Extremophiles (2002) 6:469–477
Digital Object Identifier (DOI) 10.1007/s00792-002-0284-5

ORIGINAL PAPER

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Biochemical characterization of an ATP-dependent DNA ligase from the hyperthermophilic crenarchaeon *Sulfolobus shibatae*

Received: November 13, 2001 / Accepted: May 21, 2002 / Published online: July 30, 2002

Abstract A gene encoding a putative ATP-dependent DNA ligase was identified in the genome of the hyperthermophilic archaeon *Sulfolobus shibatae* and expressed in *Escherichia coli*. The 601 amino acid recombinant polypeptide was a monomeric protein capable of strand joining on a singly nicked DNA substrate in the presence of ATP (K_m = 34 μM) and a divalent cation (Mn^{2+}, Mg^{2+}, or Ca^{2+}). dATP was partially active in supporting ligation catalyzed by the protein, but GTP, CTP, UTP, dGTP, dCTP, dTTP, and NAD^+ were inactive. The cloned *Ssh* ligase showed an unusual metal cofactor requirement; it was significantly more active in the presence of Mn^{2+} than in the presence of Mg^{2+} or Ca^{2+}. Unexpectedly, the native *Ssh* ligase preferred Mg^{2+} and Ca^{2+} rather than Mn^{2+}. Both native and recombinant enzymes displayed optimal nick-joining activity at 60–80°C. *Ssh* ligase discriminated against substrates containing mismatches on the 3′-side of nick junction and was more tolerant of mismatches at the 5′-end than of those at the penultimate 5′-end. The enzyme showed little activity on a 1-nucleotide gapped substrate. This is the first biochemical study of a DNA ligase from the crenarchaeotal branch of the archaea domain.

Key words DNA ligase · Crenarchaeota · *Sulfolobus shibatae* · ATP · Metal cofactor · Thermostability

Communicated by G. Antranikian

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Introduction

DNA ligases catalyze the formation of a phosphodiester bond between adjacent 3′-hydroxyl and 5′-phosphoryl groups at a single-stranded break in double-stranded DNA (Lehman 1974). These enzymes are an essential component in DNA replication, recombination, and repair systems and are therefore indispensable in all organisms (Lindahl and Barnes 1992). Based on energy cofactor requirement, DNA ligases fall into two groups: those requiring ATP for activity and those requiring NAD^+ for activity. ATP- and NAD^+-dependent DNA ligases employ a common three-step mechanism in ligation reaction (Engler and Richardson 1982). In the first step the enzyme is activated through the covalent addition of the adenylate group from ATP or NAD^+ to the conserved active site lysine. The second step involves the transfer of AMP from the enzyme to the 5′-phosphoryl group at a nick site on the DNA strand. In the final step the nick is sealed with the release of AMP from the adenylated DNA intermediate.

NAD^+-dependent ligases are found only in bacteria, whereas ATP-dependent ligases are encoded in eukarya, archaea, bacteriophages as well as bacteria. The two classes of ligases share limited sequence homology but have a common core architecture (Doherty and Suh 2000). These enzymes are characterized structurally by a set of six short motifs (I, III, IIIa, IV, V, and VI) conserved in sequence, order and spacing (Aravind and Koonin 1999). The conserved motifs play critical roles in nucleotide binding, nick recognition, and nucleotidyl transfer, as revealed in structural and mutational studies (Doherty and Dafforn 2000; Doherty and Wigley 1999; Lee et al. 2000; Odell et al. 2000; Singleton et al. 1999; Sriskanda and Shuman 1998; Subramanya et al. 1996).

Biochemical and genetic studies have been performed on ligases from eukarya, bacteria, viruses, and recently archaea. As the third domain of life, archaea have been shown to be similar to eukarya in many aspects of DNA metabolism despite their morphological and structural resemblance to bacteria (Edgell and Doolittle 1997; Olsen and Woese 1997). Culturable archaea are divided into two

kingdoms: the Crenarchaeota and the Euryarchaeota. Kletzin (1992) identified the first archaeal ligase gene from a hyperthermophilic crenarchaeon, *Desulfurolobus ambivalens*, and showed that the gene encoded a protein similar at the amino acid sequence level to eukaryal ATP-dependent DNA ligases. In recent years a number of genes encoding putative ATP-dependent ligases have been identified from sequenced archaeal genomes. Phylogenetic analysis indicates that all archaeal DNA ligases are closely related to DNA ligase I from eukarya (Nakatani et al. 2000). The availability of these putative genes has offered good opportunities for studying DNA ligases in archaea. However, only two archaeal DNA ligases have been biochemically characterized so far. An ATP-dependent DNA ligase from the thermophilic euryarchaeon *Methanobacterium thermoautotrophicum* was the first DNA ligase to be characterized in archaea (Sriskanda et al. 2000). This was followed by the biochemical study of a DNA ligase from the hyperthermophilic euryarchaeon *Thermococcus kodakaraensis* (Nakatani et al. 2000). Both enzymes are capable of closing nicks in an ATP-dependent manner at elevated temperatures characteristic of those required for the optimal growth of the host organisms. Interestingly, the *T. kodakaraensis* enzyme appears to have a low but significant activity when using NAD^+ as the cofactor. No biochemical studies have been conducted on DNA ligases from crenarchaeota, which have been shown to differ from euryarchaeota in DNA replication and cell cycle process (She et al. 2001).

In this study we identified a gene encoding a putative ATP-dependent DNA ligase in the genome of the hyperthermophilic crenarchaeon *Sulfolobus shibatae*. The putative protein shares sequence homology with known ATP-dependent DNA ligases and consists of the six conserved sequence motifs common to all ATP-dependent DNA ligases. Sequence comparison shows that putative ligases encoded by *S. shibatae* and other crenarchaeotal species are more closely related at the amino acid sequence level to eukaryal ATP-dependent ligases than their euryarchaeotal counterparts. In order to investigate the enzymatic properties of the *S. shibatae* protein and compare them with those of other ligases we overexpressed the gene coding for the protein in *Escherichia coli* and purified the recombinant protein to apparent homogeneity. We show that the protein displays optimal nick-joining activity at temperatures between 60° and 80°C in the presence of ATP and therefore is indeed an ATP-dependent DNA ligase. We also demonstrate that the identified ligase gene is expressed in *S. shibatae*. A biochemical characterization of *Ssh* ligase is presented.

Materials and methods

Growth of *Sulfolobus shibatae*

S. shibatae 51178 was purchased from the American Type Culture Collection (Rockville, Md., USA). The organism was grown at 75°C in Brock's medium (Brock et al. 1972) supplemented with 0.2% tryptone and 0.1% yeast extract in a 5-l Bioflo fermentor (New Brunswick Scientific, Edison, N.J., USA).

Computer analysis of protein sequences

Protein sequences were retrieved from public sequence databases. The ClustalW program (Thompson et al. 1994) was employed to produce a multiple sequence alignment and to identify conserved regions.

Isolation of the putative *Ssh* ligase gene

Based on motif I and VI sequences in selected known archaeal and eukaryal ATP-dependent DNA ligases, the following pair of degenerate oligonucleotide primers were synthesized: 5′-AARTARGATGGWRHRMGWGKWCA RRTWCA/5′-GGRAATCKYADNGMDWANCC (R: A/G, Y: C/T, M: A/C, K: G/T, W: A/T, H: A/C/T, D: A/G/T, N: A/C/G/T). A PCR reaction was carried out with the *S. shibatae* genomic DNA as the template, using the above primers, in the presence of 40 µM digoxigenin-11-dUTP (DIG; Boehringer-Mannheim, Mannheim, Germany). A *Hin*dIII digest of the *S. shibatae* genomic DNA was resolved by electrophoresis in an agarose gel. DNA fragments identified by hybridization with the DIG-labeled probe were recovered and cloned into the *Hin*dIII site of pUC18. After transformation into *E. coli* strain DH5α, recombinant plasmids were screened by colony hybridization with the DIG-labeled probe. Inserts in plasmids isolated from positive clones were sequenced in both directions.

Expression of the *Ssh* ligase gene

The putative *Ssh* ligase gene was amplified by PCR using a pair of primers (5′-CCGGAATTCATATGGAGTTTAAA GTTAT/5′-CCCGGGATCCGATATATTTTAAACGCTC) derived from the 5′- and 3′-ends of the gene. The primers were designed to introduce *Nde*I and *Bam*HI sites at the 5′- and 3′-ends, respectively, of the *Ssh* ligase gene. *S. shibatae* genomic DNA was used as the template for PCR. The PCR product was cleaved with *Nde*I and *Bam*HI, and cloned into the *Nde*I and *Bam*HI sites of the T7-based expression plasmid pET-30a(+) (Novagen, Madison, Wis., USA). The sequence of the insert was verified by sequencing in both directions. The recombinant plasmid was transformed into *E. coli* BL21(DE3) (Stratagene, La Jolla, Calif., USA). The transformant was grown in Luria-Bertani medium containing 30 µg kanamycin/ml at 37°C until the optical density at 600 nm reached approx. 0.5. isopropyl-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. Incubation was continued for 3 h.

Purification of recombinant *Ssh* ligase

Fully induced cells of the constructed *Ssh* ligase overproducer were harvested by centrifugation (5,000 *g*, 10 min,

4°C), and resuspended in lysis buffer containing 20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride. The cells were sonicated, and the cell debris was removed by centrifugation (15,000 *g*, 20 min, 4°C). The supernatant was heat-treated at 80°C for 20 min and then placed on ice for 30 min. The sample was clarified by centrifugation (15,000 g, 20 min, 4°C), and dialyzed against buffer A (50 mM Tris-HCl, pH 7.8, 0.1 mM EDTA, 0.1 mM DTT, 10% glycerol). The dialyzed material was loaded onto a Mono Q HR 5/5 column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with buffer A. The column was washed with buffer A (5 ml), and bound proteins were eluted with a KCl gradient (0–1 M) in buffer A (10 ml). Peak fractions containing *Ssh* ligase, which eluted at approx. 0.34 M KCl, were pooled, dialyzed against buffer A and concentrated using a NANOSEP 10 K microconcentrator (Pall-Gelman, Ann Arbor, Mich., USA). The sample was applied to a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech) equilibrated with buffer A. Fractions containing *Ssh* ligase were pooled. All purification steps were carried out at 4°C. The protein concentrations of the *Ssh* ligase preparations were determined using the Bradford (1976) method with bovine serum albumin (BSA) as the standard.

Preparation of a crude ligase fraction from *S. shibatae*

The lysate of *S. shibatae* was prepared as described previously (Mai et al. 1998). Solid $(NH_4)_2SO_4$ was added slowly with stirring to the lysate kept at 4°C to 30% saturation. The mixture was stirred for 30 min at 4°C, and centrifuged at 15,000 *g* for 15 min at 4°C. The concentration of $(NH_4)_2SO_4$ in the supernatant was adjusted to 60% saturation. After centrifugation (15,000 *g*, 15 min, 4°C), the pellet was resuspended in and dialyzed against buffer A.

Ligase substrates

A perfect match ligase substrate was formed by annealing two short oligonucleotides (a 36-bp donor strand and a 30-bp acceptor strand) with the polycloning site on M13mp19 ssDNA. The sequences of the donor and acceptor strands were 5′-CTCTAGAGTCGACCTGCAGGCATGCAAGC TTGGCGT and 5′-CAGTGAATTCGAGCTCGGTACC CGGGGAT C, respectively. Substrates containing a mismatched base pair at or 1 bp from the nick junction on either 5′- or 3′-side were made by substituting the corresponding base in the donor or acceptor strand of the perfect match substrate with the rest of four bases mixed at an equal molar ratio. A substrate containing a 1-nt gap was prepared by removing the 3′-end base from the acceptor strand of the perfect match substrate. The 36-bp donor strand in the above substrates was labeled at the 5′-end with [γ-^{32}P]ATP and purified using a MicroSpin G-25 column (Amersham Pharmacia Biotech, Piscataway, N.J., USA). In annealing reactions ^{32}P-labeled 36mer, 30mer, and M13mp19 ssDNA were mixed at a molar ratio of 1 : 3 : 2. The mixture was heated at 95°C for 5 min in 0.1 M NaCl and cooled slowly to room temperature. Singly nicked pUC18 plasmid employed in ligase assays was prepared as described previously (Clark and Felsenfeld 1991).

DNA ligation

Standard DNA ligase assays were performed by incubating *Ssh* ligase with the ^{32}P-labeled DNA substrate (1 pmol) for 10 min at 65°C in 50 mM Bis-Tris-HCl, pH 6.5 (at 22°C), 1 mM ATP, 5 mM DTT, 5 mM $MnCl_2$, and 50 µg BSA/ml in a total volume of 20 µl. The reaction was terminated by the addition of 20 µl loading buffer [98% (vol/vol) formamide, 10 mM EDTA, 0.01% (wt/vol) bromophenol blue]. Samples were heated at 95°C for 5 min and electrophoresed through an 8% polyacrylamide 7 M urea gel in 1× Tris-borate-EDTA buffer. The gel was dried and exposed to X-ray film or analyzed with a Phosphoimager (Molecular Dynamics, Sunnyvale, Calif., USA).

DNA ligase assays with nicked plasmid were conducted by incubating *Ssh* ligase with pUC18 DNA (500 ng) containing a single nick per circular molecule for 10 min at a specified temperature in 50 mM Bis-Tris-HCl, pH 6.5 (at 22°C), 1 mM ATP, 5 mM DTT, 5 mM $MnCl_2$ (for recombinant enzyme) or $MgCl_2$ (for native enzyme), 100 mM KCl, and 50 µg BSA/ml in a total volume of 20 µl. The ligation mixtures were electrophoresed through a 0.8% agarose gel containing 0.5 µg ethidium bromide/ml in 1× Tris-acetate EDTA buffer. The gel was photographed under UV light.

Preparation and purification of antibodies and immunoblotting

Antiserum against recombinant *Ssh* ligase was raised in a rabbit. Specific antibody to *Ssh* ligase was prepared as described previously (Olmsted 1981). For immunoblotting, protein samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 10%), electroblotted onto a polyvinylidene fluoride membrane (Bio-Rad), and reacted with the specific antibody to *Ssh* ligase. The blot was incubated with goat anti-rabbit IgG antibody conjugated to alkaline phosphotase for colorimetric detection.

Results

Identification of the DNA ligase gene in *S. shibatae*

To identify the DNA ligase encoded by *S. shibatae* we designed two degenerate oligonucleotide primers on the basis of the conserved sequences of motifs I and VI in known eukaryal and archaeal DNA ligases as described above. PCR reactions using the primers amplified from the *S. shibatae* genomic DNA a DNA fragment of the size close to that expected from the spacing between the two motifs in an ATP-dependent DNA ligase. The PCR fragment was internally labeled with DIG and used to probe genomic

blots of restriction enzyme-digested *S. shibatae* DNA. Two *Hin*dIII fragments (approx. 2.7 and 2.0 kb) were identified and cloned into pUC18. Sequencing of the two fragments revealed a putative ligase-encoding open reading frame (GenBank accession number: AF242877) containing an internal *Hin*dIII site. The sequence of the identified open reading frame was verified by sequencing a PCR product prepared from the *S. shibatae* genomic DNA using primers designed on the basis of the 5′- and 3′-ends of the putative ligase gene.

The putative *Ssh* ligase gene encodes a 601 amino acid polypeptide with a calculated molecular weight of 67,647 and an isoelectric point of 5.86. The protein shares the highest sequence homology with other putative crenarchaeal ATP-dependent DNA ligases, being 96%, 74%, and 56% identical to homologues from *S. solfataricus* P2 (GenBank accession number: AAK40535), a species closely related to *S. shibatae*, *D. ambivalens* (Q02093), and *Aeropyrum pernix* (NP_147713), respectively. Sequence identities of the putative *Ssh* ligase with euryarchaeal and eukaryal ligases are significantly lower, for example, 33% and 38% with ligases from *M. thermoautotrophicum* (H69077) and *Pyrococcus abyssi* (B75173), respectively; 27% and 21% with *Saccharomyces cerevisiae* ligase I (P04819) and human DNA ligase I (NP_000225), respectively. Phylogenetic analysis further revealed that crenarchaeal ligases are more closely related to eukaryal ligases than euryarchaeal ligases. All six conserved sequence motifs typical of an ATP-dependent DNA ligase exist in *Ssh* ligase. It is worth noting, however, that although ligases from both archaeal branches contain the six conserved motifs, they differ in the following aspects (Fig. 1). First, motif V is conserved in crenarchaeal ligases including *Ssh* ligase whereas only the N-terminal half of the site is found in euryarchaeal ligases. Second, the spacing between motifs V and VI is significantly smaller in euryarchaeal ligases than that in crenarchaeal enzymes. Crenarchaeal ligases are more similar to eukaryal counterparts in both of these aspects than euryarchaeal enzymes.

Expression and purification of recombinant *Ssh* ligase

To characterize the putative *S. shibatae* ligase we cloned the putative *Ssh* ligase gene into the expression plasmid pET-30a(+). Following induction with IPTG cells harboring the recombinant plasmid produced a prominent 62-kDa polypeptide and a number of minor polypeptides that were not present in uninduced cells (Fig. 2). When the lysate of the induced cells was centrifuged, these proteins remained soluble. Most of the *E. coli* proteins were subsequently removed by a heat treatment step. Chromatography of the remaining material on a Mono Q column yielded a fraction containing the 62-kDa protein and two smaller polypeptides. The 62-kDa protein was purified by gel filtration on Superdex 200 (Fig. 2). After calibrating the gel filtration column with protein standards we found that the 62-kDa protein came off the column as a monomer (data not shown). N-terminal sequencing of the protein produced the following sequence: MEFKV, indicating that the 62-kDa polypeptide is the product of the cloned *Ssh* ligase gene. To probe the identity of the two other polypeptides (52 and 26 kDa in molecular mass, respectively) that were induced and coeluted from the Mono Q column with the 62-kDa protein we prepared specific antibody against the 62-kDa polypeptide.

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        I          III           IIIa          IV          V              VI
Ssh   KYDG -41- FIIEGEIV -33- NVFLFDLMYYED -51- EGVMV -17- WIKLKRDYQSE -118- PRFI-RWRDDK
Dam   KYDG -41- FIVEGEII -33- NVFLFDLMYYEG -51- EGVMV -17- WIKFKRDYQSE -117- PRFI-RWRPDK
Pae   KYDG -41- AILEGEIV -33- VLYLFDVLYVDG -51- EGLIC -15- WIKYKRDYRSE -118- PRFTGRYRSDK

Mja   KYDG -40- LIVEGECV -34- RVYLFDILYKDG -68- EGVMI -15- MYKFK-----P -100- PRVV-RFRFDK
Mth   KYDG -41- YIVEGEII -32- SLFLFDVLYHRE -53- EGIMI -15- MLKFK-----A -101- PVVK-RIRDDL
Afu   KFDG -40- VILDGEVI -32- EAHFFDILYHDG -50- EGVMI -15- WLKVK-----A -101- PRFV-RLRDDK
Pab   KYDG -44- AIVEGELV -33- ELNLFDVLYVDG -51- EGLMA -15- WLKIK-----P -101- PRYV-ALREDK

Hu1   KYDG -44- FILDTEAV -32- CLYAFDLIYLNG -52- EGLMV -17- WLKLKKDYLDG -120- PRFI-RVREDK
Mou1  KYDG -44- FILDTEAV -32- CLYAFDLIYLNG -52- EGLMV -17- WLKLKKDYLDG -120- PRFI-RVRKDK
Hu3   KYDG -43- MILDSEVL -28- CLFVFDCIYFND -52- EGLVI -13- WLKVKKDYLNE -121- PRCT-RIRDDK
Mou3  KYDG -43- MILDSEVL -28- CLFVFDCIYFND -52- EGLVL -13- WLKVKKDYLNE -122- PRCT-RIRDDK
Sce   KYDG -44- LILDCEAV -32- CLFAFDILCYND -52- EGLMV -18- WLKLKKDYLEG -118- PRFL-RIREDK

T7    KYDG -50- FMLDGELM -49- HIKLYAILPLHI -60- EGLIV -14- WWKMK-----P -101- PSFV-MFRGTE
ChV   KIDG -31- EGSDGEIS -24- SYYWFDYVTDDP -55- EGVMI -18- LLKMK-----Q -103- FVFI-GIRHEE
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Fig. 1. Sequence motifs conserved in characterized and putative ATP-dependent DNA ligases from archaea, eukarya, and viruses. *Ssh Sulfolobus shibatae* (GenBank accession number: AAF61267); *Dam Desulfurolobus ambivalens* (S26383); *Pae Pyrobacterium aerophilum* (AAD00532); *Mja Methanococcus jannaschii* (Q57635); *Mth Methanobacterium thermoautotrophicum* (H69077); *Afu Archaeoglobus fulgidus* (NP_069457); *Pab Pyrococcus abyssi* (B75173); *Hu1* human ligase I (NP_000225); *Mou1* mouse ligase I (NP_034845); *Hu3* human ligase III (P49916); *Mou3* mouse ligase III (P97386); *Sce Saccharomyces cerevisiae* (P04819); *T7* bacteriophage T7 DNA ligase (P00969); *ChV Chlorella* virus PBCV-1 DNA ligase (NP_048900). *S. shibatae*, *D. ambivalens*, and *P. aerophilum* are members of the kingdom Crenarchaeota; *M. jannaschii*, *M. thermoautotrophicum*, *A. fulgidus*, and *P. abyssi* belong to the kingdom Euryarchaeota. Sequences of conserved motifs (I, III, IIIa, IV, V, and VI) and the numbers of amino acid residues separating the motifs are indicated

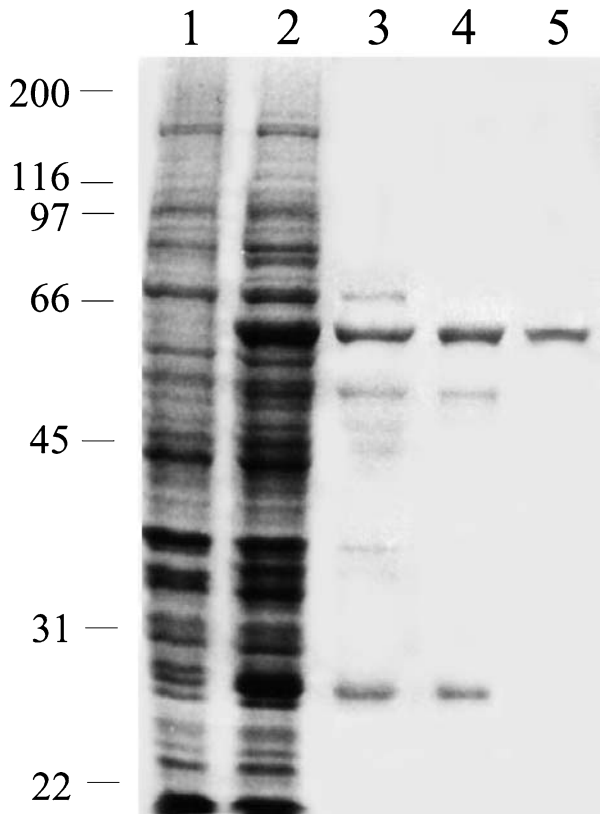

Fig. 2. Purification of recombinant *Ssh* ligase. *Lane 1* Extract of uninduced cells of the *Ssh* ligase overproducer; *lane 2* extract of IPTG-induced cells of the *Ssh* ligase overproducer; *lane 3* supernatant of the extract of induced cells after heat treatment at 80°C for 20 min; *lane 4* Mono Q column peak fractions; *lane 5* Superdex 200 column peak fractions. After electrophoresis the gel was stained with Coomassie brilliant blue. *Left* Positions and sizes (in kDa) of molecular weight standards

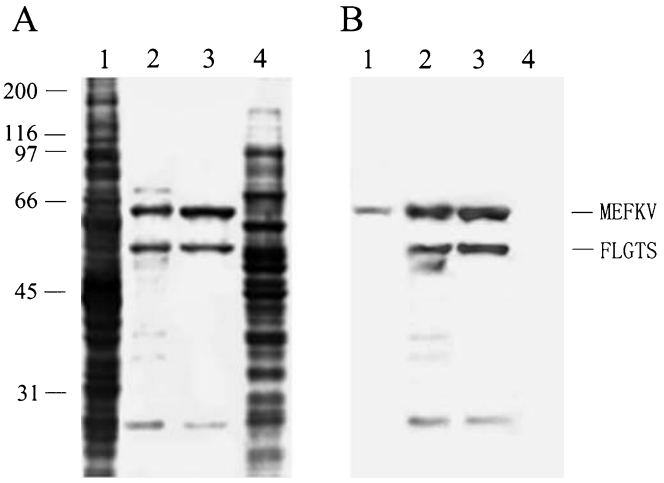

Fig. 3A, B. Analysis of recombinant and native *Ssh* ligases by SDS-PAGE and immunoblotting. *Lane 1* Cell extract of *S. shibatae*; *lane 2* supernatant of the heat-treated cell extract of *E. coli* cells overproducing *Ssh* ligase; *lane 3* peak *Ssh* ligase fractions from Mono Q column; *lane 4* cell extract of *E. coli* BL21 (DE3). **A** Samples were resolved by SDS-PAGE, and the gel was stained with Coomassie brilliant blue. *Left* Positions and sizes (in kDa) of molecular weight standards. **B** Same samples as those in (**A**) were resolved by SDS-PAGE. Proteins in the gel were electrotransferred to a polyvinylidene fluoride membrane and reacted with specific antibody to *Ssh* ligase. The blot was then incubated with goat anti-rabbit IgG antibody conjugated to alkaline phosphotase for colorimetric detection. In a separate experiment, proteins in the Mono Q column fractions were resolved by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. After staining with Coomassie brilliant blue, slices of the membrane containing the 62- and 52-kDa polypeptide bands, recognized by antibody to *Ssh* ligase, were excised. The two polypeptides were subjected to microsequencing. The N-terminal amino acid sequences are denoted on the right

Immunoblotting experiments showed that these polypeptides were recognized by the antibody and therefore were probably proteolytic products of the 62-kDa protein (Fig. 3). Analysis of the 52-kDa polypeptide by N-terminal sequencing revealed that the protein was indeed a truncated version of the 62-kDa ligase lacking the first 109 amino acid residues at the N terminus.

Catalytic properties of *Ssh* ligase

We examined the ability of putative *Ssh* ligase to seal a nick between two oligonucleotides (36mer and 30mer) annealed to single-stranded M13 DNA. In our standard assays the molar ratio of *Ssh* ligase to the labeled substrate was approx. 1 : 4. Ligation reactions were conducted at 65°C in the presence of ATP (1 mM) and Mn^{2+} (5 mM). Under these conditions ligation reached a 50% point at 3 min and was nearly completed within 10 min. The 5′-^{32}P-labeled 36mer was joined to the 30mer, producing a labeled 66mer (Fig. 4). Over 80% of the input 36mer was converted into the 66mer at maximum. The incomplete conversion of the 36mer presumably resulted from incomplete annealing of the oligonucleotides to single-stranded M13 DNA during the preparation of the substrate. No ligation product was detectable when either ATP or the divalent cation was omitted from the reaction mixture. We noticed, however, that ligation did occur in the absence of added ATP when the enzyme/substrate molar ratio was increased to approx. 100 : 1 (data not shown). It appears that a fraction of purified *Ssh* ligase was preadenylated. Our results indicate that the *S. shibatae* protein is an ATP-dependent DNA ligase. The K_m of *Ssh* ligase for ATP was calculated to be 34 µM by the method of Lineweaver and Burk (1934). This value is approx. 30-fold higher than that reported for the ATP-dependent ligase from *M. thermoautotrophicum* (Sriskanda et al. 2000). *Ssh* ligase was inactive when ATP was substituted by CTP, GTP, UTP, dCTP, dGTP, dTTP, or NAD^+. However, the enzyme displayed a low but detectable activity in the presence of dATP (Fig. 4). A similar observation was also made on the *M. thermoautotrophicum* DNA ligase (Sriskanda et al. 2000). Consistent with the nick sealing activity in the presence of dATP, *Ssh* ligase became adenylated after incubation with [α-^{32}P]dATP (data not shown). Formation of a covalent enzyme-adenylate intermediate represents the first step in the pathway of DNA ligation.

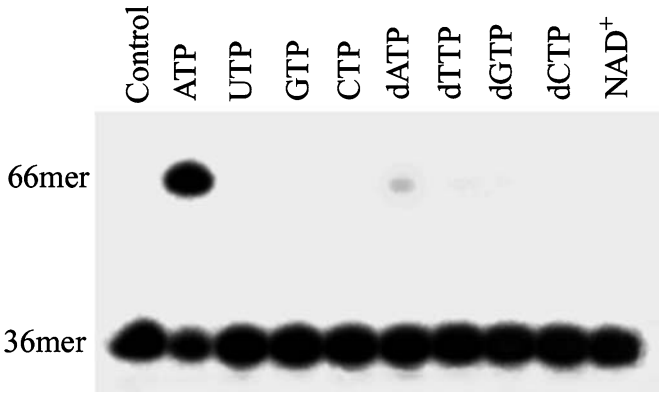

Fig. 4. Nucleotide cofactor specificity of recombinant *Ssh* ligase. Ligation reactions were carried out for 10 min at 65°C in the presence of 1 mM NTP, dNTP, or NAD^+, or in the absence of a cofactor (*control*). Reaction mixtures were subjected to denaturing PAGE. The gel was dried and exposed to X-ray film

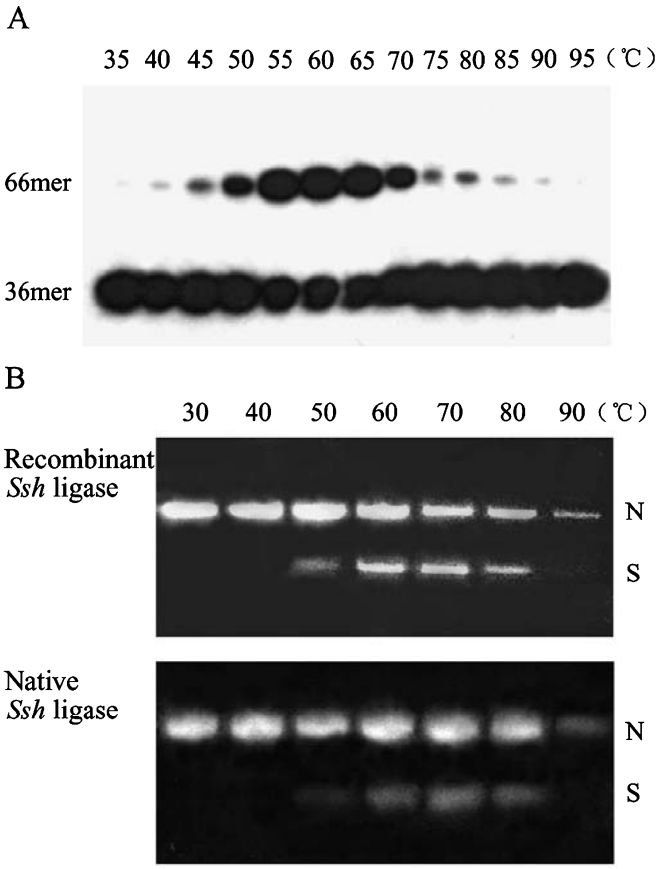

Fig. 5A,B. Effect of temperature on the activity of *Ssh* ligase. **A** Ligation reactions using the labeled substrate were carried out with recombinant *Ssh* ligase for 10 min at various temperatures. Reaction mixtures were subjected to denaturing PAGE. The gel was dried and exposed to X-ray film. **B** Ligation reactions using the singly nicked plasmid were carried out with recombinant or native *Ssh* ligase for 10 min at various temperatures. Reaction mixtures were electrophoresed through an agarose gel containing 0.5 µg ethidium bromide/ml in 1× Tris-acetate EDTA buffer. The gel was photographed under UV light. *N* Nicked plasmid; *S* supercoiled plasmid

Detection and partial purification of native *Ssh* ligase

To determine whether the *Ssh* ligase gene was expressed in native cells we performed an immunoblot analysis on a *S. shibatae* cell extract (Fig. 3, lane 1). A protein band with the same mobility as that of recombinant *Ssh* ligase was specifically recognized by the antibody to *Ssh* ligase. We therefore conclude that *Ssh* ligase is synthesized in *S. shibatae*, and that the recombinant and native versions of *Ssh* ligase are indistinguishable in size. However, we were unable to assay the activity of the native enzyme in the cell extract because of the interference by nucleases. To separate the ligase from nucleases we subjected the *S. shibatae* cell extract to $(NH_4)SO_4$ fractionation. The native ligase precipitated when $(NH_4)SO_4$ was added to approx. 60% saturation. This crude enzyme preparation was active in nick joining, displaying the same nucleotide cofactor requirement as the recombinant enzyme. We also found that the native and recombinant *Ssh* ligases had similar elution profiles on a Superdex 200 gel filtration column. Thus the two enzymes are probably similar in size and conformation in their native form.

Effects of temperature, pH, and salts on ligation

In the standard ligase assay using the labeled substrate, recombinant *Ssh* ligase was most active in nick joining at 50–70°C and remained partially active at temperatures up to at least 80°C (Fig. 5A). However, it is possible that this measurement was an underestimate since the substrate might become unstable at high temperatures. To obtain a more accurate measurement of the temperature optimum of *Ssh* ligase we set up a different nick closing assay in which a singly nicked plasmid was employed as the ligase substrate. Ligation was carried out in the presence of 100 mM KCl. This level of salt was found to inhibit the activity of *Ssh* ligase (see below) but stabilize the nicked plasmid, allowing the assay to be performed at temperatures up to 90°C. In this assay the covalently closed ligation product would migrate faster than the nicked substrate during electrophoresis in agarose in the presence of ethidium bromide. The recombinant *Ssh* ligase activity measured in this assay peaked at temperatures between 60° and 80°C (Fig. 5B), which were higher than those obtained in the assay using the radiolabeled substrate. We also examined the effect of temperature on the activity of the partially purified native ligase in the same manner except that $MnCl_2$ was replaced by $MgCl_2$, a preferred metal cofactor for the native enzyme. As shown in Fig. 5B, native *Ssh* ligase had a temperature optimum similar to that of the recombinant protein.

To examine the thermostability of *Ssh* ligase we incubated the lysate of *E. coli* cells overproducing *Ssh* ligase at a given temperature for various lengths of time. The ligase activity of the treated samples was then determined in the standard assay. We found that the ligase retained approximately 85% and 71% of the original activity after incuba-

tion for 5 h at 80° and 85°C, respectively, but lost one-half of its activity in slightly longer than 10 min at 90°C (Fig. 6). Therefore the enzyme was stable at temperatures up to 85°C.

Ssh ligase was optimally active at pH 6.0–7.0. The enzyme was sensitive to inhibition by salts at moderate concentrations. Ligase activity was not significantly affected by KCl or NaCl at concentrations below 50 m M, but was inhibited by approx. 85% in 100 mM KCl or NaCl.

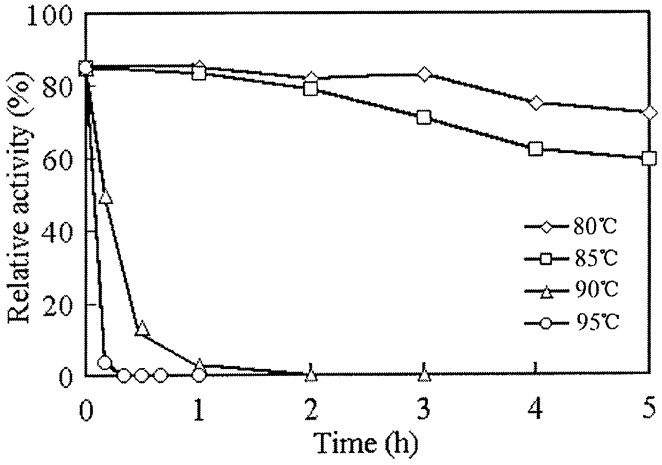

Fig. 6. Thermostability of *Ssh* ligase. The lysate of *E. coli* cells overproducing *Ssh* ligase was incubated at a given temperature for various lengths of time. The ligase activity of the treated samples was determined in the standard assay using the labeled substrate

Divalent cation cofactor requirement

A divalent cation is indispensable for the activity of a ligase. Among divalent cations tested, Mn^{2+} and to a lesser extent Mg^{2+} and Ca^{2+} were able to satisfy the metal cofactor requirement of recombinant *Ssh* ligase (Fig. 7A, B). The enzyme was consistently more active with Mn^{2+} than with Mg^{2+} over a range of cation concentrations from 1 to 25 mM (Fig. 7C). The optimal concentrations of Mn^{2+} and Mg^{2+} for the recombinant enzyme were similar: 2–5 and 5 mM, respectively. Ligation was ten times more efficient in the presence of Mn^{2+} than in the presence of Mg^{2+} when both cations were used at their optimal concentrations. To our knowledge, the cloned *S. shibatae* enzyme shows the greatest preference for Mn^{2+} rather than Mg^{2+} among characterized ligases. We also determined the metal cofactor requirement of native *Ssh* ligase. Surprisingly, the native enzyme differed strikingly from the recombinant enzyme in metal cofactor specificity. Although native *Ssh* ligase was also able to use Mg^{2+}, Mn^{2+}, or Ca^{2+} as a cofactor, it was more active with Mg^{2+} and Ca^{2+} (Fig. 7B). Since the recombinant and native forms of *Ssh* ligase were apparently similar in molecular properties, the above observation suggests that subtle structural differences may exist in the metal coordination site of the two enzymes.

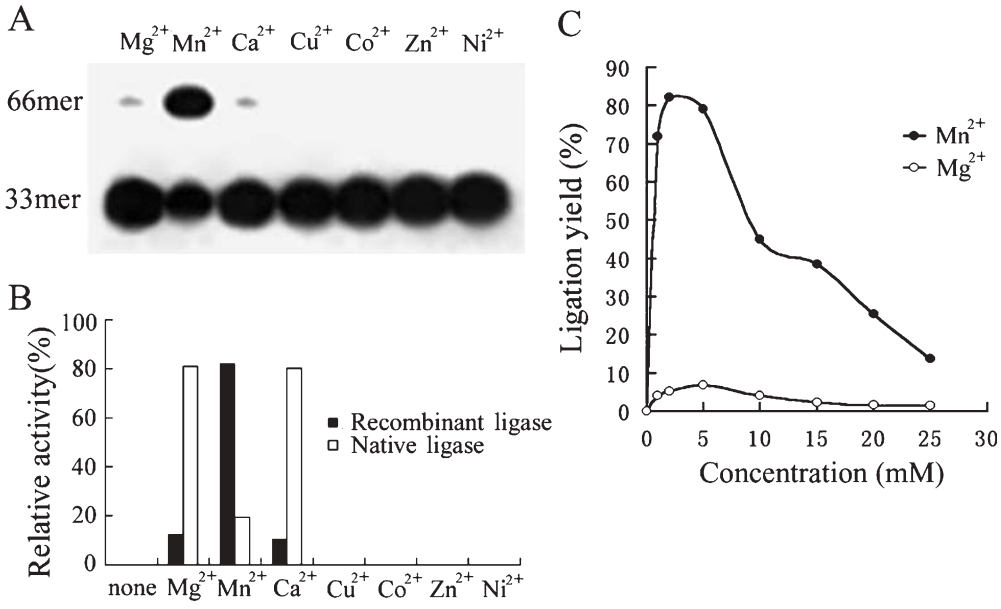

Fig. 7A–C. Metal cofactor requirement of *Ssh* ligase. **A** A ligation reaction was carried out with recombinant *Ssh* ligase for 10 min at 65°C in the presence of 10 mM $MgCl_2$, $MnCl_2$, $CaCl_2$, $CuCl_2$, $CoCl_2$, $ZnCl_2$, or $NiSO_4$. Reaction mixtures were subjected to denaturing PAGE. The gel was dried and exposed to X-ray film. **B** A ligation reaction was carried out with recombinant or native *Ssh* ligase for 10 min at 65°C in the presence of 10 mM $MgCl_2$, $MnCl_2$, $CaCl_2$, $CuCl_2$, $CoCl_2$, $ZnCl_2$, or $NiSO_4$, or in the absence of a cofactor (*none*). Ligation products were resolved by denaturing PAGE and quantified using a Phosphoimager. (C) A ligation reaction was carried out with recombinant *Ssh* ligase for 10 min at 65°C in the presence of various concentrations of $MgCl_2$ or $MnCl_2$. Ligation products were resolved by denaturing PAGE and quantified using a Phosphoimager

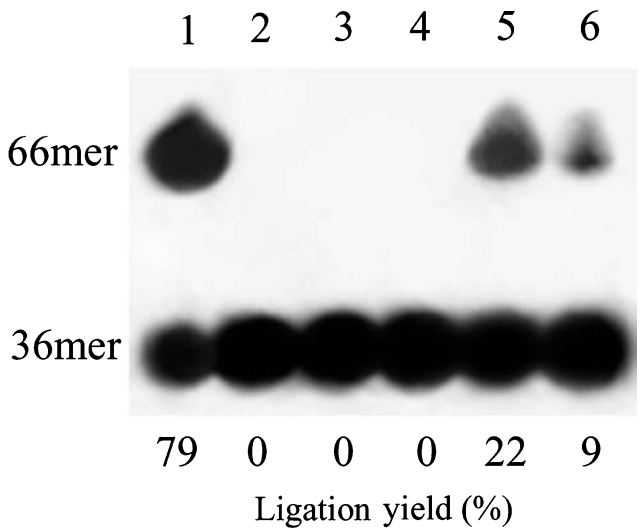

Fig. 8. Substrate specificity of recombinant *Ssh* ligase. Ligation reactions were carried out for 10 min at 65°C with matched, mismatched, and gapped substrates. Reaction mixtures were subjected to denaturing PAGE. The gel was dried and exposed to X-ray film. Ligation products were also quantified using a Phosphoimager. *Lane 1* Matched substrate; *lane 2* mismatches at the 3′-side of the nick; *lane 3* mismatches at the penultimate 3′-side; *lane 4* 1-nt gapped substrate; *lane 5* mismatches at the 5′-side of the nick; *lane 6* mismatches at the penultimate 5′-side. Bottom Ligation yields

DNA substrate specificity

To study the substrate discrimination of *Ssh* ligase we prepared a series of mismatched substrates which contained in one of the two oligonucleotides an equal molar mixture of all three possible mismatched bases at or 1 bp away from the 5′- or 3′-side of the nick junction. Recombinant *Ssh* ligase was significantly more sensitive to mismatches on the 3′-side than to those on the 5′-side of the nick (Fig. 8). No ligation was detectable in reactions using 3′ mismatched substrates even when incubation was lengthened to 20 min. Although 5′ mismatched substrates were tolerated, ligation efficiency decreased considerably when the mismatched base pair was moved from the 5′-end to the penultimate position from the 5′-end. No ligation was detected on a 1-nt gapped substrate under our experimental conditions. We also evaluated the ligation fidelity of native *Ssh* ligase using the same set of mismatched and gapped substrates. We found that the native enzyme resembled the recombinant enzyme in substrate specificity (data not shown).

Discussion

In this contribution we present the first biochemical characterization of an ATP-dependent DNA ligase from the crenarchaeotal branch of the domain archaea. The gene encoding the enzyme was identified in the genome of the hyperthermophilic archaeon *S. shibatae* on the basis of its similarity to those of known ATP-dependent DNA ligases and expressed in bacteria. Characterization of the enzymatic properties of the 601-amino acid gene product demonstrated that the protein is indeed an ATP-dependent DNA ligase. We also showed by immunoblotting and activity assays that the enzyme is synthesized in native cells. This protein is probably the only ligase encoded in *S. shibatae* since no ligases other than its homologue were found in the recently published genome sequence of closely related *S. solfataricus* (She et al. 2001). Sequence comparison revealed that despite their overall similarity DNA ligases from crenarchaeota and euryarchaeota differ in the sequence of motif V as well as in the spacing between motifs V and VI. Motif V is a component of the ATP binding site (Subramanya et al. 1996), whereas motif VI has been implicated in the adenylation reaction of ligases (Sriskanda and Shuman 1998).

A unique feature of ligases from thermophiles is their thermophilic nature. To determine accurately the temperature dependence of the activity of *Ssh* ligase we employed a ligation assay based on the closure of singly nicked plasmid by the enzyme. This assay can be carried out at higher temperatures than the nucleotide joining based standard assay. Our data show that both recombinant and native *Ssh* ligases are most active at 60–80°C. We also found that *Ssh* ligase is stable at temperatures up to 85°C. The temperature optimum and thermostability of the *S. shibatae* ligase suggest that the enzyme is adapted to functioning in host cells thriving in high temperature habitats (Grogan 1989).

ATP-dependent DNA ligases are conserved in sequence and have a common catalytic core structure. However, they are diverse in cofactor specificity. *Ssh* ligase utilized ATP as the nucleotide cofactor. dATP was a weak effector. Therefore *Ssh* ligase resembles the ligase from the thermophilic euryarchaeon *M. thermoautotrophicum* in exhibiting relaxed discrimination of the NTP sugar moiety (Sriskanda et al. 2000). By comparison, ATP-dependent ligases from *Haemophilus influenzae* T4 vaccinia virus and eukarya are highly specific for ATP (Cheng and Shuman 1997; Montecucco et al. 1990; Shuman 1995). Nakatani et al. (2000) recently reported a surprising observation that *T. kodakaraensis* DNA ligase is able to use NAD^+, in addition to ATP, as the cofactor. In addition to *Ssh* ligase, the *T. kodakaraensis* enzyme is the only ligase from a hyperthermophilic archaeon that has been biochemically characterized so far. It was speculated that cofactor specificity of the ligase is relaxed at high temperature. The *S. shibatae* ligase, however, was inactive with NAD^+.

Both ATP- and NAD^+-dependent DNA ligases generally are most active when Mg^{2+} is used as the divalent metal cofactor although they are able to use alternative metal cofactors. Some ligases (e.g., ligases from *Aquifex aeolicus*, *M. thermoautotrophicum*, *Thermus thermophilus* HB8) are similarly active with either Mg^{2+} or Mn^{2+} (Sriskanda et al. 2000; Takahashi et al. 1984; Tong et al. 2000). Recombinant *Ssh* ligase, however, displayed far greater activity with Mn^{2+} than with Mg^{2+}. Surprisingly, this property of the cloned ligase is not shared by native *Ssh* ligase, which preferred Mg^{2+}, although the activity of both enzymes was supported by the same spectrum of metal cofactors (Mn^{2+},

Mg^{2+}, and Ca^{2+}). The structural basis for the different metal-coordinating properties of the enzymes remains to be determined.

DNA ligases from eukarya, bacteria, and viruses show great variation in their discrimination against mismatches at or near the nick junction. While some viral ATP-dependent ligases, represented by the T4 enzyme (Wu and Wallace 1989), are tolerant of mismatches on both 3′- and 5′-sides of the nick, most ATP-dependent ligases (e.g., ligases I, II, and III from mammalian cells, vaccinia ligase) are more sensitive to mismatches on the 3′-side than to those on the 5′-side (Husain et al. 1995; Shuman 1995). Little is known about the substrate specificity of archaeal ligases. In this study we examined the ability of *Ssh* ligase to ligate mismatched substrates. We found that *Ssh* ligase is more tolerant of mismatches on the 5′-side of the nick junction than of those on the 3′-side. In addition, mismatches located at the penultimate 5′-end has a greater inhibitory effect on ligation than those at the nick. It appears that a ligase may not necessarily be more sensitive to a mismatched base pair at the nick than to that located away from the nick.

Because of their high thermostability ligases from thermophiles are well suited for structural and mechanistic studies and hold great potential in the development of new technologies. The availability of a rapidly increasing number of genome sequences of thermophilic archaea and bacteria provides unprecedented opportunities for comparative studies of thermophilic ligases. These studies will help understand the unique properties of this group of enzymes and explore their potential biotechnological applications.

Acknowledgments This research was supported by grants from the National Science Foundation of China (39925001 and 30030010) and from the Chinese Academy of Sciences (KSCX2-3-01-02).

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