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Characterization of an ATP-Dependent DNA Ligase Encoded by Chlorella Virus PBCV-1

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We report that Chlorella virus PBCV-1 encodes a 298-amino-acid ATP-dependent DNA ligase. The PBCV-1 enzyme is the smallest member of the covalent nucleotidyl transferase superfamily, which includes the ATP-dependent polynucleotide ligases and the GTP-dependent RNA capping enzymes. The specificity of PBCV-1 DNA ligase was investigated by using purified recombinant protein. The enzyme catalyzed efficient strand joining on a singly nicked DNA in the presence of magnesium and ATP (Km, 75 μM). Other nucleoside triphosphates or deoxynucleoside triphosphates could not substitute for ATP. PBCV-1 ligase was unable to ligate across a 2-nucleotide gap and ligated poorly across a 1-nucleotide gap. A native gel mobility shift assay showed that PBCV-1 DNA ligase discriminated between nicked and gapped DNAs at the substrate-binding step. These findings underscore the importance of a properly positioned 3′ OH acceptor terminus in substrate recognition and reaction chemistry.

The ATP-dependent DNA ligases catalyze the joining of 5′ phosphate-terminated donor strands to 3′ hydroxyl-terminated acceptor strands via three sequential nucleotidyl transfer reactions (10). In the first step, nucleophilic attack on the α-phosphate of ATP by ligase results in liberation of pyrophosphate (Pp) and formation of a covalent intermediate in which AMP is linked to the ε-amino group of a lysine. The nucleotidyl is then transferred to the 5′ end of the donor polynucleotide to form DNA-adenylate—an inverted (5′)-(5′) bridge structure, AppN. Attack by the 3′ OH of the acceptor strand on the DNA-adenylate joins the two polynucleotides and liberates AMP.

Animal cells contain multiple DNA ligase isozymes encoded by separate genes (1, 28, 29). ATP-dependent DNA ligases are also encoded by animal DNA viruses, e.g., the poxviruses and African swine fever virus (ASFV), by the T-odd and T-even bacteriophages (T4, T6, T3, and T7), by yeasts, plants, and archaea (7). The ATP-dependent DNA ligases belong to a superfamily of covalent nucleotidyl transferases that includes the GTP-dependent eukaryotic mRNA capping enzymes (20, 22). The ligase-capping enzyme superfamily is defined by a set of six short motifs (Fig. 1). The lysine within motif I (KxDG) is the active site of AMP transfer by the ligases (6, 25, 28) and GMP transfer by the capping enzymes (2, 4, 15, 18). Conserved residues within motifs I, III, IV, and V are critical for covalent nucleotidyl transfer, as shown by mutational analyses (2–4, 6, 8, 18, 20, 21). The recently reported crystal structure of T7 DNA ligase shows that the ATP-binding site is made up of conserved motifs I, III, IIIa, IV, and V (24).

The bacteriophage T7 and T3 enzymes (359- and 346-amino-acid polypeptides, respectively) are the smallest ATP-dependent ligases described to date (7). Cellular DNA ligases are much larger; for example, human ligases I, III, and IV are 919-, 922-, and 844-amino-acid polypeptides, respectively (1, 29). Vaccinia virus and ASFV ligases are intermediate in size (552 and 419 amino acids, respectively) (5, 23). Sequence comparisons of cellular and virus-encoded proteins suggest that a catalytic domain common to all ATP-dependent ligases is embellished by additional isozyme-specific protein segments situated at their amino or carboxyl termini. Virus-encoded enzymes, by virtue of their small size, may well define the catalytic core of the ligase family.

Here, we report a new viral DNA ligase encoded by Paramecium bursaria Chlorella virus 1 (PBCV-1). PBCV-1 is the prototype of a family of large, polyhedral DNA viruses that replicate in unicellular eukaryotic Chlorella-like green algae (27). The PBCV-1 genome, like the genomes of the poxviruses and ASFV, is a linear, double-strand DNA molecule with inverted terminal repeats and covalently closed hairpin telomeres. The sequence of the 330-kbp PBCV-1 genome has been determined (9, 11–13); PBCV-1 encodes ∼380 polypeptides.

An open reading frame encoding a ligase-like protein was encountered between nucleotide coordinates 264,000 and 265,000 of the PBCV-1 genome (11a; Genbank accession number is U77663). The predicted gene product includes the six motifs shared among the cellular and DNA virus-encoded ATP-dependent DNA ligases (Fig. 2). The order and spacing of these motifs in the PBCV-1 ligase-like protein are similar to those seen in other ligase family members (Fig. 1). The PBCV-1 polypeptide, at 298 amino acids, is smaller than any known ligase or capping enzyme. We have expressed the PBCV-1 protein in Escherichia coli and purified it to apparent homogeneity. We report that the recombinant protein is indeed an ATP-dependent DNA ligase. A biochemical characterization of the PBCV-1 ligase is presented.

MATERIALS AND METHODS

T7-based vector for expression of PBCV-1 DNA ligase. Oligonucleotide primers complementary to the 5′ and 3′ ends of the putative PBCV-1 ligase gene were used to amplify the 298-amino-acid open reading frame. Total PBCV-1 genomic DNA was used as the template for a PCR catalyzed by Pfu DNA polymerase (Stratagene). The sequence of the 5′ flanking primer was 5′-CATGAAATTTAC GTTGTCATAATGGGCAACAAAGCC: that of the 3′ flanking primer was 5′-CAAGACCTTGTAAGACCGATCTCAGTCCAGGGATAGA. These primers were designed to introduce NdeI and BamHI restriction sites at the 5′ and 3′ ends, respectively, of the ligase gene. The 0.9-kbp PCR product was digested with NdeI and BamHI and then cloned into the NdeI and BamHI sites of T7-based expression plasmid pET3c (Novagen). The resulting plasmid, pET-ChV-ligase, was transformed into Escherichia coli BL21(DE3). Dideoxy sequencing of the entire insert of pET-ChV-ligase confirmed that no alterations of the genomic DNA sequence were introduced during PCR amplification and cloning of the ligase gene.

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Expression and purification of recombinant PBCV-1 ligase. A 500-ml culture of E. coli BL21(DE3)/pET-ChV-ligase was grown at 37°C in Luria-Bertani medium containing 0.1 mg of ampicillin per ml until the A600 reached 0.5. The culture was adjusted to 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and incubation was continued at 37°C for 2 h. Cellswere harvested by centrifugation, and the pellet was stored at −80°C. All subsequent procedures were performed at 4°C. Thawed bacteria were resuspended in 50 ml of buffer A (50 mM Tris-HCl [pH 7.5], 2 mM dithiothreitol [DTT], 10% glycerol) containing 0.1, 0.15, 0.2, 0.3, 0.5, and 1.0 M NaCl. Lysozymewas added to a final concentration of 2 mg/ml, and the sample was sonicated for 30 s. Triton X-100 was added to a 0.1% final concentration. The samples were electrophoresed through a 12% polyacrylamide gel containing 7 M urea in 0.5 M NaCl. Label transfer to the 34-kDa PBCV-1 polypeptide was detected by autoradiography of the dried gel and was quantitated by scanning the gel with a FUJIX BAS1000 Bio-Imaging Analyzer.

Results

Expression of the PBCV-1 ligase-like protein in bacteria. The PBCV-1 open reading frame encoding a ligase-like protein was PCR amplified from viral genomic DNA and cloned into a T7 RNA polymerase-based bacterial expression vector.
The pET-ChV-ligase plasmid was introduced into *E. coli* BL21(DE3), a strain that contains the T7 RNA polymerase gene under the control of a lacUV5 promoter. A prominent 34-kDa polypeptide was detectable by SDS-PAGE in whole-cell extracts of IPTG-induced bacteria (Fig. 3A, lane 1). This polypeptide was not present when bacteria containing the pET vector alone were induced with IPTG (data not shown). After centrifugal separation of the crude lysate, the PBCV-1 protein was recovered in the soluble supernatant fraction (Fig. 3A, lane 2).

**Recombinant PBCV-1 34-kDa protein forms a covalent enzyme-adenylate complex.** The initial step in DNA ligation involves formation of a covalent enzyme-adenylate intermediate, EpA. EpA formation by DNA ligases can be detected with a valent enzyme-adenylate complex.

*The initial step in DNA ligation involves formation of a covalent enzyme-adenylate intermediate. 

We assayed the ability of the recombinant PBCV-1 protein at sequential stages of purification to react with [*α-32P*]ATP to the enzyme. To assay the adenylyltransferase activity of the expressed PBCV-1 protein, we incubated either whole-cell or soluble extracts of IPTG-induced BL21(DE3)/pET-ChV-ligase cells in the presence of [*α-32P*]ATP and a divalent cation. This resulted in the formation of a nucleotidyl-protein adduct that migrated as a single 34-kDa species during SDS-PAGE (Fig. 3B, lanes 1 and 2). Labeling of this polypeptide was not detected in extracts prepared from bacteria that lacked the PBCV-1 gene (data not shown). We concluded that the expressed 34-kDa PBCV-1 protein is active in nucleotidyl transfer.

**Purification of recombinant PBCV-1 ligase.** The 34-kDa polypeptide was purified from the soluble bacterial extract by sequential DEAE-cellulose and phosphocellulose column chromatography steps. The recombinant protein did not bind to DEAE-cellulose at low ionic strength (50 mM NaCl). SDS-PAGE analysis of the DEAE flowthrough fraction (Fig. 3A, lane 3) showed that many of the bacterial polypeptides were eliminated during this step. The PBCV-1 protein adsorbed to phosphocellulose and was recovered during step elution with 0.3 M NaCl (Fig. 3A, lane 8). The phosphocellulose preparation was highly enriched with respect to the 34-kDa polypeptide; approximately 7 mg was obtained from a 500-ml culture of IPTG-induced bacteria. The adenylyltransferase activity coincided with the 34-kDa polypeptide during column chromatography (Fig. 3B and other data not shown).

When the phosphocellulose fraction was centrifuged through a 15 to 30% glycerol gradient in 0.5 M NaCl, a single peak of adenylyltransferase activity was detected that coincided with the 34-kDa polypeptide (Fig. 4). We estimated a sedimentation coefficient of 3.1S relative to marker proteins sedimented in a parallel gradient. This suggested that the PBCV-1 adenylyltransferase is a monomer of the 34-kDa protein.

**DNA ligation.** We assayed the ability of the recombinant PBCV-1 protein to seal a 36-mer synthetic duplex DNA substrate containing a single nick. The structure of the substrate is shown in Fig. 5. Ligase activity was evinced by conversion of the 5'-32P-labeled 18-mer donor strand into an internally labeled 36-mer product. The DNA ligase activity profile across the glycerol gradient paralleled that of enzyme-adenylate complex formation (Fig. 4B). These results demonstrate that the 34-kDa PBCV-1 protein is indeed a DNA ligase. Further characterization of the PBCV-1 ligase was performed by using the glycerol gradient preparation (peak fraction 19).

The extent of ligation of the nicked duplex (added at a 50 nM concentration with respect to the 5'-labeled donor strand) during a 10-min incubation in the presence of 1 mM ATP increased linearly with the concentration of PBCV-1 ligase from 50 to 500 pM (Fig. 6A). In the linear range of enzyme dependence in this experiment, the recombinant ligase joined about 100 to 120 fmol of DNA ends per fmol of enzyme. To estimate the ratio of product to enzyme, the enzyme molarity was calculated based on total protein concentration, assuming enzyme homogeneity. It was also assumed that all of the enzyme molecules in the preparation were catalytically active. The reaction saturated at >500 pM enzyme with 85% of the labeled donor strand converted to 36-mer in 10 min. This upper limit of ligation probably reflected incomplete annealing of all three component strands to form the nicked substrate.

Ligation could be detected in the absence of added ATP, but only at high levels of input enzyme (Fig. 6B). ATP-independent ligation was attributed to preadenylated ligase in the enzyme preparation. The linear dependence of ATP-independent strand joining on enzyme indicated that about 0.9 mol of
ends was sealed per mol of ligase (Fig. 6B), implying that 90% of the enzyme molecules had AMP bound at the active site.

**Kinetics, ATP dependence, and nucleotide cofactor specificity of ligation.** The kinetics of ligation were examined in DNA excess (50 nM) in the presence of 1 mM ATP and 10 mM MgCl₂. The initial rate was proportional to enzyme concentration in the range of 125 to 500 pM (Fig. 7A). In subsequent experiments, ligation assay mixtures contained 250 pM enzyme and activity was determined after a 2 min incubation at 22°C (unless otherwise specified). Under these conditions, ligation activity in 50 mM Tris-HCl buffer was optimal between pHs 7.0 and 7.5. Activity at pH 9.5 was ~50% of that at pH 7.5 (data not shown). The rate of ligation increased with ATP concentration from 10 to 200 μM and leveled off at 0.5 to 2 mM (Fig. 7B). A $K_m$ of 75 μM ATP was calculated from a double-reciprocal plot of the data. Other ribonucleoside triphosphates or deoxynucleoside triphosphates at a 0.5 mM concentration could not substitute for ATP (Fig. 8).

**Divalent cation dependence and specificity.** Ligation depended on a divalent cation in excess of the input ATP; activity was enhanced steadily as Mg was increased from 2 to 20 mM (Fig. 9B). The divalent cation requirement was satisfied by Mn and, to a lesser extent, by Co but not by Ca, Cu, or Zn (Fig. 9A and B).

**DNA substrate specificity—nicks versus gaps.** The structure of the ligation substrate was altered such that the 3’ hydroxyl-terminated acceptor strand was separated from the 5’ phosphatedonor terminus by a 2- or 1-nucleotide (nt) gap (Fig. 5). The specific activity of PBCV-1 ligase on a 1-nt gap substrate was 1% of the activity of a nicked duplex DNA (Fig. 10). PBCV-1 ligase was incapable of joining strands across the 2-nt gap. The implication is that the 3’ OH must be positioned fairly precisely relative to the 5’ phosphate donor terminus for ligation to occur.

**Specificity of ligase binding to DNA—nicks versus gap.** A native gel mobility shift assay was employed to examine the binding of PBCV-1 ligase to the 32P-labeled, nicked duplex DNA (19). Binding reactions were performed in the absence of magnesium and ATP to preclude conversion of substrate to product during the incubation. Control experiments verified that ATP-independent ligation of the nicked DNA substrate by stoichiometric amounts of PBCV-1 ligase required a divalent cation. No strand joining occurred under the reaction conditions employed in our gel shift experiments (data not shown).

Mixing the ligase with nicked substrate resulted in the formation of a discrete protein-DNA complex that migrated more slowly than the free DNA during electrophoresis through a 6% gel (Fig. 11).
native polyacrylamide gel (Fig. 11). The abundance of this complex increased in proportion to the amount of input ligase.

To estimate binding affinity, the gel was scanned with a PhosphorImager; the apparent dissociation constant, calculated as described by Riggset al. (17), was 15 nM.

Little or no specific complex was detected when PBCV-1 ligase was incubated with 1- or 2-nt gap DNA (Fig. 11). A diffuse smear of shifted material was detected at a 200 nM ligase concentration. Thus, PBCV-1 DNA ligase binds specifically at a DNA nick and was capable of discriminating between nicked and 2-nt-gapped DNAs at the substrate-binding step. This affirms the importance of the 3'-OH acceptor strand in substrate recognition.

Analysis of enzyme-AMP complex formation. The PBCV-1 ligase reacted specifically with \([\alpha-^{32}P]ATP\). The amount of enzyme-AMP complex formed during a 5-min incubation at 37°C in the presence of 10 μM \([\alpha-^{32}P]ATP\) was proportional to the amount of added protein (data not shown). EpA formation increased as a function of ATP concentration and reached saturation near 20 μM \([\alpha-^{32}P]ATP\) (Fig. 12A). Half saturation was achieved at ~5 μM ATP. \([\alpha-^{32}P]dATP\) was an extremely poor substrate for EpA formation.

We estimated from the NTP titration experiment in Fig. 12A that ATP was 100-fold more effective than dATP in EpA formation. Hence, PBCV-1 ligase, which utilized ATP, but not dATP, as a cofactor in the strand-joining reaction, discriminated between ribose and deoxyribose sugars at the step of EpA formation. No protein-nucleotide complex was formed with \([\alpha-^{32}P]GTP\) (data not shown).

We calculated, based on the molar amount of AMP label transfer versus the molar amount of ligase added, that ~5% of the input protein was converted to ligase-[32P]adenylate. This was consistent with the estimate (based on ATP-independent ligation activity) that 90% of the enzyme molecules in the preparation were preadenylated and, hence, incapable of EpA formation. We observed a fivefold increase in the extent of label transfer from \([\alpha-^{32}P]ATP\) to protein when a nicked DNA substrate was added to the reaction mixture (data not shown). This would be expected if transfer of unlabeled AMP from preadenylated enzyme to the 5'-phosphate of the donor strand (step 2 of the ligase reaction) regenerates unadenylated enzyme that can then react with \([\alpha-^{32}P]ATP\).

EpA formation depended on a divalent cation cofactor. This requirement was satisfied by either 5 mM magnesium or 5 mM manganese and, to lesser extent, by 5 mM cobalt (Fig. 12B). Calcium, copper, and zinc were inactive at this concentration (Fig. 12B). The yield of EpA was proportional to the magne-
sium concentration from 0.1 to 1 mM and plateaued at 2 to 10 mM (Fig. 12C). Manganese was a more effective cofactor than magnesium at 0.5 mM but was progressively less active at higher concentrations (Fig. 12C).

**DISCUSSION**

A *Chlorella* virus PBCV-1 gene encoding a putative DNA ligase was identified during sequencing of the viral DNA genome. We show that the 298-amino-acid gene product is an ATP-dependent DNA ligase. This was achieved by expressing the PBCV-1 protein in bacteria, purifying the protein to homogeneity, and characterizing its enzymatic properties. PBCV-1 ligase, like other cellular and virus-encoded DNA ligases, catalyzes strand joining via an enzyme-AMP intermediate. The PBCV-1 enzyme displays strict specificity for ATP as the nucleotide cofactor; dATP is inactive. PBCV-1 ligase thus resembles the T4, vaccinia virus, and cellular type I enzymes in its discrimination of the nucleoside triphosphate sugar moiety (14, 16, 19, 21, 26).

The high efficiency of PBCV-1 ligase in strand joining across a nick in duplex DNA contrasts sharply with the low efficiency of ligation across a 1-nt gap and the inability to seal strands across a 2-nt gap. Vaccinia virus ligase and yeast CDC9 ligase display similar properties. The latter two enzymes synthesize substantial levels of the DNA-adenylate intermediate on a 1-nt gap DNA substrate (19, 26). Similar results were obtained when stoichiometric levels of PBCV-1 ligase were reacted with the 1-nt gap DNA (6a).

A native gel mobility shift assay was used to demonstrate that formation of a stable complex between PBCV-1 ligase and DNA depends on a DNA nick. The PBCV-1 enzyme discriminates clearly at the DNA-binding step between nicked DNA molecules that can be sealed versus 2-nt-gapped molecules that cannot. Even a 1-nt gap significantly reduces the affinity of ligase for the DNA. This implies that the protein initially contacts both the donor and acceptor DNA strands on either side of the nick prior to any covalent modification of the DNA substrate. Similar specificity for binding to DNA nicks has been documented for the vaccinia virus ligase (19). Thus, both of these virus-encoded enzymes have an intrinsic ability to bind...
preferentially to DNA sites where their action is required. Electrophoretic resolution of the ligase-DNA complex from unbound DNA will permit the use of chemical and enzymatic footprinting techniques to delineate the interface between PBCV-1 ligase and the DNA substrate, a subject about which almost nothing is known for any DNA ligase. Of particular interest is identification of the region(s) of the ligase polypeptide that mediates nick recognition.

Because the Chlorella viruses are not amenable to genetic manipulation, it is not possible to determine the biological function of the DNA ligase during the PBCV-1 replication cycle. A role during viral DNA replication, repair, or recombination is plausible, although the molecular mechanisms of these transactions are largely unexplored for PBCV-1. Available insights come largely from analysis of the genomic DNA sequence, which identifies by sequence homology several potential replication and repair proteins. These include, in addition to the DNA ligase, a DNA polymerase, a polymerase processivity factor, a helicase, a DNA glycosylase-apyrimidinic lyase, and a type II topoisomerase (9, 11–13). The DNA ligase is the first instance in which it has been shown that a purified recombinant PBCV-1 DNA replication protein actually has the enzymatic properties attributed to it on the basis of sequence homology.

The PBCV-1 ligase is the smallest DNA ligase described to date. It may well represent the minimum catalytic unit of an ATP-dependent ligase. Insofar as PBCV-1 ligase is also smaller than any known mRNA capping enzyme, it may constitute the catalytic core of the nucleotidyl transferase superfamily. PBCV-1 ligase includes the six conserved motifs that constitute the catalytic core of the nucleotidyl transferase superfamily but contains no additional sequence at the carboxyl terminus downstream of motif VI. It contains only 26 amino acids N terminal to the presumptive active site, Lys-27, within motif I. In this light, the PBCV-1 ligase emerges as an excellent model for further structural and functional studies of a eukaryotic ligase. The fact that the recombinant PBCV-1 ligase is purified in high yield as ligase-adenylate offers an opportunity to solve the structure of the covalent reaction intermediate. Realization of this goal would extend and complement the insights provided by the crystal structure of the bacteriophage T7 enzyme bound noncovalently to ATP (24).

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