotide (A and C) at the nick. The distribution of ligated 24-mer product and the AppDNA intermediate, expressed as the fraction of the total 32P-labeled material, was plotted as a function of time. The structure of the annealed three-piece substrate is illustrated below the graphs.
RNA Ligase Mechanism

Analysis of Phosphodiester Synthesis at a Preadenylated Nick—Step 3 of the Rnl2 ligation reaction was assayed in isolation using a preadenylated nicked duplex substrate (Fig. 2). The adenylated strand used to form this substrate was synthesized by ligase-mediated AMP transfer from the H37A mutant to the 5'-adenylated 12-mer DNA strand at a nick containing a 3'-OH/2'-H terminus, i.e., using conditions that we showed were conducive to capturing high levels of the AppDNA intermediate. The radiolabeled AppDNA strand was then gel-purified and annealed to a 24-mer DNA template strand and a 12-mer 3'-OH strand containing two ribonucleotides at its 3'-end. Wild-type Rnl2 reacted with this preadenylated substrate in the absence of ATP to form a ligated 24-mer product (Fig. 2A, ○). Although the reaction was efficient (95% of the input AppDNA was sealed in 1 min), the rate of the isolated step 3 reaction ($k_{\text{obs}} = 0.042 \text{ s}^{-1}$) was paradoxically slower than the rate of single-turnover ligation at a 3'-OH/5'-PO$_4$ nick (0.37...

Do the RNA 2'-OH and His-37 promote phosphodiester synthesis through a common mechanism, or do they separately affect the step 3 reaction? To answer this question, we performed a “biochemical epistasis” experiment in which we examined the kinetics of single-turnover sealing of the 2'-OH nicked substrate by the H37A mutant. The logic is as follows. If the 2'-OH and His-37 affect the same phase of the step 3 reaction then combining the nick modification with the protein mutation should not elicit a further decrement in the step 3 rate constant, whereas, if the two step 3 catalysts act via independent mechanisms, we can expect to see additive effects on step 3 when both moieties are removed. We observed (Fig. 1C) that the combination of H37A and a 2'-H drastically slowed the overall nick-sealing reaction (note the x axis units are minutes) without affecting the shape of the product distribution curve. The DNA-adenylate intermediate comprised 79% of the total labeled DNA at 15 s and increased to 90% at 0.5–2 min before decaying steadily to 4% after 120 min. From a simulation of the data in Fig. 1C, we determined the 5'-adenylation and phosphodiester formation rate constants of 0.06 s$^{-1}$ and 0.0004 s$^{-1}$, respectively. The H37A + 2'-H combination resulted in a 2250-fold decrement in step 3 catalysis, a value that agrees well with the theoretical additive effects (110 × 25 = 2750) of the individual 2'-OH and H37A modifications. We surmise that the 2'-OH and His-37 function as step 3 catalysts through distinct mechanisms.

Analysis of Phosphodiester Synthesis at a Preadenylated Nick—Step 3 of the Rnl2 ligation reaction was assayed in isolation using a preadenylated nicked duplex substrate (Fig. 2). The adenylated strand used to form this substrate was synthesized by ligase-mediated AMP transfer from the H37A mutant to the 5'-adenylated 12-mer DNA strand at a nick containing a 3'-OH/2'-H terminus, i.e., using conditions that we showed were conducive to capturing high levels of the AppDNA intermediate. The radiolabeled AppDNA strand was then gel-purified and annealed to a 24-mer DNA template strand and a 12-mer 3'-OH strand containing two ribonucleotides at its 3'-end. Wild-type Rnl2 reacted with this preadenylated substrate in the absence of ATP to form a ligated 24-mer product (Fig. 2A, ○). Although the reaction was efficient (95% of the input AppDNA was sealed in 1 min), the rate of the isolated step 3 reaction ($k_{\text{obs}} = 0.042 \text{ s}^{-1}$) was paradoxically slower than the rate of single-turnover ligation at a 3'-OH/5'-PO$_4$ nick (0.37...

Do the RNA 2'-OH and His-37 promote phosphodiester synthesis through a common mechanism, or do they separately affect the step 3 reaction? To answer this question, we performed a “biochemical epistasis” experiment in which we examined the kinetics of single-turnover sealing of the 2'-OH nicked substrate by the H37A mutant. The logic is as follows. If the 2'-OH and His-37 affect the same phase of the step 3 reaction then combining the nick modification with the protein mutation should not elicit a further decrement in the step 3 rate constant, whereas, if the two step 3 catalysts act via independent mechanisms, we can expect to see additive effects on step 3 when both moieties are removed. We observed (Fig. 1C) that the combination of H37A and a 2'-H drastically slowed the overall nick-sealing reaction (note the x axis units are minutes) without affecting the shape of the product distribution curve. The DNA-adenylate intermediate comprised 79% of the total labeled DNA at 15 s and increased to 90% at 0.5–2 min before decaying steadily to 4% after 120 min. From a simulation of the data in Fig. 1C, we determined the 5'-adenylation and phosphodiester formation rate constants of 0.06 s$^{-1}$ and 0.0004 s$^{-1}$, respectively. The H37A + 2'-H combination resulted in a 2250-fold decrement in step 3 catalysis, a value that agrees well with the theoretical additive effects (110 × 25 = 2750) of the individual 2'-OH and H37A modifications. We surmise that the 2'-OH and His-37 function as step 3 catalysts through distinct mechanisms.

Analysis of Phosphodiester Synthesis at a Preadenylated Nick—Step 3 of the Rnl2 ligation reaction was assayed in isolation using a preadenylated nicked duplex substrate (Fig. 2). The adenylated strand used to form this substrate was synthesized by ligase-mediated AMP transfer from the H37A mutant to the 5'-adenylated 12-mer DNA strand at a nick containing a 3'-OH/2'-H terminus, i.e., using conditions that we showed were conducive to capturing high levels of the AppDNA intermediate. The radiolabeled AppDNA strand was then gel-purified and annealed to a 24-mer DNA template strand and a 12-mer 3'-OH strand containing two ribonucleotides at its 3'-end. Wild-type Rnl2 reacted with this preadenylated substrate in the absence of ATP to form a ligated 24-mer product (Fig. 2A, ○). Although the reaction was efficient (95% of the input AppDNA was sealed in 1 min), the rate of the isolated step 3 reaction ($k_{\text{obs}} = 0.042 \text{ s}^{-1}$) was paradoxically slower than the rate of single-turnover ligation at a 3'-OH/5'-PO$_4$ nick (0.37...

Do the RNA 2'-OH and His-37 promote phosphodiester synthesis through a common mechanism, or do they separately affect the step 3 reaction? To answer this question, we performed a “biochemical epistasis” experiment in which we examined the kinetics of single-turnover sealing of the 2'-OH nicked substrate by the H37A mutant. The logic is as follows. If the 2'-OH and His-37 affect the same phase of the step 3 reaction then combining the nick modification with the protein mutation should not elicit a further decrement in the step 3 rate constant, whereas, if the two step 3 catalysts act via independent mechanisms, we can expect to see additive effects on step 3 when both moieties are removed. We observed (Fig. 1C) that the combination of H37A and a 2'-H drastically slowed the overall nick-sealing reaction (note the x axis units are minutes) without affecting the shape of the product distribution curve. The DNA-adenylate intermediate comprised 79% of the total labeled DNA at 15 s and increased to 90% at 0.5–2 min before decaying steadily to 4% after 120 min. From a simulation of the data in Fig. 1C, we determined the 5'-adenylation and phosphodiester formation rate constants of 0.06 s$^{-1}$ and 0.0004 s$^{-1}$, respectively. The H37A + 2'-H combination resulted in a 2250-fold decrement in step 3 catalysis, a value that agrees well with the theoretical additive effects (110 × 25 = 2750) of the individual 2'-OH and H37A modifications. We surmise that the 2'-OH and His-37 function as step 3 catalysts through distinct mechanisms.

Analysis of Phosphodiester Synthesis at a Preadenylated Nick—Step 3 of the Rnl2 ligation reaction was assayed in isolation using a preadenylated nicked duplex substrate (Fig. 2). The adenylated strand used to form this substrate was synthesized by ligase-mediated AMP transfer from the H37A mutant to the 5'-adenylated 12-mer DNA strand at a nick containing a 3'-OH/2'-H terminus, i.e., using conditions that we showed were conducive to capturing high levels of the AppDNA intermediate. The radiolabeled AppDNA strand was then gel-purified and annealed to a 24-mer DNA template strand and a 12-mer 3'-OH strand containing two ribonucleotides at its 3'-end. Wild-type Rnl2 reacted with this preadenylated substrate in the absence of ATP to form a ligated 24-mer product (Fig. 2A, ○). Although the reaction was efficient (95% of the input AppDNA was sealed in 1 min), the rate of the isolated step 3 reaction ($k_{\text{obs}} = 0.042 \text{ s}^{-1}$) was paradoxically slower than the rate of single-turnover ligation at a 3'-OH/5'-PO$_4$ nick (0.37...
s\(^{-1}\)). The slow step 3 problem in sealing a preadenylated nick was noted previously for DNA ligases (15, 16) and attributed to the imposition of a new rate-limiting conformational step for productive binding of the exogenous AppDNA, which does not pertain when the AppDNA intermediate is formed in situ on the ligase by catalysis of step 2.

An instructive finding was that the slow step 3 phenotype was alleviated by deleting the C-domain of Rnl2 (Fig. 2A, □). Sealing of the preadenylated nick by the isolated N-terminal domain Rnl2-(1–249) was efficient (95% ligation in 15 s) and rapid (90% of the end point value attained in 5 s). We calculated an apparent step 3 rate constant of 0.4 s\(^{-1}\) for Rnl2-(1–249).

This value, which is a lower limit estimate, is ~10-fold faster than the rate observed for full-length Rnl2. We concluded that the C-terminal domain is an impediment to step 3 catalysis on an exogenous preadenylated substrate. Previously, we identified two mutations in the C-terminal domain, R266A and D292A, that phenocopied the C-domain deletion with respect to loss of overall activity in nick joining, despite retention of adenylyltransferase activity (9). Here we found that the R266A and D292A mutations elicited an acceleration of step 3 catalysis identical to that observed for Rnl2-(1–249) (Fig. 2A).

To gauge the contribution of His-37 to the isolated step 3 reaction, we introduced the H37A change into the truncated Rnl2-(1–249) protein and performed a kinetic analysis of its ability to catalyze sealing at a preadenylated nick (Fig. 2B). The apparent step 3 rate constant of 0.0082 s\(^{-1}\) for the Rnl2-(1–249)-H37A mutant was in excellent agreement with the step 3 rate constant of 0.008 s\(^{-1}\) determined for full-length H37A-AMP in the single-turnover step 2 + 3 reaction (Fig. 1B). In parallel, we tested the role of the motif I lysine in the step of phosphodiester formation by introducing the K35A mutation into the Rnl2-(1–249) protein. The apparent step 3 rate constant of the Rnl2-(1–249)-K35A mutant was 0.011 s\(^{-1}\) (Fig. 2B).

This result shows that the lysine adenylylation site, which is strictly essential for steps 1 and 2 of the ligation pathway, is not absolutely required for step 3, although it does contribute a ~35-fold rate enhancement to phosphodiester synthesis.

Earlier studies highlighted the importance of a 2'-OH at the penultimate nucleotide on the 3’-OH side of the nick for nick recognition and catalysis of 5'-adenylylation (10). Here we addressed what contributions, if any, are made by the penultimate 2'-OH during the sealing of a preadenylated nick by preparing an otherwise identical 24-bp 5'-adenylated nicked substrate that contained only a single ribonucleotide on the 3’-OH side of the nick (Fig. 2C, ○). The penultimate 2'-H substitution had no effect on the extent of the step 3 reaction of Rnl2-(1–249) and exerted only a 2-fold effect on the step 3 rate constant (k\(_{\text{obs}}\) = 0.17 s\(^{-1}\)) (Fig. 2C). We conclude that the penultimate 2’-OH is not involved directly in step 3 catalysis.

The Terminal 2’-OH Accelerates Phosphodiester Synthesis at a Preadenylated Nick—To gauge the contribution of the terminal 2'-OH to the isolated step 3 reaction, we prepared preadenylated nicked duplexes composed of a radiolabeled 24-mer AppDNA strand, a bridging DNA template strand, and either an all RNA 3’-OH strand (Fig. 3, ■) or a 3’-OH RNA strand containing a single terminal 2’-H nucleoside (Fig. 3, ○). Although RNA-to-AppDNA joining by Rnl2-(1–249) was efficient and rapid (k\(_{\text{obs}}\) = 0.32 s\(^{-1}\)), a single deoxyribonucleotide at the 3’ terminus of the nick slowed step 3 by a factor of 35 (k\(_{\text{obs}}\) = 0.009 s\(^{-1}\)) without affecting the end point (Fig. 3). The 35-fold rate enhancement by the 2’-OH in the catalysis of step 3 in isolation was in good agreement with the rate acceleration of 25-fold determined for phosphodiester synthesis starting with a 5’-PO\(_{4}\) nick.

Contributions of the Divalent Cation during Catalysis of Steps 2 and 3—The composite Rnl2 ligation reaction requires a divalent cation cofactor. To assess the contributions of the metal to catalysis of steps 2 and 3, we reacted excess Rnl2-AMP with the 24-bp substrate containing a 3’-ribonucleotide/5’-PO\(_{4}\) nick in the absence of exogenous divalent cation. The Rnl2 enzyme preparation used in this experiment had been dialyzed against a buffer containing EDTA. In addition, we included 10 mM EDTA in the reaction mixture. We found that Rnl2 was capable of adenylylating the nicked substrate to form AppDNA under these conditions (Fig. 4A). The extent of the step 2 reaction was high (95% of the input pDNA strand was converted to AppDNA in 4 h), but the rate was very slow. The data in Fig. 4A are fit to a single exponential with a step 2 rate constant of 0.00097 s\(^{-1}\). By comparing this value to the rate of single-turnover nick sealing in the presence of magnesium (0.34 s\(^{-1}\)), we concluded that the metal cofactor accelerates step 2 by a factor of 380. We detected no formation of ligated 24-mer in the −Mg\(^{2+}\)/EDTA reaction, even when the incubation was extended to 24 h (data not shown). Given that virtually all of the nicks were adenylylated by 2–4 h, this result suggests that the ligase has a more stringent requirement for a metal cofactor during the step of phosphodiester formation than during the 5’-adenylylation step. We confirmed this inference by analyzing the ability of excess Rnl2-(1–249) to seal a preadenylated nicked 24-bp duplex in the absence of added magnesium, whereby we detected no ligation product even after a 5-h reaction (data not shown).

The Nick 3’-OH Is Critical for Catalysis of the 5’-Adenylylation Reaction—The experiment in Fig. 4B shows that elimination of the 3’-OH at the nick completely blocked step 3 catalysis (as expected) but still permitted the execution of step 2, with a conversion of >90% of the input 5’-PO\(_{4}\) DNA strand to the AppDNA intermediate. However, the rate constant of the step 2 reaction at a 3’-H terminus (3.4 × 10\(^{-4}\) s\(^{-1}\)) was slower by a factor of 1000 than the step 2 rate at a nick containing a 3’-terminal ribonucleotide. This result is revealing mechanistically, insofar as the 3’-OH is itself not chemically reactive
or partially, when the nick is preadenylated. This is illustrated most clearly by the ability of the N-terminal domain, which is defective in binding and ligation of a 3'-OH/5'-PO₄ nick (2, 10), to rapidly seal a nicked polynucleotide-adenylate substrate. We surmised from this result that occupancy of the adenylate binding pocket of Rnl2-(1–249) suffices to position the covalently tethered nicked duplex in the active site for phosphodiester synthesis, which resides entirely with the N-terminal domain. Indeed, we observed that the isolated N-terminal domain was more active on the exogenous nicked polynucleotide-adenylate than was full-length Rnl2. Point mutations R266A or D292A in the C-terminal domain of Rnl2 elicited the same gain of function in sealing a preadenylated nick observed for a complete deletion of the domain. Thus, the C-terminal domain of Rnl2 is responsible for much of the rate-limiting impediment to binding and sealing the exogenous 5'-adenylate substrate. A rate-limiting impediment to the isolated step 3 reaction by the C-terminal protein segment was also observed for the Chlorella virus DNA ligase (15).

Second, the transfer of the adenylate from Rnl2-AMP to the 5-PO₄ end of the nicked duplex is critically dependent on the terminal 3'-OH of the nick. Loss of the 3'-OH group slows the rate of step 2 by three orders of magnitude (without affecting the final extent of 5'-adenylation), even though the 3'-OH is not chemically transformed during the step 2 reaction. This phenomenon can be viewed as a kinetic checkpoint mechanism, which helps ensure that 5'-end activation is confined to sites where a reactive 3'-OH is in place and the ligation reaction can continue through the step of phosphodiester synthesis. Absent this checkpoint, Rnl2 (or other ligases) would pull the risk of adenylating 5'-PO₄ termini at gaps in duplex structures or at single-stranded ends. The resulting AppDNA or AppRNA ends might be difficult to repair, given that (i) polynucleotide ligases are predominantly in the adenylated state at physiological ATP concentrations, (ii) ligase-adenylate can neither seal nor deadenylate an AppDNA/RNA terminus, and (iii) the AppDNA/RNA end would be resistant to processing by 5'-exonucleases. Thus, substrate-assisted catalysis of step 2 by the 3'-OH focuses Rnl2 function on nucleic acid repair and diverts it from generating potentially deleterious 5'-lesions.

The 3'-OH step 2 checkpoint mechanism invoked for Rnl2 likely extends to DNA ligases, insofar as the 5'-adenylation reactions of Chlorella virus, vaccinia virus, and human DNA ligases were blocked by a 3'-H substitution at the nick (17, 18). Although an extended kinetic analysis of the reaction of the viral and human DNA ligases with a 3'-H nick was not conducted, early studies of E. coli DNA ligase noted that the rate of 5'-adenylation of a substrate containing a 3'-H end was at least three orders of magnitude lower than the rate of strand joining with a 3'-OH substrate (16). Thus, Rnl2 and bacterial DNA ligase display a similar kinetic dependence on the 3'-OH for substrate-assisted catalysis of step 2. The recent report that the nicked DNA-adenylate intermediate was captured during crystallization of human DNA ligase I in the presence of ATP, magnesium, and a nicked ligand containing a 3'-H terminus suggests that the human enzyme was able to slowly adenylate a 3'-H nick (19).

Third, Rnl2 relies on the terminal 2'-OH for catalysis of phosphodiester synthesis. This phase of substrate-assisted repair is unique to RNA ligase. Loss of the 2'-OH results in an accumulation of the polynucleotide 5'-adenylate intermediate during single-turnover ligation of a 3'-OH/5'-PO₄ nick by Rnl2, reflecting at least a 25-fold decrement in the rate of step 3 compared with the lower limit estimate of the step 3 rate constant determined previously (10). Our finding that a single deoxy substitution at the 3' nick terminus slowed the rate of
phosphodiester synthesis by Rn2-(1–249) by a factor of 35 provides clear evidence that RNA specificity is inherent to the N-terminal catalytic domain.

His-37 in motif I contributes an ~100-fold rate enhancement of step 3 catalysis. An epistasis experiment showed that the 2’-OH and His-37 act independently to promote phosphodiester synthesis. It is noteworthy that the corresponding motif I Asp side chain of *Chlorella* virus DNA ligases accelerates phosphodiester synthesis by a factor of 60 (20), which attests to the conservation of function of the motif I Asp/His groups during step 3 of the RNA and DNA ligase reactions. However, Rnl2 and *Chlorella* virus DNA ligase diverge sharply in their reliance on the motif I Asp/His during step 2. Although loss of His-37 had almost no impact on the rate of the 5’-adenylylation reaction of Rnl2, the equivalent mutation of the *Chlorella* virus DNA ligase motif I Asp slowed the rate of single-turnover ligation by a factor of 6000 (17).

Finally, our experiments highlighted a quantitatively distinct dependence of the 5’-adenylylation and phosphodiester synthesis reactions on an exogenous divalent cation. In the absence of magnesium, step 2 catalysis is slowed by a factor of 380, whereas step 3 catalysis was undetectable. An epistasis experiment showed that the metal ion and the nick 3’-OH act independently to promote the 5’-adenylylation reaction.

REFERENCES

1. Ho, C. K., and Shuman, S. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 12709–12714
2. Ho, C. K., Wang, L. K., Lima, C. D., and Shuman, S. (2004) *Structure* **12**, 327–339
3. Deng, J., Schnauffer, A., Salavati, R., Stuart, K. D., and Hol, W. G. J. (2004) *J. Mol. Biol.* **343**, 601–613
4. Yin, S., Ho, C. K., Miller, E. S., and Shuman, S. (2004) *Virology* **319**, 141–151
5. Martins, A., and Shuman, S. (2004) *J. Biol. Chem.* **279**, 50654–50661
6. Yin, S., Ho, C. K., and Shuman, S. (2003) *J. Biol. Chem.* **278**, 17601–17608
7. Blanc, V., Alfonso, J. D., Aphasizheva, R., and Simpson, L. (1999) *J. Biol. Chem.* **274**, 24289–24296
8. Palazzo, S. S., Pamigrahi, A. K., Igo, R. P., Salavati, R., and Stuart, K. (2003) *Mol. Biochem. Parasitol.* **127**, 161–167
9. Nandakumar, J., Ho, C. K., Lima, C. D., and Shuman, S. (2004) *J. Biol. Chem.* **279**, 31337–31347
10. Nandakumar, J., and Shuman, S. (2004) *Mol. Cell** **16**, 211–221
11. Shuman, S., and Lima, C. D. (2004) *Curr. Opin. Struct. Biol.* **14**, 757–764
12. Sekiguchi, J., and Shuman, S. (1997) *J. Virol.* **71**, 9679–9684
13. Sriskanda, V., and Shuman, S. (1998) *Nucleic Acids Res.* **26**, 525–531
14. Odell, M., Sriskanda, V., Shuman, S., and Nikolov, D. (2000) *Mol. Cell* **6**, 1185–1193
15. Sriskanda, V., and Shuman, S. (1988) *Nucleic Acids Res.* **26**, 4618–4625
16. Modrich, P., and Lehman, I. R. (1973) *J. Biol. Chem.* **248**, 7502–7511
17. Odell, M., and Shuman, S. (1999) *J. Biol. Chem.* **274**, 14032–14039
18. Yang, S. W., and Chan, J. Y. H. (1992) *J. Biol. Chem.* **267**, 8117–8122
19. Paschal, J. M., O’Brien, P. J., Tomkinson, A. E., and Ellenberger, T. (2004) *Nature* **432**, 473–478
20. Sriskanda, V., and Shuman, S. (2002) *Nucleic Acids Res.* **30**, 903–911
21. Lehman, I. R. (1974) *Science* **186**, 790–797
22. Dall’Aqua, W., and Carter, P. (2000) *Protein Sci.* **9**, 1–9
23. Kosloff, M., and Selinger, Z. (2001) *Trends Biochem. Sci.* **26**, 161–166
24. Lahiri, S. D., Zhang, G., Dunaway-Mariano, D., and Allen, K. N. (2003) *Science* **299**, 2067–2071
25. Jiang, Y. L., Ichikawa, Y., Song, F., and Stivers, J. T. (2003) *Biochemistry* **42**, 1922–1929
26. Weinger, J. S., Parnell, K. M., Dorner, S., Green R., and Strobel, S. A. (2004) *Nat. Struct. Mol. Biol.* **11**, 1101–1106