Substrate Specificity and Structure-Function Analysis of the 3′-Phosphoesterase Component of the Bacterial NHEJ Protein, DNA Ligase D*

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DNA ligase D (LigD) performs end remodeling and end sealing reactions 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 phosphoesterase (PE) module. The PE domain catalyzes manganese-dependent phosphodiesterase and phosphomonoesterase reactions at the 3′ end of the primer strand of a primer-template. The phosphodiesterase cleaves a 3′-terminal diribonucleotide to yield a primer strand with a ribonucleoside 3′-PO4 terminus. The phosphomonoesterase converts a terminal ribonucleotide 3′-PO4 or deoxynucleoside 3′-PO4 of a primer-template to a 3′-OH. Here we report that the phosphodiesterase and phosphomonoesterase activities are both dependent on the presence and length of the 5′ single-strand tail of the primer-template substrate. Although the phosphodiesterase activity is strictly dependent on the 2′-OH of the penultimate ribose, it is indifferent to a 2′-OH versus a 2′-H on the terminal nucleoside. Incision at the ribonucleotide linkage is suppressed when the 2′-OH is moved by 1 nucleotide in the 5′ direction, suggesting that LigD is an exoribonuclease that cleaves the 3′-terminal phosphodiester. We report the effects of conservative amino acid substitutions at residues: (i) His42, His48, Asp50, Arg52, His84, and Tyr88, which are essential for both the ribonuclease and phosphatase activities; (ii) Arg14, Asp15, Glu21, and Glu82, which are critical for 3′-phosphatase activity but not 3′-ribonuclease removal; and (iii) at Lys66 and Arg76, which participate selectively in the 3′-ribonuclease reaction. The results suggest roles for individual functional groups in metal binding and/or phosphoesterase chemistry.

DNA ligase D (LigD)2 is a multifunctional DNA repair enzyme implicated in a recently described bacterial pathway of nonhomologous end joining (NHEJ) (1–4). Pseudomonas aeruginosa LigD consists of a central DNA ligase catalytic domain (Lig) fused to a C-terminal polymerase domain (Pol) and an N-terminal phosphoesterase module (PE) (5–7). The Pol and PE domains are suggested to remodel the 3′-end of the broken strands prior to sealing. These activities could contribute to the error-prone character of NHEJ in vivo (2). Genetic evidence in mycobacteria implicates the Pol activity of LigD as the direct agent of the high incidence of +1 frameshift mutations at blunt ends during NHEJ in vivo (8). The LigD Pol domain, which is structurally related to archaean DNA primases (8), catalyzes two types of primer extension synthesis reactions in vitro: (i) templated fill-in of a 5′-overhang and (ii) nontemplated addition of single nucleotides at a blunt end (5, 6). The Pol domain can use either rNTPs or dNTPs as substrates for the primer extensions.

The 187-amino acid PE domain of Pseudomonas LigD was described initially as a nuclease capable of resecting a short tract of 3′-ribonucleotides on a primer-template substrate to the point that the primer strand had a single 3′-ribonucleotide remaining (6). The failure to digest into the DNA strand beyond this point reflects a stringent requirement for a 2′-OH group on the penultimate nucleoside of the primer strand (6). The ribonuclease resection activity is the result of two component steps: (i) the 3′-terminal nucleoside is first removed to yield a primer strand with a ribonucleoside 3′-PO4 terminus; (ii) the 3′-PO4 is hydrolyzed to a 3′-OH. The 3′-ribonuclease and 3′-phosphatase activities are both dependent on manganese (6). The PE domain also catalyzes manganese-dependent hydrolysis of the 3′-PO4 of an all-DNA primer-template substrate (7), signifying 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 (6, 7).

The PE domain of LigD has no apparent structural or mechanistic similarity to previously characterized nucleases or 3′-phosphatases. Thus, we speculated that it exemplifies a novel phosphoesterase family (6). An initial alanine scan of 16 residues of Pseudomonas LigD identified six side chains (His42, His48, Asp50, Arg52, His84, and Tyr88) as essential for both the 3′-ribonuclease and 3′-phosphatase activities, five residues (Arg14, Asp15, Glu21, Glu82, and Lys66) that were important for 3′-phosphatase activity, but not 3′-ribonucleoside removal, and two amino acids (Lys66 and Arg76) that were required selectively for the 3′-ribonuclease (6, 7). These results indicated that the active sites for the ribonuclease and phosphatase functions overlap but are not identical.

Here we conduct further studies of the substrate specificity of the PE domain, focusing on the contributions of the template DNA strand to the remodeling of the 3′ end of the primer strand. We find that the single-strand tail of the template strand accelerates the rate of the ribonuclease resection and 3′-phosphatase reactions by factors of 20 and 28, respectively, with optimal activation occurring when the tail length is 8–12 nucleotides. By varying the sugar at the end of a primer strand containing a single penultimate ribonucleotide, we show that the phosphodiesterase reaction does not depend on a terminal 2′-OH. By varying the position of the single ribonucleotide, we demonstrate that LigD acts specifically at the terminal phosphodiester. Furthermore, we characterize the effects of 26 conservative mutants in the 13 amino acids of the PE domain that were implicated via alanine scanning in the phosphodiesterase or phosphomonoesterase reactions. The present findings emphasize the unique structural and mechanistic features of LigD vis à vis other 3′-processing enzymes.

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2 The abbreviations used are: LigD, ligase D; NHEJ, nonhomologous end joining; Pol, polymerase domain; PE, phosphoesterase; Phk, polynucleotide kinase-phosphatase; WT, wild-type.
Pseudomonas DNA Ligase D

EXPERIMENTAL PROCEDURES

PE Domain Mutants—Missense mutations were introduced into the pET-PaeLigD-PE plasmid as described previously (6, 7). The inserts of the mutant plasmids were 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 β-D-thiogalactopyranoside, preparation of soluble bacterial lysates, and purification of the His_{60}-tagged PE proteins by nickel-agarose affinity chromatography were performed as described (6). The 200 mM imidazole eluate fractions containing the PE domain were stored at −80 °C. Protein concentrations were determined by using the Bio-Rad dye reagent with bovine serum albumin as the standard.

3′-Ribonuclease Assay—The 5′-32P-labeled 12-mer D10R2 strand was annealed to a 4-fold excess of unlabeled complementary 24-mer, 20-mer, 16-mer, or 12-mer DNA strands to form the primer-templates depicted in Fig. 1A. The 3′-ribonuclease reaction mixtures (80 μl) containing 50 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol, 0.5 mM MnCl₂, 50 mM 5′-32P-labeled D10R2 primer-template, 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 electrophoresis through a 40-cm 18% polyacrylamide gel containing 7 M urea in TBE (90 mM Tris borate, 2.5 mM 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 (6). 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 dithiothreitol, 0.5 mM MnCl₂, 50 mM 5′-32P-labeled D9R1-p primer-template (see Fig. 3C), 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′-PO₄ 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 (5′-cAATTGGCCGcC) was annealed to a 4-fold excess of unlabeled complementary 24-mer, 20-mer, 16-mer, or 12-mer DNA strands to form the primer-templates depicted in Fig. 1C. The DNA 3′-phosphatase reaction mixtures (80 μl) containing 50 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol, 0.5 mM MnCl₂, 50 mM 5′-32P-labeled D11-p primer-template, 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 and quantified as described above.

RESULTS AND DISCUSSION

3′-Ribonuclease and 3′-Phosphatase Activities at a Primer Terminus Depend on the 5′ Single-strand Tail of a Template DNA Strand—Pseudomonas LigD prefers to remodel the recessed 3′ ends of the primer strand of a duplex primer-template substrate compared with a single-strand of identical composition (6, 7). The substrate for the 3′-ribonuclease reaction consists of a 12-nucleotide 5′-32P-labeled primer strand (D10R2, composed of 10 deoxynucleotides and a 3′-terminal diribonucleotide) annealed to a 24-mer DNA strand to form a 12-bp duplex with a 12-nucleotide 5′ tail (Fig. 1A, left panel). To gauge the contributions, if any, of the single-strand tail to the 3′-phosphodiesterase activity, we annealed the D10R2 primer to a series of incrementally shortened template strands to form substrates with 8- or 4-nucleotide 5′ tails attached to identical 12-bp duplex segments (Fig. 1A, middle panels), and a substrate consisting only of the 12-bp duplex with no single-strand tail (Fig. 1A, right panel). We examined the kinetics of the reaction of the LigD PE domain with this set of primer-templates under conditions of enzyme excess (Fig. 1A). The labeled D10R2 strand of the 12-mer/24-mer substrate was converted first to a 3′-phosphorylated species, D10R1-p, which was then converted to a more slowly migrating 3′-OH end-product, D10R1 (Fig. 1A). The rate of the 3′-phosphodiesterase reaction was quantified in Fig. 1B 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. The reaction was completed in 10 min and the data fit to a single exponential with an apparent rate constant of 0.01 s⁻¹, which agrees with value reported previously (6).

The extent of substrate decay and the distribution of the reaction products were essentially unchanged for the primer-template containing an 8-nucleotide 5′ tail (Fig. 1A, A and B). However, further shortening of the tail to 4 nucleotides slowed both the conversion of D10R2 to D10R1-p and the appearance of the dephosphorylated D10R1 end-product (Fig. 1A); the apparent rate constant for the phosphodiesterase step (0.0018 s⁻¹) was slowed by a factor of 5.5 compared with the substrates with longer 5′ tails (Fig. 1B). The reaction of the PE domain with the blunt-end 12-bp duplex was even more feeble, and there was virtually no D10R1 end product formed at a time when ~60% of the input primer had been converted to D10R1-p (Fig. 1, A and B). We estimated a phosphodiesterase rate constant of 0.00053 s⁻¹ for the 12-bp duplex, which indicated that the 5′ tail accelerates the rate of the 3′-ribonucleoside removal by a factor of 20.

The 3′-phosphatase step can be studied in isolation using a substrate composed of a 5′-32P-labeled 11-mer DNA strand with a 3′-PO₄ terminus (D11-p) annealed to a 24-mer DNA strand to form the primer-template shown in Fig. 1C. This molecule, which has a 13-nucleotide 5′ tail attached to an 11-bp duplex, was rapidly converted by excess PE domain to a single D11 product (Fig. 1C). The reaction was complete in 2–5 min and attained 77% of the end point value in 1 min. From this datum, we calculated a DNA 3′-phosphatase rate constant of 0.025 s⁻¹, which agreed with the value reported previously (7). Annealing of the D11-p strand to complementary 20-mer, 16-mer, and 12-mer strands yielded a series of primer-templates containing an 11-bp duplex segment and 5′ tails of 9, 5, or 1 nucleotide (Fig. 1C). Whereas the rate and extent of 3′-dephosphorylation of the D11-p/20-mer primer template was identical to that of the D11-p/24-mer, shortening the tail to 5 nucleotides in the D11-p/16-mer substrate caused a 2.5-fold decrement in the rate constant to 0.01 s⁻¹ (Fig. 1C). When the 5′ tail was retracted to only 1 nucleotide in the D11-p/12-mer duplex, the rate was reduced more dramatically, to 0.00088 s⁻¹. The kinetic profile for 3′-dephosphorylation of the D11-p/12-mer duplex was almost identical to that observed for removing the 3′-phosphate from the D11-p strand alone (Fig. 1C). These data underscore that the 5′ tail accelerates the rate of the DNA 3′-phosphatase reaction by a factor of 28.

To our knowledge, the strong dependence of the 3′-phosphatase activity of LigD on a complementary template strand with a 5′ tail has not been noted previously for other biochemically characterized polynucleotide 3′-phosphatase enzymes, which include bacteriophage T4 polynucleotide kinase-phosphatase (PnkP) (9), mammalian PnkP (10), the budding yeast DNA 3′-phosphatase Tp11 (11), and E. coli exonuclease III (12). For example, the DNA 3′-phosphatase reaction of
mammalian Pnkp is equally effective on nicked or gapped double-stranded substrates or with single-stranded oligonucleotides of lengths varying from 3 to 21 nucleotides (10). Yeast Tpp1, although unable to dephosphorylate a 3′-PO4-terminated single strand, is equally adept at removing the 3′-PO4 from an internal nick or gap in duplex DNA, a recessed 3′ end of a 5′-tailed primer-template, or a blunt DNA duplex with no single-strand tail (11). The phosphatase component of T4 Pnkp hydrolyzes a 3′-PO4 from either single-stranded polynucleotides or free 3′-mononucleotides (9). Mycobacteriophage and vibriophage Pnkp enzymes also hydrolyze a 3′-mononucleotide substrate (13).

3′-Phosphodiesterase Activity Does Not Require a Terminal 2′-OH—Whereas the 3′-phosphodiesterase activity of LigD is strictly dependent on a 2′-OH of the penultimate nucleoside (6), the role of the terminal 2′-OH has not been tested. To address this issue, we exploited a new primer strand, D10RD, composed of 10 deoxynucleotides, a single penultimate ribonucleotide, and a 3′-terminal deoxynucleotide. D10RD differs from the standard D10R2 primer only insofar as it lacks a terminal 2′-OH group. 5′-32P-labeled D10R2 and D10RD were annealed to a complementary 24-mer strand to form the primer-templates depicted in Fig. 2A. The differences in the terminal sugar resulted in a difference of electrophoretic mobility of the strands, whereby D10RD migrated slightly ahead of D10R2 (Fig. 2A, − lanes). Both substrates were converted quantitatively by the LigD PE domain to the D10R1 end-product (Fig. 2A, + lanes). To more finely compare how well the LigD PE domain processes these substrates, we performed an enzyme titration experiment shown in Fig. 2B. The extent of product formation increased with PE concentration. At limiting enzyme levels, the products consisted of a mixture of D10R1-p and D10R1 strands. At the highest level of enzyme, nearly all the substrate was converted to D10R1.

The titration profiles were virtually identical for the D10R2 and D10RD substrates. We conclude that cleavage of the 3′-phosphodiester linkage is indifferent to a ribose versus deoxyribose sugar on the departing 5′-OH nucleoside.

LigD Measures and Cleaves the Terminal Ribonucleotide Phosphodiester—We showed previously that LigD could resect 2′ribonucleotides from the 3′ end of a D9R3 primer-template to form a D9R1 end product (6). The kinetic analysis of the processing of the D9R3 substrate was complicated by the fact that a potential reaction intermediate, D9R2-p, comigrated with the D9R1 end product. Thus, we could not distinguish between two alternative reaction schemes in which: (i) LigD initially incises the phosphodiester of the terminal (rC)p(rC) dinucleotide to release cytidine and leave the 11-mer D9R2-p, which is converted to D9R1-p, and then dephosphorylated to form the D9R1 end product; or (ii) LigD initially cleaves at the second phosphodiester from the 3′ terminus to release a diribonucleotide and leave the 10-mer D9R1-p, which is then dephosphorylated to D9R1. The key mechanistic issue is whether LigD is obliged to cleave the terminal phosphodiester.

We addressed this question by using primer strand D9RD2, which consists of nine 5′-deoxynucleotides, a single ribonucleotide, and two 3′-terminal deoxynucleotides (Fig. 2A). The D9RD2 primer-template...
contains a single potential cleavage site for the LigD phosphodiesterase, which is phased 1 nucleotide inward from the 3' end compared with the D10RD substrate (Fig. 2A). Whereas D10RD was quantitatively processed by the PE domain, the D9RD2 substrate was highly refractory, such that 91% of the primer strand remained intact (Fig. 2A). Scant amounts of two products were formed (denoted by the arrowheads in Fig. 2A) that migrated ahead of D10R1 and apparently correspond to D9R1-p and D9R1. We surmise that the LigD phosphodiesterase active site is poised specifically over the terminal ribonucleoside phosphodiester and is not very effective at reaching inward by one nucleotide to cleave a penultimate ribonucleoside phosphodiester. Thus, we designate LigD as a 3'-exonuclease that releases 5'-OH mononucleoside products.

**Structure-Activity Relationships at Residues Essential for 3'-Phosphodiesterase and Monoesterase Activity**—Six amino acids were identified previously as essential for both the 3'-ribonuclease and 3'-phosphatase functions. Alanine mutations at His42, His48, Asp50, Arg52, and His84 abolished activity and mutation of Tyr88 reduced ribonuclease and 3'-phosphatase activity by 65 and 91%, respectively (6, 7). Because limited tryptic digestion of the Ala mutant proteins revealed a pattern of resistant fragments that was virtually identical to that of the wild-type PE protein, it was inferred that the six essential residues are not critical for global folding (7). Rather, we proposed that they are catalytic residues at a shared active site that perform functions common to both the phosphodiesterase and phosphomonoesterase reactions.

To illuminate structure-activity relationships for the six putative active site residues, we introduced two conservative substitutions at each position. Histidine was replaced by asparagine and glutamine, aspartate by glutamate and asparagine, arginine by lysine and glutamine, and tyrosine by phenylalanine and serine. The wild-type PE domain and the 12 conservative mutants were produced in *E. coli* as His10 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, as noted previously, migrated anomalously at 30 kDa (predicted size: 24 kDa) (Fig. 3A).

**Mutational effects on 3'-ribonucleotide resection** were gauged by analysis of the reaction of the PE protein with the 32P-labeled D10R2 primer-template under conditions of enzyme excess. Wild-type PE quantitatively converted the labeled strand to a 3'-OH end product, D10R1 (Fig. 3B). The H42N, H42Q, H48N, H48Q, D50E, D50N, R52K, R52Q, H84N, and H84Q mutants were virtually inert. We conclude that His42, His48, Asp50, Arg52, and His84 are strictly essential for the 3'-phosphodiesterase function of LigD. Whereas the Y88S mutant displayed feeble 3'-ribonucleotide resection activity resulting in the formation of the D10R1-p intermediate only, the Y88F mutant consumed all of the input substrate to generate predominantly D10R1-p and only a small
amount of the D10R1 end product (Fig. 3B). The results emphasize that the aromatic ring of Tyr88, not the hydroxyl, is the relevant contributor to the 3′-phosphodiesterase reaction, although a role for the tyrosine hydroxyl in the 3′-phosphatase step is suggested.

Mutational effects on the 3′-phosphatase were assayed directly by reacting the PE proteins with a 32P-labeled D9R1-p primer-template under conditions of enzyme excess (Fig. 3C). Wild-type PE converted nearly all of the input D9R1-p labeled strand to a slower migrating D9R1 species. All 12 mutants were virtually inert in the isolated 3′-phosphatase reaction (Fig. 3C). (The trace amounts of radiolabeled oligonucleotide comigrating with the D9R1 species were present at similar levels in a control reaction from which PE domain was omitted; data not shown). Thus, His42, His48, Asp50, Arg52, and His84 are strictly essential for the 3′-phosphomonoesterase and the 3′-phosphodiesterase.

Coordination of the manganese cofactor and stabilization of the transition state on the reactive phosphate are the likely essential contributions of the enzyme to both the phosphodiesterase and phosphomonoesterase reactions. We speculate that Arg52 coordinates the reactive phosphate and stabilizes the extra charge developed on a presumptive associative transition state. The finding that lysine could not functionally substitute for Arg52 suggests that this side chain makes a bidentate contact with two of the phosphate oxygens. Any of the essential histidines are also candidates to contact the reactive phosphate, although it might be thought that a simple hydrogen bond between a histidine and the phosphate could have been attained by conservative substitutions with asparagine or glutamine (which mimic the histidine Nδ and Ne, respectively).

We suggest instead that His42, His48, and His84, together with Asp50, comprise a coordination complex for the manganese ion(s) that are required for catalysis. Aspartates are routine components of the metal-binding sites of many enzymes involved in phosphoryl transfer. Yet, histidines are also commonly involved in metal binding, especially the coordination of manganese, as in the binuclear metallophosphoesterase superfamily that includes lambda phosphatase and the DNA 3′-exonuclease Mre11, where either three or four histidines serve to chelate two manganese ions at the active site (14, 15). It is worth emphasizing that there is no apparent conservation of primary structure between the binuclear metallophosphoesterases (or any other phosphotransferase family) and the LigD PE domain. Nor is it clear, at present, whether the LigD PE domain uses a one-metal or a two-metal mechanism.

The fact that none of the three essential histidines could be substituted functionally by Asn or Gln raises an alternative possibility: that...
one or more of the histidines might serve as general acid-base catalysts. The putative role of a general acid would be to donate a proton to the 5′-O of the leaving nucleoside in the phosphodiesterase reaction or to the sugar 3′-O of the primer strand during the phosphomonoesterase reaction. It is not obvious how a single histidine could serve both roles, given that the position of the leaving group at the reactive phosphorus center is different in the two reactions. The role of a general base would be to abstract a proton from the attacking nucleophile. Although this nucleophile is clearly water in the phosphomonoesterase reaction, it is not yet established whether the phosphodiesterase reaction entails a nucleophile other than water. For example, glutamic acid has been implicated in the phosphodiesterase reaction, but this has not been shown definitively.

Conservative Mutations That Selectively Impede the Phosphodiesterase Activity of Lg2D—In addition to their reliance on the same side chains analyzed above, the phosphodiesterase and phosphomonoesterase activities depend on distinct structural components of the PE domain, as revealed by separation-of-function mutations described previously (6, 7). For example, alanine substitutions for conserved residues Arg14, Asp15, and Glu21 (located within the protease-sensitive N-terminal segment of the PE domain) selectively impaired the 3′-phosphomonoesterase activity. Indeed, a deletion of the protease-sensitive N terminus elicited similar loss of phosphomonoesterase activity, with relatively little impact on the phosphodiesterase (7). Mutations at two residues within the protease-resistant body of the PE domain, Gln60 and Gln82, also selectively affected the 3′-phosphatase reaction. In contrast, alanine substitutions for Lys96 and Arg26 impaired the 3′-ribonuclease activity but had only a modest effect on 3′-phosphatase.

To further probe the unique requirements for the phosphomonoesterase and phosphodiesterase reactions, we introduced two conservative substitutions at each of the seven residues at which alanine changes differentially affected the diesterase and monoesterase activities. Arginine and aspartate were substituted as described above; glutamate was replaced by glutamine and aspartate, glutamine by glutamate and asparagine, and lysine by arginine and glutamine. The 14 conservative mutants were produced as His10 fusions and purified from soluble E. coli lysates by nickel-affinity chromatography. Their purity was equivalent to that of the proteins shown in Fig. 3A (data not shown).

A kinetic analysis of the reaction of the PE proteins with the 32p-labeled D10R2 primer-template under conditions of enzyme excess is shown in Fig. 4. Distinct classes of mutational effects on 3′-ribonuclease activity; (ii) the rates of substrate consumption by the R14K, R14Q, D15E, E21D, E21Q, E82D, and E82Q mutants were similar to wild-type and the normally transient D10R1 p intermediate accumulated as a predominant product; (iii) the rates of substrate decay were similar to (D15N) or moderately slower than (K66R,R76K) the wild-type PE domain, and the D10R1-p intermediate was efficiently converted to the D10R1 end product; (iv) Q40N consumed the substrate slowly, yet D10R1-p was converted to D10R1.

The impact of the conservative mutations on the rate of the phosphodiesterase reaction was quantified in Fig. 5 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 most severe effects were exerted by mutations Q40E, R76Q, and K66Q, which reduced the initial rate to 0.3, 0.6, and 1.2% of the wild-type value, respectively (Table 1). The R76Q and K66Q defects in the rate of 3′-ribonucleoside removal were similar to those reported previously for R76A and K66A (Table 1), and they contrast with the significant restoration of activity (37–47% of wild-type initial rate) seen with the R76K and K66R proteins (Fig. 5; Table 1). We conclude that a pos-

| Protein   | 3′-RNase (D10R2) | 3′-Pase (D9R1-p) | 3′-Pase (D11-p) |
|-----------|-----------------|-----------------|-----------------|
| WT        | 100             | 100             | 100             |
| R14A*     | 77              | 0.2             | 0.1             |
| R14K      | 160             | 1.3             | ≤0.1            |
| R14Q      | 130             | 0.7             | 0.2             |
| D15A*     | 110             | 3.3             | 4.9             |
| D15E      | 130             | 6.0             | 14              |
| D15N      | 140             | 54              | 79              |
| E21A*     | 130             | ≤0.1            | ≤0.1            |
| E21D      | 140             | ≤0.1            | ≤0.1            |
| E21Q      | 130             | 0.8             | 0.1             |
| Q40A*     | 41              | 6.7             | 8.6             |
| Q40E      | 0.3             | 0.6             | 1.0             |
| Q40N      | 8.2             | 38              | 72              |
| K66A*     | 2.9             | 28              | 48              |
| K66R      | 37              | 53              | 95              |
| K66Q      | 1.2             | 13              | 33              |
| R76A*     | 1.1             | 16              | 44              |
| R76K      | 47              | 38              | 98              |
| R76Q      | 0.6             | 9.7             | 21              |
| E82A*     | 110             | ND              | ND              |
| E82D      | 140             | 1.7             | 1.9             |
| E82Q      | 150             | 0.2             | ≤0.1            |

a The kinetic data for the alanine mutants are from previous studies (6, 7).
itive charge at positions 66 and 76 is critical for the phosphodiesterase reaction. The severity of the Q40E change contrasts with the relatively benign effect of the Q40A substitution noted previously (41% of wild-type initial rate; Table 1), which suggests that the negative charge of the Glu40 side chain exerts a directly deleterious effect. The Q40N mutation (8.2% of wild-type rate) was also more deleterious than Q40A, although not as severe as glutamate.

The rates and extents of 3'-ribonucleoside resection by the R14K, R14Q, D15E, D15N, E21D, E21Q, E82D, and E82Q proteins were equal to or slightly higher than that of the wild-type PE domain (Fig. 5; Table 1). The findings are consistent with the results obtained previously for the corresponding alanine changes, thereby underscoring that Arg14, Asp15, Glu21, and Glu82 play no significant role in the phosphodiesterase reaction.

Conservative Mutations That Selectively Affect the Phosphomonooesterase Activity—A kinetic analysis of the 3'-phosphatase reaction of the PE proteins with a 32P-labeled D9R1-p primer-template under conditions of enzyme excess is shown in Fig. 6 and quantified in Fig. 7. As expected, wild-type PE converted nearly all of the input labeled D9R1-p strand to a slower-migrating D9R1 species. Mutants R14K, R14Q, E21Q, E21D, E82Q, and E82D, all of which had wild-type levels of 3'-phosphodiesterase activity, were severely impaired with respect to 3'-phosphomonoesterase function (Fig. 6). Their rates of 3'-phosphate removal were 1.3, 0.7, 0.8, 0.1, 0.2, and 1.7% of the wild-type value, respectively (Fig. 7; Table 1) and were in accord with the rates of the respective alanine mutations reported previously (Table 1). These results underscore stringent requirements for the Arg14, Glu21, and Glu82 functional groups in the 3'-phosphomonoesterase reaction. This contrasts with the situation at Asp15, where conservative replacement by asparagine restored activity to 54% of wild-type, compared with 3.3 and 6% for D15A and D15E, respectively (Fig. 6; Table 1). The finding that the carboxylate moiety is not strictly essential, and can be replaced by the isosteric amide of asparagine, suggests that hydrogen bonding is the relevant contribution of this side chain to the 3'-phosphatase reaction.

We also tested mutational effects on the kinetics of the DNA 3'-phosphatase reaction using the D11-p primer template (Fig. 8). The initial rates were quantified, and initial rates of the conservative mutants were normalized to that of the wild-type enzyme (Table 1). The mutational effects at Arg14, Asp15, Glu21, and Glu82 on DNA 3'-phosphatase activity agreed with the results obtained for phosphomonoesterase activity on the D9R1-p primer-template (Table 1), which suggests that: (i) the PE domain exploits the same active site for 3'-phosphate resection.
hydrolysis at a ribonucleotide or deoxyribonucleotide end; and (ii) Arg^{14}, Asp^{15}, Glu^{32}, and Glu^{82} are specific constituents of the monoesterase active site.

The initial description of Gln^{40} as a candidate "phosphomonoesterase-specific" residue was based on the finding that the Q40A mutation reduced the rate of the 3'-phosphatase reaction to 6.7 and 8.6% of wild-type on the D91R-p and D11-p substrates, while having only a modest effect on the 3'-ribonuclease reaction (41% of wild type). The Q40E substitution, which abolished 3'-ribonucleoside removal, also suppressed the 3'-phosphatase activity with the D91R-p and D11-p primer-templates (0.6–1.0% of the wild-type initial rates; Table 1). This interference of glutamate with the diesterase and monoesterase reactions is not consistent with Gln^{40} being a specific component of a phosphomonoesterase active site distinct from that of the phosphodiesterase. This conclusion is reinforced by the observation that the conservative Q40N mutant displays a gain of function in 3'-phosphatase relative to Q40A (to 38 and 72% with the D91R-p and D11-p substrates, respectively) while suffering a loss-of-function in 3'-ribonucleoside removal than in 3'-phosphate hydrolysis. The K66Q and R76Q proteins were 13 and 9.7% as active as wild-type with D91R-p and 33 and 21% as active with D11-p (Table 1). The relative activities of the glutamine mutants were 25–50% lower than those of K66A and R76A, which was consistent with the glutamine versus alanine effect on the 3'-ribonuclease reaction. Whereas the K66R and R76K changes elicited a large gain of function (13–43-fold) relative to alanine in ribonucleotide resection, they also resulted in modest (2-fold) gains in 3'-phosphatase activity, to fully wild-type levels for the D11-p substrate.

Possible Functions of the Monoesterase-specific Components of the PE Domain—How do Arg^{14}, Asp^{15}, Glu^{32}, and Glu^{82} contribute to catalysis of 3'-phosphate removal and why might the 3'-phosphomonoesterase reaction call upon additional active site residues that are not required for the 3'-ribonuclease? One possibility has to do with differences in electrostatics in the two reactions. The $-2$ charge on the ground state and the $-3$ charge on the associative transition state of the 3'-phosphate are more negative than the ground state charge of $-1$ and the transition state charge of $-2$ on a 3'-terminal phosphodiester. This difference might engender a need for an additional basic residue to neutralize the extra negative charge. We speculate that Arg^{14} plays this role via a bidentate interaction with two of the phosphate oxygens (which would account for why lysine was unable to substitute for Arg^{14}).

The acidic side chains Asp^{15}, Glu^{32}, or 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 fact that Asp^{15} can be functionally substituted by asparagine tended to rule out this side chain as a general acid-base catalyst but does not weigh against Asp^{15} helping to simply orient the attacking water via hydrogen bonding, without abstracting a proton. Conservative substitutions were not tolerated at Glu^{32} and Glu^{82}, indicating that the carboxylate functional group and the side chain to main chain distance are both critical parameters for 3'-phosphate hydrolysis. These results raise an additional possibility: that these two glutamates could coordinate a second divalent cation required for the phosphomonoesterase activity but not for the phosphodiesterase. Yet another alternative is that one of the essential glutamates fixes the position of Arg^{14} in the phosphomonoesterase active site via a salt bridge.

In summary, the present analysis of the PE domain of LigD suggests how overlapping but nonidentical constellations of protein functional groups might promote catalysis of the mechanistically distinct phosphodiesterase and phosphomonoesterase reactions. The requirement for a 5' single-strand tail on the duplex substrate distinguishes LigD PE from other well characterized 3' end-processing enzymes. A fuller description of the reaction scheme will depend on obtaining an atomic structure of the PE domain. The monoesterase-specific loss-of-function mutations characterized here provide a potentially useful means to capture the structures of intermediates in the 3'-processing reactions of LigD. They also afford tools to assess genetically the role of the 3'-phosphomonoesterase activity in DNA repair in vivo.

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