DNA ligase D (LigD) catalyzes end-healing and end-sealing steps during nonhomologous end joining in bacteria. *Pseudomonas aeruginosa* LigD consists of a central ATP-dependent ligase domain fused to a C-terminal polymerase domain and an N-terminal 3′-phosphoesterase (PE) module. The PE domain catalyzes manganese-dependent phosphodiesterase and phosphomonoesterase reactions at a duplex primer-template with a short 3′-ribonucleotide tract. The phosphodiesterase, which cleaves a 3′-terminal diribonucleotide to yield a primer strand with a ribonucleoside 3′-PO₄ terminus, requires the vicinal 2′-OH of the penultimate ribose. The phosphomonoesterase converts the terminal ribonucleoside 3′-PO₄ to a 3′-OH. Here we show that the PE domain has a 3′-phosphatase activity on an all-DNA primer-template, signifying that the phosphomonoesterase reaction does not depend on a 2′-OH. The distinctions between the phosphodiesterase and phosphomonoesterase activities are underscored by the results of alanine-scanning, limited proteolysis, and deletion analysis, which show that the two reactions depend on overlapping but nonidentical ensembles of protein functional groups, including: (i) side chains essential for both ribonuclease and phosphatase activity (His-42, His-48, Asp-50, Arg-52, His-84, and Tyr-88); (ii) side chains important for 3′-phosphatase activity but not for 3′-ribonucleoside removal (Arg-14, Asp-15, Glu-21, Gln-40, and Glu-82); and (iii) side chains required selectively for the 3′-ribonuclease (Lys-66 and Arg-76). These constellations of critical residues are unique to LigD-like proteins, which we propose comprise a new bifunctional phosphoesterase family.

The presumption that bacteria rely solely on homologous recombination to repair DNA double strand breaks was overturned recently by evidence that *Mycobacteria* exploit a nonhomologous end-joining (NHEJ)³ system driven by a bacterial Ku homolog and a specialized polyfunctional ATP-dependent DNA ligase (LigD) (1–4). The fact that Ku and LigD are jointly encoded by many diverse bacterial genera (5, 6) hints that NHEJ is broadly relevant to bacterial physiology. LigD proteins contain a DNA ligase catalytic domain (Lig) fused to a polymerase domain (Pol) and a phosphoesterase (PE) module (1–4, 7, 8). The domain order within the LigD primary structure is variable. For example, *Mycobacterium* LigD consists of an N-terminal Pol domain, a central PE domain, and a C-terminal Lig domain, whereas *Pseudomonas* LigD is composed of an N-terminal PE domain, a central Lig domain, and a C-terminal Pol domain. The need for a DNA ligase in NHEJ is self-evident. The LigD Pol domain catalyzes *in vitro* both template-dependent fill-in of 5′-overhangs and the nontemplate addition of single nucleotides at blunt ends (2, 4, 7), which are the predominant outcomes of the highly mutagenic mycobacterial NHEJ pathway *in vivo* (2). Thus, it is likely that the Pol domain is responsible for insertional mutagenesis during NHEJ.

A significant fraction of the mutagenic NHEJ events cataloged *in vivo* entailed nucleolytic resection at one or both ends prior to the resealing step (2). It is not yet clear whether LigD is the direct agent of DNA end resection during NHEJ. Initial informatics analysis of LigD highlighted clusters of conserved histidines and aspartates within the PE domain that were predicted to form a metal-coordinating cluster composed of β-strands. On this basis, a deoxyribonuclease function was suggested (5). *Mycobacterium tuberculosis* LigD was reported to have an associated metal-dependent DNA 3′-exonuclease activity (4).

Recently, we demonstrated a 3′-ribonuclease/3′-phosphatase activity of *Pseudomonas* LigD, whereby it resects a short tract of 3′-ribonucleotides on a primer-template substrate to the point at which the primer strand has a single 3′-ribonucleotide remaining (8). The failure to digest beyond this point reflects a requirement for a 2′-OH group on the penultimate nucleoside of the primer strand. Replacing the 2′-OH by a 2′-F, 2′-NH₂, 2′-OCH₃, or 2′-H abolishes the resection activity (8). The ribonucleotide resection activity resides within the 187-amino-acid N-terminal PE domain and is the result of at least two component steps: (i) the 3′-terminal nucleoside is first removed to yield a primer strand with a ribonucleoside 3′-PO₄ terminus and (ii) the 3′-PO₄ is hydrolyzed to a 3′-OH. The 3′-ribonuclease and 3′-phosphatase activities are both dependent on manganese. Here we show that the PE domain also catalyzes hydrolysis of the 3′-PO₄ of an all-DNA primer-template substrate, thereby establishing that the 2′-OH of the terminal nucleoside is not required for the phosphomonoesterase reaction. LigD preferentially cleaves the 3′-PO₄ of a duplex primer-template substrate rather than a single-stranded DNA of identical composition.

The PE domain of *Pseudomonas* LigD and its bacterial homologs has no apparent structural or mechanistic similarity to previously characterized nucleases or 3′-phosphatases. Thus, we speculated that it exemplifies a novel phosphoesterase family (8). An initial alanine scan of five residues of *Pseudomonas* LigD identified Asp-50, Arg-52, and His-84 as essential for both the 3′-ribonuclease and 3′-phosphatase reactions; Glu-82 was specifically required for the 3′-phosphatase activity but not for initial ribonuclease resection. Here we extended the alanine scan to 11 other conserved amino acids. We focused on: (i) histidines (His-42 and His-48) as potential general acid/base catalysts or ligands for manganese, (ii) basic residues (Arg-14, Arg-46, Lys-66, and Arg-76) as candidate ligands for the reactive phosphodiester and 3′-phosphate moieties, (iii) acidic groups (Asp-15, Glu-21, and Asp-83) as potential metal...
Pseudomonas DNA Ligase D

ligands, and (iv) polar side chains (Gln-40 and Tyr-88) with potential for hydrogen bonding to the primer-template substrate. Mutational effects fell into several classes: (i) those that abolished or severely impeded both the phosphodiesterase and phosphomonoesterase functions, (ii) those that selectively impaired the phosphodiesterase, and (iii) those that selectively affected the phosphomonoesterase. These results indicate that the active sites for the ribonuclease and phosphatase functions overlap but are not identical.

EXPERIMENTAL PROCEDURES

**PE Domain of LigD**—A gene fragment encoding LigD-(1–187) (PE domain) was amplified by PCR with sense strand primers that introduced an NdeI restriction site at the beginning of the open reading frame and an antisense primer that introduced a new stop codon and a flanking BamHI site. The PCR product was digested with NdeI and BamHI and inserted into pET16b to generate an expression plasmid pET-PeLigD-PE that encodes a His$_{10}$-tagged version of the PE domain. Alanine mutations and N-terminal deletions were introduced into the plasmid as described previously (8). The insert of the mutant plasmids was sequenced completely to exclude the acquisition of unwanted changes during amplification and cloning. The plasmids were transformed into *Escherichia coli* BL21(DE3). Induction of protein expression with isopropyl 1-thio-β-D-galactopyranoside, preparation of soluble bacterial lysates, and purification of the recombinant PE proteins by nickel-agarose affinity chromatography were performed as described previously (8). The 200 mM imidazole eluate fractions containing the PE domain were stored at −80 °C. Protein concentrations were determined using the Bio-Rad dye reagent with bovine serum albumin as the standard.

**3'-Ribonuclease Assay**—Reaction mixtures (80 μl) containing 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 0.5 mM MnCl$_2$, 50 nM 5'-32P-labeled D10R2 primer-template (see Fig. 2), and 5 μM LigD PE domain were incubated at 37 °C. Aliquots (10 μl) were withdrawn at the times specified and quenched with 7 mM EDTA and 31% formamide. The products were resolved by electrophoresis through a 40-cm 18% polyacrylamide gel containing 7 M urea in Tris-borate/EDTA. The products were visualized by autoradiography and quantified by scanning the gel with a Fujifilm BAS-2500 imaging apparatus.

**3'-Phosphatase Assay**—The 5'-32P-labeled, 3'-phosphate-terminated D9R1-p strand was prepared by digesting a 5'-32P-labeled D9R3 primer oligonucleotide with ribonuclease A as described previously (8). The labeled D9R1-p oligonucleotide was annealed to a 4-fold excess of an unlabeled complementary 24-mer DNA strand. The 3'-phosphatase reaction mixtures (80 μl) containing 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 0.5 mM MnCl$_2$, 25 nM 5'-32P-labeled D9R1-p primer-template (see Fig. 4), and 5 μM LigD PE domain were incubated at 37 °C. Aliquots (10 μl) were withdrawn at the times specified and quenched with EDTA/formamide. The products were resolved by denaturing gel electrophoresis as described above. The extent of conversion of the 3'-PO$_4$ oligonucleotide to the slower migrating 3'-OH product was determined by scanning the gel with a Fujifilm BAS-2500 imaging apparatus.

**DNA 3'-Phosphatase Assay**—The 5'-32P-labeled, 3'-phosphate-terminated D11-p strand was prepared by reaction of the 11-mer 5'-OH, 3'-PO$_4$, oligodeoxynucleotide 5'-CAATTGCGACCp (purchased from BIOSOURCE) with the N-terminal 5'-kinase domain of T4 polynucleotide kinase (33) and [γ-32P]ATP. The labeled 11-mer was purified by native gel electrophoresis and then annealed to a 4-fold excess of an unlabeled complementary 24-mer DNA strand. The DNA 3'-phosphatase reaction mixtures (80 μl) containing 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 0.5 mM MnCl$_2$, 50 nM 5'-32P-labeled D11-p primer-template (see Fig. 5), and 5 μM LigD PE domain were incubated at 37 °C. Aliquots (10 μl) were withdrawn at the times specified and quenched with EDTA/formamide. The products were resolved by denaturing gel electrophoresis as described above. The extent of conversion of the 3'-PO$_4$ oligonucleotide to the slower migrating 3'-OH product was determined by scanning the gel.

**Limited Proteolytic enzyme**—To prepare the native tag-free PE domain, we initially produced LigD-(1–187) in *E. coli* as a His$_{10}$-Smt3 fusion, purified the fusion protein from a soluble extract by nickel-agarose chromatography, removed the His$_{10}$-Smt3 tag by digestion with the Smt3-specific protease Ulp1 (9), and then purified the tag-free PE domain away from His$_{10}$-Smt3 by nickel-agarose chromatography. Aliquots (10 μg) of the native PE domain were digested for 15 min at 22 °C with 0, 25, 50, 100, or 200 ng of trypsin or chymotrypsin. The digests were resolved by SDS-PAGE (15% polyacrylamide), and the polypeptides were transferred electrophoretically to a polyvinylidene difluoride membrane and then stained with Coomassie Blue. Proteolytic fragments were excised from the membrane and subjected to at least six cycles of automated Edman chemistry to determine the N-terminal amino acid sequence.

**RESULTS**

**Mutational Effects on Ribonucleotide Resection**—Wild-type and mutated versions of the N-terminal PE domain of *Pseudomonas* LigD were produced in *E. coli* as His$_{10}$ fusions and purified from soluble bacterial lysates by nickel-agarose chromatography. SDS-PAGE analysis verified that the preparations were enriched to the same extent with respect to the PE polypeptide, which migrated anomalously at ∼29 kDa (predicted size 24 kDa) (Fig. 1). Mutational effects on 3'-ribonucleotide resection were gauged by kinetic analysis of the reaction of the PE protein with a 5'-32P-labeled D10R2 primer-template under conditions of enzyme excess (Fig. 2). Wild-type PE initially converted the labeled strand to a 3'-phosphorylated species, D10R1-p, which was subsequently converted to a more slowly migrating 3'-OH end product, D10R1 (Fig. 2).

Distinct classes of mutational effects were apparent from the extent of substrate decay and the distribution of the reaction products: (i) the H42A and H48A changes effectively abolished the 3' resection activity; (ii) the rates of substrate decay by the K66A, R76A, and Y88A mutants were much slower than wild-type; (iii) the rates of substrate consumption by the R14A, D15A, E21A, and Q40A mutants were similar to wild-type, and the normally transient D10R1-p intermediate accumulated as a predominant product; and (iv) the rates of substrate decay by
the D83A and R46A mutants were similar to, or moderately slower than, the wild-type PE domain, and the D10R1-p intermediate was efficiently converted to the D10R1 end product. These results implicate Arg-14, Asp-15, Glu-21, and Gln-40 as candidate catalysts of the 3’/H11032 phosphatase reaction, insofar as alanine substitutions had qualitatively selective effects on the 3’/H11032-PO4 hydrolysis step of the overall ribonucleotide resection reaction.

The impact of Ala mutations on the rate of the phosphodiesterase reaction was quantified in Fig. 3A by plotting the sum of the D10R1-p and D10R1 reaction products (expressed as the fraction of total radiolabeled material) as a function of time. This analysis revealed that the Arg-14, Asp-15, Glu-21, and Asp-83 mutants were similar to wild-type with respect to the initial rate (see TABLE ONE) and extent of 3’-ribonucleoside removal. We infer that these side chains, although conserved among the PE domain proteins, do not contribute to the phosphodiesterase active site. The H42A and H48A proteins displayed ≤0.01% of the wild-type rate and are thereby candidate components of the 3’-ribonuclease catalytic site. The initial rates of the Y88A, K66A, and R76A proteins were 7.1, 2.9, and 1.1% of the wild-type rate, respectively. Our working definition of a functionally important residue is one for which alanine substitution reduced activity to ≤10% of wild-type. We therefore regard Tyr-88, Lys-66, and Arg-76 as important for 3’-ribonucleoside removal. The modest effects of the Q40A and R46A mutations on the reaction rate (41 and 16% of wild-type) do not meet the criterion for designating either Gln-40 or Arg-46 as candidate catalytic residues for the 3’-ribonuclease reaction.

Mutational Effects on 3’-Phosphatase Activity—A kinetic analysis of the reaction of the PE proteins with a 5’/H11541 32P-labeled D9R1-p primer-template (shown at the bottom, with ribonucleotides highlighted in shaded box) and 5 μM wild-type (WT) or Ala-mutant PE domain were incubated at 37 °C. Aliquots were withdrawn at the times indicated and quenched immediately with EDTA/formamide. The time 0 sample was withdrawn prior to adding the PE domain. The products were resolved by PAGE and visualized by autoradiography. The labeled species corresponding to the D10R2 substrate, the D10R1-p intermediate, and the D10R1 end product are indicated by arrowheads on the right.
template under conditions of enzyme excess is shown in Fig. 4 and quantified in Fig. 3B. Wild-type PE converted nearly all of the input labeled strand to a slower migrating D9R1-OH species; the kinetic profile fit well to a single exponential with an apparent rate constant of 0.004 s\(^{-1}\). Mutants R14A, E21A, H42A, H48A, and Y88A were virtually inert in the isolated 3\(^{\prime}\)-phosphatase reaction (<1% of the wild-type rate; see TABLE ONE). Mutants D15A and Q40A were weakly active, with initial rates being 3.3 and 6.7% of wild-type, respectively. The R46A, K66A, R76A, and D83A mutants retained substantial 3\(^{\prime}\)-phosphatase activity, their initial rates being 19, 28, 16, and 51% of the wild-type, respectively.

Taken together, the kinetic analyses of 3\(^{\prime}\)-ribonucleotide resection and the isolated 3\(^{\prime}\)-phosphatase reaction (TABLE ONE) highlight three classes of functionally relevant side chains: (i) those that are essential/important for both ribonuclease and phosphatase activity (His-42, His-48, and Tyr-88); (ii) those essential/important for 3\(^{\prime}\)-phosphatase activity but not for 3\(^{\prime}\)-ribonucleoside removal (Arg-14, Asp-15, Glu-21, and Gln-40); (iii) those that are important for 3\(^{\prime}\)-ribonuclease but have only a modest effect on 3\(^{\prime}\)-phosphatase (Lys-66 and Arg-76).

**DNA 3\(^{\prime}\)-Phosphatase Activity of LigD**—Although the 3\(^{\prime}\)-phosphodiesterase activity of LigD is strictly dependent on a 2\(^{\prime}\)-OH of the penultimate nucleoside (8), the role of the terminal 2\(^{\prime}\)-OH in the 3\(^{\prime}\)-phosphomonoesterase reaction has not been tested. To address this issue, we prepared an all-DNA primer-template composed of an 11-mer \(^{32}\)P-labeled, 3\(^{\prime}\)-PO\(_4\) oligodeoxyribonucleotide (D11-p) annealed to a
24-mer oligonucleotide to form the substrate shown in Fig. 5. The LigD PE domain converted the labeled D11-p strand to a slower migrating species, D11-OH. The extent of dephosphorylation of the D11-p primer-template was proportional to the amount of input PE domain (Fig. 5A) and required a divalent cation, specifically manganese (not shown). We estimated from the slope of the titration curve (Fig. 5C) that ~1.3 pmol of ends were repaired/pmol of PE domain. The specific activity of the PE domain on the D11-p primer-template was ~16-fold higher than its specific activity on the D9R1-p primer-template (not shown). A kinetic analysis of the DNA 3'-phosphatase reaction under conditions of enzyme excess is shown in Figs. 5B and 6. The reaction was complete in 2–5 min; the data fit well to a single exponential with an apparent rate constant of 0.024 s⁻¹ (compared with 0.004 s⁻¹ for dephosphorylation of D9R1-p under identical conditions). These results demonstrated that a terminal 2'-OH is not required for the 3'-phosphomonoesterase activity and that a DNA 3'-PO₄ primer-template is a more effective substrate than a structurally similar primer-template with a ribonucleoside 3'-PO₄ end.

The dependence of the DNA 3'-phosphatase activity on the DNA template strand was gauged by comparing the D11-p primer-template to the D11-p single strand (Fig. 5C). The 3'-phosphatase specific activity on the primer-template substrate was ~130-fold greater than on the primer alone. Thus, the DNA 3'-phosphatase reaction, similar to the reaction at a ribonucleoside 3'-phosphate end (8), displays a fairly stringent requirement for the template DNA strand.

A kinetic analysis of the reaction of the collection of mutant PE proteins with the 5'-3₂P-labeled D11-p primer-template under conditions of enzyme excess is shown in Fig. 6; initial rates were quantified (Fig. 3C) and normalized to the rate of the wild-type enzyme (TABLE ONE). The R14A, E21A, H42A, H48A, and Y88A proteins were inactive (<1% of

### TABLE ONE

| Protein | 3'-RNase (D10R2) | 3'-Pase (D9R1-p) | 3'-Pase (D11-p) |
|---------|-----------------|-----------------|-----------------|
| WT      | 100             | 100             | 100             |
| R14A    | 77              | 0.2             | 0.1             |
| D15A    | 110             | 3.3             | 4.9             |
| E21A    | 130             | ≤0.1            | ≤0.1            |
| Q40A    | 41              | 6.7             | 8.6             |
| H42A    | ≤0.1            | ≤0.1            | 0.2             |
| R46A    | 16              | 19              | 67              |
| H48A    | ≤0.1            | 0.8             | 0.2             |
| R66A    | 2.9             | 28              | 48              |
| R76A    | 1.1             | 16              | 44              |
| D83A    | 57              | 51              | 70              |
| Y88A    | 7.1             | 0.2             | ≤0.1            |

![FIGURE 4. Mutational effects on 3'-phosphatase activity.](image)

The dependence of the DNA 3'-phosphatase activity on the DNA template strand was gauged by comparing the D11-p primer-template to the D11-p single strand (Fig. 5C). The 3'-phosphatase specific activity on the primer-template substrate was ~130-fold greater than on the primer alone. Thus, the DNA 3'-phosphatase reaction, similar to the reaction at a ribonucleoside 3'-phosphate end (8), displays a fairly stringent requirement for the template DNA strand.

A kinetic analysis of the reaction of the collection of mutant PE proteins with the 5'-3₂P-labeled D11-p primer-template under conditions of enzyme excess is shown in Fig. 6; initial rates were quantified (Fig. 3C) and normalized to the rate of the wild-type enzyme (TABLE ONE). The R14A, E21A, H42A, H48A, and Y88A proteins were inactive (<1% of
**Pseudomonas DNA Ligase D**

**DISCUSSION**

**Pseudomonas LigD** is composed of phosphoesterase, ligase, and polymerase modules. It is homologous to mycobacterial LigD, which plays a critical role in an NHEJ pathway of DNA repair *in vivo* (2). Here we showed via several criteria the separation of the phosphodiesterase and phosphomonomoesterase functions of the PE domain. The key mechanistic distinction between the two activities is that the 3’-phosphodiesterase requires a 2’-OH, whereas the 3’-phosphomonomoesterase does not. The available data are consistent with a mechanism whereby the 2’-OH is a direct participant in the catalysis of the phosphomonomoesterase reaction but not required for the phosphodiesterase reaction.

**Structure Probing by Limited Proteolysis**—Native tag-free PE domain was subjected to proteolysis with increasing amounts of trypsin or chymotrypsin, and the products were analyzed by SDS-PAGE (Fig. 7). Initial scission by trypsin yielded two major fragments, T1 and T2. N-terminal sequencing revealed that the T1 fragment arose via cleavage between Arg-17 and Gln-18; the T2 fragment resulted from scission between Arg-28 and Lys-29 (cleavage sites indicated by arrows in Fig. 7). Although the T1 fragment decayed completely as the trypsin concentration was increased, the T2 fragment persisted at up to 200 ng of trypsin, a level at least 8-fold higher than that sufficient to digest all of the input native protein (Fig. 7). Thus, we surmised that the T2 fragment comprises a trypsin-resistant domain. Minor trypptic fragments T3 and T4 had the same N termini as the T2 fragment, indicating that they arose via trimming of C-terminal peptides.

Treatment with chymotrypsin yielded two major products, C1 and C2, which were generated by cleavage at the Tyr-10/Ala-11 (C1) and Phe-16/Arg-17 (C2) dipeptides, respectively. The C1 fragment was consumed as the chymotrypsin concentration was increased, but the C2 fragment was stable (Fig. 7). Note that the C2 cleavage site is 1 residue upstream from the T2 tryptic site (Fig. 7). These results suggest that the N-terminal segment of the PE domain is either disordered or exposed on the protein surface.

**N-terminal Deletions of the PE Domain**—The protease-sensitive N-terminal segment of the PE domain is hydrophilic; 13 of the first 30 side chains are either positively or negatively charged (Fig. 7). Included within the N-terminal segment are three functional groups (Arg-14, Asp-16, and Glu-21), that were identified in the alanine scan as important for 3’-phosphatase activity. To further probe the function of the N terminus, we produced a series of four truncated versions of the PE domain, with new N-terminal sites corresponding to the N termini of proteolytic fragments C1, C2, T1, and T2. The proteins were produced in bacteria as His$_{10}$ fusions and purified from soluble extracts by nickel-agarose chromatography. SDS-PAGE analysis revealed similar purities and the expected decrements in electrophoretic mobility (Fig. 8A).

Assay of the truncated proteins for 3’ resection of the D10R2 primer-template showed that they readily cleaved the terminal phosphodiester to form D10R1-p but were slowed in removing the 3’-PO$_4$ to form the D10R1 end product (Fig. 8B). A plot of the sum of the D10R1-p and D10R1 reaction products (expressed as the fraction of total labeled material) as a function of time showed that the initial rates of the C1, C2, T1, and T2 proteins were 83, 78, 100, and 100% of the wild-type rate, respectively (Fig. 8C).

Assays of 3’-phosphomonomoesterase activity with the D11-p primer-template showed that the initial rates of the C1, C2, T1, and T2 proteins were 26, 0.2, 0.1, and 0.2% of the wild-type rate, respectively (Fig. 8D). Thus, deletion of the N-terminal protease-sensitive fragment phenocopied the separation-of-function effects of point mutations within the N-terminal segment. We conclude that the N-terminal peptide of LigD is implicated in catalysis of the phosphomonomoesterase reaction but not required for the phosphodiesterase reaction.
topoisomerase-DNA adducts (16–21). Exonuclease III and mammalian polynucleotide kinase-phosphatase are well studied enzymes that have intrinsic DNA 3‘-phosphomonoesterase activities implicated in DNA break repair (14, 22–28). The 3‘-phosphomonoesterase of bacteriophage T4 polynucleotide kinase-phosphatase (29–31) plays a role in RNA 3‘ end healing during tRNA restriction/repair in phage-infected E. coli (32). Because the PE domain of LigD has no apparent structural or mechanistic similarity to either exonuclease III or mammalian/T4 polynucleotide kinase-phosphatase, we propose that it exemplifies a new family of 3‘-end-healing enzymes.

Our mutational analysis provides additional evidence that the phosphodiesterase and phosphomonoesterase activities rely on distinct structural components of the PE domain. In particular, mutations of a constellation of three conserved residues (Arg-14, Asp-15, and Glu-21) located within the protease-sensitive N-terminal segment selectively impair the 3‘-phosphomonoesterase activity of LigD. Deletion of the protease-sensitive N terminus elicited a similar loss of phosphomonoesterase activity, with relatively little impact on the phosphodiesterase. Mutations at two residues within the protease-resistant body of the PE domain, Gln-40 and Glu-82 (8), also selectively affected the 3‘-phosphate reaction. Therefore, we surmise that the active sites for the phosphomonoesterase and phosphodiesterase are not identical, although they likely overlap. Although we cannot conclude from the present results exactly how Arg-14, Asp-15, Glu-21, and Glu-82 contribute to the catalysis of 3‘-phosphate removal, we can infer from the concordant mutational effects with the D11-p and D9R1-p primer-templates that there is likely a single active site for 3‘-phosphate removal at a deoxynucleoside or ribonucleoside terminus. Why might the 3‘-phosphomonoesterase reaction call upon additional active site residues that are not required for the 3‘-ribonuclease? Conceivably, the extra charge on the ground state and transition state of the 3‘-phosphate compared with that of the 3‘-terminal phosphodiester engenders a need for an additional basic residue, such as Arg-14, or a polar functional group, such as Gln-40. The acidic side chains Asp-15, Glu-21, or

FIGURE 6. Mutational effects on DNA 3‘-phosphatase activity. Reaction mixtures containing 50 nM 32P-labeled D11-p primer-template (shown at the bottom) and 5 μM wild-type (WT) or Ala-mutant PE domain were incubated at 37 °C. Aliquots were withdrawn at the times indicated and quenched immediately with EDTA/formamide. The time 0 sample was withdrawn prior to adding the PE domain. The products were resolved by PAGE and visualized by autoradiography. The labeled species corresponding to the D11-p substrate (3‘-P) and the D11 product (3‘-OH) are indicated by arrowheads on the right.

FIGURE 7. Limited proteolysis of the PE domain. Aliquots (10 μg) of the tag-free PE domain were digested with the indicated amounts of trypsin or chymotrypsin. The digests were resolved by SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. The Coomassie Blue-stained membrane is shown. Slices containing tryptic fragments T1, T2, T3, and T4 and chymotryptic fragments C1 and C2 were excised and subjected to automated Edman sequencing. The amino acid sequence of the N-terminal 100-amino-acid segment of LigD is shown at the bottom, with the amino termini of the C1, C2, T1, T2, T3, and T4 proteolytic fragments indicated by arrows. The 11 conserved residues that were changed to alanine in the present study are indicated by ●. Four essential residues identified previously (8) are highlighted in shaded boxes. Nonessential residue glu-54 is denoted by •.
Glu-82 might play a role in either orienting or activating the water nucleophile or in donating a proton to the 3'-O-leaving group.

The effects of alanine mutations at Arg-14 and Glu-21 (and of N-terminal deletions C2, T1, and T2) on the isolated 3'-phosphatase reactions with either the D9R1-p or D11-p primer-templates were apparently more severe than their impact on the 3'-PO4 removal step of the ribonucleotide resection mechanism assayed using the D10R2 primer-template. For example, although the R14A, E21A, and T1 proteins generated virtually no 3'-OH products during a 30-min reaction with the 3'-PO4 primer-template substrates (Figs. 4, 7, and 8D), they clearly did convert a fraction of the D10R1-p intermediate to D10R1-OH during the 30-min ribonucleotide resection assay (Figs. 2 and 8B). A simple interpretation of the "leakiness" of the phosphatase-defective mutations using the D10R2 primer-template substrate is that the requirement for certain constituens of the phosphatase active site is more stringent when the 3'-PO4 terminus is formed in situ on the enzyme by the action of the phosphodiesterase catalytic center. This line of thinking posits that, under the reaction conditions employed in our studies, the 3'-PO4 intermediate in the ribonucleotide resection reaction is retained at the active site of the enzyme, where it is immediately accessible to the phosphomonoesterase (without the need for dissociation and rebinding).

Six amino acids have now been identified as essential/important for both the 3'-ribonuclease and 3'-phosphatase functions. Mutations of His-42, His-48, Asp-50, Arg-52, and His-84 abolish virtually all activity, while mutation of Tyr-88 reduces ribonuclease and phosphatase activity to 7% and ≤1% of wild-type, respectively. Either these six side chains are essential for global folding of the PE domain or they contribute essential components of an overlapping active site for the phosphodiesterase and phosphomonoesterase functions. Limited tryptic digestion of the H42A, H48A, D50A, R52A, H84A, and Y88A proteins revealed a pattern of resistant fragments that was virtually identical to that of the wild-type PE protein (data not shown); thus we suspect that the essential residues are not critical for global folding. Rather, we propose that they are catalytic residues at a shared active site that perform functions common to both the phosphodiesterase and phosphomonoesterase reactions, e.g., coordination of the essential manganese cofactor (possibly by His-42, His-48, Asp-50, and His-84) and stabilization of the transition state on the reactive phosphate (by Arg-52 and perhaps one of the histidines).

In summary, our biochemical analysis of the PE domain of LigD reveals unexpected complexity whereby overlapping but nonidentical protein functional groups are responsible for catalysis of the mechanistically distinct phosphodiesterase and phosphomonoesterase reactions. Although a detailed reaction scheme will hinge on obtaining an atomic structure of the PE domain, the loss-of-function and separation-of-function mutations characterized here provide a set of genetic tools to gauge the roles of the phosphomonoesterase and phosphodiesterase reactions in LigD-dependent DNA repair pathways in vivo.

REFERENCES

1. Gong, C., Martins, A., Bongiorno, P., Glickman, M., and Shuman, S. (2004) J. Biol. Chem. 279, 20594–20606
2. Gong, C., Bongiorno, P., Martins, A., Stephanou, N. C., Zhu, H., Shuman, S., and Glickman, M. S. (2005) Nat. Struct. Mol. Biol. 12, 304–312
3. Weller, G. R., Kyvelou, B., Roy, R., Tonkin, L. M., Scanlan, E., Della, M., Devine, S. K., Day, J. P., Wilkinson, A., di Fagagna, F., Devine, K. M., Bowater, R. P., Jeggo, P. A., Jackson, S. P., and Doherty, A. J. (2002) Science 297, 1686–1689
4. Della, M., Palmbos, P. L., Tseng, H. M., Tonkin, L. M., Daley, J. M., Topper, L. M.,
Pitcher, R. S., Tomkinson, A. E., Wilson, T. E., and Doherty, A. J. (2004) *Science* **306**, 683–685

5. Aravind, L., and Koonin, E. V. (2001) *Genome Res.* **11**, 1365–1374

6. Doherty, A. J., Jackson, S. P., and Weller, G. R. (2001) *FEBS Lett.* **500**, 186–188

7. Zhu, H., and Shuman, S. (2001) *J. Biol. Chem.* **276**, 25973–25981

8. Mosesso, E., and Lima, C. D. (2000) *Mol. Cell* **5**, 865–876

9. Evans, C. J., and Aguilara, R. J. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102**, 1–15

10. Weber, D. J., Gittis, A. G., Mullen, G. P., Abeygunawardana, C., Lattman, E. E., and Mildvan, A. S. (1992) *Proteins* **13**, 275–287

11. Henner, W. D., Grunberg, S. M., and Heseltine, W. A. (1982) *J. Biol. Chem.* **257**, 11749–11754

12. Henner, W. D., Rodriguez, L. O., Hecht, S. M., and Heseltine, W. A. (1983) *J. Biol. Chem.* **258**, 711–713

13. Wiederhold, L., Leppard, J. B., Kedar, P., Karimi-Busheri, F., Rasouli-Nia, A., Weinfield, M., Tomkinson, A. E., Izumi, T., Prasad, R., Wilson, S. H., Mitra, S., and Haza, T. K. (2004) *Mol. Cell* **15**, 209–220

14. Zharkov, D. O., Shoham, G., and Grollman, A. P. (2003) *DNA Repair (Amst.)* **2**, 839–862

15. Yang, S., Burgin, A. B., Huizenga, B. N., Robertson, C. A., Yao, K. C., and Nash, H. A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 11534–11539

16. Davies, D. R., Interalth, H., Champoux, J. J., and Hol, W. G. I. (2003) *Chem. Rev.* **10**, 139–147

17. Interalth, H., Chen, H. J., Kehl-Fie, T. E., Zotzmann, J., Leppard, J. B., and Champoux, J. J. (2005) *EMBO J.* **24**, 2224–2233

18. El-Khamisy, S. F., Saifl, G. M., Weinfield, M., Johansson, F., Hellday, T., Lupsik, J. R., and Caldecott, K. W. (2005) *Nature* **434**, 108–113

19. Lisby, M., Krogh, B. O., Boege, F., Westergaard, O., and Knudsen, B. (1998) *Biochemistry* **37**, 10815–10829

20. Krogh, B. O., and Shuman, S. (2000) *Biochemistry* **39**, 6422–6423

21. Bernstein, N. K., Williams, R. S., Rakovszky, M. L., Cui, D., Green, R., Karimi-Busheri, F., Mani, R. S., Galicia, S., Koch, C. A., Cass, C. E., Durocher, D., Weinfield, M., and Glover, J. N. M. (2005) *Mol. Cell* **17**, 657–670

22. Rasouli-Nia, A., Karimi-Busheri, F., and Weinfield, M. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 6905–6910

23. Henner, W. D., Grunberg, S. M., and Heseltine, W. A. (1983) *J. Biol. Chem.* **258**, 15198–15205

24. Cunningham, R. P., Saporito, S. M., Spitzer, S. G., and Weiss, B. (1986) *J. Bacteriol.* **168**, 1120–1127

25. Cameron, V., and Uhlenbeck, O. C. (1977) *Biochemistry* **16**, 5120–5126

26. Wang, L. K., and Shuman, S. (2002) *Nucleic Acids Res.* **30**, 1073–1080

27. Gallburt, E. A., Pelletier, J., Wilson, G., and Stoddard, B. L. (2002) *Structure* **10**, 1249–1260

28. Amitsur, M., Levitz, R., and Kaufman, G. (1988) *EMBO J.* **6**, 2499–2503

29. Wang, L. K., and Shuman, S. (2001) *J. Biol. Chem.* **276**, 26868–26874