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# A DNA Ligase from a Hyperthermophilic Archaeon with Unique Cofactor Specificity

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**A gene encoding DNA ligase ($lig_{Tk}$) from a hyperthermophilic archaeon, *Thermococcus kodakaraensis* KOD1, has been cloned and sequenced, and its protein product has been characterized. $lig_{Tk}$ consists of 1,686 bp, corresponding to a polypeptide of 562 amino acids with a predicted molecular mass of 64,079 Da. Sequence comparison with previously reported DNA ligases and the presence of conserved motifs suggested that $Lig_{Tk}$ was an ATP-dependent DNA ligase. Phylogenetic analysis indicated that $Lig_{Tk}$ was closely related to the ATP-dependent DNA ligase from *Methanobacterium thermoautotrophicum* ΔH, a moderate thermophilic archaeon, along with putative DNA ligases from *Euryarchaeota* and *Crenarchaeota*. We expressed $lig_{Tk}$ in *Escherichia coli* and purified the recombinant protein. Recombinant $Lig_{Tk}$ was monomeric, as is the case for other DNA ligases. The protein displayed DNA ligase activity in the presence of ATP and $Mg^{2+}$. The optimum pH of $Lig_{Tk}$ was 8.0, the optimum concentration of $Mg^{2+}$, which was indispensable for the enzyme activity, was 14 to 18 mM, and the optimum concentration of $K^+$ was 10 to 30 mM. $Lig_{Tk}$ did not display single-stranded DNA ligase activity. At enzyme concentrations of 200 nM, we observed significant DNA ligase activity even at 100°C. Unexpectedly, $Lig_{Tk}$ displayed a relatively small, but significant, DNA ligase activity when $NAD^+$ was added as the cofactor. Treatment of $NAD^+$ with hexokinase did not affect this activity, excluding the possibility of contaminant ATP in the $NAD^+$ solution. This unique cofactor specificity was also supported by the observation of adenylation of $Lig_{Tk}$ with $NAD^+$. This is the first biochemical study of a DNA ligase from a hyperthermophilic archaeon.**

DNA ligases (EC 6.5.1.1 and EC 6.5.1.2) catalyze the phosphodiester bond formation between adjacent 3′-hydroxyl and 5′-phosphoryl groups at a single-strand break in double-stranded DNA (22). They are essential enzymes for maintaining the integrity of the genome during DNA replication (24), DNA excision repair (48), and DNA recombination (16). DNA strand breaks are commonly generated as reaction intermediates in these events, and the sealing of these breaks solely depends on the proper function of DNA ligase. Therefore, DNA ligases are indispensable enzymes in all organisms.

DNA ligases fall into two groups on the basis of the required cofactor for activity: the group requiring ATP (8) and the group requiring $NAD^+$ (43). There is high similarity among the ligases within the ATP-dependent group (19) or $NAD^+$-dependent group (42). ATP-dependent DNA ligase I from humans and *Saccharomyces cerevisiae* are 42% identical, and $NAD^+$-dependent enzymes from *Escherichia coli* and *Thermus thermophilus* are 46% identical. However, enzymes between the two groups show no similarity, with the exception of the KXDG motif, which includes the active-site lysine (19). Furthermore, biochemical investigations have indicated that there is strict specificity towards the respective cofactors. This suggests that the two groups have evolved through completely different pathways.

It is now accepted that both ATP-dependent and $NAD^+$-dependent DNA ligases catalyze their reactions through a common mechanism (7). The ligation reaction proceeds through three steps: (i) activation of the enzyme through the covalent addition of AMP to the conserved active-site lysine of the protein, accompanied by the release of $PP_i$ or nicotinamide mononucleotide from the cofactor (ATP or $NAD^+$), (ii) transfer of AMP from the protein to the 5′-phosphoryl group of the nick on the DNA, and (iii) phosphodiester bond formation with concomitant release of free AMP from the adenylated DNA intermediate.

Biochemical and genetic studies have been performed for DNA ligases from various organisms. It has been shown that eukaryotes (17, 33, 46), viruses (29, 30), and bacteriophages (7, 20) harbor ATP-dependent enzymes. Although only a single type of DNA ligase has been reported for viruses and bacteriophages, eukaryotic organisms have multiple enzymes. Five distinct DNA ligases have been reported from mammalian cells (17, 33, 45, 46). ATP-dependent enzymes show a wide range in molecular mass, from 41 kDa (bacteriophage T7) (7) to 102 kDa (human DNA ligase I) (2). This considerable difference in size is mainly due to the diversity of the N-terminal region of each DNA ligase. DNA ligase I from humans includes a regulatory domain of 216 amino acid residues in its N-terminal region, which is dispensable for DNA ligase activity in vitro (5, 31). On the other hand, the C-terminal catalytic domain of DNA ligase I is highly similar among ATP-dependent DNA ligases (19, 47). The active-site lysine of these DNA ligases is mostly located at a distance of 332 ± 20 residues from the C terminus (47).

Various DNA ligases from bacteria (3, 15, 41, 42) have also been characterized and have been shown to utilize $NAD^+$ as a cofactor. These enzymes include those from mesophiles, such as *E. coli* (11), and thermophilic bacteria, such as *Bacillus stearothermophilus* (3), *T. thermophilus* (41), *Thermus scotoductus* (42), and *Rhodothermus marinus* (42). The enzyme from *T.*

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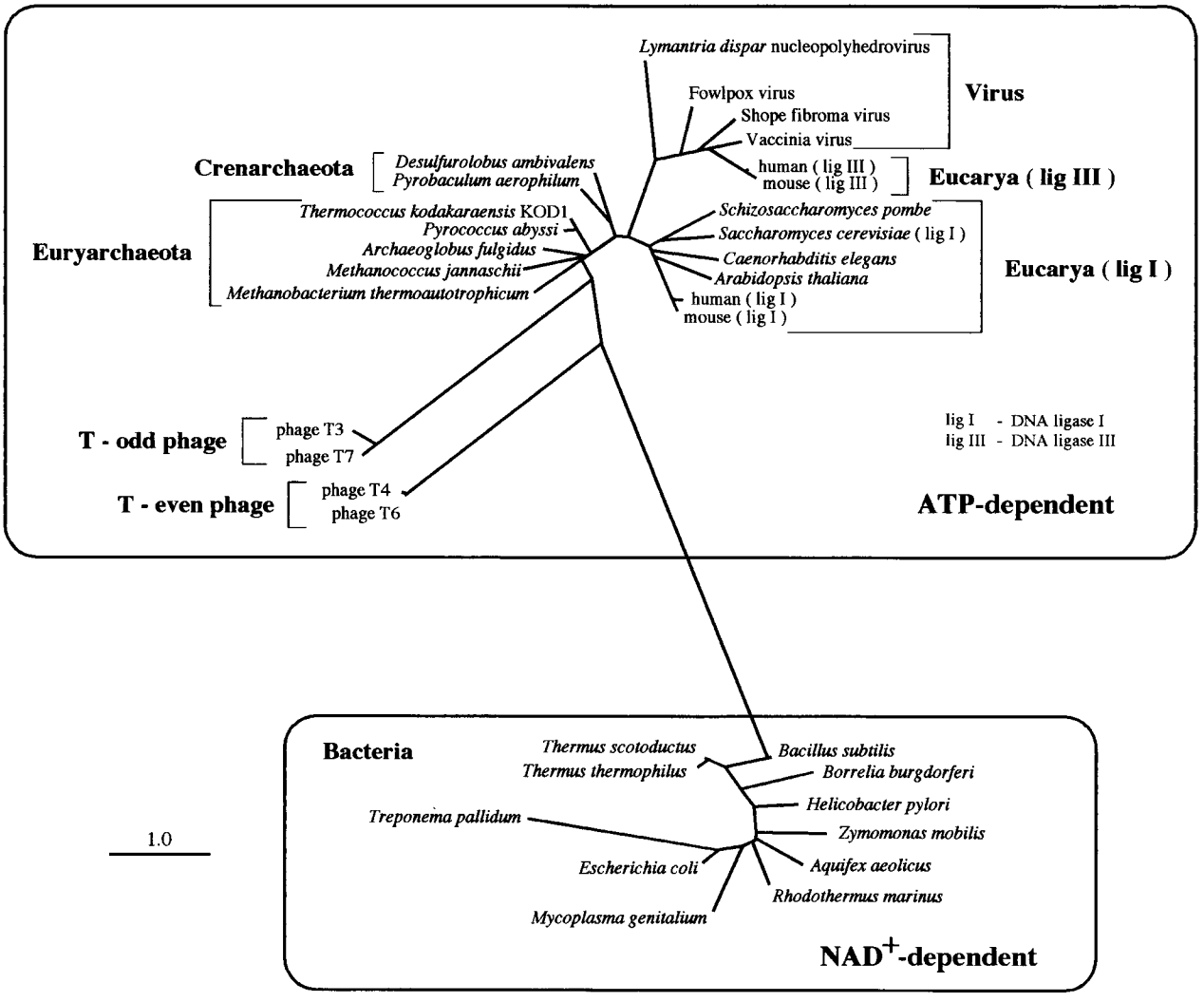
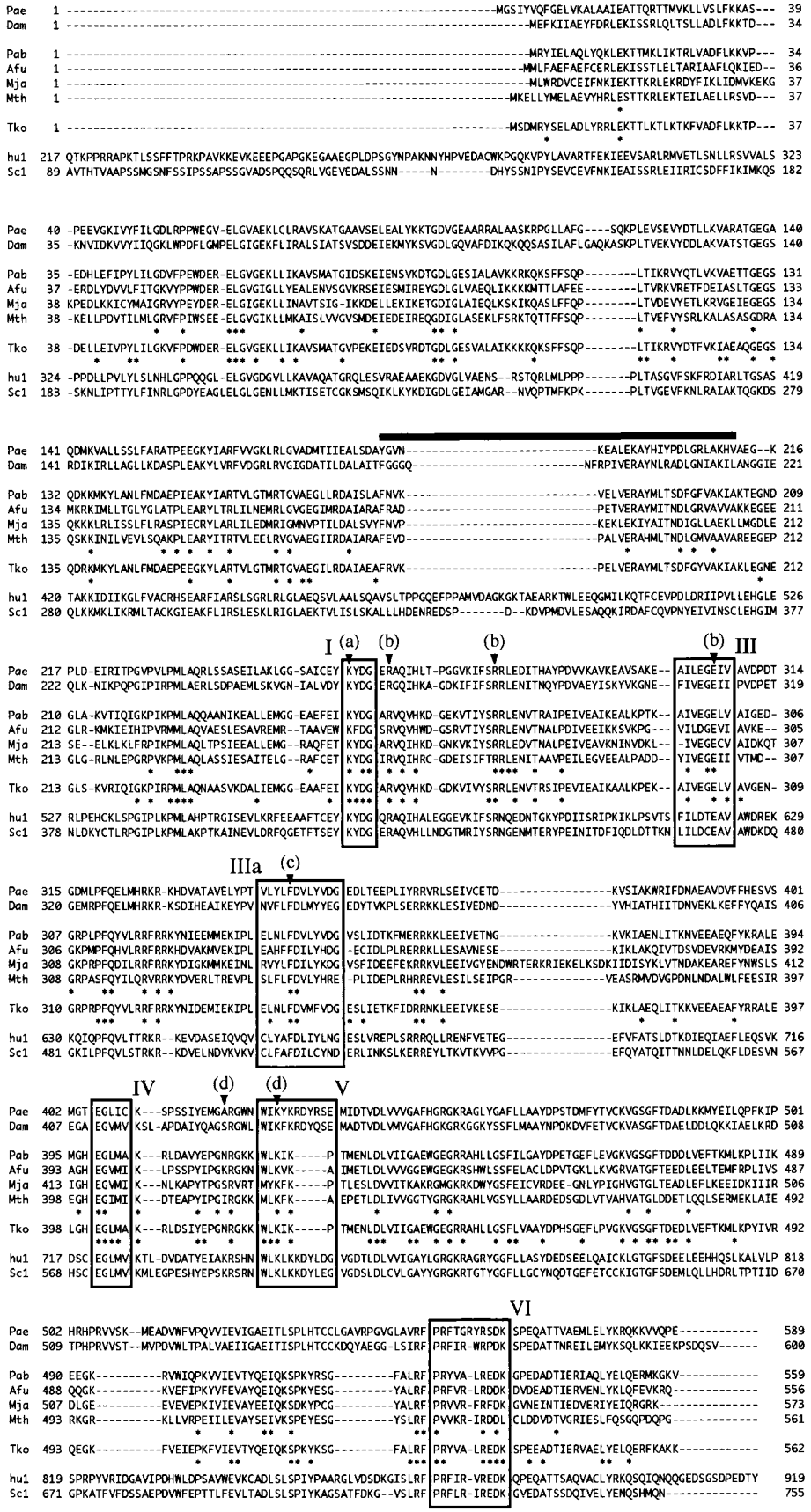



FIG. 1. Phylogenetic tree of DNA ligases. Multiple sequence alignments were conducted using ClustalW. Tree topology and evolutionary distance estimations were done by the neighbor-joining method. The accession numbers for each protein sequence are as follows, by source: *Aquifex aeolicus* (AE000699-7), *Arabidopsis thaliana* (X97924-1), *Archaeoglobus fulgidus* (O29632), *Bacillus subtilis* (Z99107-110), *Borrelia burgdorferi* (G70168), *Caenorhabditis elegans* (Q27474), *Desulfurolobus ambivalens* (Q02093), *Escherichia coli* (P15042), fowlpox virus (Z29716-4), *Helicobacter pylori* (O25336), human ligase I (NP_000225), human ligase III (P49916), *Lymantria dispar* nucleopolyhedrovirus (AF081810-22), *Methanobacterium thermoautotrophicum* (U51624-4), *Methanococcus jannaschii* (U67474-4), mouse ligase I (NP_034845), mouse ligase III (U66058-1), *Mycoplasma genitalium* (U39704-5), phage T3 (P07717), phage T4 (P00970), phage T6 (P19088), phage T7 (P00969), *Pyrobaculum aerophilum* (U82370), *Pyrococcus abyssi* (B75173), *Rhodothermus marinus* (P49421), *Saccharomyces cerevisiae* ligase I (Z74212-1), *Schizosaccharomyces pombe* (P12000), Shope fibroma virus (U00761-1), *Thermococcus kodakaraensis* KOD1 (AB042527), *Thermus scotoductus* (P49422), *Thermus thermophilus* (P26996), *Treponema pallidum* (O83642), vaccinia virus (P16272), *Zymomonas mobilis* (P28719).

*thermophilus* has been reported to show DNA ligase activity at temperatures of up to 85°C, with an optimal reaction temperature of approximately 70°C (41). Interestingly, genome analysis has indicated the presence of an ATP-dependent DNA ligase gene on the chromosome of the bacterium *Aquifex aeolicus* VF5 (6).

Little is known about DNA ligases from *Archaea*, the third kingdom of life. The recently reported ATP-dependent DNA ligase (*Mth* ligase) from *Methanobacterium thermoautotrophicum* ΔH, a moderate thermophilic archaeon, is the only enzyme that has been characterized (38). Genome analyses of hyperthermophilic archaeal strains, such as *Pyrococcus abyssi* (http://www.genoscope.cns.fr/Pab/), *Archaeoglobus fulgidus* (18), and *Methanococcus jannaschii* (4), have revealed the presence of putative DNA ligase genes on their genomes. The sequence of the gene from *Desulfurolobus ambivalens* has also been reported and compared with eukaryotic DNA ligases (19). Sequence comparison of these putative genes, along with the data from *Mth* ligase, indicates that archaeal enzymes are ATP dependent (19). *Thermococcus kodakaraensis* KOD1 (previously reported as *Pyrococcus kodakaraensis* KOD1) is a sulfur-reducing hyperthermophilic archaeon that was isolated from a geothermal spring in Kodakara Island, Kagoshima, Japan (28). We have been focusing on the biochemical and structural characterization of protein products of genes with putative functions, including archaeal DNA polymerase (14, 40), archaeal ribulose 1,5-bisphosphate carboxylase/oxygenase (9, 26), $O^6$-methylguanine-DNA methyltransferase (13, 21), and archaeal aspartyl-tRNA synthetase (12, 35). As there is no information concerning DNA ligases from hyperthermophilic archaea, we performed detailed characterization of the protein product of a DNA ligase gene from *T. kodakaraensis* KOD1. This is the


I
(a)
(b)
(b)
(b)
III
IIIa
(c)
IV
(d)
(d)
V
VI

first characterization of a DNA ligase from a hyperthermophilic archaeon.

## MATERIALS AND METHODS

**Microbial strains, plasmids, phages, and media.** *T. kodakaraensis* KOD1 was cultivated using a medium described in the previous report (28). *E. coli* DH5α and the vector pUC19 were used for cloning and gene manipulation. *E. coli* XL1-Blue MRA (P2) (Stratagene, La Jolla, Calif.) was used as a host strain for λEMBL4 phage (Toyobo, Osaka, Japan). *E. coli* BL21(DE3)pLysS (Stratagene) and the vector pET21a(+) (Novagen, Madison, Wis.) were used for overexpression of $lig_{Tk}$. Luria-Bertani (LB) medium was used for the cultivation of *E. coli*, and NZYM medium was used for the amplification of phage (27).

**Isolation of $lig_{Tk}$.** A genomic library was constructed from *T. kodakaraensis* KOD1 by using the λEMBL4 phage vector system. A DNA fragment containing a part of the $lig_{Tk}$ gene was obtained through sequence analysis of the genome of *T. kodakaraensis* KOD1. A phage clone which carried the complete $lig_{Tk}$ gene was screened from the genomic library by plaque hybridization using the DNA fragment as a probe.

**DNA manipulation and sequencing.** For isolation of plasmid DNA, Plasmid Mini-, Midi-, and Lambda Kits (QIAGEN, Hilden, Germany) were used along with the alkaline extraction method (27). Restriction enzymes and modifying enzymes were purchased from Toyobo, Takara (Kyoto, Japan), and Boehringer GmbH (Mannheim, Germany). DNA sequencing on both strands of DNA was conducted using the ABI PRISM kit and Model 310 capillary DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, Calif.). Sequence data were analyzed and compared using DNASIS software package (Hitachi Software Engineering, Yokohama, Japan).

**Phylogenetic analysis.** The multiple alignment of protein sequences and the identity and similarity between sequences were obtained with the program ALIGN contained within the ClustalW program provided by DNA Data Bank of Japan (DDBJ). The phylogenetic tree was constructed by the neighbor-joining method after alignment. Bootstrap resampling was performed with the BSTRAP program 2,000 times.

**Cloning and expression of the $lig_{Tk}$ gene.** Two oligonucleotides derived from the $lig_{Tk}$ gene sequence were designed: a 5′-primer containing an *Nde*I site (in bold), 5′ CGGTGGTG**CATATG**AGCGATATGCGCTACTCTGAACTGG 3′, and a 3′ primer containing a *Bam*HI site (in bold), 5′ CTCG**GGATCC**CTGGG AGGGAAAAGGAATCTCACTCGCC 3′. PCR was performed with these primers, along with the phage DNA as a template. The PCR product, cleaved with *Nde*I and *Bam*HI, was inserted into an *Nde*I-*Bam*HI site of pET-21a(+) (Novagen). The resulting plasmid pET-lig was transferred to *E. coli* BL21 (DE3)pLysS. The transformants were cultivated in LB medium containing 50 μg of ampicillin/ml at 37°C until the optical density at 660 nm reached 1.0. Isopropyl-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM to induce $lig_{Tk}$ gene expression for 6 h.

**Purification of recombinant $Lig_{Tk}$.** The cells were harvested by centrifugation (5,000 × *g*, 15 min, 4°C), washed with buffer A (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$), and then resuspended in buffer A. The cells were disrupted by sonication, and the supernatant was obtained by centrifugation (12,000 × *g*, 30 min, 4°C). The soluble fraction of cell-free extract was heat treated at 80°C for 30 min, and the precipitate was removed by centrifugation (12,000 × *g*, 30 min, 4°C) to obtain thermostable proteins. The supernatant was applied to a ResourceQ column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with buffer A. $Lig_{Tk}$ did not bind to the resin, and the flowthrough fractions were collected and applied to a cation-exchange column (ResourceS; Amersham Pharmacia Biotech) equilibrated with buffer B (50 mM Tris-HCl, pH 7.5). After washing with buffer B, the enzyme was eluted with a 0 to 1.0 M $MgCl_2$ linear gradient with buffer B. The peak fractions containing $Lig_{Tk}$, which eluted between 0.09 and 0.10 M $MgCl_2$, were concentrated by using Centricon-30 (Millipore, Bedford, Mass.). The enzyme solution was applied to a gel filtration column (Superdex 200HR 10/30; Amersham Pharmacia Biotech) equilibrated with buffer C (50 mM Tris-HCl [pH 7.5], 100 mM $MgCl_2$) and eluted with the same buffer. The active fractions were desalted with buffer B and used as purified $Lig_{Tk}$ in the following experiments. The protein concentration was determined by the Bio-Rad protein assay system (Bio-Rad, Hercules, Calif.) with bovine serum albumin as a standard. The N-terminal amino acid sequence of purified $Lig_{Tk}$ was determined by a protein sequencer (Model 270; Perkin-Elmer Applied Biosystems).

**DNA substrates.** DNA ligase activity measurements were carried out with synthesized oligonucleotides consisting of a 5′-phosphorylated 30-mer (5′ P-CA GAGGATTGTTGACCGGCCCGTTTGTCAG 3′) and a 40-mer (5′ CGCACC GTGACGCCAAGCTTGCATTCCTACAGGTCGACTC-OH 3′) annealed to a complementary 80-mer (5′ CGTTGCTGACAAACGGGCCGGTCAACAATC CTCTGGAGTCGACCTGTAGGAATGCAAGCTTGGCGTCACGGTGCGC CAAC 3′).

**DNA ligase assays.** Nick joining activity was measured by using the DNA substrates described above. Unless otherwise stated, ligation reaction mixtures (20 μl) contained 20 mM Bicine-KOH, pH 8.0, 15 mM $MgCl_2$, 20 mM KCl, 1 mM ATP, 10 μM concentration of the 30-mer, 10 μM concentration of the 40-mer, 5 μM concentration of the 80-mer, and 200 nM $Lig_{Tk}$. The enzyme and other constituents of the reaction mixture were incubated separately at the desired temperature, and reactions were initiated by mixing the two solutions. Standard reactions were carried out at 80°C for 15 min. The reactions were stopped by addition of 30 μl of loading buffer (98% [vol/vol] formamide, 10 mM EDTA, 0.05% [wt/vol] xylene cyanol FF) and cooling in ice water. The products (12 μl) were heated at 95°C for 3 min and then electrophoresed through a denaturing 6% polyacrylamide–7 M urea gel (18 cm [width] × 20 cm [height] × 0.5 mm). Super Reading DNA Sequence PreMix Solution (6%) (Toyobo) and Gel-Mix Running Mate TBE buffer (GIBCO BRL, Rockville, Md.) were used for electrophoresis. The gel was stained with ethidium bromide, and the 70-mer ligation product was quantified by densitometric analysis and Quantity One software (*pdi*; Huntington Station, N.Y.).

**DNA ligase assays with labeled oligonucleotide.** A nonphosphorylated 30-mer oligonucleotide (5′ CAGAGGATTGTTGACCGGCCCGTTTGTCAG 3′) was synthesized and phosphorylated at its 5′ terminus by using [γ-$^{32}$P]ATP. The oligonucleotide (10 pmol) was phosphorylated and radiolabeled by incubation with 1.85 MBq of [γ-$^{32}$P]ATP (Amersham Pharmacia Biotech) and 10 U of T4 polynucleotide kinase (MEGALABEL; Takara) at 37°C for 30 min. The reaction product was purified by centrifugation through a CENTRI-SEP Spin Column (Perkin-Elmer Applied Biosystems). Nick joining activity was measured by using the DNA substrates described above. Ligation reaction mixtures (20 μl) contained 20 mM Bicine-KOH, pH 8.0, 15 mM $MgCl_2$, 20 mM KCl, 1 mM ATP, 0.1 μM concentration of the labeled 30-mer, 0.1 μM concentration of the 40-mer, 0.1 μM concentration of the 80-mer, and 200 nM $Lig_{Tk}$. The reaction was carried out at 80°C for 15 min and stopped by addition of 30 μl of loading buffer (98% [vol/vol] formamide, 10 mM EDTA, 0.05% [wt/vol] xylene cyanol FF) and cooling in ice water. The products (10 μl) were heated at 95°C for 3 min and then electrophoresed through a denaturing 6% polyacrylamide–7 M urea gel. Super Reading DNA Sequence PreMix Solution (6%) (Toyobo) and Gel-Mix Running Mate TBE buffer (GIBCO BRL) were used for electrophoresis. The gel was dried, and labeled oligonucleotides were detected by autoradiography.

**Measurement of DNA ligase activity with other cofactors.** $NAD^+$ and ADP were added at a concentration of 0.1 mM each, and reactions were carried out under the same conditions as when ATP was used as a cofactor. Methods used for detailed analysis of $NAD^+$ utilization as a cofactor are described below.

**Elimination of ATP from $NAD^+$ solution.** $NAD^+$ of the highest grade commercially available (99+%; Sigma, St. Louis, Mo.) was used for initial examination of cofactor specificity. We further eliminated any small traces of contaminant ATP enzymatically. ATP was eliminated from $NAD^+$ solution by treating the solution with hexokinase (Sigma) and D-glucose. As a control, ATP solution was also treated with hexokinase. $NAD^+$ solution (1 ml) contained 20 mM Tris-HCl (pH 7.5), 10 mM D-glucose, 10 mM $MgCl_2$, 20 mM $NAD^+$, and 0.2 U of hexokinase. ATP solution (1 ml) contained 20 mM Tris-HCl (pH 7.5), 10 mM D-glucose, 10 mM $MgCl_2$, 2 mM ATP, and 0.2 U of hexokinase. One unit of hexokinase consumes 1.0 μmol of ATP to phosphorylate 1.0 μmol of D-glucose per min. Optimal conditions for the enzyme are pH 7.6 and 25°C. These solutions were incubated at 25°C for 2 h. After incubation, the enzyme was excluded from these solutions by using Centricon-10 (Millipore).

**Adenylation of $Lig_{Tk}$.** Adenylation of $Lig_{Tk}$ was performed with [α-$^{32}$P]ATP or [adenylate-$^{32}$P]$NAD^+$, both with $^{32}$P in the phosphate group of the AMP moieties. The adenylation reaction mixture (20 μl) contained 20 mM Bicine-KOH, pH 8.0, 15 mM $MgCl_2$, 200 nM $Lig_{Tk}$, and 740 kBq of [α-$^{32}$P]ATP (Amersham Pharmacia Biotech) or 258 kBq of [adenylate-$^{32}$P]$NAD^+$ (ICN Pharmaceuticals, Costa Mesa, Calif.). Reactions were carried out at 80°C for 2 h. The proteins were applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and after gels were dried, adenylated proteins were detected by autoradiography.

FIG. 2. Sequence alignment of ATP-dependent DNA ligases from eukaryotes and DNA ligase sequences from archaea. Enzyme, source, and accession number are as follows: Pae, *Pyrobaculum aerophilum* (U82370); Dam, *Desulfurolobus ambivalens* (Q02093); Pab, *Pyrococcus abyssi* (B75173); Afu, *Archaeoglobus fulgidus* (O29632); Mja, *Methanococcus jannaschii* (U67474-4); Mth, *Methanobacterium thermoautotrophicum* (U51624-4); Tko, *Thermococcus kodakaraensis* KOD1 (this work); hu1, DNA ligase I from humans (NP_000225); and Sc1, DNA ligase I from *Saccharomyces cerevisiae* (Z74212-1). *P. aerophilum* and *D. ambivalens* belong to *Crenarchaeota. A. fulgidus*, *M. jannaschii*, *M. thermoautotrophicum*, and *T. kodakaraensis* KOD1 belong to *Euryarchaeota*. Boxes I to VI represent the six motifs commonly found in ATP-dependent DNA ligases mentioned in the text. Arrowheads indicate AMP-binding site (a), ribose binding residues (b), purine ring-stacking residue (c), and phosphate-binding residues (d). The thick bar indicates a region distinct among eukaryotic and archaeal sequences. Asterisks above the Tko sequence indicate conserved residues in archaeal sequences, and those below the Tko sequence indicate conserved residues among Tko, hu1, and Sc1.

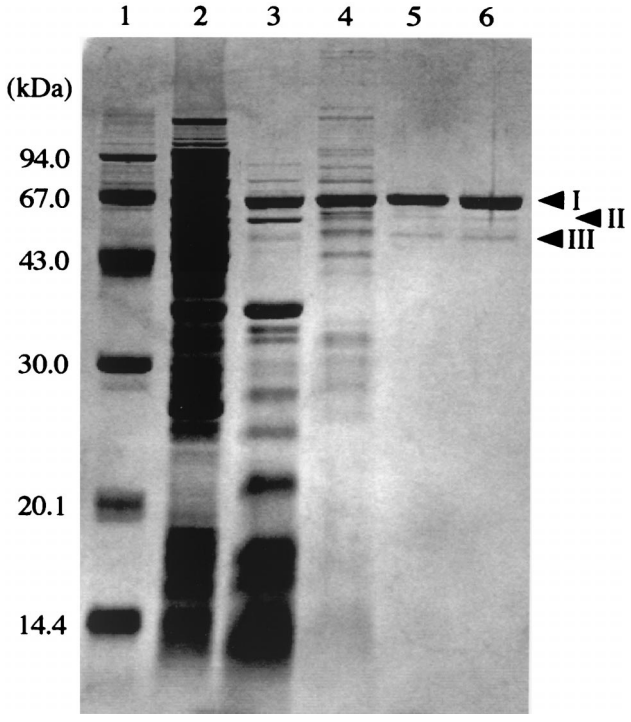



FIG. 3. Expression of *lig*$_{Tk}$ in *E. coli* and purification of the recombinant protein. Lane 1, molecular mass marker; lane 2, cell-free extract of *E. coli* transformant harboring *lig*$_{Tk}$ after 6 h of induction with IPTG; lane 3, supernatant of cell-free extract after heat treatment at 80°C for 30 min; lane 4, flowthrough fraction after anion-exchange chromatography; lane 5, peak fraction after cation-exchange chromatography; lane 6, peak fraction after gel filtration chromatography. Arrowheads represent the major band I and minor bands II and III.

**Nucleotide sequence accession number.** The gene sequence and deduced amino acid sequence of *lig*$_{Tk}$ are available under the accession no. AB042527.

## RESULTS

**Cloning and nucleotide sequence of the DNA ligase gene (*lig*$_{Tk}$).** Through sequence analysis of the genome of *T. kodakaraensis* KOD1, we found a sequence with similarity to the DNA ligase gene of *M. thermoautotrophicum*. A genomic library was constructed from *T. kodakaraensis* KOD1 by using the λEMBL4 phage vector system. A phage clone harboring the complete open reading frame (*lig*$_{Tk}$) was isolated by plaque hybridization. We determined the nucleotide sequence of approximately 7 kbp within the isolated DNA fragment, in which the complete *lig*$_{Tk}$ gene was included. *lig*$_{Tk}$ consists of 1,686 bp, corresponding to a polypeptide of 562 amino acids with a predicted molecular mass of 64,079 Da.

**Phylogenetic analysis of Lig$_{Tk}$.** An unrooted phylogenetic tree of DNA ligases from various sources was constructed by the neighbor-joining method (Fig. 1). Phylogenetic analysis indicated that Lig$_{Tk}$ was closely related to *Mth* ligase, along with putative DNA ligases from *Euryarchaeota* and *Crenarchaeota*. These archaeal sequences showed close relationships with DNA ligase I from eukaryotes, suggesting that all archaeal DNA ligases, including Lig$_{Tk}$, belong to the ATP-dependent group (19). The $NAD^+$-dependent DNA ligases were considerably distant in the tree from ATP-dependent enzymes, indicating that ATP-dependent and $NAD^+$-dependent enzymes are clearly different in primary structure.

**Sequence comparison of Lig$_{Tk}$ with other ATP-dependent DNA ligases.** We compared the deduced amino acid sequence of Lig$_{Tk}$ with other previously reported ATP-dependent enzymes (Fig. 2). Lig$_{Tk}$ showed high identity with other archaeal DNA ligases: *P. abysii* (81%), *A. fulgidus* (53%) (18), *M. jannaschii* (43%) (4), *M. thermoautotrophicum* (43%) (37), *Pyrobaculum aerophilum* (40%) (http://genome.caltech.edu/pyrobaculum/), and *D. ambivalens* (39%) (19). Identity of Lig$_{Tk}$ with eukaryotic DNA ligase I was as follows: human (30%) (2)

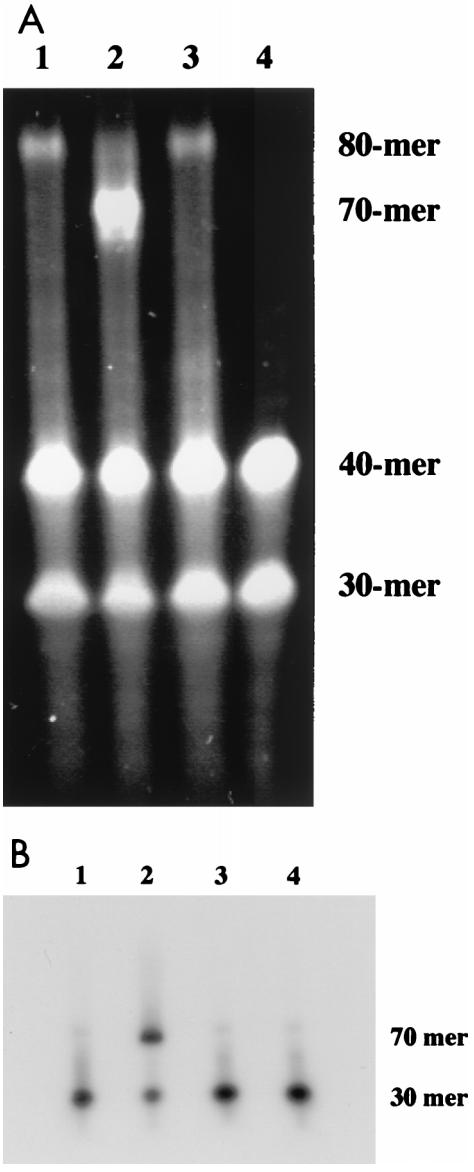



FIG. 4. ATP-dependent DNA ligase activity of Lig$_{Tk}$. (A) DNA substrates used for DNA ligase activity measurements were 80-mer as a template and complementary 30-mer and 40-mer. The 30-mer was phosphorylated at its 5′ terminus. DNA ligase joins the nick between the 40-mer and 30-mer, leading to a 70-mer. This change in size was monitored on denaturing 6% polyacrylamide–7 M urea gels. Lane 1, oligonucleotides with Lig$_{Tk}$ and ATP before reaction; lane 2, same as lane 1, but after 15 min at 80°C; lane 3, same as lane 2 but without ATP; lane 4, same as in lane 2 but without 80-mer template. (B) ATP-dependent DNA ligase activity was measured with oligonucleotide substrates, including a $^{32}$P-labeled 30-mer. Lane 1, before reaction with Lig$_{Tk}$; lane 2, after reaction with Lig$_{Tk}$ at 80°C for 15 min; lane 3, same as lane 1 but without Lig$_{Tk}$; lane 4, same as lane 2 but without Lig$_{Tk}$.

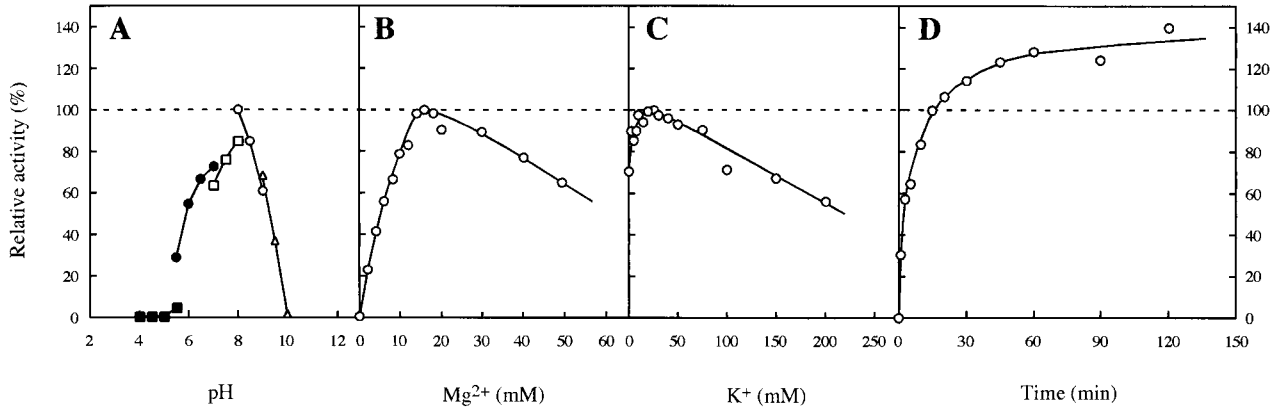



FIG. 5. Biochemical characterization of $Lig_{Tk}$. (A) Effect of pH on purified $Lig_{Tk}$. Buffers used were as follows: 20 mM citrate buffer (pH 4.0 to 5.5), 20 mM MES (morpholineethanesulfonic acid) buffer (pH 5.5 to 7.0), 20 mM HEPES buffer (pH 7.0 to 8.0), 20 mM Bicine buffer (pH 8.0 to 9.0), and 20 mM CHES buffer (pH 9.0 to 10.0). (B) Effect of $Mg^{2+}$ concentration on activity of $Lig_{Tk}$. Reactions were carried out at 80°C for 15 min at various concentrations of $Mg^{2+}$. (C) Effect of $K^+$ on ligation activity of $Lig_{Tk}$. Reactions were carried out at 80°C for 15 min at various concentrations of $K^+$. (D) Kinetic analysis of $Lig_{Tk}$ toward the nick ligation. All quantifications were performed as described in Materials and Methods.

and *S. cerevisiae* (29%) (1). The C-terminal domain of human DNA ligase I has been reported to be responsible for enzymatic activity (44). As shown in Fig. 2, alignment of $Lig_{Tk}$ with human DNA ligase I indicated that $Lig_{Tk}$ corresponds to the C-terminal catalytic domain of human DNA ligase I. Other archaeal DNA ligases also showed the same tendency. Most residues that were conserved among all archaeal sequences were also conserved in the eukaryotic sequences. The only major region which was characteristic of the archaeal sequences was the region from Phe182 to Ala206 in $Lig_{Tk}$. Thirteen of the 25 residues compared were similar (4 identical residues) in all archaeal sequences, while only five positions were similarly conserved in the eukaryotic ligases. There was also a 29-residue insertion in the human DNA ligase I and a 20-residue insertion in the enzyme from *S. cerevisiae*. Six motifs (I, III, IIIa, IV, V, VI) have been previously proposed to be conserved in various ATP-dependent DNA ligases (39). All motifs were found in $Lig_{Tk}$ and other archaeal sequences with the exception of motif V. Although motif V was conserved in the sequences from *Crenarchaeota*, half of the motif was lacking in all enzymes from *Euryarchaeota*, including $Lig_{Tk}$. The crystal structure of the ATP-dependent DNA ligase from bacteriophage T7 complexed with ATP has been reported, and residues directly interacting with the ATP molecule have been identified (36, 39). The corresponding residues in the human DNA ligase I are Lys568, which covalently binds with AMP, Arg573, Arg589, and Glu621, which form hydrogen bonds with the ribose ring, Phe660, which stacks on the purine base, and Lys737 and Lys744, which contact the phosphate groups. Corresponding residues Lys252 (AMP binding), Arg257, Arg272, and Glu302 (ribose binding), Phe342 (purine stacking), and Lys423 (phosphate binding) were conserved in $Lig_{Tk}$. However, an Asn416 residue replaced the position corresponding to Lys737 in the human enzyme.

**Overexpression of *lig*$_{Tk}$ and purification of the recombinant protein.** In order to examine the enzymatic properties of $Lig_{Tk}$, we expressed *lig*$_{Tk}$ in *E. coli*. Cells harboring the expression plasmid pET-lig were induced by IPTG. The cells were disrupted by sonication, and the recombinant protein was purified by heat treatment and column chromatography with ResourceQ, ResourceS, and Superdex-200 as described in Materials and Methods. Most proteins from *E. coli* were precipitated by heat treatment. ResourceQ column chromatography removed nucleic acids as well as proteins, and ResourceS and Superdex-200 chromatography provided the purified recombinant protein (Fig. 3). We could observe one major and two minor protein bands with apparent molecular masses of 62 kDa (band I), 56 kDa (band II), and 50 kDa (band III), respectively. As we have encountered some cases in which archaeal proteins showed aberrant migration rates on SDS-PAGE gels (10), the protein was denatured under various SDS and reducing-agent concentrations (data not shown). We found that the presence of the minor band II was inconsistent, and stronger denaturation conditions led to an increase in this band. This indicates that band II is a modified product of the intact protein during denaturation with SDS and reducing agents. We also determined the N-terminal amino acid sequence of band III. The sequence, FFSQPLTIKR, corresponded to residues 109 to 118 of $Lig_{Tk}$, indicating that band III was a cleaved peptide fragment. The N-terminal amino acid sequence of major band I was SDMRYSELADLYRRLEK, identical to amino acid residues 2 to 18 in the deduced sequence of $Lig_{Tk}$. Supported by these results, we performed further biochemical characterization of $Lig_{Tk}$ by using this enzyme fraction after gel filtration.

**Subunit composition of recombinant Lig$_{Tk}$.** We investigated the subunit composition of the purified enzyme by gel filtration chromatography. The native enzyme showed a molecular mass of 52.1 kDa (data not shown), indicating that $Lig_{Tk}$ was a monomeric enzyme.

**Catalytic properties of Lig$_{Tk}$.** We investigated the DNA ligase activity of recombinant $Lig_{Tk}$. Enzymatic activity was measured by using complementary oligonucleotides shown in Materials and Methods (an 80-mer as a template and a complementary 30-mer and 40-mer). When the DNA substrates and $Lig_{Tk}$ were added to the reaction mixture, including 1 mM ATP, 15 mM $MgCl_2$ and 20 mM KCl, we could clearly detect the 70-mer product after incubation at 80°C for 15 min (Fig. 4A, lane 2). The 70-mer was not detected prior to incubation and could not be observed when ATP was depleted from the reaction mixture (Fig. 4A, lanes 1 and 3). The results indicate that $Lig_{Tk}$ is an ATP-dependent DNA ligase. We also found that $Lig_{Tk}$ did not show ligase activity on single-stranded DNA substrates without template DNA, as no reaction product could be detected when the 80-mer oligonucleotide was not included in the reaction (Fig. 4A, lane 4). In order to confirm

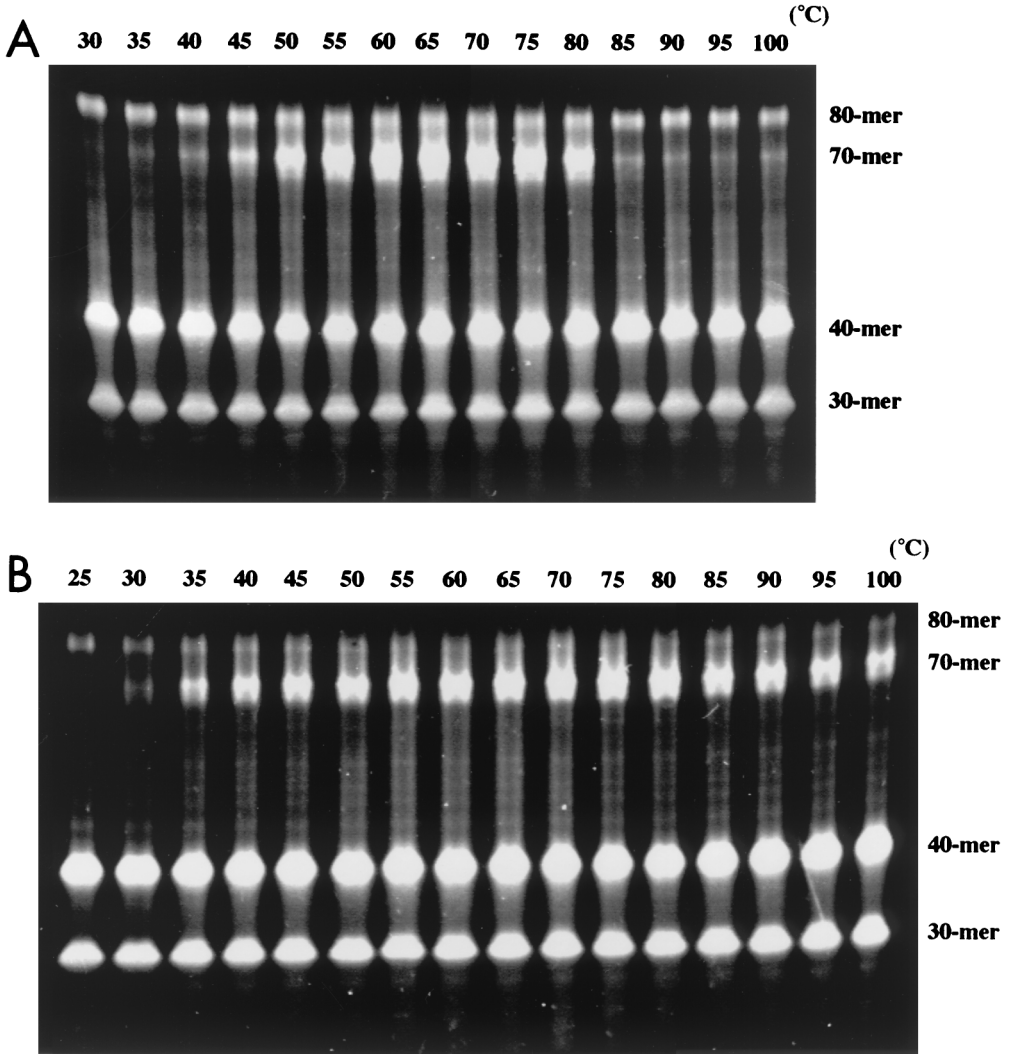



FIG. 6. Effect of temperature on DNA ligase activity of $Lig_{Tk}$ at different enzyme concentrations. (A) Denaturing 6% polyacrylamide–7 M urea gels after reaction at various temperatures for 15 min with 20 nM enzyme. Temperatures are indicated above each lane. (B) Same as panel A, but reactions were carried out for 10 min with 200 nM enzyme.

that the 70-mer product derived from the 30-mer and 40-mer substrates, we performed the same experiments with labeled substrates. The 5′ terminus of the 30-mer was phosphorylated using [$\gamma$-$^{32}$P]ATP. We could clearly detect an enzyme-dependent $^{32}$P-labeled 70-mer product after the reaction, coinciding with a decrease in the $^{32}$P-labeled 30-mer substrate (Fig. 4B, lane 2).

We performed detailed investigations on the reaction conditions for the ligation activity of $Lig_{Tk}$. The optimum pH was 8.0 (Fig. 5A). The optimum concentration of $Mg^{2+}$, which was indispensable for enzyme activity, was 14 to 18 mM (Fig. 5B). A low concentration (0 to 100 mM) of a monovalent cation, $K^+$, significantly stimulated the enzyme activity, with an optimum concentration of 10 to 30 mM (Fig. 5C). Under these optimum conditions, we performed a kinetic analysis of the ligation reaction (Fig. 5D). The reaction was linear for approximately 5 min and nearly complete at 60 min.

**Effects of temperature and enzyme concentration on DNA ligase activity of $Lig_{Tk}$.** We measured the DNA ligase activity of $Lig_{Tk}$ at various temperatures. When enzyme concentration was 20 nM (molar ratio of substrate to enzyme = 500:1), $Lig_{Tk}$ showed significant activity from 35 to 80°C, with an optimal temperature of 65°C (Fig. 6A). A drastic decrease in activity was observed between 80 and 85°C. However, when enzyme concentration was elevated to 200 nM (molar ratio of substrate to enzyme = 50:1), the drastic decrease in activity at temperatures above 80°C could not be observed (Fig. 6B). $Lig_{Tk}$ showed activity from 30 to 100°C, and the optimal temperature shifted to 70 to 80°C. It should be stressed that in these reactions, $Lig_{Tk}$ and DNA substrates were preincubated separately and mixed after reaching the respective temperatures.

**Cofactor specificity of $Lig_{Tk}$.** We have previously reported biochemical investigations on a number of archaeal enzymes from *T. kodakaraensis* KOD1 whose cofactor specificities differed from their eukaryotic or bacterial counterparts. Namely, aspartyl-tRNA synthetase utilized GTP and UTP as well as the usual ATP (12), while glutamine synthetase was found to utilize not only ATP but also GTP (32). We investigated the DNA ligase activity of $Lig_{Tk}$ using cofactors other than ATP. $Lig_{Tk}$ could not utilize ADP as a cofactor. To our surprise, we found that $Lig_{Tk}$ was able to utilize $NAD^+$ as a cofactor (Fig. 7A). Although the enzymatic activity was lower than when ATP was

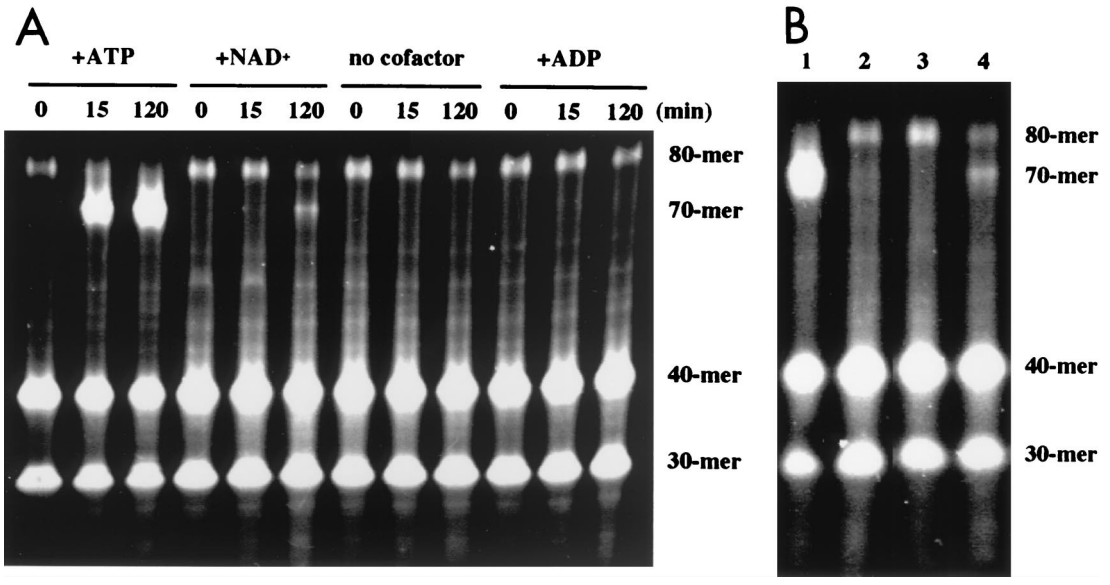



FIG. 7. $NAD^+$-dependent DNA ligase activity of $Lig_{Tk}$. (A) Ligation reactions were carried out at 80°C with 0.1 mM ATP, with 0.1 mM $NAD^+$, without cofactor, or with 0.1 mM ADP for 0, 15, and 120 min. (B) Ligation reaction measurements using hexokinase-treated $NAD^+$. All ligation reactions were carried out at 80°C for 2 h. Lane 1, 0.1 mM ATP without hexokinase treatment was added to the ligation reaction mixture; lane 2, 0.1 mM ATP treated with hexokinase for 2 h was added to the ligation reaction mixture; lanes 3 and 4, 0.1 mM $NAD^+$ treated with hexokinase for 2 h was added to the ligation reaction mixture. The mixture was applied to the gel prior to the ligation reaction (lane 3) and after the ligation reaction (lane 4).

used, a significant amount of 70-mer product could be observed with 0.1 mM $NAD^+$ after 120 min. We could not detect such a product when no cofactor was added. We also detected DNA ligase activity with $NAD^+$ at concentrations of 1 and 0.01 mM (data not shown). As DNA ligases have been reported to have strict cofactor specificity, we further examined the possibilities of contaminant ATP in our $NAD^+$ solution. In the experiments above, we used $NAD^+$ of the highest grade commercially available (99+%; Sigma). Although the possibilities of enzyme activity due to ATP contamination were low, we ensured the depletion of ATP by treating the $NAD^+$ solution with hexokinase (Sigma) and D-glucose. We first examined the extent of ATP degradation with hexokinase. ATP was incubated with glucose and hexokinase at 25°C for 2 h, and after removal of hexokinase, ligation reactions were carried out. The 70-mer ligation product could not be detected using the hexokinase-treated ATP (Fig. 7B, lane 2). This indicated that the treatment with hexokinase was sufficient to degrade any contaminant ATP. We therefore performed the same hexokinase treatment with $NAD^+$ prior to the ligation reaction. Using the hexokinase-treated $NAD^+$, $Lig_{Tk}$ was still able to ligate the 30-mer and 40-mer, producing the 70-mer product (Fig. 7B, lane 4). ATP-dependent T4 DNA ligase did not show any activity when $NAD^+$ solution with or without hexokinase treatment was added to the reaction mixture (data not shown). The results strongly indicate that $Lig_{Tk}$, a predominantly ATP-dependent DNA ligase, can also utilize $NAD^+$ as a cofactor.

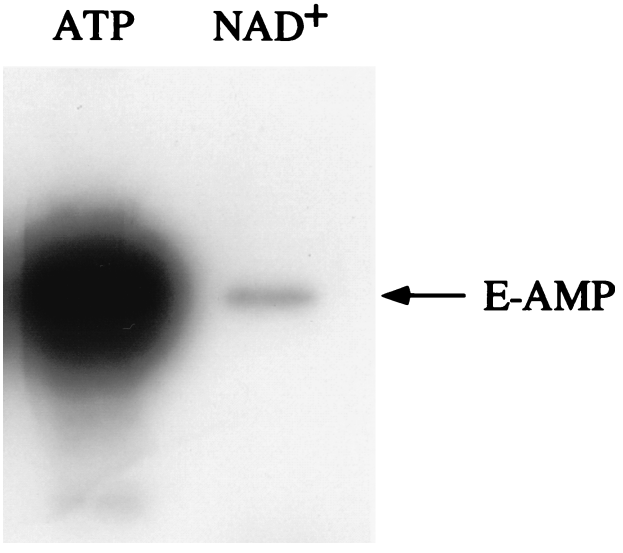



FIG. 8. Adenylation of $Lig_{Tk}$ with ATP or $NAD^+$ as a cofactor. $Lig_{Tk}$ was incubated at 80°C for 2 h with [$\alpha$-$^{32}$P]ATP or [adenylate-$^{32}$P]$NAD^+$, both with $^{32}$P in the phosphate group of the AMP moieties. After incubation, the proteins were applied to SDS-PAGE gels and detected by autoradiography.

We further pursued the utilization of $NAD^+$ as a cofactor by examining the adenylation activity of $Lig_{Tk}$. [$\alpha$-$^{32}$P]ATP and [adenylate-$^{32}$P]$NAD^+$, both with $^{32}$P in the phosphate group of the AMP moieties, were used to detect the enzyme-AMP complex. The radiolabeled cofactors were incubated along with the enzyme for 2 h at 80°C. Although the efficiency of adenylation was much lower than in the case of ATP, we found that $NAD^+$ could also be utilized as an AMP donor for adenylation of $Lig_{Tk}$ (Fig. 8). This further strongly supports the utilization of $NAD^+$ as a cofactor for DNA ligase activity of $Lig_{Tk}$.

## DISCUSSION

In this paper, we describe the biochemical characterization of a DNA ligase from a hyperthermophilic archaeon. Determination of the primary structure, expression of the *lig*$_{Tk}$ gene, purification of the recombinant protein, and evaluation of its enzymatic properties have been performed. Through these studies, we have clearly indicated that $Lig_{Tk}$ harbors DNA ligase activity and that the activity is mainly dependent on ATP as a cofactor. The biochemical characterization of $Lig_{Tk}$ and

*Mth* ligase (38), along with the high similarity among these enzymes and other putative archaeal DNA ligases, suggests that archaeal DNA ligases are predominantly ATP dependent. However, at present, information concerning archaeal DNA ligases is limited to those from thermophilic organisms, and we have no structural or biochemical information on DNA ligases from mesophilic archaea, such as halophiles.

Genome analyses of *P. abyssi* and *M. jannaschii* (4) have indicated the presence of only one DNA ligase gene in each organism. The genome of *A. fulgidus* (18) additionally harbors a short putative open reading frame (939 bp corresponding to 313 amino acid residues) slightly resembling a truncated DNA ligase gene, but there is only a single gene with high similarity to those of known DNA ligases. Judging from structural comparison, there seems to be only a single DNA ligase in hyperthermophilic archaea. In contrast, eukaryotic organisms have multiple DNA ligases which function to seal nicks in specific cellular events, such as DNA replication, DNA excision repair, and DNA recombination. The archaeal DNA ligases would have to function in all of these events.

Comparison of primary structures revealed that $Lig_{Tk}$ corresponded to the C-terminal domain (703 residues) of human DNA ligase I. DNA ligase I, which is involved in nick sealing during DNA replication, is often cleaved into two fragments during enzyme purification (25, 44). The C-terminal fragment alone displays DNA ligase activity in vitro. The N-terminal region of 118 amino acid residues has been reported to directly interact with the proliferating cell nuclear antigen (PCNA) (23, 34). PCNA binds to DNA polymerase δ during DNA replication, and after completion of the Okazaki fragment, it is left alone in this position after DNA polymerase δ is detached. The interaction between PCNA and the N-terminal region of DNA ligase I explains why DNA ligase I is uniquely able to function in DNA replication (23, 34). As $Lig_{Tk}$ corresponds to the C-terminal domain of DNA ligase I, it is of interest as to how the enzyme is recruited to the sites where it should function in vivo. Interestingly, putative genes with similarity to the eukaryotic PCNA gene have been identified in *P. abyssi*, *M. jannaschii*, and *A. fulgidus*.

In this study, we have found two unique features in $Lig_{Tk}$. One is the ability to ligate DNA fragments at temperatures up to 100°C, which is supposed to be well above the calculated $T_m$ values of the oligonucleotides. This could clearly be observed at high enzyme concentration (Fig. 6B). As $Lig_{Tk}$ showed no activity when template DNA was depleted, annealing of the substrates, in other words, a double-stranded DNA substrate, is necessary before the ligation reaction can occur. As we could detect a significant amount of 70-mer in the reaction at 100°C with $Lig_{Tk}$ at high concentration, this suggests that excess $Lig_{Tk}$ may somehow increase the population of double-stranded DNA substrates. This may occur by binding to double-stranded DNA substrates and lengthening their half-lives. Further studies will be necessary to elucidate this mechanism.

The most unique and unexpected property of $Lig_{Tk}$ is that the enzyme can utilize $NAD^+$ as a cofactor. Experiments with hexokinase to eliminate contaminant ATP strongly confirm this unique property. Furthermore, we have also detected adenylation of $Lig_{Tk}$ using $NAD^+$. Although specificity towards ATP is much higher, this is the first observation of an ATP-dependent DNA ligase able to utilize $NAD^+$. The *Mth* ligase did not show activity with $NAD^+$ as a cofactor (38). Comparison of primary structure and phylogenetic analysis of $Lig_{Tk}$ strongly indicate that the enzyme is an ATP-dependent DNA ligase. We found no significant similarity between $Lig_{Tk}$ and bacterial $NAD^+$-dependent enzymes. At present, as we cannot identify a primary structural basis for utilization of $NAD^+$, this unique cofactor specificity may be due to the fact that we were able to measure activities at high temperature, at which cofactor and/or substrate specificities may be less strict. Nevertheless, 80°C is a temperature at which the native host cell shows rapid growth, and the physiological relevance of this cofactor specificity should be addressed in future studies. Three-dimensional structural analysis should also provide the physical basis as to how $Lig_{Tk}$ utilizes $NAD^+$.

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