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Characterization of a thermophilic DNA ligase from the archaeon *Thermococcus fumicolans*

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Abstract

A PCR protocol was used to identify and sequence a gene encoding a DNA ligase from *Thermococcus fumicolans* (*Tfu*). The recombinant enzyme, expressed in *Escherichia coli* BL21(DE3) pLysS, was purified to homogeneity and characterized. The optimum temperature and pH of *Tfu* DNA ligase were 65 °C and 7.0, respectively. The optimum concentration of $MgCl_2$, which is indispensable for the enzyme activity, was 2 mM. We showed that *Tfu* DNA ligase displayed nick joining and blunt-end ligation activity using either ATP or NAD^+, as a cofactor. In addition, our results would suggest that *Tfu* DNA ligase is likely to use the same catalytic residues with the two cofactors. The ability for DNA ligases, to use either ATP or NAD^+, as a cofactor, appears to be specific of DNA ligases from Thermococcales, an order of hyperthermophilic microorganisms that belongs to the euryarchaeotal branch of the archaea domain.

Keywords: DNA ligase; Archaea; *Thermococcus fumicolans*; Hyperthermophile

1. Introduction

DNA ligases play an essential role in cellular DNA replication, recombination and repair [1]. They are universally found in eucarya, bacteria and archaea and classified into two families, according to the cofactor required for ligase adenylate formation, ATP-dependent DNA ligases (EC 6.5.1.1) and NAD^+-dependent ligases (EC 6.5.1.2). DNA ligases catalyze the joining of 5′ phosphate-terminated donor strands to 3′-hydroxyl-terminated acceptor strands via a common three sequential nucleotidyl transfer reactions [1,2]. In the first step, nucleophilic attack by the ligase on ATP or NAD^+ results in formation of a covalent intermediate in which AMP is covalently attached to the active site lysine residue, with release of pyrophosphate or nicotinamide mononucleotide. In the second step, the AMP is transfered to the 5′-end of the 5′-phosphate-terminated DNA strand to form DNA-adenylate, an inverted (5′)–(5′) pyrophosphate bridge structure, AppN. In the final step, ligase catalyses attack by the 3′-OH of the acceptor strand on the DNA-adenylate to join the two polynucleotides, thus liberating AMP.

All eukaryal and archaeal DNA ligases known to date, as well as those from bacteriophages and viruses share amino acid sequence similarity with ATP-dependent DNA ligases [3]. They all utilize ATP as a cofactor and have been classified in the ATP-dependent DNA ligase family (EC 6.5.1.1). Interestingly, the ATP dependant DNA ligase from the hyperthermophilic archaea, *Thermococcus kodakaraensis* KOD1, was demonstrated to have a low but significant activity when

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using NAD^+ instead of ATP as the cofactor [4]. However, this cofactor specificity seems unique to *T. kodakaraensis* and not common to all archaeal DNA ligases. Indeed, NAD^+ cannot be substituted for ATP with *Sulfolobus shibatae* [5] and *Methanobacterium thermoautotrophicum* [6] DNA ligases. On the other hand, most bacterial DNA ligases have been classified in the NAD^+-dependent DNA ligase family (EC 6.5.1.2), although two bacteria *Haemophilus influenzae* [7] and *Aquifex aeolicus* [8] were shown to possess ATP-dependent DNA ligases; possibly the result of horizontal gene transfer and integration of viral genes in the bacterial genome [9].

Here, we report the characterization of a DNA ligase from *Thermococcus fumicolans* (*Tfu*), a chemoorganotrophic hyperthermophilic archaeon that is able to grow optimally at 85 °C [10]. The *Tfu* open reading frame displays strong similarity to the ATP-dependant DNA ligase and contains the core catalytic domain common to all ATP-dependant DNA ligases. To characterize the enzymatic activity of *Tfu* DNA ligase, we produced the polypeptide in *Escherichia coli* and purified the recombinant protein. We showed that the *Tfu* DNA ligase, indeed displays nick joining activity using ATP as a cofactor, but is also able to drive the reaction using NAD^+.

2. Materials and methods

2.1. Organisms and growth conditions

Thermococcus fumicolans is a hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent in the North Fiji Basin [10]. This strain is a strictly anaerobic coccus, that grows at an optimum temperature of 85 °C. *E. coli* DH5α and *E. coli* BL21 (DE3) harboring pLysS were used for cloning and over-expression of the *Tfu* DNA ligase. A *T. fumicolans* genomic bank was made in pBluescriptII SK(+) phagemid vector (Stratagene) and plasmid pET-26b(+) (Stratagene) was used for over-expression. *E. coli* was grown in 2×YT medium at a rotary shaker at 37 °C. Ampicillin was added to 2×YT medium (Bacto Peptone 16g/l, yeast extract 10 g/l, and NaCl 10 g/l) at a final concentration of 100 g/ml. Isopropyl-D-thiogalactyopyranoside (IPTG) was added in a final concentration of 1 mM, for the induction of gene expression.

2.2. Cloning of Tfu DNA ligase gene

2.2.1. Consensus PCR

An ATP-dependent DNA ligase gene fragment was amplified from *Tfu* genomic DNA by using the O1–O3 consensus primer pair, as shown in Fig. 1. The PCR was performed for 35 cycles consisting of 94 °C for 1 min, 37 °C for 1 min, and 72 °C for 1 min. The O1–O3 primers pair gave amplification products that were approximately 530 bp long.

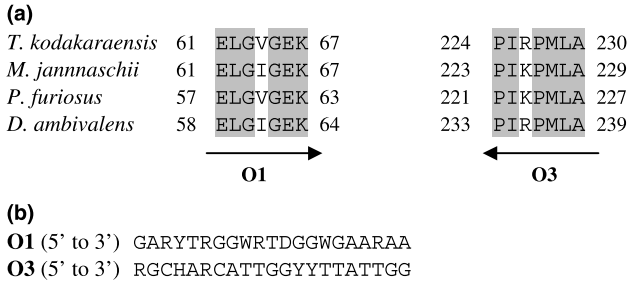

Fig. 1. Design of consensus PCR primers for the identification of *Thermococcus fumicolans* DNA ligase. (a) Conserved motifs in archaeal DNA ligases. (b) Deduced archaeal DNA ligase consensus primers.

2.2.2. Tfu library construction

Restriction fragments were generated by incubating 150 μg of Rnase A-treated *Tfu* genomic DNA 1h at 37 °C with 5U of restriction endonuclease *Sau*3A. *Tfu* restriction fragment library was then prepared by ligating 1 μg of digested genomic DNA with 0.5 μg of the *Bam*HI cohesive end pBluescript SK(+) plasmid in a 20 μl overnight reaction mixture using T4 DNA ligase (Boehringer Mannheim). *Tfu* genomic library was transformed into *E. coli* DH5α $CaCl_2$ competent cells using standard procedures and plated on 2×YT medium.

2.2.3. Tfu library screening

The genomic library was plated, transferred onto Hybond N+ membrane filters (Amersham) and hybrydized with the [α-^{32}P]dCTP labeled O1–O3 PCR amplification fragment (Random Prime Labelling System, Amersham–Pharmacia Biotech).

2.3. Construction of the recombinant Tfu DNA ligase plasmid

Based on the sequence of the *Tfu* DNA ligase, primers were designed to amplify the gene by the polymerase chain reaction on a DNA Thermal Cycler (Stratagene). The two primers (with *Nde*I and *Bam*HI restriction sites in boldface) were as follows: LIG1: 5′-GGTGGTGAGCATGGGC**CATATG**AAGTACTCCGAGC-3′, sense and LIG2: 5′-CGCATGCCCAATAAAC**GGATCC**GGGGAAGTCCCC-3′, antisense. In addition to the template and the primers, the 50 l reaction mixture contained 1 μg of genomic DNA, 0.2 mM dNTPs, *Taq* DNA polymerase buffer, 5 U *Taq* DNA polymerase (Q-biogene) and was subjected to eight cycles of amplification (1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C). A PCR product of the expected size was digested with *Nde*I and *Bam*HI, cloned in a pET-26b(+) expression vector and transformed into *E. coli* BL21 (DE3) pLysS.

2.4. Expression and purification of Tfu DNA ligase

An overnight culture of *E. coli* BL21 (DE3), pLys S, harboring *Tfu* DNA ligase gene, was diluted 1:20 and grown until the absorbance at 600 nm reached 0.6. The culture was induced with 1 mM of IPTG for 16 h. Cells were harvested by centrifugation, resuspended in lysis buffer (50 mM phosphate buffer pH 7.5, 10 mM 2-mercaptoethanol, 1 mM EDTA, 200 μg/ml lysozyme) and sonicated using a Vibracell sonifier (375W, 40% amplitude). Cell debris were removed by centrifugation (10,000*g* for 10 min). The resulting supernatant was heated twice for 10 min at 75 °C and precipitated proteins were removed by two additional centrifugations. The supernatant was diluted fivefold in Tris–HCl buffer (50 mM Tris–HCl, pH 8.0, DTT 1 mM, EDTA 1 mM, Tween 20 0.1%, and glycerol 10%), loaded on a Resource Q (Pharmacia) column that was previously equilibrated with the same buffer. Bound proteins were eluted by a linear gradient of NaCl (0–1 M in Tris–HCl buffer, pH 8). Active fractions eluted around 0.2 M NaCl. The fractions containing DNA ligase activity were combined, concentrated to 1 mL (Centricon 10 kDa, Millipore) and loaded onto a Superdex-200 column (50 cm × 16 mm diameter, Pharmacia) that was equilibrated with Tris–HCl buffer (50 mM Tris–HCl, pH 6.5, DTT 1 mM, EDTA 1 mM, Tween 20 0.1%, and glycerol 10%). The purified enzyme was then dialysed against Tris–HCl buffer (50 mM Tris–HCl, pH 6.5, DTT 1 mM, EDTA 1 mM, Tween 20 0.1%, and glycerol 50%) and stored at −20 °C.

Protein samples were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and protein concentration was determined using the Bradford reagents (BioRad) with BSA as the standard.

2.5. DNA ligase assays

The DNA ligase substrate, [γ-^{32}P]oligo(dT)$_{16}$–poly(dA)$_{500}$ was prepared as followed. 10 nmol of [γ-^{32}P]oligo(dT)$_{16}$ was used per 20 μl assay which contained 25 mM Tris–HCl pH 6.5, 20 mM $MgCl_2$, 1 mM ATP or NAD^+, 1 mM DTT and 10 pmol poly(dA)$_{500}$. The reaction was started by addition of 20 ng of purified *Tfu* DNA ligase and incubated for 15 min at 55 °C. The reaction was stopped by heating at 100 °C for 5 min. Reaction mixtures were removed and treated for 15 min at 80 °C with 0.2 unit of bacterial alkaline phosphatase (Eurogentec). The reaction products were precipitated in 10% TCA, filtered through GF/C paper and washed five times with TCA-PPi (5% trichloroacetic acid, 40 mM sodium pyrophosphate). Radioactivity was determined by liquid scintillation counting. One unit of DNA ligase converts 1 nmol of [γ-^{32}P]oligo(dT)$_{16}$ into an alkaline phosphatase resistant form in 15 min at 55 °C. The temperature optimum was determined by running the DNA ligase assay at temperatures from 40 to 90 °C. The pH optimum was determined at 55C, using Tris–HCl buffer (0.1 M) over the pH range 6.5–8 (the $\Delta pK_a/\Delta T$ of Tris buffer was taken into consideration to draw the Fig. 3(a)). Michaelis–Menten constants were determined from Lineweaver–Burk representations of data obtained by determining the initial rate of the ligation reactions under the standard assay conditions. DNA ligase assays for thermostability properties of *Tfu* DNA ligase were performed with labeled oligonucleotide, as described by Nakatani et al. [4], after incubating, for different times, the enzyme at 80 °C and 90 °C. DNA ligase activity measurements were carried out with synthetized oligonucleotides consisting of a 5′-[γ-^{32}P]ATP phosphorylated 35-mer (5′P-GCAGTCACATAGTAGTCGTCGTACGTCCAGCATGG) and a 35-mer (5′-GCTATGACAGAGCGACTTCGATGAGTGACCATGCG) annealed to a complementary 75-mer (5′ATCGCATGGTCACTCATCGAAGTCGCTCTGTCATAGCCCATGCTGGACGTACGACGA CTACTATGTGACTGCACG) [4].

Blunt ended DNA ligation was assayed in a 10 μl reaction containing 25 mM Tris–HCl, pH 6.5, 20 mM $MgCl_2$, 1 mM DTT, 1 mM ATP or NAD^+, 2 mg/ml BSA, 50% PEG 6000, 0.25 μg *Eco*RV digested pBluescript SK(+) plasmid and 20 ng of *Tfu* DNA ligase. After incubation at 55 °C for 15 min the reaction was analysed by electrophoresis on 0.8% agarose gel.

2.6. Formation of the DNA ligase adenylated complex

The DNA ligase-[^{32}P]AMP intermediate complex was prepared as reported by Rossi et al. [11]. Purified enzyme was incubated at 37 °C for 2 h in 50 mM Tris–HCl, pH 7.0, $MgCl_2$ 10 mM, DTT 2 mM, 0.2 μM ATP containing 5 μCi [α-^{32}P]ATP or [α-^{32}P]NAD in a total volume of 60 μl. Reactions were stopped by boiling, after the addition of 10 μl sample buffer (Tris–HCl, pH 6.8, 20% SDS, 15% 2-mercaptoethanol, and 0.003% bromophenol blue), for 5 min and products were analysed by SDS–10% PAGE. The gels were dried and adenylated polypeptides were detected by autoradiography on Kodak biomax MR film.

2.7. Nucleotide sequence Accession Number

The reported nucleotide sequence has been submitted to the GenBank/EMBL Data Bank with Accession No. AJ133713.

3. Results and discussion

3.1. Cloning, expression and purification of Tfu DNA ligase

To identify the DNA ligase gene, two PCR primers (O1 and O3, Fig. 1) were designed, based on the con-

served motifs in the sequences of the known archaeal DNA ligases. A 530 bp fragment was amplified by PCR from *Tfu* genomic DNA using these primers. The sequence of this fragment was highly homologous to the sequences of other archaea ATP-dependent DNA ligases in the GenBank and EMBL databases. This fragment was then used as a probe to screen a *Tfu* genomic library. A positive clone harboring a 5 kb insert yielded a full length DNA ligase gene as shown by DNA sequencing. The full-lengh *Tfu* DNA ligase gene was amplified from *Tfu* genomic DNA and cloned in a pET26b(+) vector and expressed in *E. coli*. The protein was expressed to approximately 30% of the total cellular proteins and was separated from most *E. coli* host proteins by thermal denaturation. An anion exchange chromatography followed by a gel filtration step yielded a preparation that was homogeneous by coomassie brillant blue staining (Fig. 2).

3.2. Analysis of the Tfu DNA ligase sequence

The *Tfu* DNA ligase consists of 559 amino acids, which is within the range of eucaryal, bacteriophage and archaeal DNA ligases (500–900 residues) [12], and possesses the six conserved sequence motifs common to all ATP-dependent DNA ligases. The *Tfu* ATP-dependent DNA ligase also contains the conserved motifs that are common between DNA ligases, RNA ligases, tRNA ligases and capping enzymes (for a review, see Shuman and Shwer [13]). These motifs are considered to be essential for both classes of enzymes and also present in the nucleotydyltransferase superfamily [14–16]. The *Tfu* enzyme shares 87%, 78% and 77% amino acid sequence identity with the ATP dependent ligases from the archaea *T. kodakaraensis* KOD1, *Pyrococcus abyssi* and *Pyrococcus furiosus*, respectively; 44% with *M. thermoautotrophicum*; and 33% identity with the DNA ligase from eucarya (*Saccharomyces cerevisiae*). The domain archaea is now recognized as constituting a third major branch of life, together with eubacteria and eukarya [17]. Archaeal proteins involved in gene expression, such as those for DNA replication, transcription, and translation, have been found to be similar to those from eukarya, although the cellular appearance and organization of archaea are more like those of bacteria. All of the sequences of the archaeal DNA-ligases thus far are classified in the ATP-dependent DNA ligase family (EC 6.5.1.1). This finding has tempted us to speculate as it has been done from DNA polymerases studies [18], that the DNA replication machinery in archaea may be a prototype of the eukaryotic machinery [19,20] .

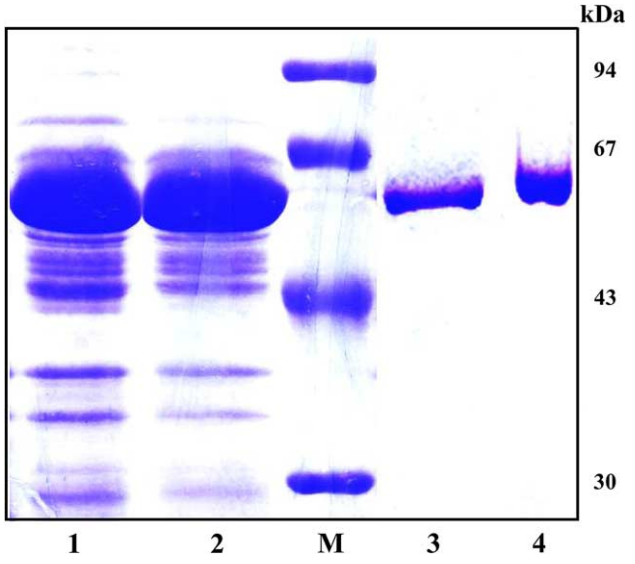

Fig. 2. SDS–PAGE of *E. coli* BL21(DE3) p*Lys*S producing the recombinant *Thermococcus fumicolans* DNA ligase. Lanes: 1, crude extract of induced cells (1 mM IPTG, 16 h); 2, crude extract of induced cells after heat treatment (10 min at 75 °C); 3, ResourceQ pooled active fractions; 4, purified fraction after Superdex-200 chromatography; M, low-molecular-mass standards (Pharmacia Biotech).

3.3. Effect of pH and salt on ligation

The *Tfu* DNA ligase is active within a rather narrow pH range, the optimal pH being 7.0 (Fig. 3(a)). The activity could not be measured accurately for pH values below 6.5 because the poly(dA) substrate tends to precipitate at acidic pH. The activity of the enzyme is strongly dependent on the $MgCl_2$ concentration (Fig. 3). While optimal activity is obtained with 2 mM, it is almost totally inhibited with 10 mM $MgCl_2$. The *Tfu* DNA ligase does not require monovalent cations to be active, but 2 mM KCl or up to 5 mM of NaCl increase its activity up to 70%. Higher concentrations of these cations are inhibitory, but the enzyme still retains 50% activity with 100 mM NaCl or 100 mM KCl (Fig. 3(c)). The *Tfu* ligase sensitivity to cations is quite similar to that of other ATP-dependent DNA ligases such as the *M. thermoautotrophicum* DNA ligase [6] and yeast DNA ligase Cdc9p [21] although it is more sensitive to magnesium concentration than has been previously reported.

3.4. Thermoactivity and thermostability

The *Tfu* DNA ligase is active over a wide range of temperatures. Although the optimal temperature was shown to be 65 °C, 50% of the activity is still retained at either 40 °C or 80 °C (Fig. 3(b)). For temperatures higher than 70 °C, denaturation of the poly(dA)/oligo(dT) complex interfered with the reaction. The thermostability was evaluated by incubating the enzyme in 50 mM Tris–HCl buffer, pH 6.5 at 80 °C and 90 °C. *Tfu* DNA ligase retained 100% of the original activity after

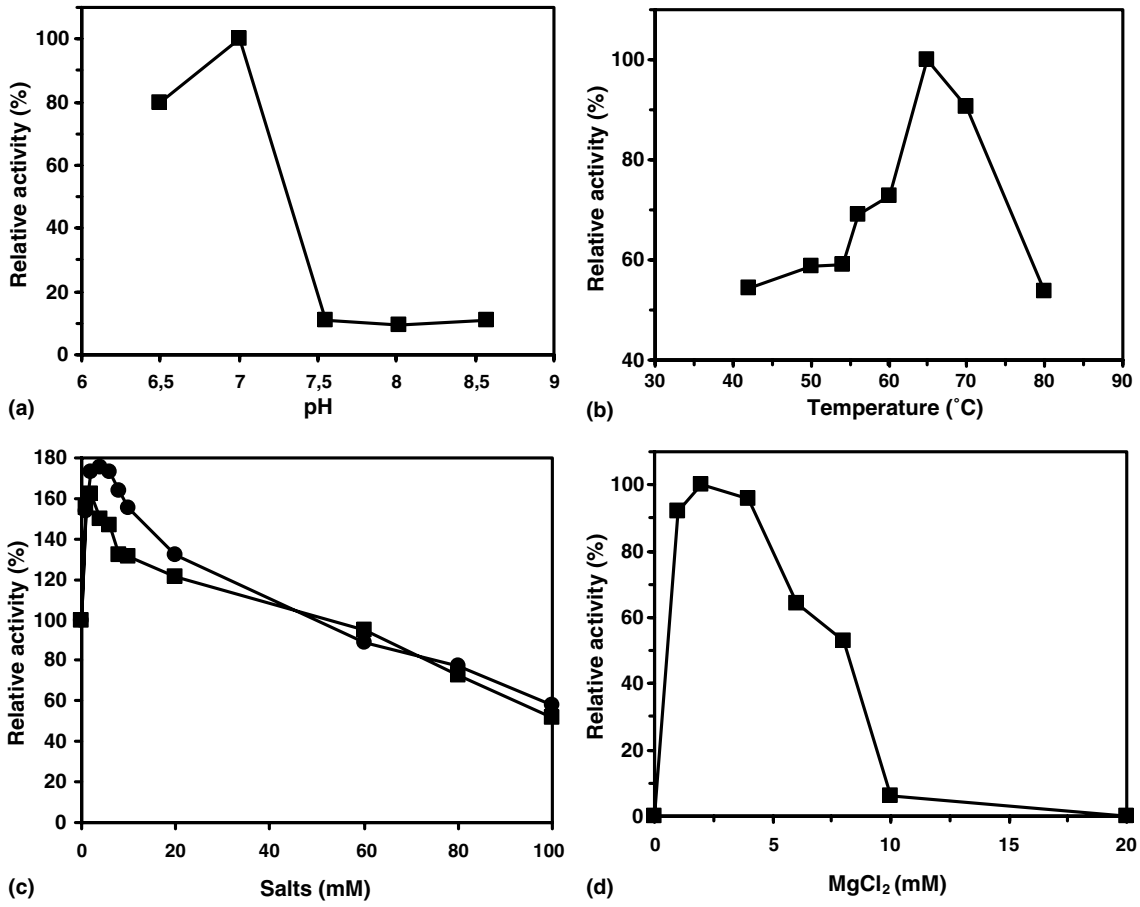

Fig. 3. Enzymatic properties of purified recombinant *T. fumicolans* DNA ligase. (a) Effect of pH on the activity of *Tfu* DNA ligase. The optimum pH was determined at 55 °C and the assay was buffered using 25 mM Tris–HCl buffer (pH 6.5–8.5). (b) Effect of temperature on the activity of *Tfu* DNA ligase. (c) Effect of NaCl (■) and KCl (●) concentration on the activity of *Tfu* DNA ligase. (d) Effect of $MgCl_2$ concentration on the activity of *Tfu* DNA ligase. Results were quantified as described in Section 2.

incubation for 3 hours at 80 °C, but lost half of its activity in 15 min at 90 °C (data not shown). The *Tfu* DNA ligase thermostability is comparable to already described thermosatble DNA ligases from *T. thermophilus* and *S. shibatae* [5] (half life of 26 min at 91 °C and 10 min at 90 °C, respectively) and less thermotable compared to *P. furiosus* DNA ligases [22]. Such thermosable DNA ligase are particularly suited to reactions requiring thermal cycling such as the ligase chain reaction technique or the padlock probe circularization prior to rolling circle amplification. Thus far, two thermostable DNA ligases, *Pyrococcus furiosus* enzyme [22] and Ampligase (from *Tebu*) have been applied to diagnostic methods.

3.5. Cofactor specificity and kinetics of the Tfu DNA ligase

The *Tfu* DNA ligase is readily adenylated when incubated with [α-^{32}P] ATP in a manner similar to T4 DNA ligase (Fig. 4), which is in line with enzyme belonging to the ATP-dependent DNA ligase family. More surprisingly, the formation of the enzyme-adenylate intermediate was also observed with NAD (Fig. 4) as it was shown for the DNA ligase from *T. kodakaraensis* [4]. With both classes of DNA ligases (NAD or ATP), the first step of the ligation reaction involves covalent addition of an AMP group to a lysine residue in the active site of the enzyme (Fig. 1). In ATP-dependent ligases, the AMP donor is ATP whereas the NAD-dependent enzyme uses NAD^+. In our case, the purified *Tfu* DNA ligase presents adenyl transferase activity using either one of the two cofactors.

To know if the enzyme-adenylate intermediate, observed with either NAD^+ or ATP cofactors, is due to the covalent addition of the AMP group to the lysine residue of the active site of the enzyme, we studied the formation of the protein-adenylate intermediate after preincubation for 2 hours at 37 °C of the enzyme with ATP (12.5 mM) or NAD (12.5 mM). When *Tfu* DNA ligase was preincubated with ATP, the ability to form the protein-adenylate intermediate with [α-^{32}P]NAD was not observed, suggesting that the lysine active site

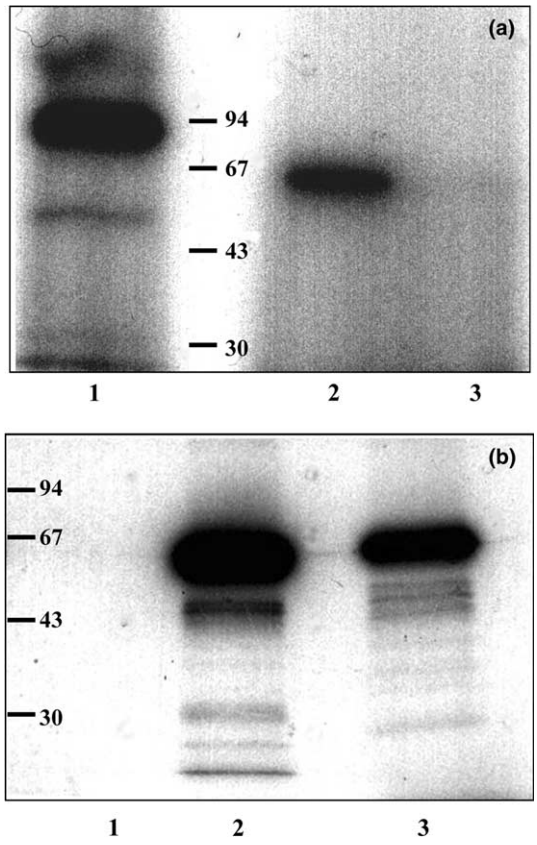

Fig. 4. Adenylation of *T. fumicolans* DNA ligase with (a) NAD^+ or (b) ATP as cofactor. *Tfu* DNA ligase was incubated at 37 °C for 2 h with [α-^{32}P]ATP or [α-^{32}P]NAD^+, applied to SDS–PAGE gels and the complex was detected by autoradiography. The position and sizes (in kDa) of marker proteins are indicated. Lanes: 1, Ampligase (NAD-dependent DNA ligase from *Tebu*); 2, *Tfu* DNA ligase; 3, T4 DNA ligase (T4 Bacteriophage ATP-dependent DNA ligase from Boehringer Mannheim).

residue involved in the reaction is saturated by ATP prior the adenylation assay with [α-^{32}P]NAD (data not shown). The same experiment was performed with a preincubation of the *Tfu* DNA ligase with NAD^+ prior to an adenylation assay with [α-^{32}P]ATP and the same absence of labelling of the protein was observed (data not shown). These results would suggest that *Tfu* DNA ligase is likely to use the same catalytic residues with the two cofactors. Three dimensional structure analysis should provide relevant information on the basis of the observed cofactor specificity.

To confirm the ability of *Tfu* DNA ligase to use either ATP or NAD as cofactor for the ligation reaction, we studied kinetic parameters. The enzyme exhibited typical Michaelis–Menten steady-state kinetics with a K_m of 1.16 mM for ATP and 690 μM for NAD^+ in the standard assay conditions. The K_m of the *Tfu* ligase for ATP is 10-fold higher than that of the other ATP-dependent DNA ligases and contrary to what was observed for the *T. kodakaraensis* enzyme, the *Tfu* DNA ligase seems to have a higher affinity towards NAD^+ than towards

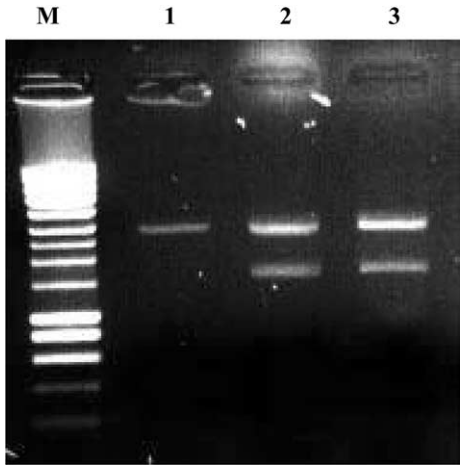

Fig. 5. Blunt-end joining activity of *T. fumicolans* DNA ligase. *Eco*RV-digested pBluscript SK(+) was incubated 15 min with *Tfu* DNA ligase, 50% PEG 6000 and ATP or NAD^+ as cofactor. The reaction was analysed by electrophoresis on a 0.8% agarose gel. Lanes: 1, no cofactor; 2, ATP as a cofactor; 3, NAD^+ as a cofactor.

ATP. The same V_{max} (26.5 pmol/min) was obtained for the ligation reaction with the two cofactors.

3.6. Blunt-end joining of DNA

Under conventional assay conditions, only the T4 DNA ligase efficiently catalyses blunt-end ligation. However, in the presence of a high concentration of polyethylene glycol, NAD-dependent and ATP-dependent DNA ligases are also able to catalyse blunt-end ligation [3]. The *Tfu* DNA ligase can join blunt-end DNA fragments with ATP or NAD^+ as cofactors at 55 °C (Fig. 5). This function is less efficient than the sealing of single-strand interupted DNA and must be promoted in reaction mixtures by macromolecular crowding conditions, by addition of 50 % polyethylene glycol. In the absence of polyethylene glycol, no blunt-end ligation was observed (data not shown). These results confirm the ability of this ligase to use either ATP or NAD for its activity. The blunt-end activity of the archaea DNA ligase is described for *Pfu* DNA ligase and other archaea, but *Tfu* DNA ligase is the first ATP-dependent DNA ligase that was shown to have blunt-end activity using NAD^+ as cofactor.

4. Concluding remarks

We report here the biochemical characterization of *Tfu* DNA ligase. As predicted from sequence comparisons, *Tfu* DNA ligase displays nick-joining activity in an ATP-dependent manner at elevated temperatures, characteristic of those required for the growth of the *T. fumicolans* strain. As it was reported for *T. kodakaraensis* DNA ligase, *Tfu* enzyme is able to use NAD, instead of ATP as the cofactor. However, the difference with

T. kodakaraensis is that, *Tfu* DNA ligase showed as much activity with NAD^+ as with ATP as a cofactor, and that its K_m for NAD^+ is higher than its K_m for ATP. Nakatani et al. [4] proposed that the observed cofactor specificity of the enzyme is less strict at high temperature. However, the *Sulfolobus shibatae* ATP-dependent DNA ligase was inactive using NAD^+ even at high temperature, demonstrating that this ability to use ATP or NAD^+ as cofactor is not a general characterisitc of hyperthermophilic archaea [5]. We also biochemically characterized *P. abyssi* DNA ligase (unpublished results) and observed that the enzyme showed nick-joining activity using ATP or NAD^+, as a cofactor. This ability for DNA ligases from *Thermococcus* and *Pyrococcus* strain, to use either ATP or NAD, as a cofactor, seems to be specific to DNA ligases from Thermococcales, an order of hyperthermophilic microorganisms that belong to the euryarchaeotal branch of the archaea domain. Based on these experimental results, we suggest that the classification of DNA ligases into two families based on the cofactor required for ligase-adenylate formation [6,23] should be modified. In addition, these results raise potentially interesting questions about the evolution of the DNA ligases and their role in DNA replication in general.

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