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Original Article

Effects of 2′-*O*-methyl nucleotide on ligation capability of T4 DNA ligase

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To further understand the ligation mechanism, effects of 2′-*O*-methyl nucleotide (2′-OMeN) on the T4 DNA ligation efficiency were investigated. Fluorescence resonance energy transfer assay was used to monitor the nick-joining process by T4 DNA ligase. Results showed that substitutions at 5′- and 3′-ends of the nick decreased the ligation efficiency by 48.7% ± 6.7% and 70.6% ± 4.0%, respectively. Substitutions at both 5′- and 3′-ends decreased the ligation efficiency by 76.6% ± 1.3%. Corresponding kinetic parameters, V_{max}, K_m, and k_{cat}, have been determined in each case by using the Michaelis–Menten equation. The kinetic data showed that the 2′-OMeN substitutions reduced the maximal initial velocity and increased the Michaelis constant of T4 DNA ligase. Mismatches at 5′- and 3′-ends of the nick have also shown different influences on the ligation. Results here showed that the sugar pucker conformation at 3′-end impairs the ligation efficiency more profoundly than that at 5′-end. Different concentrations of Mg^{2+}, Ca^{2+}, K^+, Na^+, and ATP were also demonstrated to affect the T4 DNA ligase activity. These results enriched our knowledge about the effects of 2′-OMeN substitutions on the T4 DNA ligase.

Keywords ligation; T4 DNA ligase; 2′-*O*-methyl nucleotide; mismatch

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Introduction

DNA ligases are enzymes that catalyze the nick-joining event on DNA duplexes in DNA replication, recombination, and DNA repair processes [1,2]. Extensive investigations have been conducted to uncover the DNA ligation mechanism. It is a three-step process involving two covalent intermediates [3]. The first step initiates with an attack of the ε-amino group of a Lys residue in the active site on the adenylyl phosphorus of either NAD^+ or ATP, to form a covalent phosphoamide linkage between ligase and AMP with releasing either NMN or PPi, respectively. In the second step, the adenylated ligase intermediate transfers the AMP moiety to the 5′-phosphoryl group at the DNA nick to form the adenylated DNA intermediate. Finally, the ligase catalyzes the formation of a phosphodiester bond between the 3′-hydroxyl group of the DNA nick and the 5′-adenylated phosphate, releasing AMP.

Although DNA ligases show a wide diversity in amino acid sequences, molecular sizes, and biological properties, there are two types of DNA ligases based on their cofactor specificity in general: NAD^+-dependent and ATP-dependent. The eukaryotic and prokaryotic encoded enzymes all require ATP as the cofactor, while NAD^+-requiring DNA ligases have only been found in prokaryotic organisms [4]. Bacteriophage T4 DNA ligase is a representative ATP-dependent ligase [5–8]. The mechanism and kinetics of T4 DNA ligase-catalyzed reactions have been extensively investigated, and the ligation efficiency influenced by Mg^{2+}, ATP, pH, and temperature has been explored [9]. Influences on T4 DNA ligases by mismatching and mutation oligonucleotides have been well studied [10,11].

The catalytic mechanism of ligases for normal DNA substrates has been well documented; however, little is known about the interactions of ligases with nucleotide derivatives. Williams *et al*. [12] prepared oligonucleotides containing 2′-fluoro nucleosides with a constrained sugar pucker conformation at the 3′-end nucleotide to be ligated. The nucleoside 2′-deoxy-2′-fluoroarabinofuranosyl uracil [$U^{2'F(ara)}$] favored the 2′-endo conformation (i.e. DNA-like), while 2′-deoxy-2′-fluororibofuranosyl uracil [$U^{2'F(ribo)}$] adopted the 3′-endo conformation (i.e. RNA-like). At 37°C, the 3′-end dU and $U^{2'F(ara)}$ residues paired with dA had a similar effect on ligation efficiencies, while the 3′-end $U^{2'F(ribo)}$ residue reduced the ligation efficiency significantly [12]. This study highlighted the feasibility to use nucleotide derivatives to explore the functionality of DNA ligases.

Rapid developments in nucleic acid chemistry have offered a variety of nucleotide derivatives baring unique properties in chemical reactivity, electronegativity, hydrogen-bond formation capability, hydrophobicity, as well as special local structures [13,14]. For instance, substitution of a $-OCH_3$ group for the −H atom on the C2′ atom of native deoxynucleotide gives rise to 2′-*O*-methyl nucleotide (2′-OMeN) [15]. This substitution has effectively restricted the furanosyl ring to the C3′-endo pucker conformation and relevant antiglycosidic bond angle which are observed in A-form DNA and RNA [16]. When incorporated into oligonucleotides, 2′-OMeN provides a more favorable binding affinity toward complementary RNA nucleotides than toward DNA nucleotides [13,17]. In addition, 2′-OMeN exhibits a faster hybridization dynamics than usual nucleotides [18]. Previously, we observed that DNA substrates containing different nucleotide derivatives (LNA, 2′-OMeN, and FNA) affect the cleavage of endonucleases in a position-dependent manner [19]. Recently, methods on rapid detection of small biological molecules have been reported [20]. All these results prompt us to continue the exploration on the influence of the nucleotide derivatives on DNA ligases.

In this study, we examined the effect of the 2′-OMeN-substituted oligonucleotides on the ligation process using an MB-based fluorescence resonance energy transfer (FRET) assay (**Fig. 1**). FRET is a high-throughput method to assay the relations between two chromophores in a certain distance [21,22]. FRET involves the transfer of energy from a fluorescent group in its excited state to another excitable state acceptor. Molecular beacon (MB) provides a perfect and convenient structural model for FRET performance, which is widely used for fundamental research and practical applications [23–27]. MB is a short oligonucleotide of a loop–stem (or hairpin) structure [28]. The loop sequence is designed to be able to hybridize with a complementary target. The stem is composed of 5–10 base pairs and the two ends are labeled with a fluorophore and a quencher, respectively. When the loop hybridizes with the target sequence, the newly formed duplex creates a structure constrain which breaks the stem structure, leading to the separation of fluorophore and quencher and restoring the fluorescence intensity [28,29]. This MB-based FRET assay offers a fast, convenient, and sensitive capacity of real-time monitoring nucleic acid interaction [30]. Using the MB-based FRET assay, we examined the effects of 2′-OMeN substitutions at either 3′-end or 5′-end or both ends of the nick on the T4 ligation efficiency, and determined corresponding kinetic parameters, V_{max}, K_m, and k_{cat}. Furthermore, the mismatches at the nick and other positions have shown a great influence on the ligation efficiency. The results provide an insight into the mechanism of DNA ligation, and the conclusions highlight the application potential in designing new strand-

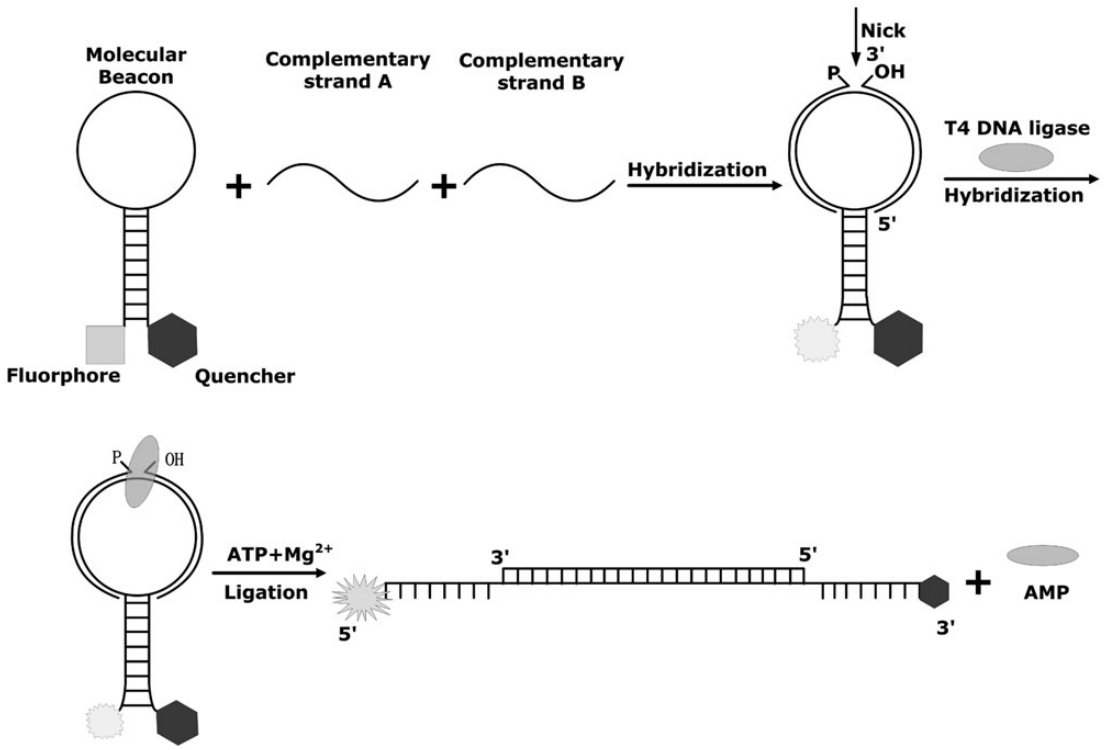

Figure 1. Principle of the FRET-based DNA ligase assay The stem–loop structure of MB quenches the fluorescence signals in the native state. Step 1: L-ON and R-ON bind hybridize with the left and right portions of the MB loop through complementary base pair, leaving behind a nick. It might destabilize the MB structure slightly. Addition of T4 DNA ligase catalyzes the nick-joining reaction, thus forming a long oligonucleotide fully complementary to the loop of MB. The structural constrain of the ligation product creates a bending force to open MB, generating measurable fluorescence signals.

sealing enzymes with unique substrate specificities for antibacterial and anticancer therapies.

Materials and Methods

Chemicals

T4 DNA ligase with a concentration of 350 U/μl was purchased from TaKaRa (Dalian, China). Ligation buffer was prepared with 66 mM Tris–HCl (pH 7.6), 6.6 mM $MgCl_2$, 10 mM DTT, and 0.1 mM ATP. The storage buffer contains 10 mM Tris–HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, and 50% glycerol. Bovine serum albumin was purchased from Sigma Chemical Co. (St Louis, USA). All oligonucleotides (**Table 1**) were purchased from TaKaRa with HPLC grade purification and used without further purification. The stock solutions of oligonucleotides at a concentration of 100 μM were prepared with deionized water that has electric resistance >18.3 MΩ. All other chemicals were products of research or analytically pure.

Preparation of oligonucleotides

Three oligonucleotides were prepared to constitute the substrate for T4 DNA ligase: MB, left oligonucleotide (L-ON) and right oligonucleotide (R-ON). The MB was 36-nucleotide long and labeled with fluorescein isothiocyanate at the 5′-end and a 4-(4¢-dimethylaminophenylazo) benzoic acid at the 3′-end (**Table 1**). Eight nucleotides at the 5′-end of MB were fully complementary to the eight nucleotides at the 3′-end, thus forming a MB with an eight-nucleotide stem and a 20-nucleotide loop. L-ON and R-ON were 10 nucleotides long, complementary to the left half portion and the right half portion of the MB loop, respectively. The 5′-end of L-ON was of a free $-PO_4^-$ group, which was required to form a phosphodiester bond with the −OH group at the 3′-end of R-ON. A negative control was an oligonucleotide of the same sequence as L-ON but was lack of $-PO_4^-$ group at the 5′-end (referred to as L′-ON). In comparison, a positive control was an oligonucleotide of 20-nucleotide long and fully complementary to the loop (referred to as C-ON). Furthermore, a group of oligonucleotides with different

Table 1. Oligonucleotides used in this study

Name	Sequence (3′-end to 5′-end)	
MB		5′-F-AATACACACTCTGCTGTGATGTCTCATCTGTGTATT-Q-3′
Complementary	L-ON	GAGACGACAC
	R-ON	TACAGAGTAG
	C-ON	GAGACGACACTACAGAGTAG
No phosphate	L′-ON	GAGACGACAC′
Length of R-ON	R-7	TACAGAG
	R-8	TACAGAGT
	R-9	TACAGAGTA
	R-11	TACAGAGTAGT
	R-12	TACAGAGTAGTG
	R-13	TACAGAGTAGTGT
OMeN substitutions	5′-OMeN	GAGACGACAC
	3′-OMeN	TACAGAGTAG
Mismatches	L1-G:g	GAGACGACAg
	L1-G:t	GAGACGACAt
	L1-G:a	GAGACGACAa
	R1-A:g	gACAGAGTAG
	R1-A:c	cACAGAGTAG
	R1-A:a	aACAGAGTAG
	R2-T:c	TcCAGAGTAG
	R3-G:a	TAaAGAGTAG
	R4-T:c	TACcGAGTAG
	R5-C:c	TACAcAGTAG
	R6-T:c	TACAGcGTAG
	R7-C:c	TACAGAcTAG
	R8-A:c	TACAGAGcAG

The nucleotide sequences are written from 3′-end to 5′-end except MB. The underlined and the lower cases represent the 2′-OMeN and mismatched nucleotides, respectively.

lengths (named as R7-R13) were used to validate the FRET assay by evaluating the structure constrains. To study the effect of 2′-OMeN substitutions on the ligation behavior, the 5′-end of L-ON and 3′-end of R-ON were substituted with 2′-OMeN, respectively (referred to as 5′-OMeN and 3′-OMeN).

Fluorescence measurements

Fluorescence spectra were recorded at 37°C in the wavelength ranging from 480 to 600 nm at a wavelength increment of 2.0 nm using a spectrofluorometer (Hitachi F4500; Tokyo, Japan). First, 2.5 μl of MB (10 μM) was added to a cuvette containing 492.5 μl ligation buffer, and the fluorescence spectrum was recorded after 600 s incubation. Equal amounts (2.5 μl, 10 μM) of L-ON and R-ON were mixed and the fluorescence spectrum was recorded. Then, 1.75 U of T4 DNA ligase was added and the fluorescence spectrum was recorded after 2400 s incubation. Fluorescence spectrum of MB hybridized with C-ON was used as control.

Real-time monitoring ligation activity

The FRET assay was used to evaluate the ligation efficiency. Fluorescence spectra were recorded on a Microplate Reader (Infinite M200; Tecan, San Jose, USA) with the excitation wavelength at 480 nm and the emission wavelength at 518 nm.

Equal volume (0.5 μl) of MB, L-ON and R-ON at a concentration of 50 nM was pre-incubated at 37°C in a 100-μl ligation buffer (66 mM Tris–HCl, pH 7.6, 6.6 mM $MgCl_2$, 10 mM DTT, and 0.1 mM ATP). Once the fluorescence intensity became stable, T4 DNA ligase of 0.35 U was added to the reaction solution. The florescence intensity was recorded every 20 s for 120 circles. The initial ligation velocity (V_0) was determined from the slope of the relative fluorescence intensity with respect to the reaction time in the initial linear ligation period between 0 and 400 s. ΔFI was calculated using the formula: $\Delta FI = (FF_t - F_1)$ [28], where F_1 is the initial fluorescence intensity at the time T4 DNA ligase was added. V_0 is normalized with respect to the normal ligation velocity (MB + L-ON + R-ON).

Enzymatic kinetics of T4 DNA ligation

To obtain kinetic parameters of T4 DNA ligation, different substrate concentrations ranging from 5 to 2000 nM were prepared. When mixing T4 DNA ligase at a concentration of 0.35 U with each sample, the initial velocities (V_0) of the ligation reaction were determined. These initial velocities were plotted with respect to the substrate concentrations, and these plots were fitted to the hyperbolic kinetic curves of the Michaelis–Menten equation using software Origin 8.5, and the kinetic parameters V_{max}, K_m were obtained in the units of nM/s and nM, respectively. k_{cat} was further calculated as $V_{max}/[E]$, where $[E]$ is the final concentration of T4 DNA ligase. Data shown here are the averaged results of three independent measurements.

Influence of other factors on the ligation

The effects of different cations on the ligation efficiency were also measured. $MgCl_2$ was prepared in the concentrations ranging from 0 to 50 mM. $CaCl_2$, KCl, and NaCl were prepared in the concentrations ranging from 0 to 300 mM. In addition, different ATP concentrations ranging from 0 to 0.5 mM influencing ligation efficiency were also tested.

Results

FRET assay of ligation

Figure 2 shows fluorescence spectra of four different ligation reactions. The fluorescence spectrum of MB in the reaction solution showed a small peak at 518 nm (**Fig. 2**, Curve a). The relatively low fluorescence background was due to the effective resonance energy transfer of a stable MB. Addition of L-ON and R-ON to the reaction solution led to a small increase in the fluorescence spectrum because their complementary hybridization with MB could lead to a slight opening of a small fraction of MB (**Fig. 2**, Curve b). Once T4 DNA ligase was added to the reaction solution, R-ON and L-ON were linked. This newly formed 20-nt oligonucleotide broke the structural constrain and opened MB efficiently. As a result, the fluorescence intensity increased significantly (**Fig. 2**, Curve c). The trend was consistent with the positive control, where mixing the 20-nt C-ON with MB caused a complete opening of the MB **(Fig. 2**, Curve d).

We also monitored the kinetic process of the T4 ligation. When MB and T4 DNA ligase were incubated together, there was no observable fluorescence signal (**Fig. 3**, Curve a). When L′-ON and R-ON were mixed with MB in the presence of T4 DNA ligase, there was still no fluorescence emission (**Fig. 3**, Curve b). Because of the absence of the PO_4^- group at the 5′-end of L′-ON, no nick-joining could take

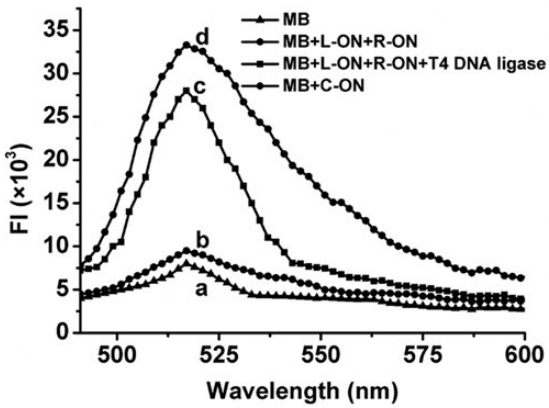

Figure 2. Fluorescence spectra of MB hybridized with different oligonucleotides Curve a, MB alone; Curve b, MB hybridized with L-ON and R-ON; Curve c, MB hybridized with L-ON and R-ON in the presence of T4 DNA ligase; Curve d, MB hybridized with a positive control C-ON. All spectra were recorded by adding the enzyme 0.35 U and the oligonucleotide concentration of 50 nM (37°C).

place even when T4 DNA ligase was present. In contrast, with the addition of T4 DNA ligase into the reaction solution of MB, R-ON, and L-ON, the fluorescence intensity increased steadily, indicating that the nick-joining process was taking place as the time was progressing (**Fig. 3**, Curve c). In case of mixing MB and C-ON in the reaction solution, the fluorescence intensity increased rapidly and became saturated (<100 s), suggesting an immediate opening of the stem–loop structure (**Fig. 3**, Curve d). These data validate the FRET-based assay.

Optimization of ligation reaction

To optimize the experimental condition for subsequent ligation reaction, the dependence of the initial ligation velocity on the T4 DNA ligase concentrations ranging from 0.175 to 35 U/ml was tested. **Figure 4** shows that the initial ligation velocity is directly proportional to the concentration of T4 DNA ligase with a good linearity ($R = 0.9914$).

As a further proof of the FRET-based assay, the effect of different lengths of R-ON on the ligation efficiency was examined using the method described above. R-ONs of different lengths from 7 to 13 nucleotides were prepared (**Table 1**). **Figure 5** shows that when R-7 was hybridized with MB, the initial velocity was relatively low. It was because that the ligated L-ON and R-7 were not long enough to open the MB efficiently. As R-ON becomes longer, the initial velocity increases gradually, and R-10 shows the fastest initial ligation velocity. Further increase of the oligonucleotide length, however, reduced the initial velocity, since the complementary hybridization has already destabilized the stem structure to some extent before the ligation. This result revealed that 10 nucleotides is the optimal length for the FRET assay.

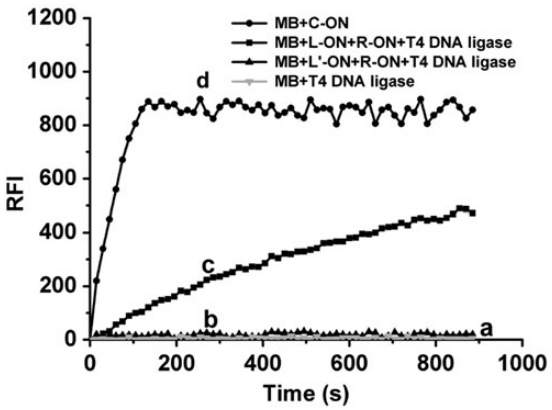

Figure 3. Florescence intensity change with respect to the reaction time Curve a, MB alone; Curve b, MB hybridized with L′-ON and R-ON in the presence of T4 DNA ligase; Curve c, MB hybridized with L-ON and R-ON in the presence of T4 DNA ligase; Curve d, MB hybridized with a positive control C-ON. All spectra at a wavelength of 518 nm were recorded by adding the enzyme 0.35 U and the oligonucleotide concentration of 50 nM (37°C).

Effects of the 2′-OMeN-substitution on the ligation efficiency

As shown in **Fig. 6A**, when the 2′-OMeN substitution was at the 5′-end on the nick, the initial ligation velocity was reduced by 48.7% ± 6.7% in comparison with no substitution, while the 2′-OMeN substitution at the 3′-end of the nick decreased the initial ligation velocity by 70.6% ± 4.0%. When both sides of the nick were substituted with 2′-OMeN, the corresponding initial ligation velocity was reduced by 76.6% ± 1.3%. These results indicate that the 2′-OMeN substitution at the 3′-end of the nick influenced the ligation efficiency more than that of the 5′-end of the nick.

The kinetic analysis of the 2′-OMeN substitution was also carried out. By varying the concentrations of the substrates ranging from 5 to 2000 nM and keeping a T4 DNA ligase concentration of 0.35 U, the initial ligation velocities were

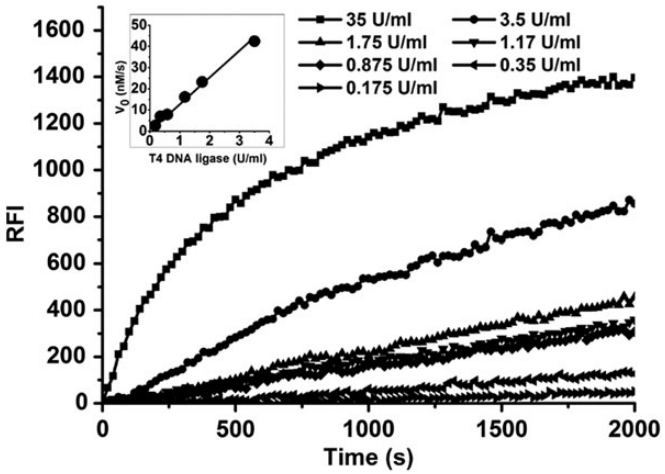

Figure 4. Fluorescence intensity changes of FRET-based DNA ligase assay for different concentrations of T4 DNA T4 DNA ligase concentrations of 0.175, 0.35, 0.583, 1.17, 1.75, 3.5 and 35 U/ml. Inserted is the concentration dependence of the initial ligation velocity of T4 DNA ligase.

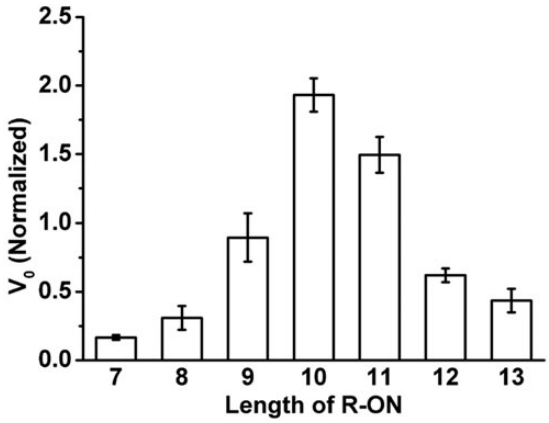

Figure 5. Effects of the length of R-ON on ligation efficiency in terms of ligation velocity (V_0) The lengths of these complementary strands are 7, 8, 9, 10, 11, 12 and 13 nucleotides, respectively.

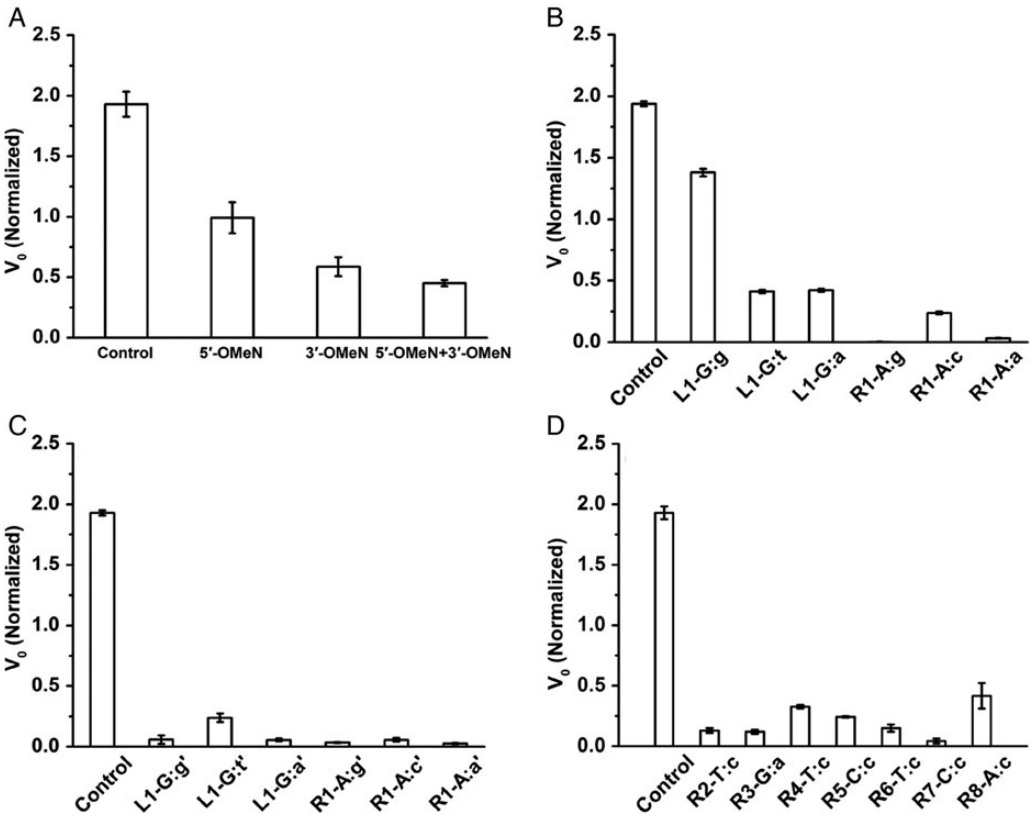

Figure 6. Influences of 2′-OMeN substitutions and mismatches on ligation efficiency (A) Effects of the 2′-OMeN substitutions on the T4 ligation efficiency in terms of the initial ligation velocity (V_0). (B) Effects of mismatched nucleotides at either 5′-end or 3′-end of the nick on the T4 ligation efficiency. (C) Effects of mismatches at one side of the nick while the other side remains 2′-OMeN substituted. (D) Effects of mismatches at different positions of R-ON when L-ON remains 2′-OMeN substituted.

measured. These initial velocities with respect to the substrate concentrations were plotted (**Fig. 7**). Kinetic parameters V_{max} and K_m were calculated, and k_{cat} was also determined. **Table 2** shows that V_{max} decreased from 7.47 ± 0.17 to 6.51 ± 0.21, 4.48 ± 0.11, and 2.51 ± 0.08 nM/s when the 2′-OMeN substitution was at the 5′-end of L-ON, 3′-end of R-ON and both ends, respectively. Correspondingly, the K_m value increased from 341.88 ± 23.87 to 579.43 ± 43.44 nM, and k_{cat} varied in the range from 0.95 ± 0.02 to 0.32 ± 0.01 s^{-1} for different substitutions.

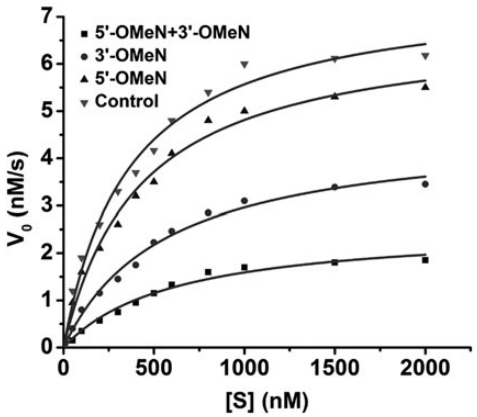

Figure 7. Non-linear fitting graph of control and 2′-OMeN-substituted substrates

Effect of mismatches on the ligation efficiency

The effect of mismatches on the ligation was explored (**Fig. 6B–D**). When the 5′-end of the nick (i.e. the 5′-end of L-ON) was mismatched with MB while keeping the 3′-end of the nick (3′-end of R-ON) correctly base-paired, the ligation efficiency of the mismatch L1-G:g was reduced by $28.8\% \pm 1.5\%$, whereas other two mismatches (L1-G:t and L1-G:a) decreased the initial ligation velocity by $78.2\% \pm 2.2\%$ and $78.2\% \pm 1.7\%$ (in the notation of L1-G:g, L1-G indicates that the nucleotide at the 5′-end of L-ON is guanine, and g represents the nucleotide at the corresponding position on MB is guanine). When the 3′-end of the nick was mismatched while keeping the 5′-end correctly base-paired, the ligation efficiency was reduced more significantly. Mismatches R1-A:g and R1-A:a could not lead to ligation at all (decreased by $99.8\% \pm 2.3\%$ and $98.3\% \pm 3.1\%$, respectively), and the mismatch R1-A:c showed a $87.8\% \pm 2.6\%$ reduction in the ligation velocity.

Figure 6C shows the effect of mismatches at one end of the nick, while the other end of the nick is 2′-OMeN substituted. The L1-G:g′, L1-G:t′, and L1-G:a′ mismatches at the

Table 2. Kinetic parameters for different 2′-OMeN substituted substrates

Substitution site	V_{max} (nM/s)	K_m (nM)	k_{cat} (s^{-1})	k_{cat}/K_m (nM^{-1} s^{-1})
Control	7.47 ± 0.17	341.88 ± 23.87	0.95 ± 0.02	2.78×10^{-3}
5′-OMeN	6.51 ± 0.21	399.79 ± 27.89	0.82 ± 0.03	2.05×10^{-3}
3′-OMeN	4.48 ± 0.11	524.66 ± 32.38	0.57 ± 0.01	1.09×10^{-3}
5′-OMeN + 3′-OMeN	2.51 ± 0.08	579.43 ± 43.44	0.32 ± 0.01	0.55×10^{-3}

5′-end of the nick and the R1-A:g′, R1-A:c′, and R1-A:a′ mismatches at the 3′-end of the nick generated a complete decreased effect. Furthermore, the effects of mismatches at other positions of R-ON were also examined (**Fig. 6D**). Mismatched positions R2-T:c, R3-G:a, R7-C:c almost decreased the ligation velocity completely, which had a greater effect than that of any other position. Mismatched position R4-T:c decreased the ligation velocity by 81.9% ± 4.4%, and R8-A:c decreased the ligation velocity by 78.2% ± 11.3%. The cytosine residue was used for creating mismatches since it could generate the most profound destructive effect [31].

Effects of cations and ATP cofactor on the ligation efficiency

Figure 8A shows that there was no ligation activity at all when Mg^{2+} was absent in the solution for both normal and substituted substrates. As the concentration of Mg^{2+} became higher, the initial ligation velocity increased and reached the maximum at 20 mM for the normal substrate. When the Mg^{2+} concentration was >50 mM, the ligase activity was significantly diminished. T4 DNA ligase also exhibited a dependence on the concentration of Mg^{2+} when the 2′-OMeN-substituted substrates were used. However, the initial ligation velocities were much lower than that of normal substrates. The optimal activity appeared at a concentration of 7.5 mM and the activity decreased completely when the Mg^{2+} concentration was >20.0 mM. Results above indicated that T4 DNA ligase was more sensitive to Mg^{2+} concentration when the substrate was 2′-OMeN substituted.

As shown in **Fig. 8B–D**, other cations, Ca^{2+}, K^+, and Na^+, could also influence the T4 DNA ligase activity. **Figure 8B** suggests that only when the Ca^{2+} concentration was <100 mM, T4 DNA ligase could show enzymatic activity; while higher Ca^{2+} concentrations diminished the T4 ligation activity under the condition of normal substrate. For 2′-OMeN substrates, the T4 DNA ligase activity decreased completely when the concentration of Ca^{2+} was >50 mM. In contrast, **Fig. 8C** and **D** shows that T4 DNA ligase could join the nick with a relatively large range of K^+ and Na^+ concentrations, although the T4 DNA ligase activity was lower than that in the Mg^{2+}-containing solution. Once the substrates were substituted with 2′-OMeN, T4 DNA ligase activity was completely lost when the concentrations of K^+ and Na^+ were >50 mM.

As expected, different concentrations of ATP also influenced T4 DNA ligase activity. **Figure 8E** shows that the ligation did not take place unless ATP is present. The initial ligation velocity increased rapidly and reached its maximum when the concentration of ATP was 0.05 mM for the normal substrate, and then changed slightly at higher ATP concentration. When the substrate was substituted at both 5′- and 3′-ends of the nick, the maximal ligation efficiency appeared at 0.075 mM, and exhibited a similar concentration dependence trend as the normal substrate.

Discussion

DNA ligases play essential roles in a DNA replication process. They catalyze the 3′-end hydroxyl group of the Okazaki fragments to the 5′-end phosphate group of the lagging DNA strand to form a covalent phosphodiester bond. DNA ligases have been shown to have a wide range of applications in DNA repair, genetic engineering, and detections.

The ligase-catalyzed nick-joining process has been extensively studied previously. The ligation capability can be influenced by ions [9], ATP [32], lengths of the oligonucleotide to be ligated [33], mismatches [33,34], and local structures around the nick [9]. Pritchard and Southern [33] examined T4 DNA ligase using a traditional approach of polyacrylamide gel electrophoresis. They determined the minimal length of the oligonucleotides for the ligation reaction and observed the effects of mismatches near the nick positions on the ligation reaction. Liu *et al.* [34] tested the effect of mismatched base pairs on the DNA ligation fidelity of Tth ligase, and found that the ligation was sensitive to the mismatches at the 3′-end of the nick, but tolerant to the mismatches on the 5′-end of the nick. They concluded that the apparent base-pair geometry was much more important than relative base-pair stability, and the major groove contacts were of little importance [34]. Cherepanov and de Vries [9] studied the Mg^{2+}-dependent adenylylation of the T4 DNA and RNA ligases. The effects of the concentrations of Mg^{2+}, ATP, and pyrophosphate have been systematically varied, and the results led to the conformation that the nucleotidyl transfer was proceeded according to a two-metal ion

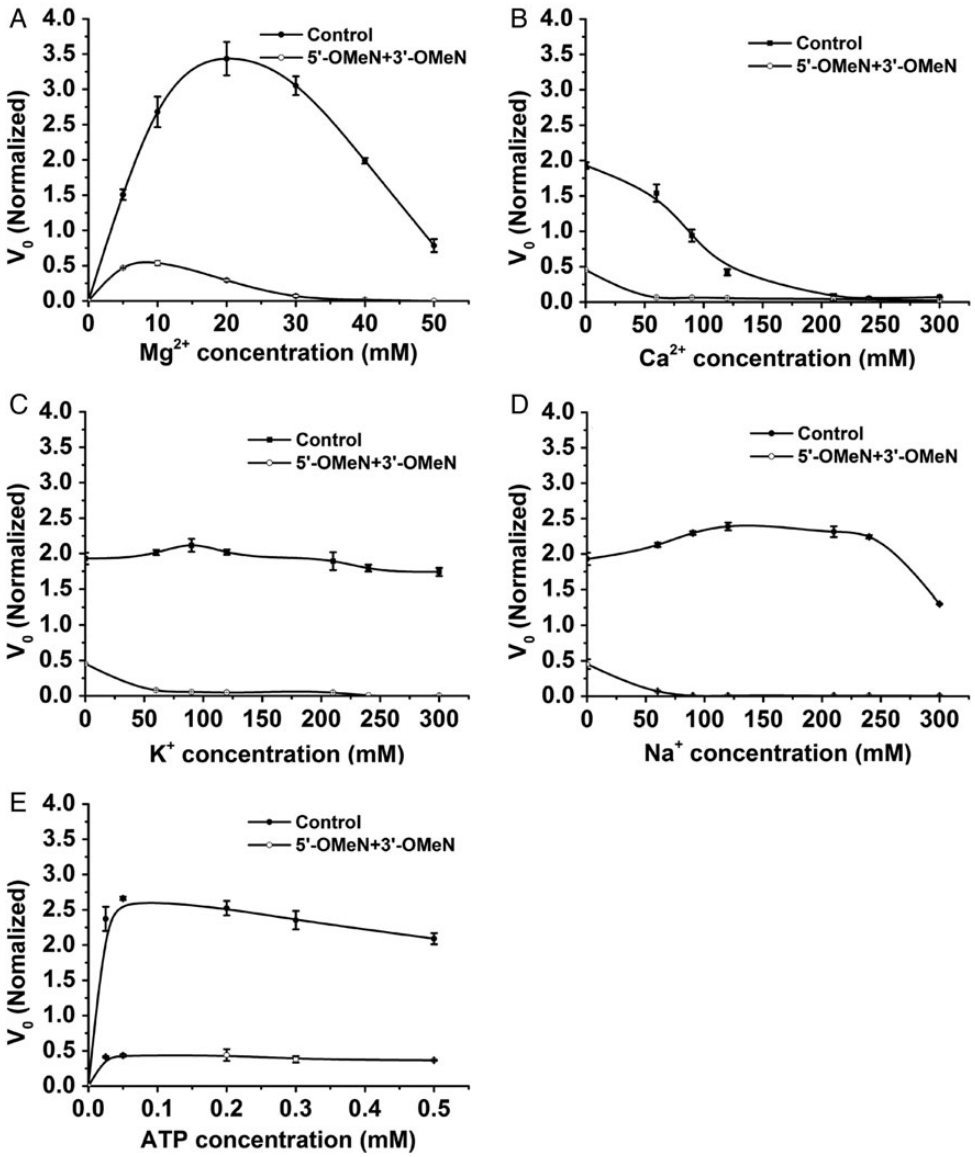

Figure 8. Effects of metal ions on the T4 ligation efficiency (A–D) The dependence curves of initial reaction velocity on the concentrations of Mg^{2+}, Ca^{2+}, K^{+} and Na^{+}, respectively. (E) The dependence curves of initial ligation velocity on the ATP concentrations.

mechanism. However, how the nucleotide derivatives affect the ligation behavior is much less investigated.

In the present study, we constructed a ligation substrate by hybridizing two 10-nt oligonucleotides with a MB (**Fig. 1**). Comparison of curves in **Fig. 3** confirmed the necessity of the 5′-PO_4^- group for the nick-joining process, and also validated the FRET-based assay. **Figure 4** shows that the initial ligation velocity was directly proportional to the concentration of T4 DNA ligase ranging from 0.175 to 3.5 U/ml. This linear relationship defined the optimal concentration for the subsequent experiments. Results obtained using different lengths of R-ON suggested that 10 nt was the optimal length to open the MB in the ligation process assayed by the FRET-based method (**Fig. 5**), which was consistent with previous conclusion that hexamer was the minimal length for the ligation reaction [33,35].

As mentioned, the ligation process is accomplished in a three-step manner. It starts with a hydrolysis of ATP to form a covalent bond between AMP and Lys residue in the active site, with a release of pyrophosphate. It is followed by a transfer of the AMP moiety from this adenylated ligase to the free 5′-phosphoryl group of the nick. Finally, the phosphodiester bond is formed with a concomitant release of AMP from the adenylated DNA intermediate. Substitution of the $-OCH_3$ group for the −H atom at the C2′-atom does constrain the ribose conformation to the C3′-endo sugar pucker conformation. Thus, it is expected that this local structure alternation at the 3′-end could have a greater impact on the phosphodiester linkage formation than that at the 5′-end.

Previous studies have highlighted the discriminative ligation behavior. Bullard and Bowater [36] conducted a study of T4 DNA ligase for different duplex substrates. They

prepared the substrates with different types of nicks. These nicks were formed by two DNA oligonucleotides (DNA : DNA), two RNA oligonucleotides (RNA : RNA), or one DNA oligonucleotide plus one RNA oligonucleotide (RNA : DNA or DNA : RNA). They observed that the ligation rate was 42 s^{-1} for DNA : DNA nick, 32 s^{-1} for RNA : DNA nick, and <0.001 s^{-1} for DNA : RNA nick, respectively, at the DNA ligase concentration of 3.0 pM and 37°C. These discriminative ligation capabilities were closely related to the sugar conformation change near the nicks: the RNA:DNA nick had a C3′-endo sugar pucker conformation at the 3′-end and C2′-endo sugar pucker conformation at the 5′-end, while the DNA : RNA had reversed sugar pucker conformations (C2′-endo at the 3′-end and C3′-endo at the 5′-end) [36]. In our study, 2′-OMeN was used to substitute the nucleotide at either the 5′-end, or the 3′-end or both ends, and similar results were observed, indicating the effect of the sugar pucker conformation on the ligation efficiency [37].

When examining their plots of initial velocity versus substrate concentration, good fitting to the hyperbolic curves of the Michaelis–Menten equation was obtained (**Fig. 7**). Corresponding V_{max} and K_m were determined consequently. Regardless of the 2′-OMeN substitutions at the 3′-end, or the 5′-end or both ends of the nick, V_{max} decreased and K_m increased. The increased K_m values implied that 2′-OMeN substitutions could decrease the binding affinity between T4 DNA ligase and the substrate [38]. Furthermore, k_{cat} and k_{cat}/K_m were calculated (**Table 2**). k_{cat} reflects the number of substrate molecules turned to product per second per T4 DNA ligase molecule under the saturated enzyme conditions. Alternatively, the reciprocal of k_{cat} is considered as the time required by a ligase molecule to 'turnover' one substrate molecule. The decrease of k_{cat} suggested that the production yield was decreased when the nucleotide was 2′-OMeN substituted. On the other hand, k_{cat}/K_m is considered as a measure of enzyme efficiency. As shown in **Table 2**, k_{cat}/K_m values were declined when the substrate were 2′-OMeN substituted, indicating a decreased ligation efficiency of T4 DNA ligase.

In comparison with the substitutions at different ends of the nick, mismatches around the nick also exhibited different effects on the initial velocity. This asymmetric decrease behavior was consistent with the fact that the 3′-end nucleotides are more sensitive than the other side. The ligation reaction requires preliminarily that the nucleotides to be linked must form a complementary base pair with the template, otherwise mismatches around the nick could repress the ligation velocity of DNA ligase [33]. Tang *et al.* [30] studied the effects of mismatches on the ligation velocity. They determined that in comparison with the complementary base pair around the nick, the mismatch -AG/TT- at the 5′-end of the nick reduced the ligation velocity by 66%, -AT/TT- by 86% and -AC/TT- by >90%, while the mismatch -GA/TT- at the 3′-end of the nick reduced the ligation velocity by 86%, mismatches -CA/TT- and -TA/TT- reduced completely the ligation velocity [30]. Similar results have also been reported previously [39].

The minimal binding size of T4 DNA ligases around the nick has been mapped upon different DNA footprinting approaches [40]. These studies established that T4 DNA ligase bound to nicks asymmetrically, seven to nine bases at the 5′-phosphate end of the nick and three to five bases at the 3′-OH end. Similarly, DNA footprinting studies showed that T7 DNA ligase bound preferentially to the phosphorylated nicks [40]. Additionally, the ligation efficiency of the mismatched substrate was reported in a position-dependent manner [33]. All the results here and previous literature suggested that the change in the 3′-end of the nick influenced the ligation process more than that of the 5′-end.

Mg^{2+} is essential for nick-joining of T4 DNA ligase, and the optimal concentration of Mg^{2+} for ligation has been determined [30,41,42]. In the present study, we observed a bell-shape ligation efficiency curve with respect to the Mg^{2+} concentration, and determined the optimal Mg^{2+} concentration to be 20 mM [30,43]. The 2′-OMeN substitutions repressed ligation and also showed Mg^{2+} concentration-dependent manner (**Fig. 8A**). The dependences of the ligation efficiency on Ca^{2+}, K^+, and Na^+ were also studied, and T4 DNA ligase was tolerant to a broad range (up to 200 mM) of K^+ and Na^+. To elucidate the role of Mg^{2+} in the nucleotidyl transfer during ligation, a transition state configuration was proposed, in which the catalytic Mg^{2+} coordinates to both reacting nucleophiles: the lysyl moiety of the enzyme that forms the phosphoramidated bond and the α–β bridging oxygen of ATP [9].

ATP acts as the energy supplier for T4 DNA ligase in the ligation process [39]. Previous investigations have reported that the optimal concentration of ATP was 0.05 mM [30]. In the present study, we observed that T4 DNA ligase activity was relatively lower for 2′-OMeN-substituted substrates even in the presence of ATP. A previous study has proposed that at low ATP concentration, the ligation efficiency became decreased, because dsDNA prevents binding of ATP to the ligase. On the other hand, when the concentration of dsDNA was low and ATP was high, ATP decreased binding of dsDNA and subsequent ligation by occupying the DNA-binding site [42]. These data indicated that the sugar structural alternation of 2′-OMeN indeed influenced the ligation significantly and demonstrated similar ATP-dependent trends for both the normal and substituted substrates.

In summary, we evaluated the ligation efficiency of T4 DNA ligase using an MB-based FRET assay when DNA duplex substrates were modified with nucleotide derivatives. 2′-OMeN substitutions at the 5′-end and the 3′-end of the nick on DNA duplex demonstrated different decrease effects, and a dual-substitution at both ends of the nick reduced the initial ligation velocity significantly. Kinetic parameters (V_{max}, K_m

and k_{cat}) were determined, and it was found that 2′-OMeN substitutions reduced the binding affinity between enzyme and substrate. This decreasing effect could be due to the sugar pucker conformation alternation. In addition, mismatches at different ends of the nick also decreased the ligation efficiency differently. These results enhance our understanding on the DNA ligation mechanism and will help us to prepare ligase substrates specific for particular applications.

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