R2D Ligase – A novel DNA ligase with unexpected DNA-to-RNA ligation activity

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Abstract

DNA ligases catalyze bond formation in the backbone of nucleic acids via the formation of a phosphodiester bond between adjacent 5' phosphates and 3' hydroxyl groups on one strand of the duplex. While DNA ligases preferentially ligate single breaks in double stranded DNA (dsDNA), they are capable of ligating a multitude of other nucleic acids substrates like blunt-ended dsDNA, TA overhangs, short overhangs and various DNA-RNA hybrids. Here we report a novel DNA ligase from Chronobacter phage CR 9 (AZ R2D Ligase) with an unexpected DNA-to-RNA ligation activity. The R2D ligase shows excellent efficiency when ligating DNA to either end of RNA molecules using a DNA template. Furthermore, we show that DNA can be ligated simultaneously to both the 5' and 3' ends of microRNA-like molecules in a single reaction mixture. Abortive adenylated side product formation is suppressed at lower ATP concentrations and the ligase reaction reaches near completion when ligating RNA to DNA or DNA to RNA. The ligation of a DNA strand to the 5'-PO4 end of RNA is unique among the commercially available ligases and may facilitate novel workflows in microRNA analysis, RNA sequencing and in the preparation of chimeric guide DNA-RNA for gene editing applications.

Introduction

Nucleic acid ligases catalyze the formation of a phosphodiester bond between the 3' OH group and an adjacent 5' phosphorylated end of nucleic acids using energy from an adenylate-donating nucleotide cofactor) [1], [2]. The reaction mechanism involves three steps that are highly conserved among all family members. In the first step, the ligase self-adenylates through bond formation between an AMP group, donated by either ATP or NAD, and an active site lysine residue. In the second step, the AMP group is transferred to the 5' phosphate of the nucleic acid, and finally the 3'OH end of the adjacent strand performs a nucleophilic attack on this activated 5' end resulting in the formation of a diester bond in the nucleic acid backbone [3].

DNA ligases are key enzymes in molecular biology workflows including molecular cloning and adapter ligation prior to sequencing [4]–[6]. These applications have traditionally been dominated by viral DNA ligases such as T3 and T7 DNA Ligases, with the T4 DNA Ligase (T4 DnL) being by far the most widely used [7]. T4 DNA ligase shows excellent efficiency when ligating nicked duplex DNA, certain chimeric DNA-RNA oligonucleotides and DNA-RNA hybrids [8], [9]. Other double-stranded substrates, however, have not been ligated efficiently by any known ligase. Here we report the novel*Chronobacter phage CR9 DNA Ligase (* Commercially known as ArcticZymes R2D Ligase) with hitherto unreported ability to ligate DNA to the

5'-end of RNA. We will also discuss possible application areas where such ligation activity may lead to new innovations.

Materials and Methods

Oligonucleotides, substrate preparation enzymes and reagents

DNA and RNA oligonucleotides used in the ligase assays were purchased from Metabion International AG (Planegg/Steinkirchen Germany) in lyophilized form. A full overview of the oligos and their sequences can be found in the supporting information (Table S1). The oligos were resuspended in Tris-EDTA (TE) buffer (pH 8.0) to prepare 100 µM stock solutions. Ligation substrates were prepared by combining acceptor, donor and template oligos in a 1:1:1 ratio and then hybridizing by heating at 95 °C for 4 minutes followed by cooling to 4°C at a rate of 1°C/min. Resulting duplexes were either used immediately or stored at -20 °C. A depiction of all substrates is shown in figure 2A and supporting information table S2. The DNA ligases R2D Ligase and T4 DNA Ligase were obtained from ArcticZymes Technologies AS (Tromsø, Norway). PBCV-1 Ligase (SplintR Ligase), T7 DNA Ligase, T3 DNA Ligase, T4 RNA Ligase 1 and T4 RNA Ligase 2 were obtained from New England Biolabs (NEB, Ipswich, Massachusetts, USA). Ligases named AZ ligase 1-4 are noncommercial prototype ligase variants of ArcticZymes Technologies AS.

Nick ligation substrate specificity assay

The substrate specificity assay was adapted from Bullard and Bowater (2006), and an identical set of oligonucleotides were used for substrate production (see supporting information). The substrate set was tested in standard reaction condition unless otherwise stated (50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1 mM ATP, 10 mM DTT, 25 mM KCl, 0.9 µM nicked substrate, 0.05 mg/ml BSA). Reactions were started by adding 5 µl enzyme to 5 µl reaction mix resulting in 10 µl in total and a final concentration of 15 µM ligase. The reactions were run at 25 °C for 15 minutes and terminated by heating the reactions to 75 °C for 10 minutes. The reactions were mixed 1:1 with 95 % formamide/10 mM EDTA to stop the reactions. Reaction products were detected by electrophoresis on 20% acrylamide/7 M Urea PAGE and imaging with GelDoc XR+ (BIO-RAD, Hercules, California, USA). Data analyses were performed in ImageLab 4.1 (BIO-RAD) and Microsoft Excel.

Characterization of optimal reaction conditions

Optimization of ATP, $MgCl_2$ and $MnCl_2$concentrations were performed similarly to assays above, using the S1 (DNA-to-DNA, DNA-splint), S7 (RNA-to-DNA, DNA-splint) and S8 substrates (DNA-to-RNA, DNA splint) (see supporting information). With respect to the linearity of the assay in the presence of different substrate compositions, the concentrations of R2D ligase chosen were 0.4 nM, 0.2 nM and 1.4 µM for substrates S1, S7 and S8, respectively. For $MnCl_2$ optima, 50 nM R2D ligase was used for the S1 and S7 substrate, and 8 nM for the S8 substrate. The standard reaction buffer was identical to that described above, and ATP, KCl and $MgCl_2$ concentrations were varied according to figure legend in the results section.

Ligation of DNA to both ends of RNA in a single reaction mixture

A single reaction mixture with DNA oligos being ligated to either side of an RNA was set up using four DNA oligos and a target 5' phosphorylated RNA (D4-D7, R4, supporting information table S2). In addition, two substrates that only ligate DNA to either end of the RNA target were prepared (S10 and S11, figure 2A). The reactions were run using 2.5 µM of T4 DNA Ligase or R2D Ligase in an altered reaction buffer (50 mM Tris-HCl pH 7.5, 5 mM $MnCl_2$, 0.1 mM ATP, 25 mM KCl, 10 mM DTT, 0.05 mg/ml BSA, 0.9 µM substrate) and incubated at 25 °C for 30 minutes, then inactivated at 75 °C for 10 minutes. The samples were detected

by Urea PAGE using a Pharos FX (BIO-RAD, Hercules, California, USA), using two channels for FAM and TAMRA respectively.

Results

R2D ligase shows excellent activity on nicked DNA/RNA hybrids

Initially, R2D ligase was tested in a substrate specificity assay adapted from Bullard and Bowater [10], along with other well-known DNA and RNA ligases. The substrate specificity assay evaluates each ligase's ability to ligate nicked duplex substrates composed of three individual oligos, where each oligo may be DNA or RNA. In the present study we focus on the DNA-splinted substrates only. For ligation performance on the full panel, see the supporting information (Figure S1-S2). Figure 1 shows that R2D was the only ligase tested that was able to ligate all four substrates S1, S6, S7 and S8 to completion. Specifically, complete attachment of DNA- to the 5'ends of RNA was only observed with R2D ligase and while both PBCV-1 DNA ligase and T3 DNA ligase carried out some ligation on this substrate, this was at a much lower level. The DNA-splinted RNA substrate was also ligated to near-completion by R2D ligase, as well as T4 DNA Ligase and T4 RNA Ligase 2. All DNA ligases were able to ligate the nicked DNA substrate, and all ligases were able to ligate RNA- to 5'DNA with a DNA splint. To our knowledge, this is the first report of a ligase that exhibits the functionality of ligating DNA to both sides of RNA efficiently [10]. Crystal structures of a range of ligases show that upon DNA binding prior to step 2, ligase enzymes enforce an RNA-like A-structure on the 3'OH terminal nucleotide of acceptor strand, while the 5'P terminal nucleotide is retained in the DNA-B conformation, providing a rationale for the high rates of joining of S7 by all ligases tested here [11], [12]. The broader tolerance of an RNA acceptor strand by R2D ligase may lie either in an ability to tolerate an A-form of the nucleotide in this position, or conversely an enhanced ability to impose a B-conformation on the 5' terminal ribonucleotide.

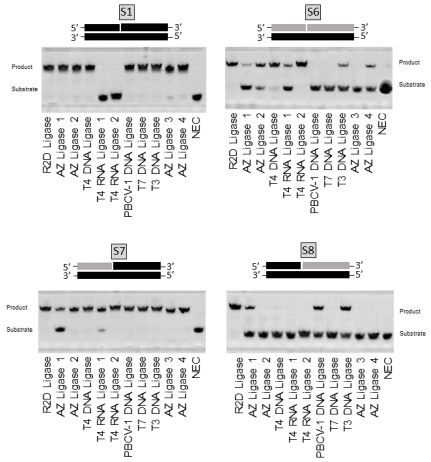

Figure 1: Initial substrate specificity panel of R2D ligase along with other commercially available ligases.

Effect of Magnesium, KCl and ATP on ligation of RNA to DNA

To investigate the effect of buffer components on ligation performance using different substrates, we tested the R2D ligase with three substrates (S1, S7 and S8, Figure 2A) with increasing amounts of $MgCl_2$, and $MnCl_2$ and ATP in the ligation mixture (figure 2B-D). In a typical ligation buffer such as for the T4 DNA Ligases these components are used at concentrations of 5 mM $MgCl_2$/$MnCl_2$ and 1 mM ATP (the arrows in the figure shows standard concentrations to which the results have been normalized).

The effect of magnesium is similar when using both DNA-DNA substrate (S1) and the DNA-RNA hybrids (S8) with an increase in activity followed by a plateau at 2 mM (S1) and 5 mM (S8) (Figure 2B and supporting information S5). With RNA-DNA the increase in activity with metal ion concentration continued until 10 mM which was the highest concentration tested. Interestingly, when substituting Mg^{2+} with Mn^{2+} as the divalent metal ligand, a shift in optimum metal concentration from more than 5 mM to 3 mM or less is observed when ligating DNA-DNA (S1) and RNA-DNA (S7) (Figure 2C). A similar shift was previously reported in archaea DNA ligases [13], as well as in human DNA Ligase I [14]. The Mn^{2+} concentration shows less impact on the activity of R2D when ligating DNA-RNA (S8), as the activity at all Mn^{2+} concentrations is within 70 % of maximum activity. Additionally, while a higher R2D ligase concentration was necessary to detect ligation of S1 and S7 with Mn^{2+}, ligation of S8 showed a 23-fold increase in efficiency compared with using Mg^{2+} (figure 2D). In the case of ATP, an increase above 0.1 mM enhanced ligation efficiency when ligating DNA-DNA (S1) and RNA-DNA (S7) (Figure 2E). When ligating DNA-RNA however, a sharp decrease was observed at ATP concentrations above 0.1 mM, indicating a clear advantage in reducing ATP with this substrate. A higher amount of side product corresponding to approximately 1 nucleotide larger than the donor oligo was also observed at higher ATP concentrations (supporting information figure S4). This observation suggests that R2D is defective in completing step 3 of strand joining with DNA-RNA (S8) relative to other substrates meaning that an increased rate of Step 1 (enzyme adenylation) with high ATP causes rapid re-adenylation of the enzyme, resulting in premature release of the 'dead-end' adenylated RNA product. This may be either due to decreased affinity for the 5'adenylated form of this duplex relative to the DNA-DNA or RNA-DNA forms, or due to decreased rate of catalysis with a ribonucleotide at the 5' end. Similar effects have been observed for other ligases acting on mixed DNA/RNA hybrids which required tuning of ATP concentration for optimal performance [12]. Reducing the ATP concentration to 0.1 mM as well as increasing $MgCl_2$concentration to 10 mM gave a 2-fold increase in ligated product when ligating S8, as well as minimizing abortive adenylation (Figure 2F and supporting figure S3).

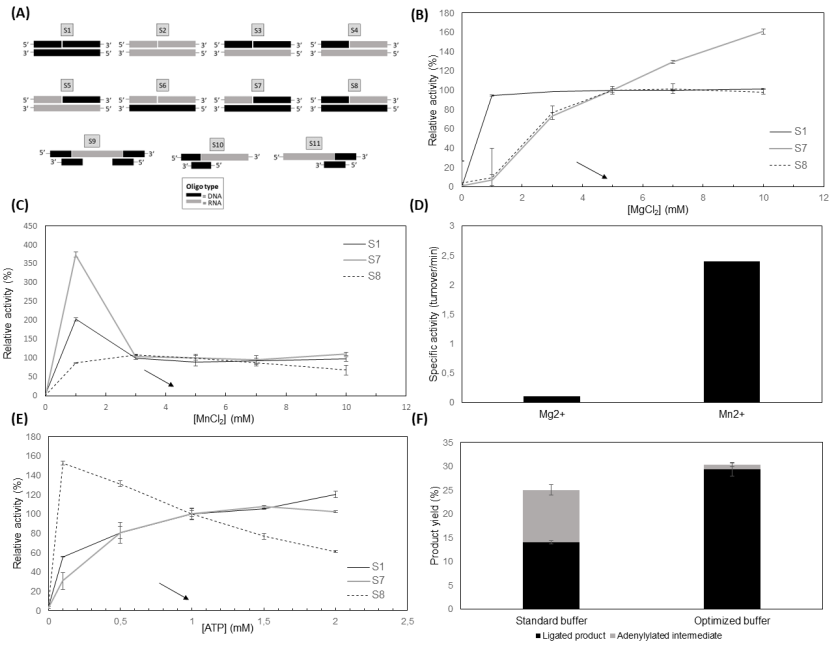

Figure 2: Biochemical characterization data of R2D Ligase using substrates S1, S7, S8 (A) Substrate panel used in this article. S1, S6, S7 and S8 were used in figure 1, S1, S7 and S8 in figure 2 and S9, S10 and S11 in figure 3. S2-S5 were used in experiments shown in supporting information. (B) $MgCl_2$ dependency. The error bars show the coefficient of variation for each data point (duplicates). (C) $MnCl_2$dependency shows a shift in optima $MnCl_2$ concentration for all three substrates compared to $MgCl_2$. (D) Replacing Mg^{2+} with Mn^{2+} in the reaction buffer with substrate S8 increases reaction efficiency with up till 23-fold. (E) ATP dependency on ligase activity. Increase in activity for S1 and S7 at higher ATP concentration, with an opposite effect of ATP on S8. (F) The standard buffer was compared with an optimized buffer (10 mM $MgCl_2$, 0.1 mM ATP, 25 mM KCl) for the S8 substrate. Improvement in overall product yield and minimization of 5' appRNA intermediate product was observed.

DNA adapters can simultaneously be ligated to both ends of RNA

To investigate the potential of DNA ligation to both ends of a 5' phosphorylated RNA molecule, we designed a proof of concept using two DNA adaptors and two DNA template strands in a single reaction mixture (Figure 3). Since the 3'RNA to DNA ligation efficiency was predicted to be more efficient than the DNA to 5'RNA ligation, we used the optimized buffer described earlier for the latter substrate. We tested this by separate reactions at either end, as well as for all components in a single mixture. The DNA adapters were marked with different fluorescent labels to differentiate between ligation to the 5' and 3' end of the target RNA after Urea/PAGE separation. Successful ligation of both adapters allowed detection of a yellow band composed of both the TAMRA and the FAM fluorophore. For the R2D Ligase, the reaction showed more than 70 % substrate turnover for both adapters, both in isolated and in simultaneous reactions. As expected, T4 DnL showed similar activity when ligating DNA to the 3' end of RNA but only trace activity (< 1 % turnover) on the 5' end of the RNA.

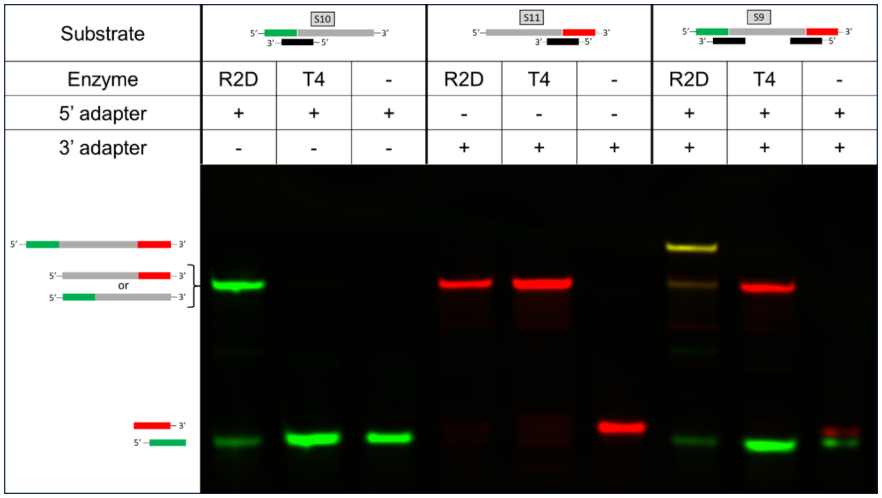

Figure 3: Ligation of DNA oligos to both ends of RNA substrate using a DNA splint. FAM fluorescence (attachment to 5' end) is illustrated indicated in green, TAMRA (attachment to 3' end) is indicated in red. When both adapters are ligated, the band becomes yellow and shifts further up.

With the current focus in biotechnology on RNA therapeutics, synthesis and analysis, there are multiple potential applications for producing chimeric RNA-DNA oligonucleotides. For example, chimeric guide DNA-RNA for CRISPR editing or synthesis of DNA-RNA chimeras for specific cutting of RNA with RNase H [15]. The novel DNA-RNA activity of R2D may also find a use in RNA analysis, e.g. for capturing microRNA or novel approaches to DNA adapter ligation of RNA. Additionally, such approaches can be used for RNA enrichment or RNA depletion workflows where there is a need to keep the 3' end of the RNA unoccupied. Finally, we envision that R2D ligase may be used for barcoding of RNA sequencing libraries prior to sample multiplexing. This could remove the need for cDNA synthesis prior to barcode attachment and facilitates multiplexing of direct RNA sequencing workflows.

Conclusion

The R2D Ligase is capable of ligating all combinations of DNA and RNA oligos using a DNA splint to completion, including DNA to 5'-end RNA which has not previously been reported. The introduction of a DNA ligase with efficient DNA to 5'RNA end ligation activity may facilitate the development of novel applications based upon this activity.

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Competing interests

BKS and OL are the inventors of the patent application. SEG, UR, EH, YP, IKR, JJS, TS, OL, and BKS are employees of ArcticZymes Technologies AS; BKS, is a board member in ArcticZymes Technologies AS. IMG is a former employee of ArcticZymes Technologies AS. AW has no competing interests.

Data and materials availability

All data supporting the results are available upon request, or can be found in the main text or in the supporting information. The R2D ligase and Ligases named AZ ligases are available from ArcticZymes Technologies AS. Contact: contact@arcticzymes.com

References

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