Structure-guided Mutational Analysis of the Nucleotidyltransferase Domain of *Escherichia coli* DNA Ligase (LigA)*

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Li Kai Wang, Hui Zhu, and Stewart Shuman

From the Molecular Biology Program, Sloan-Kettering Institute, New York, New York 10065

NAD⁺-dependent DNA ligases (LigA) are ubiquitous in bacteria, where they are essential for growth and present attractive targets for antimicrobial drug discovery. LigA has a distinctive modular structure in which a nucleotidyltransferase catalytic domain is flanked by an upstream NMN-binding module and by downstream OB-fold, zinc finger, helix-hairpin-helix, and BRCT domains. Here we conducted a structure-function analysis of the nucleotidyltransferase domain of *Escherichia coli* LigA, guided by the crystal structure of the LigA-DNA-adenylate intermediate. We tested the effects of 29 alanine and conservative mutations at 15 amino acids on ligase activity in vitro and in vivo. We thereby identified essential functional groups that coordinate the reactive phosphates (Arg¹³⁶), contact the AMP adenine (Lys²⁹⁰), engage the phosphodiester backbone flanking the nick (Arg²¹⁸, Arg¹⁰⁸, Arg³⁷ plus Arg¹⁰¹), or stabilize the active domain fold (Arg¹⁷¹). Finer analysis of the mutational effects revealed step-specific functions for Arg¹³⁶, which is essential for the reaction of LigA with NAD⁺ to form the covalent ligase-AMP intermediate (step 1) and for the transfer of AMP to the nick 5'-PO₄ to form the DNA-adenylate intermediate (step 2) but is dispensable for phosphodiester formation at a preadenylated nick (step 3).

*Escherichia coli* NAD⁺-dependent DNA ligase (LigA) is the founding member of a family of DNA replication/repair enzymes present in all known bacteria as well as certain halophilic archaea (1). LigA seals 3'-OH/5'-PO₄ DNA nicks via three nucleotidyl transfer reactions: (i) LigA reacts with NAD⁺ to form a covalent ligase-(lysyl-NTase domain) intermediate (step 1) and forms the DNA-adenylate intermediate (step 2) but is dispensable for phosphodiester formation at a preadenylated nick (step 3). The crystal structure of *E. coli* LigA bound to the nicked DNA-adenylate intermediate consolidated this point by showing that LigA encircles the DNA helix as a C-shaped protein clamp (9). The protein-DNA interface entails extensive DNA contacts by the NTase, OB, and helix-hairpin-helix domains over a 19-bp segment of duplex DNA centered about the nick (see Fig. 1). A structure-guided mutational analysis (17) pinpointed essential functional groups in the OB domain that engage the DNA phosphodiester backbone flanking the nick (Arg⁵⁵¹), penetrate the minor groove and distort the nick (Val³⁶³ and Ile⁴⁶⁵), or stabilize the OB fold (Arg⁴⁸⁷). The essential constituents of the helix-hairpin-helix domain include four glycines (Gly⁴⁵⁵, Gly⁴⁶⁹, Gly⁵²¹, and Gly⁵⁵³) that bind the phosphate backbone across the minor groove at the outer margins of the LigA-DNA interface; Arg⁴⁸⁷, which penetrates the minor groove at the outer margin on the 3'-OH side of the nick; and Arg⁴⁴⁶, which promotes protein clamp formation via contacts to the nucleotidyltransferase domain (17). The zinc finger module appears to play a purely structural role in bridging the OB and helix-hairpin-helix domains (9). Zn²⁺ coordination is critical for *E. coli* LigA activity, insofar as single alanine substitutions at three of the four cysteines (Cys⁴⁰₈, Cys⁴¹₁, and Cys⁴₃₂) suppressed nick sealing by 3 orders of magnitude (18). Deletion analysis revealed that the BRCT domain of *E. coli* LigA is required in its entirety for effective nick sealing in vitro (17). However, a structural interpretation of this result is elusive.

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**References**

1. American Cancer Society Research Professor. To whom correspondence should be addressed. E-mail: s-shuman@ski.mskcc.org.

2. The abbreviations used are: NTase, nucleotidyltransferase; FOA, 5-fluoroorotic acid.

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because no electron density was observed for this segment of DNA-bound E. coli LigA (9).

Whereas NAD$^+$ and DNA recognition are aided by the flanking domains discussed above, the chemical steps of nick sealing are performed by the NTase domain. Initial mutational analyses of the NTase domain of E. coli LigA (18, 19) focused on: (i) residues within a set of conserved peptide motifs that define the “covalent nucleotidyl-transferase superfamily,” which includes ATP-dependent DNA ligases, ATP-dependent RNA ligases, and GTP-dependent mRNA capping enzymes (20); and (ii) amino acids unique to and conserved among members of the NAD$^+$-dependent DNA ligases clade. Ten individual residues of the NTase domain were identified as critical for E. coli LigA function: Lys$^{115}$ (the site of covalent AMP attachment), Asp$^{117}$, Gly$^{118}$, Glu$^{173}$, Arg$^{200}$, Arg$^{208}$, Arg$^{277}$, Asp$^{285}$, Lys$^{290}$, and Lys$^{314}$ (19). Reference to a crystal structure of LigA bound to NAD$^+$ (7) revealed atomic contacts with the AMP moiety that could plausibly account for the essentiality of Glu$^{173}$ (which coordinates the ribose O2'), Lys$^{290}$ (which donates a hydrogen bond to adenine-N1), and Lys$^{314}$ (which coordinates the AMP phosphate (see Fig. 2C).

The roles of Arg$^{200}$ and Arg$^{208}$ were illuminated by the crystal structure of the LigA-DNA-adenylate intermediate. Arg$^{200}$ and Arg$^{208}$ reside within a conserved α-helical peptide (197ANPRNAAGSLRQ209) that engages the DNA minor groove on the 3’ side of the nick. Arg$^{200}$ is located at the nick, where it: (i) stacks over and makes van der Waals’ contacts with the terminal nucleoside sugar of the 3’-OH strand, (ii) contacts via water the 3’-OH terminal base, and (iii) forms a salt bridge to the essential Asp$^{177}$ side chain (see Fig. 2B). Arg$^{208}$ penetrates deeply into the minor groove, where it contacts several nucleoside sugars and bases (see Fig. 2B).

Here, we used the E. coli LigA-DNA crystal structure to guide a new round of mutational analysis of the NTase domain, focusing on side chains that make atomic contacts to the DNA duplex and the 5’-adenylate. We thereby identified several conserved amino acid functional groups essential for nick sealing in vitro and in vivo. New mechanistic insights were gleaned from an analysis of mutational effects on individual steps of the ligation pathway.

**EXPERIMENTAL PROCEDURES**

**Ligase Mutants**—Missense and nonsense mutations were introduced by PCR into the pET-EcoLigA expression plasmid as described previously (12). The entire ligA gene was sequenced in every case to confirm the desired mutation and exclude the acquisition of unwanted changes during PCR amplification and cloning. The expression plasmids were transformed into E. coli BL21(DE3). Mutant and wild type ligases were purified from the soluble lysates of isopropyl β-d-thiogalactopyranoside-induced BL21(DE3) cells by nickel-agarose chromatography as described (12). The protein concentrations were determined using the Bio-Rad dye reagent with bovine serum albumin as a standard.

**Nick Ligation**—Reaction mixtures (20 µl) containing 50 mM Tris-HCl (pH 7.5), 10 mM (NH$_4$)$_2$SO$_4$, 5 mM dithiothreitol, 5 mM MgCl$_2$, 20 µM NAD$^+$, 1 pmol of 5’ 32P-labeled nicked duplex DNA substrate, and aliquots of serial 2-fold dilutions of wild type or mutant ligases were incubated at 22 °C for 20 min. The products were resolved by electrophoresis through a 15-cm 18% polyacrylamide gel containing 7 M urea in 0.5× TBE (45 mM Tris borate, 1.25 mM EDTA). The extents of ligation were determined by scanning the gel with a Fuji BAS2500 imager. The specific activities of wild type and mutant ligases were determined from the slopes of the titration curves in the linear range of enzyme dependence. The activities of the mutant ligases were normalized to the specific activity of wild type LigA protein purified in parallel with that mutant and assayed in a parallel with the same preparation of radiolabeled DNA substrate. On average, the wild type LigA sealed 63 ± 21 fmol of nicks/fmol of input enzyme (average value for 20 different titration experiments with three different preparations of LigA and multiple different preparations of 32P-labeled singly nicked DNA substrate).

**Ligase Adenylylation**—Reaction mixtures (20 µl) containing 50 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol, 5 mM MgCl$_2$, 1 µM [32P-adenylate]NAD (purchased from PerkinElmer Life Sciences), and 8 pmol of wild type or mutant LigA were incubated at 22 °C for 10 min. The reactions were quenched with SDS, and the products were analyzed by SDS-PAGE. The ligase-[32P]AMP adduct was visualized by autoradiography and quantified by scanning the gel with a Fuji imager.

**Ligation at a Preadenylated Nick**—The nicked DNA-adenylate substrate was prepared as described previously (19). In brief, the 5’-adenylated 32P-labeled 18-mer strand (AppDNA) was prepared by in vitro reaction of Mycobacterium tuberculosis LigC with a singly nicked three-piece duplex DNA containing a 5’-P-adenylate-18-mer strand at the nick. The AppDNA strand was then purified by PAGE, and the nicked DNA-adenylate substrate was formed by annealing the purified AppDNA strand to the complementary 36-mer and a 3’-OH acceptor strand at a molar ratio of 1:4:4. AppDNA ligation reaction mixtures (100 µl) containing 50 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol, 5 mM MgCl$_2$, 600 fmol of nicked DNA-adenylate substrate, and 6 pmol of wild type or mutant LigA proteins were incubated at 22 °C. The sealing reactions were initiated by adding ligase. Aliquots (10 µl) were withdrawn at 0.25, 0.5, 1, 2, and 5 min and quenched immediately with EDTA and formamide. The products were resolved by denaturing PAGE, and the extents of ligation were determined by scanning the gel with a Fuji imager. At maximum, ~80% of the input AppDNA strand was ligated at the reaction end point. (This reflects incomplete annealing of the labeled AppDNA to the template and 3’-OH strands.) A plot of the kinetic profile of AppDNA sealing by wild type LigA (with each datum being the average of 11 separate experiments) fit to a single exponential with a rate constant of 0.64 ± 0.019 min$^{-1}$, as calculated in Prism by nonlinear regression curve fitting.

**Assay of LigA Activity in Vivo by Complementation of cdc9Δ**—NdeI-BamHI restriction fragments containing LigA mutant alleles were excised from their respective pET-based LigA plasmids and inserted into pYX1-His (CENTRPI). In this vector, expression of ligA is under the control of the yeast TPI1 promotor. The pYX1-LIGA plasmids were transformed into a yeast CEN TRP1—NdeI-BamHI restriction fragments containing LigA mutant alleles were excised from their respective pET-based LigA plasmids and inserted into pYX1-His (CENTRPI). In this vector, expression of ligA is under the control of the yeast TPI1 promotor. The pYX1-LIGA plasmids were transformed into a yeast CEN TRP1
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roorotic acid (FOA). Lethal mutations (− growth) were those that formed no FOA-resistant colonies at 25° or 30 °C. The viable cdc9Δ ligA yeast strains were tested for growth on YPD agar at 25 and 30 °C. + + + indicates colony size indistinguishable from a cdc9Δ strain expressing wild type LigA; + + indicates smaller colony size; and + indicates pinpoint colonies.

RESULTS

New Round of Alanine Scanning of the LigA NTase Domain—The NTase domain of E. coli LigA contains an adenylate-binding pocket composed of a cage of β strands and interstrand loops that includes the six defining motifs (I, Ia, III, IIa, IV, and V) of the covalent nucleotidyl-transferase superfamily (20). The motif I (115KLDG) lysine nucleophile is located in a loop between the two antiparallel β sheets that form the binding site for the reactive AppN dinucleotide of the nicked DNA-adenylate intermediate. Lys115 contacts the 5′-phosphate of the AppDNA strand (see Fig. 2B). Motif Ia (135TRG137) is located within an interstrand loop that contacts both sides of the nick. The pyrophosphate bridge of AppN is coordinated by motif Ia residue Arg136 and by Ser81 (see Fig. 2A). The two phosphodiester flanking the nick 5′ terminus (AppNpNpN) are engaged by Asn84, directly and via water (see Fig. 2B). The three phosphodiester flanking the 3′-OH side of the nick are coordinated by: (i) the motif Ia Thr135 side chain to NpNpNpNOM; (ii) the motif Ia Arg136 side chain to NpNpNpNOM; and (iii) Gln72 via water to NpNpNpNOM (see Fig. 2A). Asn201 engages in a network of water-mediated contacts to the nucleosides at the 3′-OH nick terminus (see Fig. 2B). Here we probed the roles of residues Gln72, Ser81, Asn84, Thr135, Arg136, and Asn201 that bind the DNA at and near the nick by replacing them individually with alanine.

The NTase domain also contacts the DNA duplex at sites remote from the nick. Arg97, Arg101, and Arg308 make electrostatic interactions with adjacent phosphates of the template DNA strand on the 5′-phosphate side of nick (Figs. 1 and 2A). Gln309 and Arg218 make direct and water-mediated contacts with two adjacent template strand phosphates on the 3′-OH side of the nick (Figs. 1 and 2B). Each of these residues was subjected presently to alanine substitution. We included three additional conserved LigA residues in the alanine scan: Arg309, situated near the nick 5′ AppN terminus (Fig. 2A); Arg171, which donates hydrogen bonds to multiple main chain and side chain atoms within the NTase domain (Fig. 2A); and Ser206, which interacts with Arg218 near the DNA minor groove (Fig. 2B).

The wild type and mutated E. coli LigA proteins were produced as N-terminal His10 fusions and purified from soluble bacterial extracts by nickel-agarose chromatography (Fig. 3A). The extent of ligation of singly nicked 3′-OH/5′PO4 DNA by wild type LigA, and each mutant was gauged as a function of input enzyme, and the specific activities were normalized to the wild type value (defined as 100%). The results are compiled in Table 1. Our operational definition of a functionally important residue is one at which alanine substitution reduced specific activity in nick joining to ≤10% of wild type LigA. By this criterion, five of the targeted residues were deemed important: Arg136, Arg171, Ser81, Arg218, and Arg308 (Table 1).

By contrast, the alanine mutations at nine other positions reduced activity to between 14 and 39% of wild type LigA. We surmise that the contacts of these residues to DNA or other protein constituents seen in the crystal structure contribute to nick sealing, although not enough to meet our criterion of significance. In some cases, an apparently “nonessential” side chain engages in potentially redundant atomic contacts. For example, the effects of loss of the hydrogen bond from Thr135 Oγ to a phosphate near the 3′-OH end might be softened by the fact that the main chain amide of nearby Gly137 donates a hydrogen bond to the same nonbridging phosphate oxygen as Thr135 (Fig. 2A). In the same vein, whereas Arg101 and Arg208...
make ionic interactions with the same phosphate oxygen atom (Fig. 2A), the R308A mutation (9% of wild type activity) is more deleterious than R101A (28%). Residues Gln209 and Arg218 share a water-mediated phosphate contact to the template strand (Fig. 2B), yet the R218A change (2% of wild type activity) was much more harmful than Q209A (33%). The effects of losing the direct hydrogen bond between Gln209 and the vicinal phosphate might be masked by the hydrogen bond to the same nonbridging oxygen atom from the main chain amide nitrogen of Leu210 (Figs. 1 and 2B).

The Arg97 and Arg101 side chains project from the same face of an $\alpha$ helix (89EESFLANKRVQDR101) and help dock the $\alpha$ helix to the template DNA strand by ionic contacts to adjacent phosphates (Fig. 2A), in which case the impact of the R97A change (17% of wild type activity) might be checked by the backup contacts of Arg101 (a seemingly inessential side chain, insofar as the R101A mutant had 70% of wild type activity). To test this idea, we produced a R97A/R101A double mutant of LigA and found that its specific activity in nick sealing was 5% of the wild type value (Table 1). This result is consistent with func-

![FIGURE 2. DNA interface of the NTase domain. A and B show stereo views of the NTase domain of E. coli LigA bound to the nicked DNA, which is rendered as a transparent surface over a stick model. Waters are depicted as red spheres. C shows a stereo view of the active site of Enterococcus faecalis LigA bound to NAD$^+$ (7). The NTase domain is colored cyan, and the Ia domain is colored yellow. The amino acid numbers indicate the equivalent positions in E. coli LigA. Ionic and hydrogen bonding interactions are denoted by dashed lines in all panels.]

![FIGURE 3. LigA mutants. Aliquots (8 $\mu$g) of the nickel-agarose preparations of wild type (WT) LigA and the indicated alanine mutants (A) or conservative mutants (B) were analyzed by SDS-PAGE. The Coomassie Blue-stained gels are shown. The positions and sizes (kDa) of marker polypeptides are indicated on the left.]

### TABLE 1

| LigA | Ligase activity % of WT | cdc9A complementation | Lig-AMP % of WT | Non-DNA atomic contacts |
|------|-------------------------|-----------------------|-----------------|------------------------|
| WT   | 100                     | ++ ++                 | 100             | NAD$^+$ $\beta$-PO$_4$ |
| Q72A | 38                      | ++ ++                 | 92              |                        |
| S81A | 6                       | ++ ++                 | 44              |                        |
| N84A | 21                      | ++ ++                 | 92              |                        |
| R97A | 17                      | ++ ++                 | 83              |                        |
| R101A| 28                      | ++ ++                 | 70              |                        |
| R97A/R101A| 5 | ++ ++ | 70 | |
| T135A| 22                      | ++ ++                 | 42              |                        |
| R136A| <0.1                    | ++ ++                 | <0.1            |                        |
| S81A/R136A| <0.1 | ++ ++ | <0.1 | |
| R171A| 1                       | ++ ++                 | 7               | Ser$^{125}$, Glu$^{169}$, Val$^{77}$, Pro$^{151}$ |
| N201A| 26                      | ++ ++                 | 19              |                        |
| S206A| 39                      | ++ ++                 | 70              | Arg$^{218}$            |
| Q209A| 33                      | ++ ++                 | 62              |                        |
| R218A| 2                       | ++ ++                 | 120             | Ser$^{206}$ Pro$^{151}$, Arg$^{61}$ |
| R305A| 14                      | ++ ++                 | 43              |                        |
| R308A| 9                       | ++ ++                 | 71              |                        |
tional redundancy of Arg97 and Arg101 at the LigA-DNA interface.

**Effects of Alanine Mutations on EcoLigA Function in Vivo in Yeast**—A deletion of the gene encoding the essential *Saccharomyces cerevisiae* ATP-dependent DNA ligase Cdc9 can be complemented by expression of LigA (18). Viability of the yeast *cdc9*Δ strain is contingent on maintenance of an extrachromosomal *CDC9* gene on a *CEN URA3* plasmid. Hence, *cdc9*Δ cells cannot grow on medium containing FOA (a drug that selects against the *URA3* *CDC9* plasmid), but they can grow on FOA if the cells have been transformed with a *CEN TRP1* plasmid expressing wild type LigA under the control of the constitutive yeast *TP11* promoter. Here we tested by the plasmid shuffle assay (18) whether the LigA-Ala mutants were functional in yeast. We found that mutations R136A, R171A, and R218A that reduced nicked sealing activity to ≤2% of wild type LigA were all lethal in *vivo*, i.e., they were unable to support growth of *cdc9*Δ on FOA at 25 or 30 °C (scored as − in Table 1). The impaired R308A mutant (9% as active as wild type LigA *in vitro*) and the R97A-R101A double mutant (5% activity *in vitro*) were also unable to complement *cdc9*Δ. By contrast, the nine alanine mutants that retained more than 10% of wild type nick joining activity *in vitro* were functional in *cdc9*Δ complementation *in vivo*. These results suggest that growth of the *cdc9*Δ strain depends on a threshold level of LigA nick sealing activity. The exception to this correlation was S81A, which had 6% of wild type nick joining activity *in vitro* but still supported yeast growth. All of the viable *cdc9*Δ ligA-Ala strains (including S81A) grew as well as the “wild type” *cdc9*Δ ligA strain on YPD agar medium at 25 and 30 °C, as gauged by colony size (scored as + + + + in Table 1).

**Structure-Activity Relationships at Key Residues of the NTase Domain**—We tested the effects of conservative lysine and glutamine substitutions for the four essential arginine residues identified above in the alanine scan (Arg136, Arg171, Arg218, and Arg308) and for Arg97, which reduced ligase activity by a factor of six. We also introduced conservative changes at Lys290, an important residue that makes a hydrogen bond to the adenine-N1 of NAD⁺ and AppDNA (Figs. 1 and 2C). We showed previously that the K290A mutant of *E. coli* LigA was lethal in yeast and had 8-fold lower nick sealing activity *in vitro* than wild type LigA (19). Here we replaced Lys290 with arginine and glutamine. Twelve conservative LigA mutants were produced in *E. coli* and purified from soluble bacterial extracts by nickel-agarose chromatography (Fig. 3B). The specific activities of the mutants in nick joining were determined. The conservative mutants were also tested for activity in yeast *cdc9*Δ complementation. The results are compiled in Table 2.

We found that Arg136 was strictly essential for LigA activity *in vitro* and *in vivo*. The R136K and R136Q mutants were 0.2 and <0.1% as active as wild type LigA in nick sealing and were lethal *in vivo* in yeast. We surmise that the multidentate contacts of Arg136 with either the NAD⁺ β phosphate (Fig. 2C) or the phosphates of the nicked DNA-adenylate (Fig. 2B) are critical for LigA activity, rather than mere positive charge on this side chain. By contrast, positive charge appears to suffice in the case of Arg308. Whereas the R308Q mutation (4% as active as wild type LigA and lethal in yeast) mimicked the effects of R308A, the lysine substitution restored nick sealing to 80% of wild type and revived activity *in vivo* in yeast (Table 2). We infer that lysine can recapitulate the monodentate contact to the DNA phosphate observed for Arg308 in the LigA crystal structure (Fig. 2A).

Glutamine substitutions for Arg171 and Arg218 had no salutary effect compared with the alanine mutations, i.e., R171Q and R218Q were lethal in yeast and were 3% and 4% as active in nick sealing as wild type LigA, respectively (Table 2). However, the introduction of lysine raised nick sealing activity above our 10% cut-off criterion (to 15% for R171K and 19% for R218K), which allowed for survival of the *cdc9*Δ R171K and *cdc9*Δ R218K yeast strains (Table 2). We surmise that LigA activity relies on a positively charged side chain at positions 171 and 218, although optimal activity is attained with arginine. Note that Arg171 makes hydrogen bonds from all three guanidinium nitrogens to main chain and side chain atoms of the NTase domain. The terminal guanidinium nitrogens of Arg218 donate hydrogen bonds to Ser206 Oγ and a DNA-bound water, respectively (Fig. 2B).

Replacing the adenine-binding Lys290 residue with glutamine elicited a 30-fold reduction in nick sealing and ablated LigA activity *in vivo* in yeast (Table 2). The K290R mutant was only slightly better at nick sealing *in vitro* (8% of wild type activity). Yeast *cdc9*Δ K290R cells were viable but grew poorly (Table 2). The decrement in nick sealing activity upon the loss of Arg171 (17% of wild type activity) was partially remedied by glutamine (30% activity) and lysine (63% activity), both of which supported growth of *cdc9*Δ (Table 2).

**Mutational Effects on Phosphodiester Formation at a Preade-nylated Nick**—The third step of the ligation pathway entails attack of the 3′-OH of the nick on the 5′-PO₄ of the DNA-adenylate to form a phosphodiester and release AMP. We assayed step 3 in isolation using a preadenylated nicked 36-bp DNA substrate labeled with 32P at the 5′-PO₄ of the 18-mer DNA-adenylate strand; this substrate was incubated with LigA in the presence of magnesium without added NAD⁺. Under condition of enzyme excess, wild type *E. coli* LigA sealed the nicked DNA-adenylate with pseudo-first order kinetics (Fig. 4). LigA mutants with alanine and conservative substitutions for Arg136, Ser41, Arg171, Arg218, and Arg308 were tested in parallel with wild type LigA (Fig. 4).

### Table 2

| LigA | Ligase activity % of WT | cdc9Δ complementation | Lig-AMP |
|------|-------------------------|------------------------|---------|
| Wild type | 100 | ++++ | 100 |
| R97K | 63 | ++++ | 108 |
| R97Q | 30 | ++++ | 122 |
| R136K | 0.2 | — | 15 |
| R136Q | <0.1 | — | 0.7 |
| S81A/R136K | 0.1 | — | 0.1 |
| R171K | 15 | ++++ | 17 |
| R171Q | 3 | — | 5 |
| R218K | 19 | +++ | 57 |
| R218Q | 4 | — | 87 |
| K290R | 8 | — | 63 |
| K290Q | 3 | — | 64 |
| R308K | 80 | +++ | 110 |
| R308Q | 4 | — | 61 |
FIGURE 4. Mutational effects on the rate of sealing of a preadenylylated nicked DNA. Reaction mixtures were constituted as described under “Experimental Procedures.” Each datum is the average of three (A) or two (B–D) separate experiments in which wild type LigA was assayed in parallel with the mutants specified in each graph. The error bars denote the standard deviation.

FIGURE 5. Effects of double-alanine mutations. A, aliquots (8 μg) of the nickel-agarose preparations of wild type (WT) LigA and the indicated double-alanine mutants were analyzed by SDS-PAGE. The Coomasie Blue-stained gel is shown. The positions and sizes (kDa) of marker polypeptides are indicated on the left. B, ligase adenylylation. Reaction mixtures contained 1 μM [γ-32P-adenylate]-NAD and 8 pmol of LigA. The extents of label transfer to LigA are shown. Each datum is the average of triplicate assays from a single experiment in which wild type LigA was tested in parallel with the mutants specified. The error bars denote the standard deviation. C, sealing a preadenylylated nick. Reaction mixtures were constituted as described under “Experimental Procedures.” Each datum is the average of three separate experiments in which wild type LigA was assayed in parallel with the mutants specified. The error bars denote the standard deviation.

A striking finding was that elimination of the motif I Arg136 side chain, which abolished overall nick sealing, had little effect on the rate of phosphodiester synthesis at a preadenylylated nick (Fig. 4A, R136A). This result was surprising to us in light of the extensive set of contacts made by Arg136 to the phosphates at the nick in the crystal structure (Fig. 2A), which is taken to mimic the Michaelis complex for the step 3 reaction (9). Moreover, the R136K change, which also abolished overall 3′-OH/5′-PO4 nick sealing, resulted in a 2-fold increase in the rate of AppDNA sealing compared with wild type LigA (Fig. 4A). The R136Q mutant (severely defective in composite nick sealing) performed the isolated step 3 reaction at about one-third the rate of wild type LigA (Fig. 4A).

Loss of the Ser81 hydroxyl group had little effect on the rate of sealing at a preadenylylated nick (Fig. 4B), compared with the significant impact of the S81A change on the complete ligation reaction (Table 1). Ser81 Oγ contacts a phosphate oxygen of the AMP leaving group in the step 3 reaction (Fig. 2A). It is conceivable that this contact is functionally redundant to that made by Arg136 with the same phosphate oxygen or the contact of the Ser81 main chain amide with the nonbridging phosphate oxygen of the adenylate (Fig. 2A). To test this idea, we produced and purified two LigA double mutants: S81A/R136A and S81A/R136K (Fig. 5A). As expected, S81A/R136A was defective in overall nick sealing (<0.1% of wild type activity) and unable to complement cdc9Δ (Table 1). The remarkable finding was that the rate of phosphodiester formation by S81A/R136A was still one-third the rate of wild type LigA (Fig. 5C). This result militates against an essential but functionally redundant role of the contacts made by Ser81 and Arg136 to the AppN anhydride bridge. Rather, we surmise that catalysis of step 3 at a preadenylylated nick does not rely on either of these amino acid side chains. This inference is reinforced by the properties of the S81A/R136K mutant, which is defective in 3′-OH/5′-PO4 nick sealing and cdc9Δ complementation (Table 2) yet is still faster than wild type LigA in sealing a preadenylylated nick (Fig. 5C). Thus, the loss of Ser81 did not diminish the vigorous step 3 activity of the R136K single mutant.

Mutations at Arg308 had disparate effects on the isolated step 3 reaction (Fig. 4C). The rates of AppDNA sealing by R308A and R308Q were reduced to 25 and 40% of wild type, respectively, whereas R308K was nearly twice as fast as wild type LigA. Similar bipolar effects were seen at Arg218, where R218A and R218Q sealed the AppDNA substrate at about one-third the wild type rate, whereas R218K was faster than wild type LigA. These results emphasize the importance of the positive charge at side chains 308 and 218 that interact with the DNA phosphodiester backbone. By contrast, mutations at Arg171 had a generally suppressive effect (Fig. 4B). The rates of AppDNA sealing by R171A, R171K, and R171Q were ~10%, 15, and 6% of wild type LigA, respectively. The R97A-R101A double mutant was one-fourth as fast as wild type LigA in AppDNA sealing (Fig. 5C).
nick. The gain of step 1 function seen with R136K attests to the importance of the ionic contacts between this side chain and the pyrophosphate bridge of NAD$^+$ during step 1 catalysis. Nonetheless, the gain of step 1 activity in R136K is nowhere reflected in its overall ligation function, which remained at 0.2% of wild type LigA, notwithstanding that R136K step 3 activity exceeds that of wild type LigA. These results implicate Arg$^{136}$ as a catalyst of step 2 of the ligation pathway (see “Discussion”).

Changing Ser$^{81}$ to alanine had relatively little effect on ligase-adenylate formation (44% of wild type), even though Ser$^{81}$ directly contacts the pyrophosphate bridge of the NAD$^+$ substrate (Fig. 2C). In particular, Ser$^{81}$ Oγ donates a hydrogen bond to the same phosphate oxygen of the NMN leaving group that receives a bidentate contact from Arg$^{136}$. This situation raised the prospect that Ser$^{81}$ is functionally redundant with Arg$^{136}$. This notion was underscored by our finding that the gain of step 1 function attendant on the R136K change (to 15% of wild type) was erased by the S81A/R136K double mutation (0.1% of wild type) (Fig. 5B and Table 2).

Mutations of Arg$^{171}$ had a generally suppressive effect on adenylyltransferase activity, whereby R171A, R171K, and R171Q yielded 7, 17, and 5% as much radiolabeled ligase-AMP adduct as wild type LigA (Fig. 6). The Arg$^{171}$ mutational effects on step 1 activity agreed reasonably well with their impact on the composite nick sealing reaction (Tables 1 and 2). Because Arg$^{171}$ makes multidentate contacts that tether secondary structure elements of NTase domain, and it makes no contacts directly to DNA or NAD$^+$, we surmise that Arg$^{171}$ plays a predominantly structural role in LigA function, e.g., in stabilizing the proper conformation of the NTase domain, especially establishing the proper position for the nearby Glu$^{173}$ side chain. Glu$^{173}$ contacts the AMP ribose 2′OH and is strictly essential for overall nick sealing and ligase adenylylation (19). An E173A mutation of LigA elicits a 30-fold decrement in the rate of phosphodiester formation at a preadenylated nick (19).

**DISCUSSION**

The present structure-guided mutational analysis of the NTase domain of *E. coli* LigA provides new insights to bacterial ligase function in the following respects: (i) by delineating Arg$^{308}$ and Arg$^{218}$ as critical constituents of the LigA-DNA interface that bind the phosphodiester backbone of the template strand at sites remote from the nick; (ii) by highlighting functionally redundant essential contacts of Arg$^{97}$ and Arg$^{101}$ with the template strand backbone on the 5′-PO$_4$ side of the nick; (iii) by discerning an essential structural role for Arg$^{171}$; and (iv) by unveiling the essential functions of the active site constituent Arg$^{136}$ in the first and second steps of the ligation pathway, albeit not in the third step.

**DNA Interface of the NTase Domain**—As illustrated in Fig. 1, the NTase domain contacts the template strand backbone at discrete trinucleotide clusters on both sides of the nick. We find here that both regional contacts with the template strand are important for LigA activity. On the 3′-OH side of the nick, Arg$^{218}$ is essential for overall nick sealing, but Gln$^{209}$ is not. The R218A and R218Q changes that suppress nick sealing have no significant impact on ligase adenylylation, consistent with the location of Arg$^{218}$ outside the active site for nucleotidyl transfer.
chemistry. We surmise that Arg218 contacts to DNA facilitate step 2, which entails initial binding of LigA-AMP to the nicked duplex and transfer of AMP to the 5′-PO₄ to form AppDNA. Arg218 mutations have a milder impact on the sealing of a preadenylated nick than they do on the composite ligation reaction, presumably because tight binding of the AppDNA adenylate within the nucleoside pocket of the NTase domain is the dominant factor in stabilizing the step 3 Michaelis complex.

The distinctive activity profile of R136K versus R136(Q/A) implicates Arg136 as a catalyst of step 2 of the ligation pathway, insofar as R136K has recovered step 1 adenyltransferase activity, is fully competent for AppDNA sealing, and yet is defective in sealing a 3′-OH/5′-PO₄ nick. We surmise that the extensive contacts of Arg136 seen in the LigA-AppDNA structure (Fig. 2A) reflect what was required to execute step 2. It appears that lysine cannot fulfill all of the phosphate interactions of arginine with both ends of the nick (involving all three guanidinium nitrogens), thereby accounting for the ultimate failure of nick sealing by R136K. Arg136 is likely to participate directly in step 2 chemistry by stabilizing the transition state of the lysyl-AMP phosphate (via contact to a nonbridging oxygen), while coordinating one of the phosphate oxygens of the attacking nick 5′-PO₄ group (Fig. 2A).

Conserved Role for Motif Ia Arg/Lys in Step 2 Catalysis—Motif Ia (consisting of a lysine or arginine flanked by serine or threonine) is conserved among NAD⁺-dependent DNA ligases, ATP-dependent DNA ligases, ATP-dependent RNA ligases, and GTP-dependent mRNA capping enzymes. A comparison of the present findings concerning the role of motif Ia Arg136 in LigA with the mutational data available for several other members of the covalent nucleotidyltransferase superfamily suggests a general role for the motif Ia arginine/lysine in DNA/RNA 5′ nucleotidylolation. On the other hand, the essentiality of the motif Ia basic side chain during enzyme nucleotidylolation and phosphodiester synthesis varies in a case-specific fashion.

Bacteriophage T4 RNA ligase 2 (Rnl2) exemplifies an ATP-dependent RNA ligase clade that repairs nicks in duplex RNAs (21). Changing its motif Ia residue Arg55 to alanine ablated overall RNA sealing and suppressed each step of the pathway (22). Replacing Arg55 with lysine revived each step and the composite pRNA sealing reaction, whereas glutamine afforded no benefit. In the crystal structure of Rnl2 bound to adenylylated nick (21), Arg55 makes a bidentate interaction with the two nonbridging oxygens of the nick 5′-PO₄ group, thereby implicating Arg55 in nick recognition and orientation of the nick 5′-PO₄ for nucleophilic attack on the lysyl-AMP (analogous to what we propose here for LigA Arg136).

T4 RNA ligase 1 (Rnl1), the prototypal tRNA repair enzyme, relies differently on its motif Ia residue, Lys119. The K119A and K119Q mutations of Rnl1 abolished the overall RNA ligation reaction but did not affect either ligase adenylylation (step 1) or sealing a preformed AppRNA (step 3) (23). Rnl1 Lys119 is thereby implicated as a specific catalyst of the RNA adenylylation reaction (step 2). The conservative K119R substitution restored overall pRNA ligation activity (23). The crystal structure of Rnl1 bound to AMPCPP reveals that Lys119 coordinates the γ phosphate (24); the observed inessentiality of Lys119 for Rnl1 adenylylation is presumed to reflect functional redundancy with several other γ phosphate contacts made by Rnl1 side chains (25). Further elucidation of the role of motif Ia during step 2 catalysis hinges on obtaining a crystal structure of Rnl1 with its broken tRNA substrate.

Deinococcus radiodurans RNA ligase (DraRnl) exemplifies yet another clade of ATP-dependent RNA sealing enzymes (26). Changing its motif Ia residue Lys86 to alanine severely impaired overall sealing and ligase adenylylation but had little
effect on sealing at a preadenylylated nick (analogous to what we find here with LigA R136A). 

*Chlorella* virus DNA ligase (ChVLig) is a minimized eukaryal ATP-dependent DNA ligase that has an intrinsic nick sensing function. Replacing the motif Ia residue Arg^{42} with alanine caused a 50-fold reduction in overall nick sealing and suppressed nick-specific DNA binding (27). Arg^{42} is proposed to directly coordinate the nick 5’-PO_{4} and thereby promote step 2 chemistry (27). In the crystal structure of ChVLig bound at a 3’-OH/5’-PO_{4} nick, Arg^{42} also engages the terminal phosphodiester -NpNOH (28). This is analogous to the nick-spanning LigA Arg^{136} contacts with the 3’-OH and AppN strands seen in Fig. 2A.

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