A novel ADP-dependent DNA ligase from *Aeropyrum pernix* K1

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Abstract A gene encoding a putative ATP-dependent DNA ligase from the aerobic hyperthermophilic archaeon *Aeropyrum pernix* K1 was cloned and the biochemical characteristics of the resulting recombinant protein were examined. The gene (accession no. APE1094) from *A. pernix* encoding a 69-kDa protein showed a 39–61% identity with other ATP-dependent DNA ligases from the archaea. Normally DNA ligase is activated by NAD^+ or ATP. There has been no report about the other activators for DNA ligase. The recombinant ligase was a monomeric protein and catalyzed strand joining on a singly nicked DNA substrate in the presence of ADP and a divalent cation (Mg^{2+}, Mn^{2+}, Ca^{2+} and Co^{2+}) at high temperature. The optimum temperature and pH for nick-closing activity were above 70°C and 7.5°C, respectively. The ligase remained stable for 60 min of treatment at 100°C, and the half-life was about 25 min at 110°C. This is the first report of a novel hyperthermostable DNA ligase that can utilize ADP to activate the enzyme.

Key words: DNA ligase; Hyperthermophile; Aerobic archaea; *Aeropyrum pernix*

1. Introduction

DNA ligases catalyze the formation of a phosphodiester bonds at single-stranded or double-stranded breaks between adjacent 3′-hydroxyl and 5′-phosphoryl groups of DNA [1]. These enzymes are essential for a number of genetic processes, such as DNA replication, recombination, and repair systems [2,3]. DNA ligases fall into two classes, ATP-dependent ligases and NAD^+-dependent ligases, based on the cofactor required for the activity. The ATP-dependent DNA ligases are found in bacteriophages, archaea, eukaryotes, eukaryotic viruses and, recently, in bacteria [4], while the NAD^+-dependent ligases appear to be produced exclusively by bacteria [5]. The sequence between these two classes shows no similarity, except for the KXDG sequence motif involved in the formation of an adenylated enzyme intermediate [6,7]. In spite of the low level in sequence homology, DNA ligases of the two classes catalyze their reactions through a three-step mechanism that is common to both classes [8]. In the first step, the enzyme is activated through the formation of a covalent phosphoamidate bond between the ε-amino group of the conserved active site lysine and the adenylate group of NAD^+ or ATP. In the second step, AMP is transferred from the ligase to the 5′-phosphoryl group of the nick on the DNA strand. The final step involves the closure of the nick with the release of AMP from the adenylated DNA intermediate.

In archaea, the first DNA ligase gene to be identified from the hyperthermophilic archaeon, *Desulfurolobus ambivalens*, showed an amino acid sequence similarity to eukaryotic ATP-dependent DNA ligases [9]. Many genes encoding putative ATP-dependent ligases have been identified from sequenced archaeal genomes and their primary structures are extensively conserved. To date, three archaeal DNA ligases from *Methanobacterium thermoautotrophicum* [10], *Thermococcus kodakaraensis* [11], and *Sulfolobus shibatae* [12] have been biochemically characterized. These enzymes are capable of joining a nick in an ATP-dependent manner at the temperature range for the growth of the host organisms. Interestingly, the ATP-dependent ligase from *T. kodakaraensis* displays a low but significant activity when NAD^+ is used as the cofactor. From the standpoint of molecular evolution, a study of cofactor requirements should be informative.

In this study, we report on the cloning of a gene encoding a putative ATP-dependent DNA ligase in the genome of the hyperthermophilic archaeon *Aeropyrum pernix* K1, its biochemical characteristics and the cofactor requirements of the ligase.

2. Materials and methods

2.1. Materials

DNA primers and substrates were prepared by Fasmac Co., Ltd (Kanagawa, Japan). The plasmid pET-3d was purchased from Novagen (Madison, WI, USA). KOD DNA polymerase and T_4 DNA polymerase were purchased from Toyobo (Osaka, Japan). ATP, ADP, AMP, and NAD^+ used were the highest grade (99+%) commercially available from Sigma (St. Louis, MO, USA).

2.2. Construction and expression of the enzymes

Chromosomal DNA of *A. pernix* K1 was prepared as described by Sako et al. [13]. The gene (APE1094) was amplified by polymerase chain reaction (PCR) using chromosomal DNA as template, and two primers Lig1: 5′-GGCTGTCTGGTTTTGGCTTCT-3′ (forward), and Lig2: 5′-GTGAAGGGATCCTTACACCTGCTCCGCCGGC-3′ (reverse), which were designed based on an open reading frame (ORF) encoding a protein of 619 amino acids. Amplification by PCR was carried out at 94°C for 30 s, 55°C for 2 s, 74°C for 30 s for 30 cycles using KOD DNA polymerase. The plasmid pET-3d was then digested with *Nco*I, treated with T_4 DNA polymerase to fill in the cohesive ends and, finally, digested with *Bam*HI again. The amplified PCR products were digested with *Bam*HI (the *Bam*HI site in primer Lig2 is underlined) and inserted into the pET-3d vector. The

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Abbreviations: PCR, polymerase chain reaction; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; ORF, open reading frame; *Ape* ligase, *Aeropyrum pernix* ligase

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nucleotide sequences of the inserted genes were confirmed using an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer). *Escherichia coli* Rosetta (DE3) cells harboring the expression plasmid were grown to an OD_{600} of approximately 0.4 and gene expression was then induced by treatment with 1 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside) for 4 h at 37°C.

2.3. Purification of the recombinant protein

E. coli cells containing the expressed recombinant enzyme were centrifuged and frozen at −70°C. The thawed cells were then disrupted by sonication in buffer A (50 mM Tris–HCl, pH 8.2, 15 mM $MgCl_2$, 0.1 mM EDTA). The suspension of disrupted cells was centrifuged at 27 000 × *g* for 30 min and the supernatant fraction was heat-treated at 85°C for 30 min followed by recentrifugation. The supernatant was loaded on a HiTrap Q column (Amersham Biosciences, Piscataway, NJ, USA) equilibrated in buffer A and the bound protein was eluted with a linear gradient of NaCl (0 to 1.0 M in the same buffer). The protein solution was concentrated using a centricon 10 filter from Amicon (Millipore, Bedford, MA, USA) and dialyzed against buffer B (50 mM Tris–HCl, pH 8.2, 150 mM NaCl, 15 mM $MgCl_2$). The dialyzed solution was loaded on a HiPrep Sephacryl S-100 HR 26/60 column (Amersham Biosciences) and eluted with buffer B. The purity of recombinant protein was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Protein concentration was determined using a Bio-Rad protein assay system (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard.

2.4. DNA substrates

To study the nick-closing activity of the DNA ligase, the oligonucleotide substrate was formed by annealing two short oligonucleotides (35-mer and 25-mer) with a 70-mer complementary oligonucleotide. The sequence of the substrate was as follows: a 5′-phosphorylated 35-mer:5′-TAAGCTCCGGATTGTCCGGGAGGTAAAGCCCTGAT-3′, 25-mer: 5′-CACAGGAAGCTCTACAGGTACTCCG-3′, 70-mer: 5′-TGGTCATCAGGGCTTTACCTCCCGGACAATCCGGAGCT-TACGGAGTACCTGTAGAGCTTCCTGTGCAAGC-3′.

2.5. DNA ligase assays

The assay for the nick-closing activity of DNA ligase was performed according to a previously published method [11]. The annealed DNA substrate (10 μM of the 35-mer, 10 μM of the 25-mer, and 5 μM of the 70-mer) described above was incubated in 20 μl of reaction mixture [50 mM Tris–HCl, pH 7.5, 5 mM KCl, 15 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 0.1 mM ATP, and 20 nM *A. pernix* ligase (*Ape* ligase)] at 70°C for 10 min. The reactions were terminated by the addition of stop buffer [98% (vol/vol) formamide, 10 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol, and 0.2% SDS] followed by heating at 95°C for 5 min. The products were subjected to electrophoresis on a 10% polyacrylamide gel containing 7 M urea in TBE buffer (90 mM Tris–borate, 2.5 mM EDTA). The gel was stained with ethidium bromide, and the ligation product was quantified by densitometric analysis (Gel-Pro Analyzer 4.0, MediaCybernetics, MD, USA).

2.6. Removal of ATP from ADP solution

Contaminating ATP was removed from the ADP solution by treatment with hexokinase and D-glucose as described by Nakatani et al. [11]. The ADP solution was then incubated in a 0.5 ml reaction mixture (20 mM Tris–HCl, pH 7.6, 10 mM D-glucose, 10 mM $MgCl_2$, 1 mM ADP, and 1 U of hexokinase) at 25°C for 2 h. After incubation, the enzyme was eliminated from these solutions by means of an Amicon centricon 10 filter from Millipore.

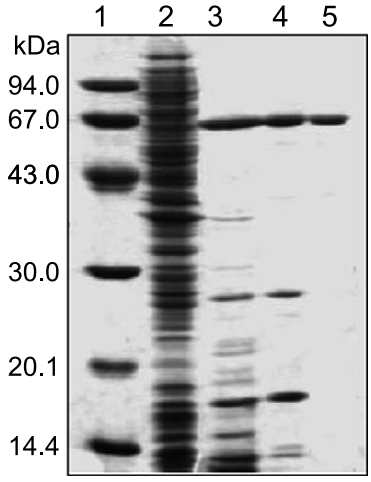

Fig. 1. Purification of recombinant *Ape* ligase. Lane 1: molecular mass marker; lane 2: crude extract induced cells; lane 3: supernatant of crude extract after heat treatment at 85°C for 30 min; lane 4: Hitrap Q column peak fractions; lane 5: Sephacryl S-100 HR 26/60 column peak fractions. The gel was stained with Coomassie brilliant blue.

3. Results and discussion

3.1. Identification of Ape DNA ligase gene

In the *A. pernix* K1 genome database (http://www.bio.nite.go.jp:8080/dogan/Top), we identified an ORF (accession no. APE1094) encoding a putative DNA ligase.

The putative *Ape* ligase gene encodes a protein of 619 amino acids with a predicted molecular mass of 69 195 Da and an isoelectric point of 9.05. The deduced amino acid sequence showed a high identity with other archaeal DNA ligases from *Sulfolobus tokodaii* (61%) [14], *S. shibatae* (57%) [12], *D. ambivalens* (56%) [9], and *T. kodakaraensis* (39%) [11]. It was also homologous to DNA ligase I (35% identity) from yeast [15] and human [16]. A phylogenetic analysis revealed that all archaeal DNA ligases are closely related to DNA ligase I from eukaryotes [11]. The putative *Ape* ligase possesses the six conserved motifs (I, III, IIIa, IV, V, VI) found in various ATP-dependent DNA ligases [17]. Motif I (KXDG) includes the lysine nucleophile that participates in a covalent link to AMP during ligase-adenylate formation. In the case

Table 1
Effects of external factors on the ligation activity of *Ape* ligase

	pH[a]	KCl (mM)[b]	ATP (mM)[c]	Mg^{2+} (mM)[d]	Mn^{2+} (mM)[d]	Temp. (°C)[e]
Optimum	7.5	5	0.1	15	7.5	70
Range (80% activity)	7.0–8.2	0–50	0.05–2.0	5.0–30	1.0–10	65–80

[a]pH effect. Reactions were performed in a 20 μl mixture containing 5 μM nicked duplex substrate, 20 nM *Ape* ligase, 50 mM Tris–HCl (pH values were determined at room temperature), 15 mM $MgCl_2$, 5 mM KCl, 5 mM DTT, and 0.1 mM ATP at 70°C for 10 min.
[b]Salt effect. Reactions were performed in a similar 20 μl mixture with 50 mM Tris–HCl (pH 7.5), 15 mM $MgCl_2$, 5 mM DTT, 0.1 mM ATP, and 0–200 mM KCl at 70°C for 10 min.
[c]ATP effect. Ligation reactions were performed in a similar 20 μl mixture with 50 mM Tris–HCl (pH 7.5), 15 mM $MgCl_2$, 5 mM KCl, 5 mM DTT, and 0–4 mM ATP at 70°C for 10 min.
[d]Metal ion effect. Reaction mixtures (20 μl) containing 5 μM of nicked duplex substrate, 20 nM *Ape* ligase, 50 mM Tris–HCl (pH 7.5), 5 mM KCl, 5 mM DTT, 0.1mM ATP, and 0–50 mM Mg^{2+} or Mn^{2+} were incubated at 70°C for 10 min.
[e]Temperature effect. Reactions were performed in a similar 20 μl mixture with 50 mM Tris–HCl (pH 7.5) at 30–90°C for 10 min.

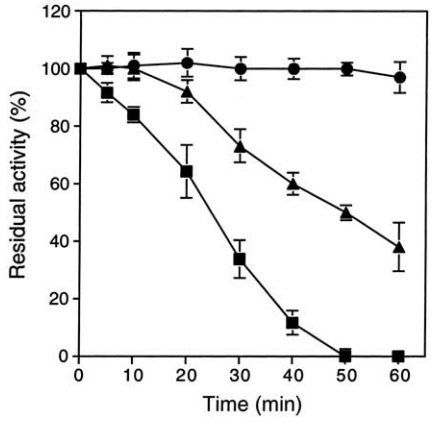

Fig. 2. Thermostability of *Ape* ligase. Enzyme (10 μg/μl in 50 mM Tris–HCl, pH 7.5, 5 mM DTT, 15 mM $MgCl_2$) was incubated for various lengths of time at 100 (circles), 105 (triangles), and 110°C (squares). The ligase activity of the treated samples was then determined by the standard assay using the nick-closing method. Error bars denote the variations from three independent experiments.

of *Ape* ligase, the presumptive nucleophile corresponds to Lys281.

The ORF (APE1094) was amplified by PCR from a *A. pernix* genomic DNA, cloned, and sequenced to confirm the sequences in the database. The gene was expressed in *E. coli* cells, and the recombinant protein was purified as described in Section 2. The molecular mass (69 kDa) calculated from the amino acid sequence was in agreement with the value obtained by SDS–PAGE (Fig. 1). The native molecular mass of the enzyme was determined to be about 70 kDa by gel filtration chromatography (data not shown), indicating that the putative *Ape* ligase was a monomeric enzyme.

3.2. Catalytic properties of Ape ligase

To investigate the catalytic properties of the recombinant protein, its activity was analyzed using a nick-closing assay described in Section 2 (a 70-mer as a template and a complementary 35-mer and 25-mer). Ligation reactions were performed at 70°C in the presence of 1 mM ATP, 15 mM $MgCl_2$ and 5 mM KCl. The 5′-phosphorylated 35-mer was joined to the 25-mer, producing a 60-mer product (Fig. 4). Under these conditions, the rate of production of the product increased linearly for approximately 4 min and the reaction was nearly complete within 10 min. No ligation product was detectable when either ATP or Mg^{2+} was absent from the reaction mixture. The results indicate that the *A. pernix* protein is an ATP-dependent DNA ligase. In the nick-closing assay, the recombinant *Ape* ligase showed more than 80% activity in the temperature range 65–80°C, with a temperature optimum of 70°C (Table 1). Ligase activity declined at temperatures below 35°C. A drastic decrease in activity was observed between 80 and 90°C. However, the optimum temperature also seems to be dependent on the stability of the substrates, because the nicked DNA substrate is unstable at high temperatures. The optimal pH for the *Ape* ligase was 7.5, with greater than 80% activity observed between pH 7.0 and 8.2 (Table 1). The *Ape* ligase activity was inhibited by added salts. The enzyme activity was not significantly affected by KCl, NaCl, or NH_4Cl at concentrations below 50 mM, but was inhibited by approximately 70% in the presence of 100 mM KCl, NaCl, or NH_4Cl (data not shown). The heat inactivation of *Ape* ligase was evaluated by measuring the residual nick-closing activity after heat treatment at three different temperatures (Fig. 2). The enzyme remained stable for 60 min at 100°C, and showed hyperthermostability as previously reported for the heat stability of other archaeal enzymes from *A. pernix* [18]. The half-life of heat inactivation was about 25 min at 110°C (Fig. 2). The heat stability of *Ape* ligase was much higher than that previously reported for other thermostable DNA ligases from *Thermus scotoductus* (26 min at 91°C) [19], *Rhodothermus marinus* (7 min at 91°C) [19], and *S. shibatae* (10 min at 90°C) [12].

3.3. Divalent cation dependence of the enzyme activity

The presence of a divalent cation is required for the ligation reaction. *Ape* ligase requires either Mg^{2+} or Mn^{2+} as a metal cofactor for activity (Fig. 3A). The concentrations of Mn^{2+} and Mg^{2+} for optimal activity were 7.5 and 15 mM, respectively. The enzyme responded sensitively to changes in the

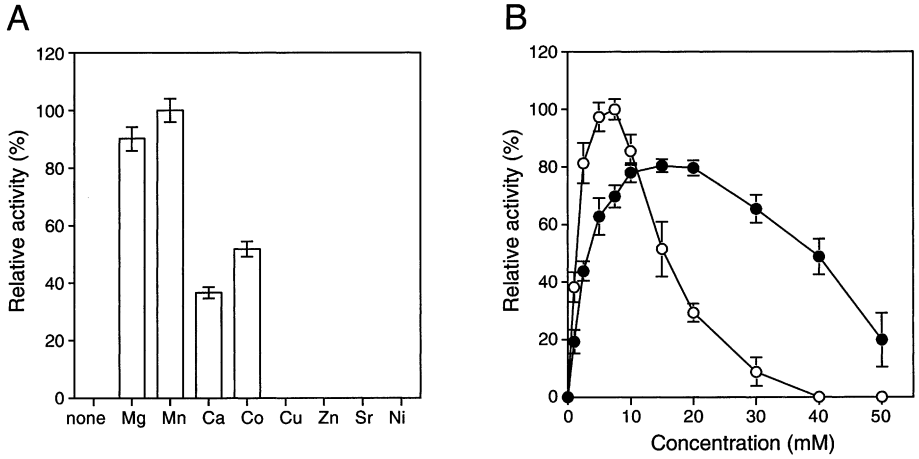

Fig. 3. Divalent cation dependence of *Ape* ligase activity. A: Ligation reaction with different divalent ions as the metal cofactor. Reaction mixtures (20 μl) containing 5 μM nicked duplex substrate, 20 nM *Ape* ligase, 50 mM Tris–HCl, pH 7.5, 5 mM KCl, 5 mM DTT, 0.1 mM ATP, and 10 mM of the indicated divalent cation were incubated at 70°C for 10 min. All divalent cations were added as the chloride salt. B: Ligase activity in the presence of various concentrations of Mg^{2+} (closed circles) or Mn^{2+} (open circles). Reactions were performed using the indicated concentrations of Mg^{2+} or Mn^{2+} at 70°C for 10 min. Error bars denote the variations from three independent experiments.

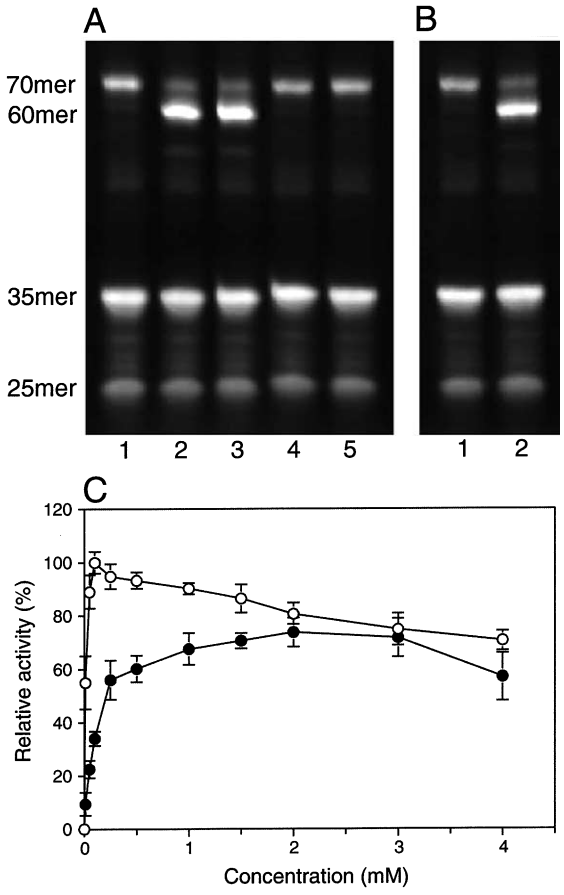

Fig. 4. ADP-dependent DNA ligase activity of *Ape* ligase. A: Ligation reactions were carried out without cofactor (lane 1), with 0.1 mM ATP (lane 2), ADP (lane 3), AMP (lane 4), or NAD^+ (lane 5) at 70°C for 30 min. B: Ligation reaction using hexokinase-treated ADP; lane 1, no cofactor; lane 2, 0.1 mM ADP treated with hexokinase was added to the ligation reaction mixture. C: Ligase activity in the presence of various concentrations of ATP or ADP. Reactions were performed using the indicated concentrations of ATP (open circles) or ADP (closed circles) at 70°C for 10 min. Error bars denote the variations from three independent experiments.

cation concentration in the reaction mixture. In particular, a drastic decrease in activity was observed at Mn^{2+} concentrations above 7.5 mM (Fig. 3B). At high cation concentrations, the high ionic strength may inhibit enzyme activity, consistent with the salt-dependence profile. The enzyme appeared to be less active when Ca^{2+} or Co^{2+} were used as cofactors, while Cu^{2+}, Zn^{2+}, Sr^{2+} or Ni^{2+} failed to support the ligation activity (Fig. 3A). On the other hand, the ligases from *S. shibatae* and *Chlorella virus* PBCV-1 were reported to utilize Mg^{2+}, Mn^{2+} and Ca^{2+} [12], and Mg^{2+}, Mn^{2+} and Co^{2+} [20] as metal cofactors for activity, respectively.

3.4. *Cofactor specificity of* Ape *ligase*

Nakatani et al. [11] recently reported a surprising observation that DNA ligase from *T. kodakaraensis* is able to use NAD^+, in addition to ATP, as a cofactor. Thus, the issue of whether *Ape* ligase is able to use cofactors other than ATP for DNA ligase activity was examined. *Ape* ligase was inactive when ATP was substituted by AMP or NAD^+ (Fig. 4A). Interestingly, however, *Ape* ligase was able to utilize ADP as a cofactor (Fig. 4A). Enzymatic activity with ADP was slightly lower than when ATP was used and was optimally active at ADP concentrations between 0.5 and 3 mM (Fig. 4C). A DNA ligase that is capable of utilizing ADP as a cofactor has not been reported to date. To exclude the possibilities of ATP contamination in the ADP solution, we ensured the removal of ATP by treating the ADP solution with hexokinase (Sigma) and D-glucose as described in Section 2. The hexokinase-treated ADP was tested for *Ape* ligase activity. *Ape* ligase was still able to join the 35-mer and 25-mer, producing the 60-mer product (Fig. 4B) using the hexokinase-treated ADP. These results indicate that *Ape* ligase, a predominantly ATP-dependent DNA ligase, can also utilize ADP as a cofactor.

Two unique features of the *Ape* ligase were found. One is an unusual metal cofactor dependence for ligation activity. Generally, all DNA ligases are most active when Mg^{2+} is used as the metal cofactor, although they are able to use alternative metal cofactors. The spectrum of divalent cations that supported nick-closing activity by *Ape* ligase (Mg^{2+}, Mn^{2+}, Ca^{2+} and Co^{2+}) was different from that reported for other ligases. The most unique property of *Ape* ligase is that the enzyme can utilize ADP as a cofactor, although specificity towards ADP is slightly lower than that for ATP. This is the first observation of an ATP-dependent DNA ligase that is able to utilize ADP. The free energy of hydrolysis of ADP is a little smaller than that of ATP. In the case of *Ape* ligase, the small free energy may be enough to activate it. The sequences of archaeal DNA ligases are conserved and have a common catalytic motif structure. However, they are diverse in cofactor specificity. ATP-dependent DNA ligases from *M. thermoautotrophicum* [10] and *S. shibatae* [12] are able to utilize dATP as a cofactor. This may be due to the fact that the cofactor specificity of the thermostable ligases is relaxed at high temperature. The structural basis for this unique cofactor specificity of the enzyme remains to be analyzed.

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