We report the production, purification, and characterization of an NAD$^+$-dependent DNA ligase encoded by the Amsacta moorei entomopoxvirus (AmEPV), the first example of an NAD$^+$ ligase from a source other than eubacteria. AmEPV ligase lacks the zinc-binding tetra-cysteine domain and the BRCT domain that are present in all eubacterial NAD$^+$ ligases. Nonetheless, the monomeric 532-amino acid AmEPV ligase catalyzed strand joining on a singly nicked DNA in the presence of a divalent cation and NAD$^+$. Neither ATP, dATP, nor any other nucleoside triphosphate could substitute for NAD$^+$. Structure probing by limited proteolysis showed that AmEPV ligase is punctuated by a surface-accessible loop between the nucleotidyltransferase domain, which is common to all ligases, and the N-terminal domain Ia, which is unique to the ligase, and the N-terminal domain Ia, which is unique to the NAD$^+$ dependent enzymes. Deletion of domain Ia of AmEPV ligase abolished the sealing of 3'-OH/5'-PO$_4$ nicks and the reaction with NAD$^+$ to form ligase-adenylate, but had no effect on phosphodiester formation at a pre-adenylated nick. Alanine substitutions at residues within domain Ia either reduced (Tyr$^{39}$, Tyr$^{40}$, Asp$^{48}$, and Asp$^{52}$) or abolished (Tyr$^{51}$) sealing of a 5'-PO$_4$ nick and adenyllyl transfer from NAD$^+$ without affecting ligase activity in the sealing reaction. We conclude that: (i) NAD$^+$-dependent ligases exist in the eukaryotic domain of the phylogenetic tree; and (ii) ligase structural domain Ia is a determinant of cofactor specificity and is likely to interact directly with the nicotinamide mononucleotide moiety of NAD$^+$.

DNA ligases catalyze the sealing of 5'-phosphate and 3'-hydroxyl termini at nicks in duplex DNA via three sequential nucleotidyl transfer reactions (1–3). In the first step, attack on the α phosphorus of ATP or NAD$^+$ by ligase results in release of pyrophosphate or nicotinamide mononucleotide (NMN) and formation of a covalent intermediate (ligase-adenylate) in which AMP is linked via a phosphoamide bond to lysine. In the second step, the AMP is transferred to the 5' end of the 5'-phosphate-terminated DNA strand to form DNA-adenylate (AppN). In the third step, ligase catalyzes attack by the 3'-OH of the nick on DNA-adenylate to join the two polynucleotides and release AMP.

DNA ligases are grouped into two families, ATP-dependent ligases and NAD$^+$-dependent ligases, according to the cofactor required for ligase-adenylate formation (1–3). The ATP-dependent DNA ligases are found in eubacteria, bacteriophages, archaea, eukarya, and eukaryotic viruses (3–5). ATP-dependent ligases are exemplified by the bacteriophage T7 and Chlorella virus enzymes, for which atomic structures have been solved by x-ray crystallography (6, 7). The viral ATP-dependent enzymes consist of an ~200 amino acid N-terminal nucleotidyltransferase domain and an ~100-amino acid C-terminal OB-fold domain (Fig. 1A). Within the N-terminal domain is an adenylate binding pocket composed of five motifs (I, III, IIIa, IV, and V) that define the polynucleotide ligase/mRNA capping enzyme superfamily of covalent nucleotidyltransferases (8). Motif I (KXN) contains the lysine nucleophile to which AMP becomes covalently linked in the first step of the ligase reaction (7, 9). Motifs III, IIIa, IV, and V contain conserved side chains that contact AMP and are essential for the nucleotidyl transfer reaction (6, 7, 10).

The C-terminal OB-fold consists of a five-stranded antiparallel β barrel and an α helix. The OB-fold domain includes nucleotidyltransferase motif VI, which contacts the β and γ phosphates of the NTP substrate (11) and which is uniquely required for step 1 of the ligase reaction (12).

The NAD$^+$-dependent DNA ligases have been described only in eubacteria. Genes encoding NAD$^+$-dependent ligases have been identified and sequenced from at least 50 eubacterial species. The NAD$^+$-dependent DNA ligase is essential for growth of Escherichia coli, Salmonella typhimurium, Bacillus subtilis, and Staphylococcus aureus (13–17). NAD$^+$-dependent ligases are of fairly uniform size (656 to 837 amino acids) and there is extensive amino acid sequence conservation throughout the entire lengths of the polypeptides. The atomic structures of NAD$^+$-dependent ligases of two species of thermophilic eubacteria (Bacillus stearothermophilus and Thermus filiformis) have been determined by x-ray crystallography (18, 19). The structure of full-length Tfi ligase reveals that NAD$^+$-dependent enzymes contain a catalytic core composed of nucleotidyltransferase and OB-fold domains (Fig. 1A). Although there is scant amino acid sequence similarity between NAD$^+$ and ATP ligases, the tertiary structures of the catalytic cores are quite well conserved and the adenylate binding pocket of NAD$^+$ ligases is composed of the same five nucleotidyltransferase motifs described originally in the ATP-dependent enzymes. (The nucleotidyltransferase motifs of the NAD$^+$-dependent ligases are highlighted in Fig. 2.) A notable distinction between ATP and NAD$^+$ ligases is that the NAD$^+$ enzymes lack a recognizable counterpart of nucleotidyltransferase motif VI within their OB-fold domain. The catalytic core of Tfi ligase is flanked by a 73-amino acid N-terminal domain (Ia) and three C-terminal domains: a tetracysteine domain that binds a single
Zn atom, a helix-hairpin-helix domain (HhH), and a BRCT domain (named after the C terminus of the breast cancer gene product BRCA1).

No NAD<sup>+</sup>-dependent DNA ligase has been identified from a eukaryotic cellular source. However, recent reports of the genomic DNA sequences of two insect poxviruses, *Melanoplus sanguinipes* entomopoxvirus and *Amsacta moorei* entomopoxvirus, identified an open reading frame in each virus that encodes a polypeptide resembling the eubacterial NAD<sup>+</sup>-dependent DNA ligases (20, 21). Alignment of the 532-amino acid *Am*EPV ligase-like protein to the Tfi, Bst, and Eco ligases reveals conservation of domain Ia, the nucleotidyltransferase domain (including the five catalytic motifs) and the OB-fold (Fig. 2) as well as the HhH domain (not shown). However, the *Am*EPV protein lacks the Zn finger and the BRCT domains that are present in all eubacterial NAD<sup>+</sup> ligases. Given that individual cysteines of the Zn finger have been shown to be essential for the nick joining activity of bacterial ligases (22, 23), and the hypothesis that the BRCT domain plays an important role in DNA binding (3), it is of considerable interest to evaluate whether the insect poxvirus gene product is a DNA ligase and whether it uses NAD<sup>+</sup> as a cofactor. Here we show that this is indeed the case and we provide new evidence that domain Ia is essential for the interaction of the *Am*EPV ligase with NAD<sup>+</sup>. Our findings have implications for the evolution of both poxviruses and the nucleotidyltransferase enzyme family, and they provide impetus and direction for efforts to identify new antibiotics that target bacterial NAD<sup>+</sup> ligases.

**EXPERIMENTAL PROCEDURES**

**T7-based Vectors for Expression of *Am*EPV DNA Ligase—Oligodeoxynucleotide primers complementary to the 5' and 3' ends were used to PCR amplify the *AmV199* open reading frame from a genomic DNA clone (21). The primers were designed to introduce *NdeI* and *BamHI* restriction sites at the 5' and 3' ends of the ligase gene. The PCR product was digested with *NdeI* and *BamHI*, then cloned into the *NdeI* and *BamHI* sites of the T7-based expression plasmid pET16b (Novagen) to yield pET-*Am*EPVLig. Dideoxynucleotide sequencing of the entire insert of pET-*Am*EPVLig confirmed that no alterations of the genomic DNA sequence were introduced during PCR amplification and cloning of the ligase gene.

The N70 deletion mutant, *Lig*(71–532), was constructed by PCR amplification of the ligase gene with a sense-strand primer that introduced an *NdeI* restriction site and a methionine codon in place of the Leu<sup>37</sup> codon. The C216 deletion mutant, *Lig*(1–316), was constructed by PCR with an antisense-strand primer that introduced a stop codon in lieu of the codon for Pro<sup>117</sup> and a *BamHI* site immediately 3' of the stop codon. *NdeI-BamHI* restriction fragments containing the truncated genes were inserted into pET16b. The resulting plasmids were sequenced to exclude the introduction of any unwanted coding changes during amplification and cloning.

Alanine mutations were introduced into the *Am*EPV ligase gene by using the two stage PCR-based overlap extension method. pET-*Am*EPVLig was used as the template for the first stage PCR reaction. *NdeI-BamHI* restriction fragments of the mutated second-stage PCR products were inserted into pET16b. The inserts of the resulting plasmids were sequenced to confirm the presence of the desired alanine mutations and the absence of any unwanted coding changes.

**Production and Purification of *Am*EPV Ligase**—The wild-type pET-*Am*EPVLig expression plasmid was transformed into *E. coli* BL21(DE3). A single ampicillin-resistant colony was inoculated into LB medium containing 0.1 mg/ml ampicillin and a 1-liter culture was grown at 37 °C until the A<sub>600</sub> reached 0.8. The culture was placed on ice for 30 min, then adjusted to 0.4 mM isopropyl-p-D-thiogalactopyranoside, and subsequently incubated at 17 °C for 16 h with continuous shaking. Cells were harvested by centrifugation and the pellets were stored at −80 °C. All subsequent procedures were performed at 4 °C. Cell lysis was achieved by treatment of thawed, resuspended cells with 0.2 mg/ml lysozyme and 0.1% Triton X-100 in 80 ml of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 0.5 mM NaCl, and 10% sucrose. The lysates were sonicated to reduce viscosity and insoluble material was removed by centrifugation at 37,000 g for 20 min. The supernatants were mixed with 2 ml of Ni-NTA-agarose resin (Qiagen) for 30 min with constant rotation. The slurries were poured into columns and the packed resins were washed with IMAC buffer (50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10% glycerol) containing 5 mM imidazole. The column was step eluted with 50 and 500 mM imidazole in IMAC buffer. The polypeptide compositions of the column fractions were monitored by SDS-PAGE. The His-tagged *Am*EPV ligase was recovered in the 500 mM imidazole eluate (14 mg of protein). The preparation was diluted 1:1 with buffer P (50 mM Tris-HCl (pH 7.5), 10% glycerol) and applied to a 2-ml phosphocellulose column that had been equilibrated with buffer P. The column was washed with 200 mM NaCl. The P and the *Am*EPV ligase was step-eluted with 600 mM NaCl in buffer P containing 1 mM EDTA. The phosphocellulose ligase preparation (8 mg of protein) was stored at −80 °C. The protein concentrations were determined by using the Bio-Rad dye reagent with bovine serum albumin as the standard. Truncated and alanine-substituted variants of *Am*EPV ligase were produced and purified from 100-ml bacterial cultures using a scaled-down version of the wild-type ligase protocol described above.

**RESULTS**

**Expression of *Am*EPV Ligase in Bacteria and Demonstration of Ligase Activity—** *Am*EPV open reading frame 199 encoding a ligase-like polypeptide was cloned into a T7 RNA polymerase-based bacterial expression vector so as to fuse the 532-amino acid *Am*EPV ligase gene with a 20-amino acid N-terminal leader peptide containing 10 tandem histidines. The expression plasmid was introduced into *E. coli* BL21(DE3), a strain that contains the T7 RNA polymerase gene under the control of a lacUV5 promoter. The recombinant His-tagged protein was purified from a soluble extract of *isopropyl-p-D-thiogalactopyranoside*-induced bacteria by nickel-agarose affinity chromatography and phosphocellulose cation exchange chromatography steps. SDS-PAGE analysis showed that the phosphocellulose preparation was highly enriched with respect to the 62-kDa *Am*EPV ligase polypeptide (Fig. 1B). The identity of the 62-kDa protein was confirmed by *N*-terminal sequence analysis (see below). In addition, the preparation contained a cluster of smaller polypeptides (35–45 kDa) corresponding to *N*-terminal fragments of the *Am*EPV ligase.

We assayed the ability of the recombinant *Am*EPV protein to seal a duplex DNA substrate containing a single nick (Fig. 1C). NAD<sup>+</sup> and magnesium were included in the assay mixtures. Ligation activity was evinced by conversion of the 5′-*<sup>32</sup>P*-labeled 18-mer substrate to 36-mer product (Fig. 1C). More than 90% of the input nicked DNA molecules were sealed. Thus the *Am*EPV protein is indeed a DNA ligase.

The initial step in DNA ligation involves formation of a covalent enzyme–adenylate intermediate. In order to assay adenylyltransferase activity, we incubated the recombinant *Am*EPV protein with [*<sup>32</sup>P*-AMP]/NAD<sup>+</sup> and magnesium. This resulted in the formation of a [*<sup>32</sup>P*-labeled] covalent nucleotidyltransferase activity of the *Am*EPV ligase (Fig. 1D, WT). Additional labeled species were formed that corresponded to *N*-terminal fragments of the *Am*EPV ligase (see below). We conclude that *Am*EPV ligase is active in covalent nucleotidyl transfer with NAD<sup>+</sup> as the AMP donor.

**Effects of Alanine Mutations in Motif I of *Am*EPV Ligase**—The KDXXG sequence (motif I) is the signature feature of the ligase/capping enzyme superfAMILY of nucleotidyltransferases that form a covalent lysyl-NMP intermediate (Fig. 2). The contributions of motif I residues Lys<sup>115</sup> and Asp<sup>117</sup> to the acyl transfer of *Am*EPV ligase were monitored by SDS-PAGE. The His-tagged *Am*EPV ligase was grafted from the effects of single alanine substitutions. Mutant proteins K115A and D117A were produced in bacteria and purified from soluble lysates by nickel affinity and phosphocellulose chromatography (Fig. 1B). The K115A and D117A mutants were both inert in nick ligation (Fig. 1C). K115A was also inert in ligase-AMP formation with
Substrate Specificity and Biochemical Characterization of AmEPV Ligase.—A low level of nick ligation could be detected in the absence of added nucleotide (Fig. 3A). Cofactor-independent ligation was attributed to pre-adenylated ligase in the enzyme preparation. The linear dependence of nucleotide-independent ligation was attributed to pre-adenylated ligase in the absence of added nucleotide. Indeed, none of the standard rNTPs or dNTPs were able to satisfy the requirement of AmEPV ligase for a high energy cofactor (Fig. 3A). NADP was also inactive (not shown). We conclude that the AmEPV enzyme is a bona fide NAD⁺-specific DNA ligase, the first such enzyme identified from the eukaryotic domain. Titration experiments showed that nick joining activity at subsaturating levels of enzyme increased with NAD⁺ concentration from 1 to 50 μM and plateaued at 50–100 μM (not shown). A $K_m$ of 9 μM NAD⁺ for AmEPV ligase was calculated from a double-reciprocal plot of the data (not shown). The $K_m$ for nick joining by recombinant E. coli DNA ligase on a similar DNA substrate is 3 μM NAD⁺ (19).

Nick joining by AmEPV ligase required a divalent cation cofactor and was optimal at 5 mM magnesium. Manganese and cobalt (5 mM) were also active, albeit less than magnesium, whereas calcium, cooper, and zinc did not support ligase activity (not shown). The AmEPV ligase was active in Tris-HCl buffer from pH 6.5 to 9.0 (not shown).

The native size of the AmEPV ligase was gauged by zonal velocity sedimentation through a 15–30% glycerol gradient containing 0.2 M NaCl. The ligation activity profile comprised a single peak centered at fraction 25–27 and coincided with the sedimentation profile of the 62-kDa AmEPV ligase polypeptide (Fig. 3B). The lower molecular weight contaminants sedi-
mented slightly slower, peaking at fraction 27. A comparison of the ligase peak to those of marker proteins catalase (248 kDa) and cytochrome C (13 kDa) that were centrifuged in a parallel gradient hinted that the AmEPV ligase was a monomer. In order to more accurately gauge the sedimentation behavior of AmEPV ligase, we mixed the recombinant enzyme with globular marker proteins catalase, ovalbumin, and cytochrome C and sedimented the mixture through a 15–30% glycerol gradient. A plot of the marker S values versus fraction number yielded a straight line (not shown). The 62-kDa ligase cosedimented precisely with ovalbumin (45 kDa). These sedimentation results suggest that the AmEPV ligase is an asymmetrically shaped monomer.

Structure Probing of AmEPV Ligase by Limited Proteolysis—Recombinant His-tagged AmEPV ligase was subjected to proteolysis with increasing amounts of trypsin and V8 proteases. N-terminal sequencing of the undigested AmEPV ligase polypeptide by automated Edman chemistry after transfer from an SDS gel to a polyvinylidene difluoride membrane confirmed that the N-terminal sequence (GHHHHH) corresponded to that of the recombinant gene product starting from the second residue of the His-tag (Fig. 4). Apparently, the ligase suffered removal of the initiating methionine during expression in E. coli. Initial scission of the ligase by 20–40 ng of

**FIG. 2.** Aligned primary structures of entomopoxvirus and eubacterial NAD+/H-dependent DNA ligases. The amino acid sequence of AmEPV ligase from amino acids 19 to 395 is aligned with the N-terminal portions of the NAD+/H-dependent ligases encoded by E. coli (Eco), B. stearothermophilus (Bst), and T. filiformis (Tfi). The alignment encompasses the Ia, nucleotidyltransferase, OB-fold, and Zn finger domains. The secondary structure of Tfi ligase is shown below the amino acid sequence. Gaps in the sequence alignment are indicated by dashes (-). Positions of side chain conservation (identity or structural similarity) in all four proteins are denoted by dots (.) above the sequence. The conserved nucleotidyltransferase motifs are denoted below the Tfi sequence; motifs I, III, IIIa, IV, and V are highlighted in shaded boxes. The four cysteines comprising the Zn finger are located near the C terminus of the alignment and are highlighted in shaded boxes. The sites of trypsin and V8 cleavage in native AmEPV ligase are indicated by arrows. The six residues in domain Ia that were targeted for alanine mutagenesis are denoted by / marks.

**FIG. 3.** Nucleotide cofactor specificity and velocity sedimentation. A, reaction mixtures (20 μl) containing 50 mM Tris-HCl (pH 7.5), 10 mM (NH₄)₂SO₄, 5 mM dithiothreitol, 5 mM MgCl₂, 1 pmol of ³²P-labeled nicked DNA, 0.5 pmol of AmEPV ligase, and 50 μM of the indicated nucleotide were incubated for 30 min at 22°C. A control reaction contained no added nucleotide cofactor (none). The reaction products were resolved by polyacrylamide gel electrophoresis. An autoradiogram of the gel is shown. B, an aliquot (0.2 ml; 200 μg) of the phosphocellulose preparation of AmEPV ligase was sedimented in a 4.8-ml 15–30% glycerol gradient containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 2 mM dithiothreitol, 0.1% Triton X-100, and 200 mM NaCl. The gradient was centrifuged for 18 h at 50,000 rpm in a Beckman SW50 rotor. Fractions (~0.13 ml) were collected from the bottom of the tube. Marker proteins catalase (246 kDa) and cytochrome C (13 kDa) were centrifuged in a parallel gradient. Aliquots (20 μl) of the odd numbered gradient fractions of AmEPV ligase were analyzed by SDS-PAGE. An aliquot of the protein sample loaded onto the gradient was indicated in lane L. The Coomassie Blue-stained gel is shown. Nick joining reaction mixtures (20 μl) containing 50 mM Tris-HCl (pH 7.5), 10 mM (NH₄)₂SO₄, 5 mM dithiothreitol, 5 mM MgCl₂, 50 μM NAD⁺, 1 pmol of ³²P-labeled nicked DNA, and 1 μl of a 1:10 dilution of the indicated gradient fractions were incubated for 30 min at 22°C. The reaction products were analyzed by PAGE. The extent of ligation (36-mer/(36-mer + 18-mer)) was determined by scanning the gel with a PhosphorImager. The positions of the marker proteins sedimented in a parallel gradient are indicated by arrows.
trypsin yielded two major products: (i) a ∼60-kDa species (sequence HXNHIK, where X is predicted to be M) resulting from tryptic cleavage of the His-tag 2 residues upstream of Met1 of the AmEPV protein, and (ii) a ∼50-kDa species (sequence IGYTPE) resulting from cleavage between Lys70 and Ile71. The latter cleavage site, denoted by an arrow above the AmEPV sequence in Fig. 2, is conserved in other NAD⁺ ligases and is located at the distal margin of a short α helix at the end of domain Ia in the crystal structures of Bst and Tfi ligases. We surmise that the tryptic site demarcates a surface loop between domain Ia and the nucleotidyltransferase domain of AmEPV ligase. The 50-kDa proteolytic fragment became more abundant as trypsin was increased to 80 ng and it remained resistant to digestion by a concentration of trypsin in excess of that sufficient to cleave all the input ligase. A lower molecular weight product accumulated at higher trypsin concentrations; this species consisted of a mixture of two peptides with overlapping N termini derived from scission at Lys70/Ile71 and Lys867/Lys70 (Fig. 4). This product apparently resulted from a discrete secondary cleavage within the nucleotidyltransferase/OB domain, however, the C terminus of the tryptic product was not determined.

Treatment of AmEPV ligase with V8 protease yielded two clusters of products that were resistant to digestion by V8 concentrations sufficient to cleavage all of the input ligase (Fig. 4). The higher molecular weight cluster consisted of a major component that retained the original N terminus of the His-tag (GHHHHH) and a minor species (sequence NSIRTV) arising from cleavage between Glu225 and Asn226 within the nucleotidyltransferase domain (see Fig. 2). The Glu/Asn site is conserved in Tfi ligase (as Glu 246/Lys247), where it is exposed on the protein surface. The lower molecular weight cluster includes a fragment that retains the His-tag and a second species (sequence PIID) generated by cleavage between Glu325 and Asn326 within the nucleotidyltransferase domain (see Fig. 2). The Glu/Asn site is conserved in Tfi ligase (as Glu246/Lys247), where it is exposed on the protein surface. The lower molecular weight cluster includes a fragment that retains the His-tag and a second species (sequence PIID) generated by cleavage between Glu325 and Asn326 within the nucleotidyltransferase domain (see Fig. 2). The Glu/Asn site is conserved in Tfi ligase (as Glu246/Lys247), where it is exposed on the protein surface. The lower molecular weight cluster includes a fragment that retains the His-tag and a second species (sequence PIID) generated by cleavage between Glu325 and Asn326 within the nucleotidyltransferase domain (see Fig. 2). The Glu/Asn site is conserved in Tfi ligase (as Glu325 and Asn326), where it is exposed on the protein surface. The lower molecular weight cluster includes a fragment that retains the His-tag and a second species (sequence PIID) generated by cleavage between Glu325 and Asn326 within the nucleotidyltransferase domain (see Fig. 2). The Glu/Asn site is conserved in Tfi ligase (as Glu325 and Asn326), where it is exposed on the protein surface. The lower molecular weight cluster includes a fragment that retains the His-tag and a second species (sequence PIID) generated by cleavage between Glu325 and Asn326 within the nucleotidyltransferase domain (see Fig. 2). The Glu/Asn site is conserved in Tfi ligase (as Glu325 and Asn326), where it is exposed on the protein surface. The lower molecular weight cluster includes a fragment that retains the His-tag and a second species (sequence PIID) generated by cleavage between Glu325 and Asn326 within the nucleotidyltransferase domain (see Fig. 2). The Glu/Asn site is conserved in Tfi ligase (as Glu325 and Asn326), where it is exposed on the protein surface. The lower molecular weight cluster includes a fragment that retains the His-tag and a second species (sequence PIID) generated by cleavage between Glu325 and Asn326 within the nucleotidyltransferase domain (see Fig. 2). The Glu/Asn site is conserved in Tfi ligase (as Glu325 and Asn326), where it is exposed on the protein surface. The lower molecular weight cluster includes a fragment that retains the His-tag and a second species (sequence PIID) generated by cleavage between Glu325 and Asn326 within the nucleotidyltransferase domain (see Fig. 2). The Glu/Asn site is conserved in Tfi ligase (as Glu325 and Asn326), where it is exposed on the protein surface. The lower molecular weight cluster includes a fragment that retains the His-tag and a second species (sequence PIID) generated by cleavage between Glu325 and Asn326 within the nucleotidyltransferase domain (see Fig. 2). The Glu/Asn site is conserved in Tfi ligase (as Glu325 and Asn326), where it is exposed on the protein surface. The lower molecular weight cluster includes a fragment that retains the His-tag and a second species (sequence PIID) generated by cleavage between Glu325 and Asn326 within the nucleotidyltransferase domain (see Fig. 2). The Glu/Asn site is conserved in Tfi ligase (as Glu325 and Asn326), where it is exposed on the protein surface. The lower molecular weight cluster includes a fragment that retains the His-tag and a second species (sequence PIID) generated by cleavage between Glu325 and Asn326 within the nucleotidyltransferase domain (see Fig. 2). The Glu/Asn site is conserved in Tfi ligase (as Glu325 and Asn326), where it is exposed on the protein surface. The lower molecular weight cluster includes a fragment that retains the His-tag and a second species (sequence PIID) generated by cleavage between Glu325 and Asn326 within the nucleotidyltransferase domain (see Fig. 2).
Characterization of N-terminal and C-terminal Domains of AmEPV Ligase—In light of the proteolysis results, we engineered the N-terminal deletion mutant Lig-(71–532) and the C-terminal truncation Lig-(1–316), referred to henceforth as NΔ70 and CΔ216, respectively. The NΔ70 protein corresponds to the major trypsin-resistant species and it lacks all of domain Ia. The CΔ216 protein is truncated at the V8-accessible site in the middle of the OB-fold and it lacks all of the HHH domain. NΔ70 and CΔ216 were produced in bacteria as N-terminal His10 fusions and purified from soluble lysates by Ni-agarose and phosphocellulose chromatography (Fig. 5A).

CΔ216 was incapable of sealing a 3′-OH/5′-PO4 nick (Fig. 5C), yet it retained the ability to react with NAD+ to form a covalent ligase-adenylate intermediate (Fig. 5B). We surmise that the OB and/or HHH domains are critical for AmEPV ligase function at a step subsequent to step 1 reaction chemistry. These findings echo those of Timson and Wigley (25) for an N-terminal domain of Bst ligase that was truncated within the first β strand of the OB-fold domain and also retained step 1 ligase-adenylation activity.

The fact that we could detect no accumulation of a DNA-adenylate intermediate during the reaction of CΔ216 with nicked DNA in the presence of NAD+ hinted that the missing C terminus played a role either in step 2 chemistry or in DNA binding. Conceivably, the C terminus may also be required for step 3 of the ligase reaction: the formation of a phosphodiester bond.

We assayed step 3 of the AmEPV ligation reaction using a pre-adenylated nicked DNA substrate. The adenyland strand used to form this substrate was synthesized using vaccinia virus DNA ligase via ligase-mediated AMP transfer to the 5′-32P-labeled strand of a DNA molecule containing a 1-nucleotide gap (12, 26). The radiolabeled AppDNA strand was purified by denaturing PAGE and then annealed to an unlabeled 36-mer template oligonucleotide and a 3′ OH 18-mer oligonucleotide to form the nicked DNA-adenylate molecule illustrated in Fig. 5C. This substrate was reacted with excess wild-type or truncated AmEPV ligase in the presence of magnesium without added NAD+. The wild-type enzyme generated a 36-mer ligation product, but CΔ216 was inert in phosphodiester formation (Fig. 5C, AppDNA). Thus, the ligation defect incurred by the loss of the C terminus could not be overcome by bypassing the DNA adenylation step.

A novel finding that emerged from the deletion analysis was that elimination of the N-terminal Ia domain abrogated nick joining by a completely different mechanism than did the loss of the C terminus (Fig. 5). NΔ70 was inert in the overall ligation reaction (Fig. 5C) and formation of a ligase-adenylate intermediate with NAD+ (Fig. 5B). However, NΔ70 was fully functional in synthesis of phosphodiester bond at a pre-adenylated nick (Fig. 5C). The latter point underscores that the step 1 defect of NΔ70 cannot be ascribed to a global folding defect, but instead reflects a specific requirement for domain Ia in the reaction of ligase with NAD+.

Several instructive points were gleaned from a kinetic analysis of phosphodiester bond formation by wild-type ligase and NΔ70 at a pre-adenylated nick under conditions of ligase excess (Fig. 6A). First, although the extent of sealing of the preadenylated nick in the absence of NAD+ was identical for wild-type ligase and NΔ70, the rate of the NΔ70 reaction was 50% faster than that of the wild-type enzyme. The implication of this result is that the presence of domain Ia constitutes a modest impediment to the interaction of the ligase with nicked DNA-adenylate (see below). Second, the inclusion of 50 μM NAD+ elicited a 5-fold decrement in the extent of step 3 ligation by the wild-type ligase, presumably by competition of NAD+ and nicked DNA-adenylate for the AMP-binding pocket within the nucleotidyltransferase domain. NAD+ had no effect whatsoever on step 3 catalysis by NΔ70, consistent with a critical role for domain Ia in NAD+ binding to AmEPV ligase.

Binding of AmEPV Ligase to DNA-adenylate—A native gel mobility shift assay was used to directly examine the binding of AmEPV ligase to the nicked DNA-adenylate substrate. Phosphodiester formation on the nicked DNA-adenylate substrate required a divalent cation cofactor (not shown); therefore the binding reactions were performed in the absence of a divalent cation so as to preclude conversion of substrate to product during the incubation. Mixing the wild-type ligase (2 pmol) with 200 fmol of nicked DNA-adenylate resulted in the formation of a discrete protein-DNA complex that migrated more slowly than the free AppDNA (Fig. 6B). Mixture with NΔ70 yielded a discrete complex that migrated just slightly faster...
than the wild-type ligase-DNA complex, consistent with loss of mass and/or charge with deletion of domain Ia. Doubling the amount of ligase to 4 pmol resulted in increased abundance of both the wild-type and N/H900470 complexes. From the amount of protein required to shift 50% of the DNA, we estimate an affinity of N/H900470 for nicked DNA-adenylate of 11011100 nM. At 2 pmol of input ligase, N/H900470 appeared to have a slightly higher affinity for the AppDNA than wild-type ligase, which seconded the inference from the step 3 kinetic analysis (Fig. 6A) that the presence of domain Ia may impede AppDNA binding. The C/H9004216 protein failed to bind AppDNA at all (Fig. 6B), suggesting that the lack of step 3 catalytic activity (Fig. 5C) can be ascribed to a primary DNA binding defect.

**DISCUSSION**

We report the characterization of AmEPV DNA ligase, the first case of an NAD+/H11001-dependent DNA ligase in the eukaryotic domain of the universal phylogenetic tree. The AmEPV ligase is smaller than any of the eubacterial NAD+/H11001 ligases because it lacks the Zn finger and BRCT structural domains present in all known eubacterial NAD+/H11001 ligases. The present study clearly establishes that NAD+ ligase activity is not inevitably depend-
ent on a Zn finger or a BRCT domain. The AmEPV ligase, and its homologue from MsEPV (which is 42% identical to the AmEPV protein), appear to comprise a new branch of the NAD\(^+\)/H\(_{11001}\) ligase family.

**Evolutionary Implications**—Poxvirus genomes encode between 150 and 280 polypeptides. Although gene number, order, and content are variable, all poxviruses share a subset of ~45 conserved proteins, half of which are required for viral mRNA synthesis and processing and viral DNA metabolism. Thus, in considering the origins of the NAD\(^+\)/H\(_{11001}\)-dependent entomopoxvirus ligases, it is remarkable that insect and vertebrate poxviruses encode completely different classes of DNA ligases. Several genera of vertebrate poxviruses (including the Orthopoxvirus, Leporipoxvirus, and Avipoxvirus) encode ATP-dependent DNA ligases that enhance virulence, facilitate DNA replication, and determine sensitivity to DNA damage (27, 28). Vertebrate poxvirus ligases are structurally similar to mammalian DNA ligase III (29, 30). The 552-amino acid vaccinia ligase is 54% identical, 73% conserved with human ligase III throughout the entire length of the vaccinia protein sequence. Indeed, human ligase III is more similar to the poxvirus ligase than it is to human DNA ligases I and IV. Eukaryotic DNA ligase III is apparently a late-evolving ligase isofrom unique to vertebrate species, i.e. there is no ligase III equivalent encoded in the complete genomes of invertebrates (Drosophila melanogaster and Caenorhabditis elegans) or fungi, although these “lower” eukaryotes do encode both ligase I and ligase IV. If cytoplasmic poxviruses acquire new genes from host cell cDNAs (which could explain the presence of a ligase III type enzyme only in chordopoxviruses), then it is quite possible that NAD\(^+\)-dependent ligases exist in certain arthropod organisms, especially the caterpillars and grasshoppers that are infected by AmEPV and MsEPV, respectively. It is also worth considering that poxviruses might pick up new genes from other viruses or microbes cohabiting the host organism, in which case the NAD\(^+\) ligases of entomopoxviruses might have originated from a eubacterium.

**Mechanistic Implications**—The selective effects of deletions and mutations in domain Ia of AmEPV ligase on the nucleotidytransferase reaction with NAD\(^+\) provide the first evidence for a structural determinant of substrate specificity for the NAD\(^+\) ligase family. Domain Ia consists principally of two antiparallel $\alpha$ helices and an intervening loop (Fig. 2). Domain
Ia is unique to NAD-dependent ligases and there is no discernible counterpart in any member of the ATP-dependent ligase family; thus, it is sensible that domain Ia is involved in NAD\(^+\) recognition. The present findings concerning the role of domain Ia in adenylate transfer from NAD\(^+\) by the \textit{Am}EPV ligase are likely to apply broadly to the eubacterial enzymes insofar as: (i) N-terminal deletions N\(_{78}\) and N\(_{38}\) of \textit{E. coli} DNA ligase, which eliminate all or part of domain Ia, result in complete loss of nick joining activity (23); (ii) the \textit{Eco} ligase N\(_{78}\) and N\(_{38}\) mutants are nonetheless able to catalyze phosphodiester formation at a pre-adenylated nick\(^2\); and (iii) single alanine substitutions within \textit{Eco} ligase domain Ia decrease or eliminate both nick joining and the step 1 ligase adenylation reaction with NAD\(^+\).

We propose a mechanistic model whereby ligase substrate specificity at the step of ligase-adenylate formation is determined by the interactions of domain Ia with the NMM moiety of NAD\(^+\) for the NAD\(^+\)-dependent enzymes (Fig. 8A) and the interactions of motif VI of the OB-fold domain with the \(\beta\) and \(\gamma\) phosphates of ATP for the ATP-dependent ligases (Fig. 8B). The crystal structures of NAD\(^+\) ligase, ATP ligases, and mRNA capping enzyme in various functional states all indicate that contacts of the enzymes with the AMP or GMP moieties are confined to the nucleotidyltransferase domain (6, 7, 11, 19). The nucleoside portion is buried within a pocket while the \(\alpha\) phosphate is exposed on the surface of the domain. The first step in ligation and capping entails the in-line attack of the motif I lysine on the nucleoside triphosphate or NAD\(^+\) substrates to form enzyme-adenylate or enzyme-guanynylate. The reaction proceeds through a pentacoordinate phosphorane transition state in which the attacking lysine nucleophile is apical to the pyrophosphate or NMN leaving group. The ground state structures of \textit{T7} ligase with ATP and capping enzyme with GTP reveal that the pyrophosphate leaving group projects out into the open cleft between the nucleotidyltransferase and OB-fold domains and that it makes few or no direct contacts with the enzyme. Indeed, the \(\beta\) and \(\gamma\) phosphates in the ground state are oriented unfavorably with respect to the motif I lysine, such that reaction chemistry is effectively precluded.

The catalysis of nucleotidyl transfer by ATP ligase and capping enzyme is believed to be facilitated by closure of the OB-fold domain over the nucleotide binding pocket such that motif VI (located at the C terminus of the OB-fold) makes direct contact with the \(\beta\) and \(\gamma\) phosphates and reorients the pyrophosphate leaving group so that it is apical to the attacking lysine (7, 11). The conformational switch is illustrated for ATP ligase in Fig. 8B. Once the proper orientation is attained, the lysyl-AMP intermediate is formed and pyrophosphate is expelled. The breaking of the \(\alpha-\beta\) phosphoanhydride bond in nucleotidyl transfer domain and triggers the adoption of a wide open domain conformation that permits the binding of the nicked DNA substrate immediately above the AMP phosphate on the surface of the nucleotidyltransferase domain (7). Motif VI, although essential for ligase-AMP formation, is dispensable for step 3 phosphodiester formation (12).

There is no equivalent of motif VI in the OB-fold of the NAD\(^+\) ligases; this is sensible insofar as they have no need for contacts with \(\gamma\) phosphate. Yet, ligase-adenylate formation by the NAD\(^+\) ligases should still require an apical orientation of the nicotinamide nucleotide phosphate moiety of NAD\(^+\) with respect to the motif I lysine nucleophile. We propose that the proper orientation of NAD\(^+\) is achieved by closure of domain Ia over the nucleotide binding pocket resulting in contacts between domain Ia and the nicotinamide nucleoside (and perhaps also the phosphate of the leaving group). The breaking of the \(\alpha-\beta\) phosphoanhydride bond of NAD\(^+\) upon enzyme-adenylate formation would release the tether of domain Ia to the nucleotidyltransferase domain and allow domain Ia to spring apart to adopt the conformation observed in the crystal structure of the \textit{T7} ligase-adenylate intermediate (19).

There is as yet no crystal structure of an NAD\(^+\) ligase bound to NAD\(^+\). However, our analysis of the effects of single alanine mutations in domain Ia of \textit{Am}EPV ligase identifies five residues (Tyr\(^39\), Tyr\(^40\), Asp\(^48\), Tyr\(^51\), and Asp\(^52\)) that are involved specifically in adenylate transfer from NAD\(^+\). These five residues are conserved in \textit{Ms}EPV ligase, in the \textit{Eco}, \textit{Bst}, and \textit{T7} ligases (Fig. 2), and in the NAD\(^+\) ligases from 25 other bacterial species (not shown). Indeed, the five side chains are tightly clustered on the same surface of domain Ia in the \textit{T7} ligase crystal structure. Accordingly, we suggest that these residues are constituents of an NMN-binding site. \textit{Am}EPV residue Asp\(^27\) in domain Ia is not important for ligase function, i.e. alanine substitution is benign. Although the corresponding position is conserved as a carboxylate in the \textit{Eco}, \textit{Bst}, and \textit{T7} ligases, it is not conserved in many other bacterial NAD\(^+\) ligases (not shown).

**Implications for Ligase Pharmacology—Inhibitors of bacterial NAD\(^+\)-dependent DNA ligases would, in principle, be outstanding candidates for effective broad spectrum antibiotic therapy, given that: (i) NAD\(^+\)-dependent ligases are present in all bacteria and are essential for bacterial growth in each case that has been studied; (ii) they are structurally conserved among bacteria, but display unique substrate specificity compared with the ATP-dependent ligases of humans and other mammals; and (iii) humans have no homolog of an NAD\(^+\) ligase. Arguably, the attractiveness of NAD\(^+\) ligases as targets for drug discovery was tempered by the crystallographic evidence that the tertiary structure of the core nucleotidyltransferase and OB domains of NAD\(^+\) ligases, as well as the active site pocket within the nucleotidyl transferase domain, are strikingly similar to those of ATP ligases, despite scant similarity in their respective amino acid sequences.

The present studies on the function of domain Ia in NAD\(^+\) ligase raise the prospects for identifying small molecules that either compete for the predicted NMN site on domain Ia (said site being absent from ATP ligases) or else interfere with the conformational movements of domain Ia that are postulated to orchestrate the adenylate transfer reaction from NAD\(^+\) (Fig. 8A). Inspection of the \textit{T7} ligase structure suggests that the domain closure step could occur by flexion of the loop that connects Ia to the nucleotidyltransferase domain without invoking a significant rearrangement within Ia. Thus, while awaiting a ligase NAD\(^+\)-cocryystal structure, it may be fruitful to model candidate ligands into the conserved and functionally important surface of domain Ia defined herein using the crystal structures that are already available.

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