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Expression, purification and biochemical characterization of *Methanocaldococcus jannaschii* DNA ligase

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ABSTRACT

We describe the biochemical characterization of *Methanocaldococcus jannaschii* (*M. jannaschii*) DNA ligase and its potential application in single nucleotide polymorphism (SNP) genotyping. The recombinant *M. jannaschii* DNA ligase is an ATP-dependent ligase. The ligase activity was dependent on metal ions of Mg^{2+} and Mn^{2+}. The optimal concentrations of ATP cofactor and Mg^{2+} ion were 0.01–2 and 10 mM, respectively. The optimal pH value for DNA ligation was 8.5. High concentrations of NaCl inhibited DNA ligation. The effects of mismatches on joining short oligonucleotides by *M. jannaschii* DNA ligase were fully characterized. The mismatches at the first position 5′ to the nick inhibited ligation more than those at the first position 3′ to the nick. The mismatches at other positions 5′ to the nick (3rd to 7th sites) exhibited less inhibition on ligation. However, the introduction of a C/C mismatch at the third position 5′ to the nick could completely inhibit the ligation of the terminal-mismatched nick of an oligonucleotide duplex by *M. jannaschii* DNA ligase. Therefore, introducing an additional mismatch at the third position 5′ to the SNP site is a more effective approach in genotyping by *M. jannaschii* DNA ligase.

Introduction

DNA ligases play an important role in genome replication and DNA damage repair. DNA ligases are classified into two families according to their nucleotide substrate requirement: ATP-dependent ligases and NAD-dependent ligases [1,2]. The ligase reaction consists of three nucleotidyl transfer steps [1]. In the first step, ligase attacks the phosphorus of ATP or NAD, resulting in the release of PPi or NMN and formation of a covalent ligase-adenylate intermediate. In the second step, the AMP is transferred to the 5′ end of the 5′ phosphate-terminated DNA strand to form DNA-adenylate. In the third step, ligase catalyzes the formation of a phosphodiester bond between 3′-OH and 5′ phosphate, resulting in the release of AMP. ATP-dependent DNA ligases are found in all three domains of life (bacteria, archaea, and eukarya), whereas NAD-dependent ligases are present only in bacteria and entomopoxviruses [1–5].

The archaea are a group of microorganisms, and many belong to extremophiles, living in extreme environments, such as those with high temperatures [6,7]. *Methanocaldococcus jannaschii*[1] is a methane-producing archaea [8]. It grows at pressures of up to more than 200 atm and at an optimum temperature of 85 °C. High temperature and nitrosative stress result in high frequency of base deamination and other types of DNA damage. DNA ligases seal the matured Okazaki fragments in DNA replication and the generated nick in DNA repair. Some DNA ligases from thermophilic bacteria and archaea have been studied [9–15]. DNA ligase is an important enzyme in molecular biology. Aside from ligating recombinant DNA, thermostable DNA ligases have been used in single nucleotide polymorphism (SNP) genotyping [16–18]. SNP is the most abundant form of genetic variation. SNP is important marker that link sequence variations to phenotypic changes. Because of the importance of SNP in the life and medical sciences, a great deal of effort has been devoted to developing accurate, rapid, and cost-effective technologies for SNP analysis. DNA ligases show preferential ligation of matched base pairs, and this then forms the molecular basis of SNP determination [16–18]. Duo to the thermostability, DNA ligases from thermophilic microbe, such as Taq and Tth DNA ligases [16,17], have some advantage over mesothermic DNA ligases, such as T4 DNA ligase [18], in SNP genotyping.

In this paper, we cloned the *lig* gene from *M. jannaschii* and characterized its biochemical properties. *M. jannaschii* DNA ligase is a typical ATP-dependent ligase. To apply *M. jannaschii* DNA ligase in SNP genotyping, the ability of *M. jannaschii* DNA ligase to discriminate terminal base mismatches on the nick was systematically characterized. We determined the best strategy for achieving the highest precision in distinguishing mismatch by *M. jannaschii* DNA ligase; that is, improving discriminative ability via the introduction of an additional mismatch into the DNA duplex. Our results provide useful data for the potential application of *M. jannaschii* DNA ligase in SNP genotyping.

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[1] *Abbreviations used: M. jannaschii*, *Methanocaldococcus jannaschii*; LCR, ligation chain reaction; ss, single-stranded; SNP, single nucleotide polymorphisms.

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Materials and methods

Materials

Pyrobest™ DNA polymerase, restriction endonucleases, and T4 DNA ligase were obtained from TaKaRa (Dalian, China). *Taq* DNA ligase was purchased from NEB. Expression vectors, *Escherichia coli* strain BL21 (DE3), and Ni–NTA His•Bind® resin were purchased from Novagen. *M. jannaschii* genomic DNA was purchased from ATCC. Oligonucleotides were synthesized by Invitrogen (Shanghai, China). All other chemicals and reagents were of analytical grade.

Preparation of DNA ligase from M. jannaschii

The *lig* gene (MJ0171) was amplified by PCR from *M. jannaschii* genomic DNA using a forward primer (5′-CCCCC**CATatg**ctttggagagatgtttg-3′) and reverse primer (5′-CCCCC**GAATTc**tatttccttcctctttggat-3′). The bold letters indicate the recognition sites of restrict endonuclease *Nde* I and *EcoR* I, respectively. The PCR product was digested with restriction endonucleases *Nde* I and *EcoR* I and inserted between the *Nde* I and *EcoR* I sites of pET28A by T4 DNA ligase. The recombinant *M. jannaschii* DNA ligase was expressed in *E. coli* BL21 (DE3) by induction with 0.5 mM IPTG. Induced bacteria were used to purify recombinant *M. jannaschii* DNA ligase using the Ni–NTA His•Bind® resin column. All eluates were collected fractionally and analyzed by 15% SDS–PAGE. The purified *M. jannaschii* DNA ligase was stored in small aliquots at −80 °C.

DNA ligation assay

The 5′ oligonucleotide to be ligated was labeled with the fluorescent group of 6-carboxyfluorescein (6-FAM) at the 5′-end, and the 3′ oligonucleotide was phosphorylated. The 5′ oligonucleotide and 3′ oligonucleotide were hybridized onto a template oligonucleotide to produce a duplex containing a single-stranded nick, which could then be sealed by DNA ligase. The pre-annealed oligonucleotide duplex containing a nick was prepared by annealing the 5′ oligonucleotide, 3′ oligonucleotide, and template oligonucleotide at a mole ratio of 1:2:1.5. The combined oligonucleotides were heated at 90 °C for 5 min, and then cooled down to room temperature and annealed for 15 min.

Enzymatic activity was determined as described in a previous study, with some modifications [19]. Before optimization, the standard reaction buffer (20 μl) contained 40 mM Tris–HCl with pH 7.5, 1 mM ATP, 5 mM DTT, 10 mM $MgCl_2$, and 0.1 μg/μl BSA. The pre-annealed oligonucleotide duplex containing a nick was incubated with DNA ligase at 50 °C for 15 min, unless otherwise specified. Reactions were stopped by adding an equal volume of loading buffer (95% formamide, 50 mM EDTA, 0.02% bromophenol blue, and 0.02% xylene cyanol). Ligated and unligated oligonucleotides were separated by electrophoresis through 15% polyacrylamide gel containing 8 M urea, and then visualized and quantified using Phosphorimager and ImageQuant software (FL5100, Fujii).

The pH value, NaCl concentration, divalent ion, and cofactor were optimized using the standard reaction buffer as basis. The following buffers (40 mM) of varying pH were used to determine the optimal pH: imidazole–HCl with pH 5.0, 5.5, 6.0, or 6.5; Tris–HCl with pH 7.0, 7.5, 8.0, or 8.5; and glycine/NaOH with pH 9.0, 9.5, 10.0, or 10.5. The optimization of NaCl concentration was performed in the presence of 40 mM Tris–HCl with pH 8.5. The effects of divalent ions and nucleotide cofactors in the absence of NaCl were determined. The concentrations of $MgCl_2$ and ATP were further optimized after confirming Mg^{2+} and ATP as the required divalent ions and cofactor, respectively. Finally, the reaction temperature, thermal stability, and kinetic parameters were characterized in an optimal buffer containing 40 mM Tris–HCl with pH 8.5, 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, and 0.1 μg/μl BSA.

Characterization of base specificity of M. jannaschii DNA ligase

The base mismatches were introduced into the oligonucleotide duplex near the nick, and their effects on ligation were determined in the optimal reaction buffer. Base mismatches included 12 mismatches at the first position both 5′ and 3′ to the nick. The effects of mismatches at other positions 5′ to the nick were also characterized. Ligations of oligonucleotide duplexes containing a nick and two mismatched base pairs were also assessed.

Ligation by ligation chain reaction

Ligation chain reaction (LCR) was performed in a 25 μl solution containing two short oligonucleotides, a template ss oligonucleotide, 40 mM Tris–HCl with pH 8.5, 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, 0.1 μg/μl BSA, and DNA ligase. The LCR protocol consisted of a 5 min preheating at 95 °C, followed by denoted cycles of denaturation at 94 °C for 30 s, ligation at 56 °C for 4 min, with a final 5 min ligation step at 56 °C. The ability of *M. jannaschii* DNA ligase to discriminate mismatch was compared with that of *Taq* thermostable DNA ligases by LCR.

Results

DNA ligase activity of M. jannaschii

The recombinant *M. jannaschii* DNA ligase was obtained after expression in *E. coli* and purification through a Ni–NTA resin column. *M. jannaschii* DNA ligase was electrophoretically homogenous as demonstrated by 15% SDS–PAGE (Fig. 1A). The DNA ligase activity was assayed using synthesized oligonucleotides as substrate. *M. jannaschii* DNA ligase could efficiently seal the nick of a pre-annealed oligonucleotide duplex (Fig. 1B).

Biochemical characterization of M. jannaschii DNA ligase

The biochemical properties of *M. jannaschii* DNA ligase were characterized using a pre-annealed oligonucleotide duplex (Fig. 2). *M. jannaschii* DNA ligase showed evident ligation activity at pH values ranging from 6.5 to 10.0, with the highest activity at pH 8.5 (Fig. 2A). NaCl exhibited some inhibition on the ligation by *M. jannaschii* DNA ligase (Fig. 2B). No ligated product was generated with a NaCl concentration of more than 100 mM (Fig. 2B, lanes 5–7). The ligation activity of *M. jannaschii* DNA ligase was strictly dependent on the divalent ions of Mg^{2+} or Mn^{2+} (Fig. 2C, lanes 2, 4, 5). Ca^{2+} also exhibited some activation on ligation (Fig. 2C, lane 6). The divalent ions of Zn^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+}, however, showed no activation on ligation by *M. jannaschii* DNA ligase (Fig. 2C, lanes 3, 7–11). *M. jannaschii* DNA ligase showed ligation activity with a 2 to 25 mM concentration of Mg^{2+} (Fig. 2D, lanes 6–9). A high concentration of Mg^{2+} inhibited ligation (Fig. 2D, lane 10). ATP was the best nucleotide cofactor for *M. jannaschii* DNA ligase (Fig. 2E, lane 3); dATP also exhibited some activation on ligation (Fig. 2E, lane 7). All other nucleotide cofactors, including NAD, NADH, UTP, CTP, GTP, dTTP, dCTP, and dGTP, played no role in ligation by *M. jannaschii* DNA ligase (Fig. 2E, lanes 1, 2, 4–6, 8–11). A 0.01 mM ATP concentration could efficiently activate ligation (Fig. 2F, lane 2). Similar to Mg^{2+}, a high concentration of ATP inhibited ligation (Fig. 2F, lanes 7, 8). *M. jannaschii* DNA ligase showed evident ligation activity at temperatures ranging from 37 to 60 °C (Fig. 2G). The *M. jannaschii* DNA ligase was thermal stable and remained about 80% activity after heating for 20 min at 90 °C