tRNA Ligase Catalyzes the GTP-Dependent Ligation of RNA with 3′-Phosphate and 5′-Hydroxyl Termini

Kevin K. Desai[†] and Ronald T. Raines*[†,‡]

[†]Department of Biochemistry and [‡]Department of Chemistry, University of Wisconsin, Madison, Wisconsin 53706, United States

Ⓢ Supporting Information

ABSTRACT: The RNA ligase RtcB is conserved in all domains of life and is essential for tRNA maturation in archaea and metazoa. Here we show that bacterial and archaeal RtcB catalyze the GTP-dependent ligation of RNA with 3′-phosphate and 5′-hydroxyl termini. Reactions with analogues of RNA and GTP suggest a mechanism in which RtcB heals the 3′-phosphate terminus by forming a 2′,3′-cyclic phosphate before joining it to the 5′-hydroxyl group of a second RNA strand. Thus, RtcB can ligate RNA cleaved by RNA endonucleases, which generate 2′,3′-cyclic phosphate and then 3′-phosphate termini on one strand, and a 5′-hydroxyl terminus on another strand.

RNA ligases catalyze the formation of a phosphodiester bond between RNA strands.[1,2] RtcB has been identified as the enzyme responsible for catalyzing the direct ligation of 2′,3′-cyclic phosphate and 5′-OH RNA termini.[3−5] This enzyme is conserved in all domains of life, and its activity is essential in archaea and metazoa for the ligation of tRNA molecules after removal of the intron by the tRNA splicing endonuclease.[6] This endonuclease generates RNA fragments analogous to those of the archetypal acid−base catalyst, bovine pancreatic ribonuclease (RNase A).[7,8] These reactions proceed in two separate steps: cleavage of RNA to generate fragments with 2′,3′-cyclic phosphate and 5′-OH termini, and hydrolysis of the 2′,3′-cyclic phosphate to form a 3′-phosphate (3′-P).[9,10]

The fate of 3′-P RNA termini in tRNA splicing intermediates is not well understood, but they are presumed to be recycled by the enigmatic RNA phosphate cyclase (RtcA).[11] RtcA is highly conserved in all three domains of life and is also postulated to function in the cyclization of the 3′-P of spliceosomal U6 snRNA.[12] Synthesis of 2′,3′-cyclic phosphate termini by the ATP-dependent RtcA occurs in three nucleotidyl transfer steps: (1) reaction of ATP with an active-site histidine residue to form a covalent enzyme−AMP intermediate and release PP_i, (2) transfer of the AMP moiety to the terminal 3′-P to form an RNA−adenylylate intermediate, and (3) attack by the terminal 2′-OH on the adenylylated 3′-P to form the 2′,3′-cyclic phosphate product and release AMP.[13] This cyclization of 3′-P RNA termini is thought to be a prerequisite for ligation to 5′-OH RNA termini.

We found that *Escherichia coli* RtcB ligase can accept 3′-P RNA termini as its substrate, consistent with a recent report.[14] To assay for ligation, we initially used two single-stranded RNA (ssRNA) oligos that were each 10 nucleotides in length. The 5′ RNA fragment was labeled with 6-carboxyfluorescein (FAM) at

its 5′ end and phosphorylated at its 3′ end. The 3′ RNA fragment had hydroxyl groups at each end. We intended to convert the 3′-P into a 2′,3′-cyclic phosphate by incubation with RtcA and ATP, and thereby to produce a suitable substrate for RtcB. Surprisingly, we found that prior cyclization of the 3′-P was not necessary for ligation to a 5′-OH RNA terminus, as long as GTP was included in reaction mixtures (Figures S1 and S2 of the Supporting Information). This result was found even with RtcB produced in an *rtcA* deletion strain of *E. coli*, eliminating the possibility of inadvertent contamination.

To explore this reactivity further, we used a substrate that mimics the broken anticodon stem−loop structure of yeast tRNA$^{Glu(UUC)}$ (Figure 1A), which was ligated more efficiently by RtcB than was ssRNA because of the proximity of its 3′-P and 5′-OH termini. We found that ATP, CTP, UTP, or NAD$^+$ was unable to substitute for GTP (Figure 1B). Ligation was dependent on Mn^{2+}, and a C78A substitution within a predicted metal-binding site abolished ligase activity, as reported previously.[14]

Then, we tested five GTP analogues as cofactors in ligation reactions and discovered that bond cleavage between the α- and β-phosphoryl groups of GTP occurs during catalysis by RtcB. These reactions were performed with high concentrations of GTP analogues to ensure saturation of RtcB. Four of the analogues, β,γ-methylene (GppCp), β,γ-imido (GppNHp), γ-thio (GTPγS), and α-thio (GTPαS), allowed ligation, albeit at a an efficiency lower than that with GTP (Figure 1C). In contrast, α,β-methylene (GpCpp) did not allow the ligation to proceed, suggesting that cleavage of the phosphoanhydride bond between the α- and β-phosphoryl groups of GTP is critical for the ligation of 3′-P and 5′-OH RNA termini. GDP and GMP allowed for low levels of ligation, which could arise from small amounts of contaminating GTP. Titrations with GTP revealed that RtcB has a K_M of <16 μM for this cofactor (Figure 1D). This value is similar to the ATP K_M values of 20 μM for *E. coli* RtcA[13] and 6 μM for human RtcA.[15]

The GTP-dependent ligation activity of RtcB requires a 3′-P and a 2′-OH on the 5′ side of the ligation junction and a 5′-OH on the 3′ side. We investigated the specificity of RNA termini for ligation by modifying the ends of our tRNA mimic substrate. First, all four combinations of either a phosphate or hydroxyl group at each termini were sampled while maintaining a 2′-OH on the 5′ side of the ligation junction. RtcB was found to be highly specific for RNA substrates that have 3′-P and 5′-
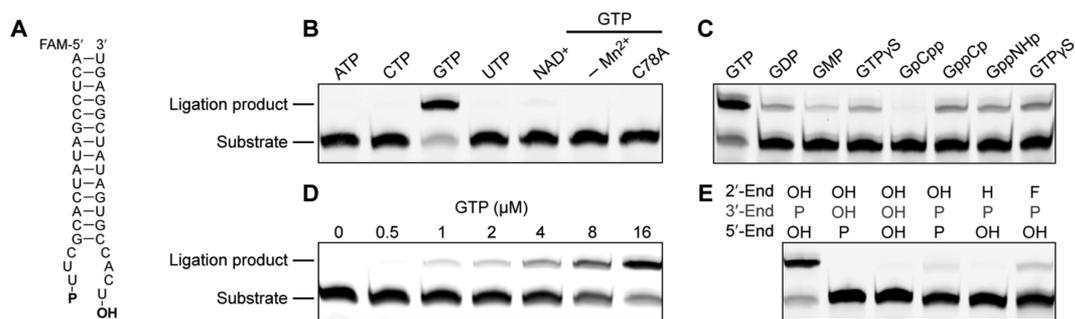
**Figure 1.** *E. coli* RtcB catalyzed GTP-dependent ligation of 3′-P and 5′-OH RNA termini. (A) RNA ligase substrate that mimics a broken tRNA anticodon loop. The FAM label allows visualization in a urea−polyacrylamide gel. Ligation of 3′-P and 5′-OH termini generates a 35-nucleotide RNA, whereas unligated substrate appears as a 17-nucleotide band. (B) Ligation assays for cofactor and metal dependence. Reaction mixtures contained Mn^{2+}, except the $-Mn^{2+}$ lane. The lane labeled C78A is a ligation reaction performed with RtcB that has a C78A substitution within the predicted metal-binding site. (C) Ligation assays using GTP analogues as cofactors. (D) Ligation assays for GTP concentration dependence, allowing estimation of a GTP K_M of <16 μM. (E) Ligation assays for specificity of RNA termini. Modifications to the ligatable ends of the tRNA mimic substrate (A) were made as indicated and tested in ligation reactions.

Scheme 1
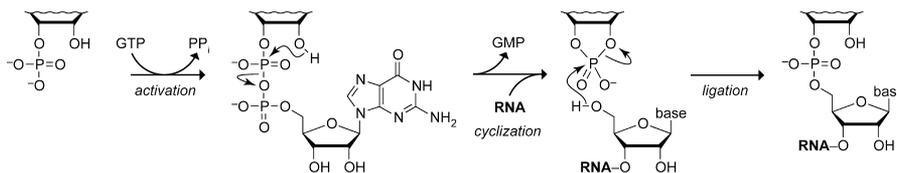
OH termini (Figure 1E). Next, the importance of the terminal ribose 2′-OH for ligation was probed by replacing this hydroxyl group with either hydrogen (2′-H) or fluorine (2′-F). The RNA substrate with a 3′-P and 2′-H terminus produced no observable ligation product, whereas the substrate with a 3′-P and 2′-F terminus yielded only a trace of product. The 2′-deoxy-2′-fluororibose will adopt a ring pucker similar to that of the substrate with 3′-P and 2′-OH[16] but will not allow for phosphate cyclization. These data suggest that the primary mechanism involves cyclization of the 3′-P as a prerequisite for ligation to a 5′-OH RNA terminus, though the appearance of a trace of ligation product with the 2′-F substrate (Figure 1E) does not allow us to rule out the possibility of direct ligation to the GMP-activated 3′-P terminus. In that mechanism, the 2′-OH could assist catalysis in a manner similar to that during ribosome-catalyzed peptide bond formation.[17]

RtcB from the archaeal domain of life likewise catalyzes the GTP-dependent ligation of 3′-P and 5′-OH RNA termini. The crystal structure of RtcB from a hyperthermophilic archaeon, *Pyrococcus horikoshii*, was determined as part of a structural genomics project.[18] This structure reveals a hydrophilic pocket with a predicted metal-binding site consisting of residues Cys98, His203, His234, and His404. The *P. horikoshii* homologue has an amino acid sequence 29% identical to that of *E. coli* RtcB and 49% identical to that of the human ligase (HSPC117).

To investigate if RtcB from archaea catalyzes the GTP-dependent ligation of 3′-P and 5′-OH RNA termini, we synthesized a *P. horikoshii rtcB* gene and produced soluble enzyme in *E. coli*. RtcB from *P. horikoshii* catalyzed the same reaction as its *E. coli* homologue and displayed identical cofactor and metal ion requirements (Figure S3 of the Supporting Information). The *P. horikoshii* ligase also required a 2′-OH on the terminal nucleotide of the 5′ RNA fragment. Although RtcB from the archaeon *Pyrobaculum aerophilum* has been reported to depend on Zn^{2+} for activity,[4] we found that
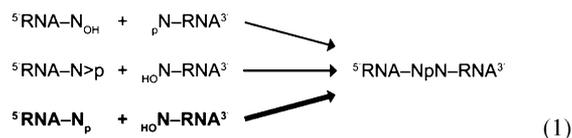
Zn^{2+} is unable to replace Mn^{2+} in active *P. horikoshii* RtcB. We did find that a C98A substitution within the predicted metal-binding site abolishes ligase activity, as reported for *P. aerophilum* RtcB.

We propose a three-step mechanism for catalysis of the ligation of 3′-P and 5′-OH RNA termini by RtcB (Scheme 1). In the activation step, cleavage of the phosphoanhydride bond between the α- and β-phosphoryl groups of GTP occurs during transfer of GMP to the 3′-P terminus of an RNA strand to form a guanylylate-activated intermediate. ATP-dependent DNA and RNA ligases that catalyze a similar activation of 5′-P RNA termini first form a covalent enzyme−AMP intermediate with an active-site lysine, prior to transfer of AMP to the 5′-P terminus.[1] Likewise, RtcB is known to be radiolabeled with $[\alpha\text{-}^{32}P]GTP$ but not $[\gamma\text{-}^{32}P]GTP$.[14] Our data with GTP analogues show without ambiguity that cleavage occurs between the α- and β-phosphoryl groups of GTP during catalysis (Figure 1C). In the cyclization step of our mechanism, the 2′-OH on the terminal ribose attacks the GMP-activated 3′-P to generate a 2′,3′-cyclic phosphate and release GMP. In the ligation step, the 5′-OH terminus of a second RNA strand attacks the cyclic phosphate to form a 3′,5′-phosphodiester bond. We note that our mechanism accommodates the known competency of an RNA strand with a 2′,3′-cyclic phosphate to be a substrate for RtcB in the absence of GTP.[3−5]

Three sets of RNA termini have been identified as competent substrates for an RNA ligase. Bacteriophage T4 RNA ligase 1 (Rnl1) and RNA ligase 2 (Rnl2) catalyze the ATP-dependent ligation of 3′-OH and 5′-P RNA termini in three nucleotidyl transfer steps similar to those of DNA ligases.[1] All three domains of life encode ligases that accept 2′,3′-cyclic phosphate and 5′-OH RNA termini[4] and are known to be essential for tRNA maturation and the unconventional splicing of *HAC1* mRNA.[2] The yeast tRNA ligase is a class I 5′-P RNA ligase and is homologous to T4 Rnl1.[2] The multifunctional yeast ligase is unable to join tRNA exon termini directly but instead has cyclic

phosphodiesterase and polynucleotide kinase activities that yield 3′-OH and 5′-P termini. The yeast ligase then seals these ends in an ATP-dependent reaction.[2] In plants, a similar mechanism catalyzed by the class II 5′-P ligase forms mature tRNAs.[19] In contrast, archaea and metazoa can form mature tRNAs via the direct ligation of 2′,3′-cyclic phosphate and 5′-OH termini in a reaction catalyzed by RtcB.[3,4] The relief of strain that accompanies the cleavage of a cyclic phosphate drives this reaction.[20] This reaction is unique in that it entails nucleophilic attack by a 5′-OH, which has a pK_a value much higher than that of 3′-OH.[21]

The presence of RtcB in bacteria is mysterious because group 1 introns in bacterial pre-tRNAs self-splice to form mature tRNAs.[22] It has been suggested that *E. coli* RtcB might serve to protect against damage from stress-induced tRNA endonucleases,[5] which cleave tRNA using a mechanism similar to that of RNase A.[23] We also note that the hairpin and hammerhead ribozymes catalyze the direct ligation of 2′,3′-cyclic phosphate and 5′-OH termini.[24,25] The discovery that RtcB is a multifunctional ligase that can prepare a 3′-P termini for ligation to a 5′-OH establishes a third pair of RNA termini that are suitable substrates for an RNA ligase (eq 1).

$$
\begin{aligned}
^5\text{RNA–N}_{\text{OH}} \; + \; {}_{\text{P}}\text{N–RNA}^3 \quad &\searrow \\
^5\text{RNA–N>p} \; + \; {}_{\text{HO}}\text{N–RNA}^3 \quad &\longrightarrow \quad ^5\text{RNA–NpN–RNA}^3 \\
^5\textbf{RNA–N}_{\textbf{p}} \; + \; {}_{\textbf{HO}}\textbf{N–RNA}^3 \quad &\nearrow
\end{aligned}
\tag{1}
$$

Recently, the human RtcB homologue was purified from HeLa cell extracts.[3] A double-stranded RNA (dsRNA) substrate containing 3′-P and 5′-OH termini was used to follow human ligase activity after the dsRNA became linked, apparently after cyclization of the 3′-P by RtcA. The covalent linking of 3′-P and 5′-OH dsRNA, upon incubation with human cell extract, has also been observed in other studies.[11,26] Our data are consistent with RtcB being the sole enzyme responsible for these previous observations. In addition, our findings call into question the importance of RtcA in maintaining the 2′,3′-cyclic phosphate termini of tRNA splicing intermediates.

A search of the National Center for Biotechnology Information database reveals that RtcB has no homology to other known RNA or DNA ligases and does not have a predictable GTP-binding site. Furthermore, RtcB does not contain the conserved KXXG sequence motif that defines a superfamily of covalent nucleotidyltransferases, which includes DNA and RNA ligases and mRNA-capping enzymes.[1] Finally, we note that RtcB is able to ligate RNA strands after cleavage by common ribonucleases or at high pH, which generates 2′,3′-cyclic phosphate and then 3′-P termini on one strand and 5′-OH termini on the other strand.[9,10] The ability of this ancient enzyme[4] to repair damaged RNA and to shuffle transcripts could have implications for organismal survival and evolution. Moreover, the ease of synthesizing RNA strands with 3′-P and 5′-OH termini suggests that RtcB could have practical applications in RNA tagging and cloning.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures S1−S3 and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
*E-mail: rtraines@wisc.edu. Telephone: (608) 262-8588.

■ REFERENCES

(1) Shuman, S., and Lima, C. D. (2004) *Curr. Opin. Struct. Biol. 14*, 757−764.
(2) Schwer, B., Sawaya, R., Ho, C. K., and Shuman, S. (2004) *Proc. Natl. Acad. Sci. U.S.A. 101*, 2788−2793.
(3) Popow, J., Englert, M., Weitzer, S., Schleiffer, A., Mierzwa, B., Mechtler, K., Trowitzsch, S., Will, C. L., Lührmann, R., Söll, D., and Martinez, J. (2011) *Science 331*, 760−764.
(4) Englert, M., Sheppard, K., Aslanian, A., Yates, J. R., and Söll, D. (2011) *Proc. Natl. Acad. Sci. U.S.A. 108*, 1290−1295.
(5) Tanaka, N., and Shuman, S. (2011) *J. Biol. Chem. 286*, 7727−7731.
(6) Abelson, J., Trotta, C. R., and Li, H. (1998) *J. Biol. Chem. 273*, 12685−12688.
(7) Calvin, K., and Li, H. (2008) *Cell. Mol. Life Sci. 65*, 1176−1185.
(8) Cuchillo, C. M., Nogués, M. V., and Raines, R. T. (2011) *Biochemistry 50*, 7835−7841.
(9) Markham, R., and Smith, J. D. (1952) *Biochem. J. 52*, 552−557.
(10) Thompson, J. E., Venegas, F. D., and Raines, R. T. (1994) *Biochemistry 33*, 7408−7414.
(11) Filipowicz, W., Konarska, M., Gross, H. J., and Shatkin, A. J. (1983) *Nucleic Acids Res. 11*, 1405−1418.
(12) Lund, E., and Dahlberg, J. E. (1992) *Science 255*, 327−330.
(13) Genschik, P., Drabikowski, K., and Filipowicz, W. (1998) *J. Biol. Chem. 273*, 25516−25526.
(14) Tanaka, N., Chakravarty, A. K., Maughan, B., and Shuman, S. (2011) *J. Biol. Chem. 286*, 43134−43143.
(15) Vicente, O., and Filipowicz, W. (1988) *Eur. J. Biochem. 176*, 431−439.
(16) Hakoshima, T., Omori, H., Tomita, K.-i., Miki, H., and Ikehara, M. (1981) *Nucleic Acids Res. 9*, 711−729.
(17) Weinger, J. S., Parnell, K. M., Dorner, S., Green, R., and Strobel, S. A. (2004) *Nat. Struct. Biol. 11*, 1101−1106.
(18) Okada, C., Maegawa, Y., Yao, M., and Tanaka, I. (2006) *Proteins 63*, 1119−1122.
(19) Englert, M., and Beier, H. (2005) *Nucleic Acids Res. 33*, 388−399.
(20) Kumamoto, J., Cox, J. R. Jr., and Westheimer, F. H. (1956) *J. Am. Chem. Soc. 78*, 4858−4860.
(21) Izatt, R. M., Hansen, L. D., Rytting, J. H., and Christensen, J. J. (1965) *J. Am. Chem. Soc. 87*, 2760−2761.
(22) Reinhold-Hurek, B., and Shub, D. A. (1992) *Nature 357*, 173−176.
(23) Tomita, K., Ogawa, T., Uozumi, T., Watanabe, K., and Masaki, H. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 8278−8283.
(24) Hertel, K. J., Herschlag, D., and Uhlenbeck, O. C. (1994) *Biochemistry 33*, 3374−3385.
(25) Hegg, L. A., and Fedor, M. J. (1995) *Biochemistry 34*, 15813−15828.
(26) Martinez, J., Patkaniowska, A., Urlaub, H., Lührmann, R., and Tuschl, T. (2002) *Cell 110*, 563−574.