Chemical ligation of oligodeoxyribonucleotides on circular DNA templates

Janardhanam Selvasekaran and Kenneth D. Turnbull*

Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR 72701, USA

ABSTRACT

We report the use of small circular DNA as a triplex-directing template for the highly efficient chemical ligation of oligodeoxyribonucleotides (ODNs) using cyanogen bromide (BrCN). These investigations compared the use of a linear homopyrimidine DNA template (17mer) and a circular pyrimidine-rich DNA template (44mer) for directing the chemical ligation of two homopurine ODNs (6mer + 11mer). The effects of substrate/template ratio, buffer, salt, ionic strength, pH and temperature have been examined in the BrCN activated ligation reactions. The optimal yield of 51% for ligation on the linear template was at pH 6.0, 200 mM MgCl₂, 4°C. In contrast, near quantitative ligation on the circular template occurred at higher pH, higher temperature, and showed less dependence on Mg²⁺ concentration (97% yield, pH 7.5, 200 mM MgCl₂, 25°C). The observed relative rate of the ligation reaction was a minimum of 35 times faster on the circular DNA template relative to the linear template at pH 7.5, 200 mM MgCl₂, 4°C. These investigations reveal that chemical ligation of short ODNs on circularized DNA templates through triplex formation is a highly efficient process over a broad range of conditions.

INTRODUCTION

The shift in emphasis of genome research from mapping to sequencing and functional analysis (1) is placing a high priority on the development of tools for functional genomic studies and impending applications (2). Natural and modified oligodeoxyribonucleotides (ODNs) have long functioned in such roles. They are used as research tools for molecular biology (3), as recognition elements in disease diagnostics (4, 5), and are of intense interest as genetic regulators and therapeutic agents (6–9). These expanding applications of ODNs have led us to explore the development of a template-directed approach for the synthesis of ODNs. We report on the use of circular DNA as a template for efficiently directing the ligation of ODNs.

The ability to non-enzymatically direct phosphodiester bond formation of two ODNs in aqueous solution through the action of a phosphate activating reagent and a nucleic acid template was first realized in 1966 (10). Numerous oligonucleotide ligation reactions in duplex– (11–13) and triplex– (14–17) directed systems have been reported since that time. Ligation strategies are advantageous for constructing circular (18–22) and modified oligonucleotides (23–34). Chemically activated template-directed ligation reactions have also gained interest for their potential role in prebiotic DNA and RNA synthesis (35–38). The higher fidelity of template-directed ligation of short oligonucleotides offers a viable alternative to template-directed mononucleotide oligomerization (39,40). The promising results seen in template-directed ligation reactions in terms of yield and reaction rates (41) has yet to be extended into oligomerization reactions. The obvious challenge for template-directed oligomerizations is to improve the substrate–template association while still allowing product turnover. While we are developing approaches to accomplish both of these objectives, this initial report focuses on a modified DNA template to improve substrate–template association.

An approach was sought for improving substrate binding to a DNA template by maximizing aromatic stacking and hydrogen bonding interactions. A pyrimidine-rich DNA template that binds to reacting substrates through both Watson–Crick and Hoogsteen hydrogen bonding would result in a triplex structure with the reacting homopyrimidine substrates bound as the central strand of the triplex (Fig. 1, top). Further improvement in binding and sequence specificity for purine-rich single-strand DNA has been demonstrated by circularizing the pyrimidine-rich strands of the triplex (42). We report the use of a pyrimidine-rich circular DNA template for directing the chemical ligation of homopurine ODNs (Fig. 1, bottom). The circular DNA template has proved far superior to a single-strand DNA template for directing the chemical activated ligation of two ODNs.

MATERIALS AND METHODS

General experimental

Phosphoramidites including 5-methylcytosine, solid supports and chemicals for DNA synthesis were obtained from Cruachem, Inc. or Peninsula Laboratories. All enzymes were purchased from Boehringer Mannheim, New England Biolabs or Gibco BRL Products. The radiolabeled 5’-[γ-32P]ATP (>6000 Ci/mmol) was obtained from Amersham. All other chemicals were of analytical or HPLC grade. Standard molecular biology techniques were used, if not mentioned otherwise (43). Analytical and preparative HPLC was performed with a Shimadzu LC-600 liquid chromatograph with SPD-6A UV using Varian 150 × 4.6 mm, 5 μm, C18, 90 Å column [acetoniitrile–0.1 M triethyl ammonium acetate (TEAA) buffer (pH 7.0) as gradient]. Sep-Pak™ was purchased from Waters. UV measurements were run with a Hitachi U-2000 spectrophotometer. Melting studies were carried out on a Wallac 1410 Liquid Scintillation Counter. Gel

*To whom correspondence should be addressed. Tel: +1 501 575 5078; Fax: +1 501 575 4049; Email: kturnbul@comp.uark.edu
images were scanned using AGFA-ARCUS II scanner at high resolution, low contrast and imported using Adobe Photoshop 4.0 software. Quantitative analyses of gel images were done with IP Lab Gel using scanned images.

**Oligonucleotide synthesis**

Oligodeoxyribonucleotides were synthesized on an Applied Biosystems 392 synthesizer using β-cyanoethyl phosphoramidite chemistry (44). Phosphorylation at the 3′-end of the pre-circle oligomer was carried out using modified solid support purchased from Peninsula Laboratories. Cleavage of the solid supports along with the base protecting groups and the phosphate protecting groups were achieved using concentrated ammonium hydroxide. Oligomers were purified by electrophoresis on 20% polyacrylamide gels with 8 M urea and Tris-borate-EDTA (TBE) buffer. The oligonucleotides were isolated from the gels by excision and elution with Tris-EDTA-NaCl (TEN) buffer. The resulting solution was desalted (using Sep-Pak™) and quantified by absorbance at 260 nm using extinction coefficients that were calculated by the nearest neighbor method (45).

**Circularization procedure**

Linear 3′-phosphorylated oligonucleotide and the complementary homopurine template were combined in a 1:1 ratio (50 mM) with MgCl₂ (20 mM) in morpholinoethanesulfonic acid-Et₃N buffer (MES) (250 mM, pH 7.5). After heating to 95 °C, the solution was cooled to 4 °C (19). The reaction was initiated by adding BrCN (500 mM) and allowed to proceed for 24 h at 4 °C. The reaction mixture was quenched with EDTA, lyophilized and redissolved in the loading buffer, and purified by 20% denaturing PAGE. The circular products migrated at 72% the rate of the linear precursor. Circular oligonucleotide 1 was isolated in 60% yield. To verify the product, melting studies were performed on circular oligonucleotide 1 with the complementary single-strand oligonucleotide and compared with the corresponding precircularized, linear oligonucleotide and the complementary single-strand oligonucleotide under identical conditions (see supplementary material). Oligonucleotides were diluted to 3 µM each with 100 mM NaCl, 10 mM MgCl₂ in 10 mM MES-Et₃N (pH 7.5). Solutions were heated to 90 °C and allowed to cool slowly to room temperature prior to the melting experiments. Absorbance (260 nM) was monitored while temperature was raised at a rate of 0.5°C/min. In all cases the complexes displayed sharp, two-state transitions. Melting temperatures (Tₘ) were taken to be the temperature of half-dissociation and were obtained from a plot of temperature versus absorbance at 260 nM. Precision in Tₘ values were estimated from variations of a minimum of two repeated experiments was ±0.5°C. An increase of 11°C in Tₘ was obtained from the closure of the linear to the circular oligonucleotide 1. Further verification that 1 was circularized was realized by showing the complete resistance of 1 to cleavage by exonucleases.

**Radiolabeling**

The gel purified oligomer B (10 pmol, without 5′-phosphate) was dissolved in 10.4 µl of sterilized, double deionized water. To this was added 2 ml of 10x kinase buffer, 6 µl of [γ-32P]ATP (60 µCi), and 2 µl of T4 polynucleotide kinase (10 000 U/ml) for a total volume of 21.4 µl. Following incubation at 37°C for 3 h, the reaction mixture was heated at 70°C for 10 min and the radiolabeled oligomer purified by chromatography.

**Ligation reactions**

A 1:1 mixture of radiolabeled oligomer B (1 pmol) and ligating fragment A (1 pmol) along with template I or II (2.0 equivalents) was dissolved in 10 µl of MES-Et₃N buffer (500 mM) and 4 µl of the appropriate concentration of MgCl₂ (1.0, 0.10 or 0.01 mM) was added. The Eppendorf tube was heated to 95°C and cooled to the reaction temperature. A solution of BrCN (2 µl, 5.0 M in CH₃CN) was added, vortexed, briefly centrifuged and the reaction was allowed to proceed at the desired temperature (4 or 25°C), (20 µl total volume). The final concentration of the ligation reaction components were: buffer (250 mM), MgCl₂ (2, 20 or 200 mM) and BrCN (500 mM). At precise intervals, an aliquot (1 µl) of the solution was taken and transferred to a tube containing 48 µl of water and 2 µl of 0.5 M EDTA (pH 7.5). For analysis of shorter reaction times (as fast as 1.0±0.5 s) during the course of the ligation reactions, the entire reaction mixture was immediately frozen by submersion of the Eppendorf tube in a liquid N₂ bath followed by addition of EDTA (20 mM, 1.0 ml) and equilibration to ambient temperature with vortexing. An aliquot (1 µl) of that resulting solution was evaporated to dryness, redissolved in the loading buffer, normalized and subjected to electrophoresis on polyacrylamide gel (8%, 8 M urea). Autoradiography was used to analyze the reaction progression. Product yields were determined from the scanned images of the autoradiograms. The yield was calculated from the densitometry ratio of ligated product C relative to the total radioactivity per lane.

**RESULTS AND DISCUSSION**

Our initial studies of template-directed reactions compared the single ligation of two homopurine ODNs on pyrimidine-rich circular DNA template I (Scheme 1) relative to homopyrimidine
linear template 2 [dTTCCTCTTTTTCTC] (43,44). Template 2 was designed for duplex-directed ligation reactions through Watson–Crick hydrogen bonding alone. Circular template 1 was designed with partial incorporation of 5-methylcytidine in order to improve selectivity for the Hoogsteen hydrogen bonding to one side of the circular template (Fig. 1, top) (46–49). Template 1 was synthesized in 60% yield by slight modification of an existing procedure (19).

![Diagram](image)

Scheme 1.

The single ligation reaction was optimized on both circular template 1 and linear template 2 using ligation ODNs A (a 6mer) and B (an 11mer) (Scheme 1). ODN B was 5'-32P-labeled (43) for analysis and quantification of the ligation reactions by autoradiogram densitometry. These investigations focused on the effects of substrate/template ratio, buffer, salt, ionic strength, pH and temperature in the BrCN activated ligation reaction to produce C (Scheme 1). Initial ligation experiments of A and B (1:1, 0.1 µM) on template 1 (2.0 equivalents) at 4°C with BrCN (125 mM) in imidazole–HCl buffer (200 mM, pH 6.0 or 7.0) in the presence of Mg$^{2+}$ (20 mM) or Ni$^{2+}$ (100 mM) afforded ligation product C in very poor yield. Greatly improved yields were realized by switching to a 4-morpholinoethanesulfonic acid buffer (MES-HCl buffer, 200 mM, pH 6.0 or 7.0) in the presence of Mg$^{2+}$ (20 mM) or Ni$^{2+}$ (100 mM) afforded ligation product C in near quantitative yield (97%, pH 7.5, 200 mM MgCl$_2$, 25°C) (Fig. 2, graph C).

Attempts were made to assess the difference in the observed rate of formation of ligated product C in the BrCN activated reaction on circular template 1 compared to linear template 2. Conventional autoradiogram densitometric analysis of the amount of ligated product C in reaction aliquots from both the single-strand and circular template directed reactions on identical conditions (pH 7.5, 200 mM MgCl$_2$, 4°C) at early time points in the reactions failed to afford a satisfactory reproducible linear correlation. While we are investigating more precise methods for comparing the observed rates of the template directed ligation reactions at early reaction times, a qualitative comparison can be made with the data acquired. A minimum yield of 3% ligated product C was required for accuracy by the densitometric analysis methods used. A relative comparison between the earliest reproducible data points (minimum of two independent experiments within ±1% yield) can be compared between the ligation on circular template I and linear template 2. Densitometric analysis of the autoradiogram of aliquots taken at 1.0 ± 0.5 s from the ligation reaction directed by circular template 1 reveal a 39% yield of radiolabeled ligation product C. This corresponds to a reaction which is ~53% complete (final yield of 73% C). The earliest data afforded sufficient product yield in the corresponding linear template 2 directed ligation was obtained at 30 ± 0.5 s, where a 3% yield of C was observed. This corresponds to a reaction which is ~11% complete (final yield of 27% C).

![Graphs](image)

Figure 2. 3-D bar graphs showing the yield (%) of ligation product C. Graph I shows ligation results at 4°C, pH 7.5 and 6.0 with MgCl$_2$ concentrations of 2, 20 and 200 mM. Graph II shows the same ligation reactions run at 25°C. Data for these graphs was obtained at a reaction time of 3 h. All reactions were reproduced at least twice to afford a % yield error of ±3.
CONCLUSION

A comparative study of the non-enzymatic ligation of two homopurine ODNs on a pyrimidine-rich circular DNA template and a homopyrimidine single-strand DNA template has been accomplished. The optimal conditions for ligation on single-strand template 2 afforded 51% yield. In contrast, near quantitative ligation (97% yield) on circular template 1 occurred at higher pH, higher temperature, and showed less dependence on Mg²⁺ concentration. The observed rate of the ligation reaction was a minimum of 35 times faster on circular template than the single-strand template. These investigations reveal that chemical ligation of short ODNs on circularized DNA templates through triplex formation is a highly efficient process over a broad range of conditions. This confirms the expected advantage of improving the substrate binding in template-directed ligation reactions by the use of circular DNA templates. The thermodynamic advantage in template binding has allowed for ligation under conditions of higher pH and higher temperatures where the ligation reaction is much more efficient. The possible advantages on the kinetics of the ligation reaction will require further investigations.

The significant yields and reaction rates found in these investigations suggest the high potential for use of circular DNA templates in development of a chemically activated template-directed methodology for the synthesis of homopurine ODNs. Multiple ligations, oligomerizations and reactions of non-natural nucleic acid derivatives are in progress. Extending this methodology beyond purine derivatives for circular template-directed reactions and improving product turnover are under development.

See supplementary material including gel densitometry images and Tm measurements for product characterization on NAR Online and at our web site http://www.uark.edu/depts/cheminfo/uarkchem

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REFERENCES