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Mutational Analyses of *Aquifex pyrophilus* DNA Ligase Define Essential Domains for Self-Adenylation and DNA Binding Activity

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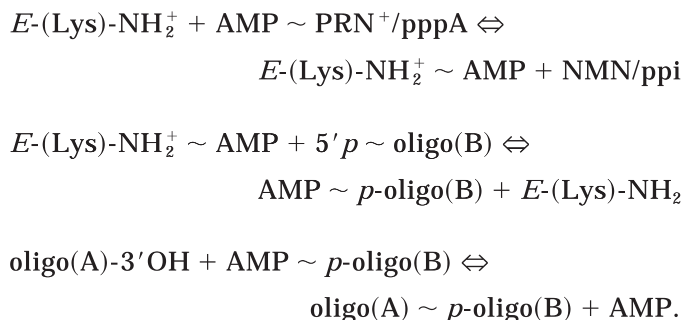
We constructed nine deletion mutants of NAD$^+$-dependent *DNA* ligase from *Aquifex pyrophilus* to characterize the functional domains. All of DNA ligase deletion mutants were analyzed in biochemical assays for NAD$^+$-dependent self-adenylation, DNA binding, and nick-closing activity. Although the mutant lsub1 (91–362) included the active site lysine (KxDG), self-adenylation was not shown. However, the mutants lsub6 (1–362), lsub7 (1–516), and lsub9 (1–635) showed the same adenylation activity as that of wild type. The lsub5 (91–719), which has the C-terminal domain (487–719) as to lsub4 (91–486), showed minimal adenylation activity. These results suggest that the presence of N-terminal 90 residues is essential for the formation of an enzyme–AMP complex, while C-terminal domain (487–719) appears to play a minimal role in adenylation. It was found that the presence of C-terminal domain (487–719) is indispensable for DNA binding activity of lsub5 (91–719). The mutant lsub9 (1–635) showed reduced DNA binding activity compared to that of wild type, suggesting the contribution of the domain (636–719) for the DNA binding activity. Thus, we concluded that the N-terminal 90 residues and C-terminal domain (487–719) of NAD$^+$-dependent *DNA* ligase from *A. pyrophilus* are mutually indispensable for binding of DNA substrate. © 2001 Academic Press

Key Words: **NAD$^+$-dependent *DNA* ligase; *Aquifex pyrophilus;* deletion mutant; self-adenylation; nick-closing activity; DNA binding activity.**

DNA ligase plays an essential role in DNA replication, repair, and/or recombination (1, 2). It catalyzes the formation of phosphodiester bonds at single-strand or double-strand breaks between the adjacent 3′-hydroxyl and 5′-phosphate terminus. DNA ligases can be divided into two groups on the basis of the required cofactors: DNA ligases from bacteriophages, archaeas, eukaryotes, and viruses utilize ATP as a cofactor, whereas those from prokaryotic organisms require NAD$^+$ (3–5).

DNA ligase catalyzes the joining of a 5′-phosphate-terminated donor strand to a 3′-hydroxyl-terminated acceptor strand via a common pathway involving three sequential nucleotide transfer reactions (6, 7):



$$E\text{-(Lys)-NH}_2^+ + \text{AMP} \sim \text{PRN}^+/\text{pppA} \Leftrightarrow E\text{-(Lys)-NH}_2^+ \sim \text{AMP} + \text{NMN/ppi}$$

$$E\text{-(Lys)-NH}_2^+ \sim \text{AMP} + 5'p \sim \text{oligo(B)} \Leftrightarrow \text{AMP} \sim p\text{-oligo(B)} + E\text{-(Lys)-NH}_2$$

$$\text{oligo(A)-}3'\text{OH} + \text{AMP} \sim p\text{-oligo(B)} \Leftrightarrow \text{oligo(A)} \sim p\text{-oligo(B)} + \text{AMP}.$$

In the first step, a covalently adenylated enzyme intermediate is formed by transfer of an adenylate group of NAD$^+$ or ATP to the ϵ-amino group of lysine in the enzyme, resulting in the formation of phosphoamide linkage between the ϵ-amino group of lysine and the phosphate of AMP. In the second step, the adenylate moiety is transferred from the enzyme to the 5′-terminal phosphate of the oligo(B) to form AMP~*p*-oligo(B) with an inverted 5′-5′ pyrophosphate bridge structure. In the final step, a phosphodiester bond is formed by a

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TABLE I

List of Primer Sequences for the Deletion Mutants of *Ap* DNA Ligase

Name	Usage	Sequences
LIG-F	Expression	5′ G C C T C A C G T T C A C A T A T G T T C A C C C C C G A A A G G G A A A G G 3′
LIG-R	Expression	5′ G C T A G G C A T G T C G G A T C C T T A A A A T A G C C T T C C C A T C T T A A C C T C 3′
Lsub1F	Mutant	5′ G C C T C A C G T T C A C C A T G G C G A G C G A G A T A A C G G G A G A G 3′
Lsub1R	Mutant	5′ G C T A G G C A T G T C G G A T C C T T A T C C A A C C T G G A A A A C A A C A T C 3′
Lsub2R	Mutant	5′ G C T A G G C A T G T C G G A T C C T T A C C C G C T T C T C T T T T C C T T T A A 3′
Lsub3R	Mutant	5′ G C T A G G C A T G T C G G A T C C T T A A G C T T C C C T G C T T G C C C A G T G 3′
Lsub4R	Mutant	5′ G C T A G G C A T G T C G G A T C C T T A C G C A T C A C C G A G A C C T C T T A T G 3′
Lsub7R	Mutant	5′ G C T A G G C A T G T C G G A T C C T T A T C C C G G A A G C C T G A G G A G G 3′
Lsub8F	Mutant	5′ G C C T C A C G T T C A C C A T G C T C A G G G C T A T A G A G G A G A G 3′
Lsub9R	Mutant	5′ G C T A G G C A T G T C G G A T C C T T A A A G G G T T C C C G T A A A G A C A A A G 3′

Note. The primers were synthesized by BioSynthesis (USA).

nucleophilic attack of the 3′-hydroxyl terminus of oligo(A) on the activated 5′-phosphoryl group of oligo(B).

DNA ligase as an enzyme was first identified in 1967 (8, 9), and the three-dimensional structure of bacteriophage T7 DNA ligase and N-terminal fragment of a NAD^+-dependent DNA ligase from *Bacillus stearothermophilus* were solved by X-ray crystallography (10, 11). T7 DNA ligase, a 359-amino-acid-polypeptide, is composed of two domains with a groove running between them. Domain 1 includes antiparallel β sheet which is the ATP binding site, whereas domain 2 consists of a twisted antiparallel β sheet which is the DNA binding site. The central core of adenylation domain of an NAD^+-dependent DNA ligase from *B. stearothermophilus* shares homology with the equivalent region of ATP-dependent DNA ligases, giving a strong evidence for the location of the NAD^+-binding site.

In order to identify the essential residues of DNA ligases, several ligases were investigated through mutational analyses using proteolysis, deletion, or base-substitution mutants (12–15). Limited proteolysis was used to characterize NAD^+ dependent DNA ligase from *B. stearothermophilus* with thermolysin (16). The N-terminal domain was found to be responsible for cofactor binding and self adenylation, while the C-terminal domain is a nicked DNA recognition unit. Crystal structure of the NAD^+-dependent DNA ligase from *Thermus filiformis* (*Tfi* DNA ligase), a 667-residue multidomain protein, has been determined (17). *Tfi* DNA ligase was found to have a unique circular arrangement of its four distinct domains—domain 1 (adenylation), domain 2 (OB-fold), domain 3 (zinc finger and HhH motif), and domain 4 (BRCT), which leads to a hole large enough to hold a double-stranded DNA.

We recently cloned and overexpressed *A. pyrophilus* DNA ligase (*Ap* DNA ligase) in *Escherichia coli. Aquifex pyrophilus* (18) is a marine hyperthermophilic bacterium that grows between 67 and 95°C, with an optimum growing temperature of 85°C. The essential domains and residues of ATP-dependent DNA ligase were almost completely determined from the studies, but not much of the NAD^+-dependent DNA ligases. Therefore, we designed total nine deletion mutants of *Ap* DNA ligases and analyzed to find the essential domains for NAD^+-dependent self-adenylation, DNA binding and nick joining activity.

EXPERIMENTAL PROCEDURES

Proteolysis of Ap DNA ligase. *Ap* DNA ligase was proteolized with subtilisin in a reaction mixture containing 200 mM sodium bicarbonate (pH 8.3) and 100 mM $CaCl_2$ for 2 h at 16°C. The proteolytic products were resolved by 12% SDS–polyacrylamide gel electrophoresis. The polypeptides were electrotransferred to PVDF[2] membrane (Millipore, USA) in transfer buffer (10 mM 3-cyclohexyl amino-1-propanesulfonic acid, pH 11, 10% methanol) at 100 mA overnight. The membrane was rinsed with deionized water and stained with 0.1% Ponceau S in 1% acetic acid. Then the membrane was destained with deionized water and air-dried. The proteolytic band was excised for N-terminal sequencing.

Cloning and purification of the deletion mutants of Ap DNA ligase. The oligonucleotides used as primers (Table I) were synthesized to clone subdomain based on nucleotide sequence of *Ap* DNA ligase. The sense primers among them were designed to introduce an *Nco*I site for the translational initiation codon, ATG, at deletion variants of DNA ligase. The DNA fragments of all nine subdomains produced using PCR were digested with *Nco*I and *Bam*HI and were ligated for further assay into pET3d. The ligation mixture was transformed into *E. coli* BL21 (DE3) containing plasmid pSJS1240 (a gift from Dr. R. Kim, UC Berkeley, CA), that codes for the rare tRNA capable of deciphering arginine (AGA) and isoleucine (ATA). The *E. coli* cells harboring the DNA ligase gene plasmid were grown at 37°C in Luria broth containing 100 μg/ml of ampicillin and 50 μg/ml of spectinomycin. The *Ap* DNA ligase protein was induced by adding 1 mM isopropyl β-D-thiogalactopyranoside to the culture at $OD_{600} \geq 0.8$ and incubation was continued for overnight at 30°C.

The cells were harvested by centrifugation for 10 min at 3000*g* and resuspended in lysis buffer A (50 mM Tris–HCl, pH 7.5, 10 mM DTT,

[2] Abbreviations used: PVDF, polyvinylidene fluoride; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; IPTG, isopropyl β-D-thiogalactoside; OB, oligonucleotide-binding.

10 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride). The resuspended cells were lysed by French press cell (SLM Instrument, Inc.) at 12,000 p.s.i. DNase I was added to the lysate at a final concentration of 20 μg/ml. The lysate was incubated for 30 min in an ice bath and then centrifuged for 40 min at 25,000*g*. The soluble extract was pooled and heated at 80°C for 40 min. After centrifugation at 25,000*g* for 30 min, the supernatant was applied to a S-Sepharose Fast Flow (40 ml, Pharmacia, Sweden) column that was previously equilibrated with buffer A without PMSF. The column was washed and eluted using a linear gradient from 0 to 1 M NaCl in buffer A. DNA ligase was eluted at 0.4 M NaCl. The fraction was applied to a heparin–Sepharose column (15 ml, Pharmacia), which had been preequilibrated with buffer A. The protein was eluted with a linear gradient of 0 to 2 M NaCl in buffer A. DNA ligase eluted at 1 M NaCl was diluted with buffer A to reduce the salt concentration to 100 mM NaCl. The proteins were applied to a HiTrap-Blue (5 ml, Pharmacia) column equilibrated with buffer A containing 100 mM NaCl. The protein was eluted with a linear gradient of NaCl in buffer A. Collected fractions were concentrated to a final protein concentration of 10 mg/ml using an Amicon concentrator. Subsequently, the concentrated protein solution was loaded on a Superdex-S200 gel filtration (Pharmacia) column equilibrated with buffer A containing 100 mM NaCl. The DNA ligase fractions were pooled and purity of the protein sample was checked by electrophoresis on a 10% SDS–polyacrylamide gel. Protein concentration of the enzyme was determined by the Bio-Rad dye reagent with BSA as a standard.

Preparation of a nicked DNA substrate. In order to check the nick-closing activity of the DNA ligase, three oligonucleotides were synthesized by BioSynthesis (USA). The substrate used in the ligase assay was a DNA duplex containing a centrally placed nick and the sequence was as follows:

LA 1: 5′-GGTAAAGCAATGGGCAAACAGGGAAGCTATG-3′ (31-mer)
LA 2: 5′-GACATAAGAGGT CTCGGTGATGACCCAGTAAAGCT-3′ (35-mer)
LA 3: 5′-GGAGCTTTACTGGGTCATCACCGAGACCTCTTATGT CCATAGCTTCCCTGTTTGCCCATTGCTTTACCCTC-3′ (71-mer).

LA-2 was radiolabeled by incubating 100 pmol of the gel-purified oligonucleotide, 100 μCi [γ-^{32}P]ATP (3000 Ci/mmol, NEN-Du Pont Co., Chadds Ford, PA) and 50 U T4 polynucleotide kinase (Promega, Madison, WI) for 60 min at 37°C. After incubation of the enzyme for 10 min at 70°C, the unincorporated radiolabeled ATP was removed by ethanol precipitation with ammonium acetate. The substrate was obtained by annealing the two oligonucleotides (LA-1, LA-2) to a complementary 71mer strand (LA-3) in annealing buffer (50 mM Tris–Cl, pH 7.5 and 200 mM NaCl). The mixture was slowly cooled to room temperature after heating at 90°C for 2 min. The molar ratio of the components, LA-1, LA-2, and LA-3 in annealing mixture was 0.9:1:1, respectively. The other DNA substrates that were used in DNA binding, gel shift, and Southwestern assay were constructed by the same method as described above.

DNA ligation assay. The assay for nick-closing activity of *Ap* DNA ligase was performed according to the previously published method (19, 20). The annealed DNA substrate (0.2 pmol, 71 mer) was incubated in a 10-μl reaction mixture (50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM NAD^+, 100 mM NaCl, 100 pmol of *Ap* DNA ligase, and mutants) for 1 h at 50°C, unless otherwise stated. After incubation, the reaction mixture was treated by protease K (0.2 mg/ml) at 37°C for 1 h and phenol extraction performed twice. Then the stop buffer (95% formamide, 1× TBE, 0.05% bromophenol blue, 0.05% Xylene cyanol, and 0.2% SDS) was added to the reaction mixture followed by heating at 95°C for 5 min. The samples were chilled on ice and analyzed by electrophoresis on a 15% polyacrylamide gel containing 7 M urea in TBE buffer (90 mM Tris–borate and 2.5 mM EDTA) at constant power of 20 W. Gels were dried under vacuum and the ligation products were visualized by autoradiography. For quantitation of the product, the dried gel was scanned using a FUJIX BAS 2000 phosphoimager.

Adenylation and DNA binding assay. The adenylation reaction was assayed by incubating 100 pmol of *Ap* DNA ligase and deletion variants with 1 μCi [^{32}P]NAD^+ (800 Ci/mmol, NEN-Du Pont Co.) in a 20-μl reaction mixture (50 mM Tris–Cl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, and 100 mM NaCl) at 65°C for 30 min. The reaction was stopped by boiling in SDS loading buffer for 5 min and was analyzed by electrophoresis on a 15% SDS–polyacrylamide gel. The gel was dried, and the adenylated protein bands were analyzed by autoradiography on Fuji RX X-ray film.

DNA binding reaction was performed by incubating 100 pmol of *Ap* DNA ligase and deletion mutants at 45°C for 30 min in the binding mixture (20 μl) that consists of 50 mM Tris–Cl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 100 mM NaCl, 1 mM NAD^+, and 0.2 pmol of ^{32}P-labeled nicked duplex as a substrate. The reacted sample was analyzed by a native 15% polyacrylamide gel electrophoresis at 15 mA for 6 h. The band of *Ap* DNA ligase–DNA complexes was visualized by autoradiography.

DNA ligase–DNA binding gel shift and Southwestern assay. DNA binding activity of *Ap* DNA ligase was tested by protein–DNA complex gel shift assay. *Ap* DNA ligase–DNA binding gel shift assay was performed by incubating 200 pmol of *Ap* DNA ligase with 1 μCi [^{32}P]NAD^+ (800 Ci/mmol, NEN-Du Pont Co.) in a 20-μl reaction mixture (50 mM Tris–Cl, 10 mM $MgCl_2$, 10 mM DTT, and 100 mM NaCl) at 65°C for 30 min. Then [^{32}P]NAD^+ labeled *Ap* DNA ligase was further incubated at 45°C for 30 min with nicked dsDNA (0.2 pmol) and ssDNA (0.2 pmol) separately that were substrates of *Ap* DNA ligase assay. The reacted samples were analyzed by a nondenaturing 15% polyacrylamide gel electrophoresis at 15 mA for 6 h. The shifted band of *Ap* DNA ligase–DNA complexes was visualized by autoradiography.

Southwestern assay was performed to confirm ssDNA binding activity of OB fold domain for the deletion mutants lsub6 (1–362) and lsub7 (1–516) (19). The proteins (5 μg) were analyzed by a 10% SDS–polyacrylamide gel electrophoresis and transferred to the PVDF membrane (Millipore) at constant 100 V for 1 h. Membrane-bound lsub 6 and lsub 7 were denatured in 6 M guanidine in hybridization buffer (20 mM Hepes, pH 7.5, 100 mM KCl, 0.1 mM EDTA, and 1 mM DTT) and renatured (20). Membrane was blocked with 5% nonfat dry milk in hybridization buffer and incubated overnight with ^{32}P-end-labeled single-strand DNA (LA3, 4×10^6 cpm/ml) in hybridization buffer with 0.25% milk at 4°C. *Ap* DNA ligase–ssDNA complex was subjected to autoradiography to visualize on an X-ray film.

RESULTS

Construction of the deletion mutants of Ap DNA ligase. *Ap* DNA ligase was digested under limiting conditions with various amounts of the protease and subtilisin. A major digestion product that has a mass of ~36 kDa was determined by SDS–PAGE (data not shown). The 36-kDa fragment was relatively resistant to high concentration of subtilisin. The N-terminal peptide sequencing of the fragment revealed that the peptide bond between V90 and A91 is sensitive to the protease.

A total of nine deletion mutants were constructed based on the results of proteolysis assay and structure of *Ap* DNA ligase (Fig. 5). To characterize the function of central domain, four mutants, lsub1 (91–362), lsub2 (91–427), lsub3 (91–477), and lsub4 (91–486), with different lengths of the central domain, were constructed. Mutant lsub5 (91–719) included the C-termi-

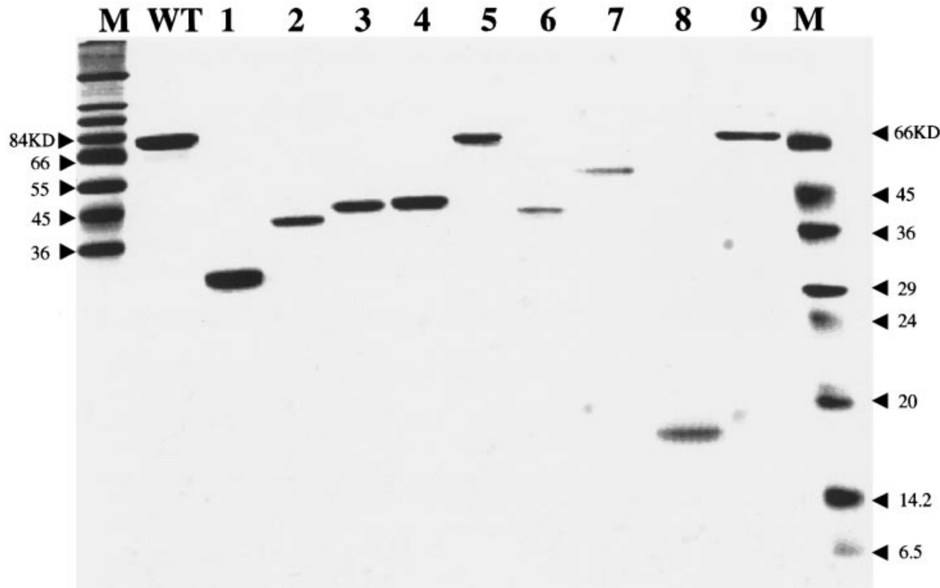

FIG. 1. SDS–electrophoresis of the *Ap* DNA ligase and deletion mutants. The purified proteins were analized on 15% SDS–polyacrylamide gel electrophoresis and stained with Commassie brilliant blue R-350. Native *Ap* DNA ligase and mutants lsub1–lsub9 were loaded on lane WT and lanes 1–9, respectively, and M was a molecular size marker.

nal region compared to lsub4. Mutants lsub6 (1–362), lsub7 (1–516), and lsub9 (1–635) were constructed to characterize the N-terminal domain, and mutant lsub8 (526–719) was designed to elucidate the role of C-terminal domain.

Each cloned mutant-expression vector was introduced to *E. coli* BL21(DE3)-[pSJS1240], a strain transformed by the T7 polymerase gene under the control of *lac*UV5 promoter. Expression of the deletion mutants was induced by IPTG. *Ap* DNA ligase mutants were purified through S-sepharose, heparin sepharose, Hi-Trap-Blue, and Superdex-S200 columns sequentially. Fractions were taken from each purification step and subjected to electrophoresis on a 15% SDS polyacrylamide gel (Fig. 1). Further analyses were performed with nine deletion variants.

Ligation properties. The assay for nick-closing activity of *Ap* DNA ligase and mutants was performed (Fig. 2). We used ^{32}P-labeled oligmer (Fig. 2A) as a substrate for the nick-joining reaction. The ligation products were loaded onto 15% polyacrylamide urea gel for electrophoresis and the efficiency of the reaction was measured by scanning with a FUJIX BAS 2000 Phosphoimager. Among the nine deletion mutants, only lsub5 (91–719) and lsub9 (1–635) showed the ligation activity. Compared to the activity of wild type, minimal ligation activity was shown by mutant lsub5 (91–719) and reduced activity for the lsub9 (1–635) (Fig. 2B).

Analysis of the enzyme–AMP formation. The adenylation of wild-type *Ap* DNA ligase and mutants were compared with one another by incubating each type of DNA ligase in the presence of [^{32}P]NAD$^+$, followed by gel electrophoresis (Fig. 3). It was shown that the wild type and four mutants (lsub5, -6, -7, and -9) formed enzyme–AMP complexes. The mutants (lsub1, -2, -3, and -4) that contain the adenylate-accepting residue Lys142 were not adenylated (21, 22). Furthermore, mutant lsub5, which retained all amino acids except the N-terminal 90 residues, was adenylated only a small amount, while mutants lsub6, lsub7, and lsub9 were adenylated almost as much as that of wild type. This result suggests that the N-terminal 90 residues are necessary for the formation of the enzyme–AMP complex.

Analysis of DNA binding. In order to characterize the DNA binding properties of the *Ap* DNA ligase and constructed mutants, gel mobility shift assay was performed. *Ap* DNA ligase and deletion mutants were incubated with ^{32}P-labeled nicked substrate as described above and applied to nondenaturing polyacrylamide gel electrophoresis (Fig. 4). Only wild type and mutants lsub5 (91–719) and lsub9 (1–635) could form enzyme–DNA complex. The DNA binding activity of lsub5 and lsub9 suggests that C-terminal region are responsible for the binding of DNA substrate. However, lsub8 (526–719) with no DNA binding activity indicates that there is another domain that is necessary for the DNA binding besides the domain 526–719. The DNA binding activity of lsub5 (91–719) was less than that of wild type, suggesting the N-terminal 90 residues may also contribute for binding of DNA sub-

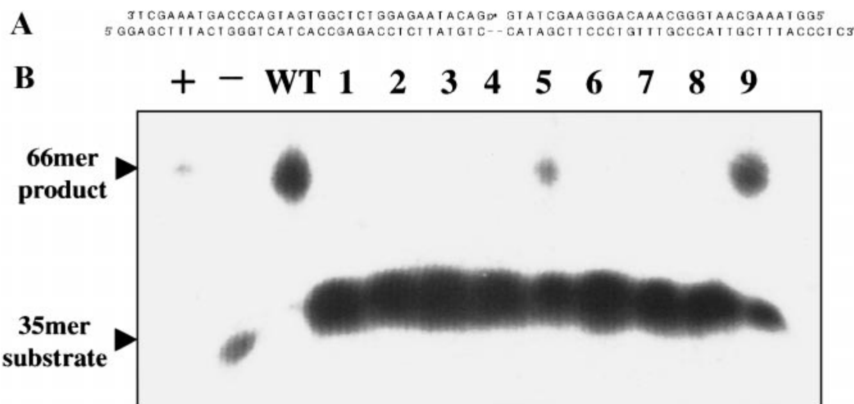

FIG. 2. Activity assay of *Ap* DNA ligase and mutant variants. The activity of *Ap* DNA ligase and mutant variants were determined by ligating ^{32}P-labeled nicked substrate. (A) The structure of nicked DNA substrate used in the activity assay is shown and the ^{32}P-labeled 5′-phosphate at the nick is indicated by the asterisk. (B) Reaction mixtures contained 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM NAD^+, 100 mM NaCl, ^{32}P-labeled nicked substrate (0.2 pmol), 100 pmol of *Ap* DNA ligase, and mutant variants. The reaction mixtures were incubated for 1 h at 50°C and were extracted by phenol extraction. The reactions were analyzed by 15% polyacrylamide gel electrophoresis containing 7 M urea in TBE buffer (90 mM Tris–borate, 2.5 mM EDTA) and were visualized on X-ray film. The substrate, 5′-phosphate-labeled LA3, was loaded on lane + and 5′-phosphate labeled LA1 was loaded on lane − (Refer to Experimental procedures). Native *Ap* DNA ligase and mutants lsub1–lsub9 were loaded on lane WT and lanes 1–9, respectively. *Ap* DNA ligase (WT), lsub5 (lane 5), and lsub9 (lane9) showed the DNA ligase activity, generating ^{32}P-labeled oligomer.

strate. These results suggest that the N-terminal 90 residues and C-terminal domain (486–719) may be mutually indispensable for binding of DNA substrate.

We found that mutant lsub5, which has the DNA binding activity, contains the Cys_4-type zinc finger motif and OB-fold (oligonucleotide/oligosaccharide binding motif) domain (23, 24), based on the sequence homology. Four cysteine residues (Cys437, Cys440, Cys455, and Cys460) of *Ap* DNA ligase were conserved among bacterial DNA ligases (data not shown). Zinc finger motif can be found in the DNA binding proteins, whereas OB-fold is common among RNA or ssDNA-binding proteins, such as the ribosomal proteins like S17 and S1 (25). The sequence of OB fold domain (350–435) for *Ap* DNA ligase has 63% of sequence identity with that of *Tfi* DNA ligase (Fig. 6). The *Ap* DNA ligase was shown to bind more specifically nicked dsDNA (Fig. 7A) than ssDNA (Fig. 7A) in a nondenaturing gel shift assay of ligase–DNA complex (Fig. 7B). SsDNA binding activity of lsub7 (1–516), containing OB-fold, was compared with that of mutant, lsub6 (1–362, no OB-fold). Only lsub7 showed ssDNA binding activity in Southwestern assay (Fig. 7C).

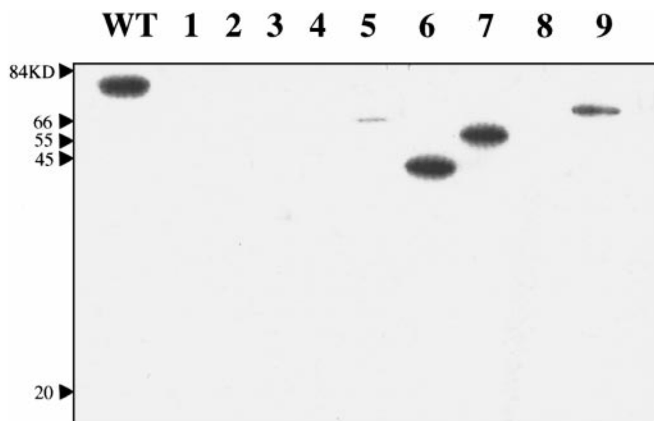

FIG. 3. Adenylation of *Ap* DNA ligase and mutant variants. Adenylation of *Ap* DNA ligase and mutant variants was determined by ligase–[^{32}P]NAD^+ product on 15% SDS–polyacrylamide gel electrophoresis. Reaction mixtures containing 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 100 mM NaCl, 1 μCi [^{32}P]NAD^+ (800 Ci/mmol, NEN-Du Pont Co., Chadds Ford, PA), 100 pmol of *Ap* DNA ligase, and mutant variants were incubated at 65°C for 30 min. The reactions were stopped by boiling in SDS loading buffer for 5 min and were analyzed by 15% SDS–polyacrylamide gel electrophoresis. Native *Ap* DNA ligase and mutants lsub1–lsub9 were loaded on lane WT and lanes 1–9, respectively. *Ap* DNA ligase (WT), lsub5 (lane5), lsub6 (lane6), lsub7 (lane7), and lsub9 (lane9) were covalently bound with ^{32}P-labeled NAD^+ and were visualized on X-ray film.

The C-terminal region of the *Ap* DNA ligase manifested a strong homology to the domain of the eukaryotic replication factor C (RF-C), that is necessary for DNA binding and helps to localize the 3′-end of DNA substrates (26, 27). The murine large subunit of activator 1 (A1, also called replication factor C) was cloned and was shown to have a DNA-binding domain similar to that of bacterial DNA ligases, suggesting that this region could be utilized by both proteins in recognizing DNA (26).

DISCUSSION

Recent demonstrations of DNA repair pathways connecting with human cancers have fueled interest in DNA repair enzymes. Because the DNA ligase can

FIG. 4. DNA binding of *Ap* DNA ligase and mutant variants. DNA binding property of *Ap* DNA ligase and mutant variants were determined by retardation bands of *Ap* DNA ligase and mutant variant–DNA complexes on nondenaturing gel electrophoresis. (A) The structure of nicked substrate used in the DNA binding assay is shown and the ^{32}P-labeled 5′-phosphate at the nick is indicated by the asterisk. (B) *Ap* DNA ligase (WT), lsub5 (lane 5), and lsub9 (lane 9) showed the DNA binding activity. Reaction mixtures contained 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM NAD^+, 100 mM NaCl, 0.2 pmol of ^{32}P-labeled nicked substrate, 100 pmol of *Ap* DNA ligase, and mutant variants. These reactions were incubated for 30 min at 45°C and were analyzed by native 15% polyacrylamide gel electrophoresis. Ligase–DNA binding products were visualized on X-ray film.

restore the continuity of the repaired DNA strand by converting nicks into phosphodiester bonds, the total loss of DNA ligase function would result in cell death. Therefore, intense research on DNA ligase was stimulated in the past few years. The basic reaction mechanism and functions of microbial DNA ligases were identified after the discovery of this enzyme in 1967, but very little of biochemical work on the catalytic properties and functional domains was accomplished.

We constructed nine deletion mutants of *Ap* DNA ligase and identified essential domains for the adenylation and DNA binding. Four different lengths of mutants–lsub1 (91–362), lsub2 (91–427), lsub3 (91–477), and lsub4 (91–486)—that retained the central domain were constructed to find out the minimum required domain for the adenylation and DNA binding. These mutants, lsub1, -2, -3, and -4, that have the AMP binding residue of Lys142 and other conserved sequences, NPRNAAAGS, did not show the adenylation and DNA binding activity, suggesting that only the central region (comprising residues 91–486) itself does not contribute to DNA ligase activity.

The self-adenylation of lsub6 (1–362), lsub7 (1–516), and lsub9 (1–635) were comparable to that of the full length protein. Mutant lsub5 (91–719), which contains the C-terminal domain compared to lsub4 (91–486), has minimal adenylation activity. It suggests that the N-terminal 90 residues are needed for the formation of the enzyme–AMP complex, and C-terminal domain also plays a minimal role in adenylation.

Aquifex pyrophilus DNA ligase contains the oligonucleotide-binding (OB) fold domain which is common

	Start	End	activity	adenylation	DNA binding
WT	1	719	+++	+++	+++
lsub1	91	362	−	−	−
lsub2	91	427	−	−	−
lsub3	91	477	−	−	−
lsub4	91	486	−	−	−
lsub5	91	719	+	+	++
lsub6	1	362	−	+++	−
lsub7	1	516	−	+++	−
lsub8	526	719	−	−	−
lsub9	1	635	++	++	+

FIG. 5. Schematic representation of the deletion mutants of *Ap* DNA ligase and functional characterization of nine mutants. The conserved residues of DNA ligases were noted on the wild-type bar. The activity of the deletion mutants similar to that of wild type was written as (+++); less activity than wild type (++ or +); no activity (−). Ligation activity, adenylation, and nicked DNA binding activity of *Ap* DNA ligase and mutant variants were quantitated using FUJIX BAS 2000 phosphoimager system.

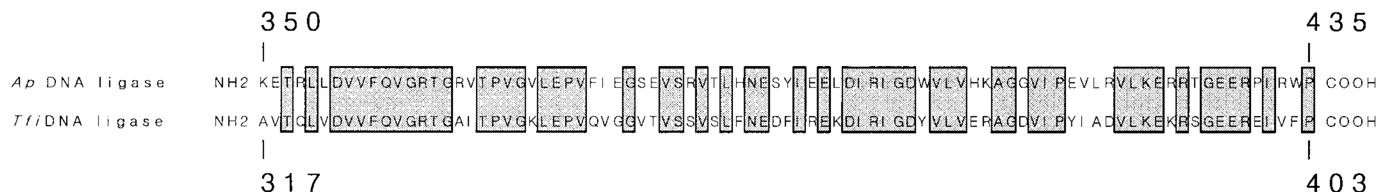

FIG. 6. Sequence alignment of OB fold domain between *Ap* DNA ligase and *Tfi* DNA ligase. A NCBI BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) was applied for sequence alignment. Identical residues are in gray boxes.

among RNA or single-stranded DNA-binding proteins. The ssDNA binding activity of lsub7 (1–562), that contains OB fold domain and zinc finger motif, was confirmed by comparing with that of lsub6 (1–362, Fig. 7C).

The DNA binding properties have been identified for the mutant lsub5 (91–719) and lsub9 (1–636). The mutant lsub5 (91–719) which retains all amino acids except for the N-terminal 90 residues showed decreased DNA binding activity compared to that of wild type, suggesting the contribution of N-terminal residues (1–90) in DNA binding activity. The mutant lsub9 (1–636) which has significantly reduced DNA binding activity suggested that the C-terminal region (637–719) was also responsible for the binding of DNA substrate. The mutant lsub8 (526–719) was designed to confirm the minimal domain for the DNA binding activities, but the DNA binding activity was not found for

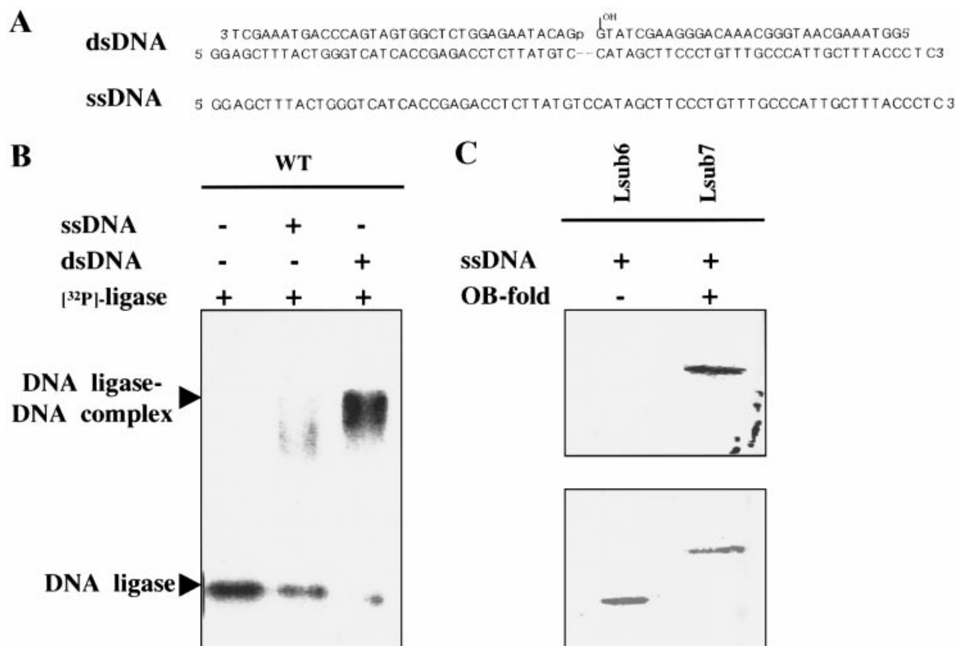

FIG. 7. *Ap* DNA ligase–DNA binding gel shift and southwestern assay. (A) The structure of double-strand (ds) and single-strand (ss) DNA substrate. (B) DNA binding property of *Ap* DNA ligase with ssDNA and dsDNA was determined by observing the shifted band of *Ap* DNA ligase–DNA complex on a nondenaturing 15% polyacrylamide gel. *Ap* DNA ligase (WT)–dsDNA complex band was more shifted compared with that of the *Ap* DNA ligase (WT)–ssDNA complex. Reaction mixtures were described under Experimental Procedures. [^{32}P]NAD$^+$-labeled *Ap* DNA ligase was further incubated at 45°C for 30 min with ssDNA and dsDNA (0.2 pmol of each). The reactions were analyzed by a nondenaturing 15% polyacrylamide gel electrophoresis at 15 mA for 6 h. (C) Southwestern assay was determined using deletion mutants—lsub6 and lsub7—as described. Equal amounts of lsub6 and lsub7 (5 μg) were loaded on a 10% SDS–polyacrylamide gel electrophoresis as shown below. Membrane-bound lsub6 and -7 were denatured in 6 M guanidine in hybridization buffer (20 mM Hepes, pH 7.5, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT) and renatured. Membrane was blocked with 5% nonfat dry milk in hybridization buffer, and incubated overnight with ^{32}P-end-labeled single strand DNA (LA3, 4×10^6 cpm/ml) in hybridization buffer with 0.25% milk at 4°C. *Ap* DNA ligase–ssDNA complex was subjected to autoradiography to visualize on a X-ray film. (Top) Only the lsub7, which has an OB-fold domain, showed ssDNA binding activity; (bottom) deletion mutants, lsub6 and lsub7, which is transferred by PDVF membrane and stained with ponceau S solution.

lsub8. Because *Ap* DNA ligase has 46% of sequence identity with *Tfi* DNA ligase, the domain of *Ap* DNA ligase could be compared with that of *Tfi* DNA ligase. The structure of *Tfi* ligase suggested that catalytic DNA binding site is a positively charged groove at the interface between domains 1 (1–317) and 2 (318–403) (17). Taken together, our results suggest that the N-terminal 90 residues and the C-terminal domain (487–719) may be mutually indispensable for binding of DNA substrate. Another domain lsub10 (350–719) which contains the OB fold, zinc finger motif, and BRCT domain was constructed to identify the minimal domain of DNA binding. However, because of the low expression level of the lsub10, we could not purify the protein.

The C-terminal region of the *Ap* DNA ligase has the homologous sequence to to BRCT domains of *Tfi* DNA ligase (17). The BRCT domain present in NAD^+-dependent DNA ligases is shared by the large subunits of eukaryotic replication C and PARP (26, 27). They could perform critical functions in the cell cycle control of organisms from bacteria to humans, acting as a signal transducer that transmits the signal from DNA damage sensors to other components of the DNA damage-responsive checkpoint machinery via specific protein–protein interactions (27).

NAD^+-dependent DNA ligase from *B. stearothermophilus* has two discrete functional domains, the N-terminal domain that is a fully functioning self-adenylation module and the C-terminal domain that is a fully functional nicked DNA recognition unit (16). In contrast, T7 DNA ligase has two structural domains that act together in both the self-adenylation and DNA binding activities of the enzyme (12). For the *Ap* DNA ligase, N-terminal 362 residues appeared to be sufficient for the self-adenylation activity of the enzyme, and the N-terminal 90 residues and the C-terminal domain (487–719) were shown to be mutually indispensable for binding of DNA substrate. Studies of functional domains of *Ap* DNA ligase using the deletion mutants defined more precisely the role of each domain.

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