Functional Dissection of the DNA Interface of the Nucleotidyltransferase Domain of Chlorella Virus DNA Ligase

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Chlorella virus DNA ligase (ChVLig) has pluripotent biological activity and an intrinsic nick-sensing function. ChVLig consists of three structural modules that envelop nicked DNA as a C-shaped protein clamp: a nucleotidyltransferase (NTase) domain and an OB domain (these two are common to all DNA ligases) as well as a distinctive β-hairpin latch module. The NTase domain, which performs the chemical steps of ligation, binds the major groove flanking the nick and the minor groove on the 3'-OH side of the nick. Here we performed a structure-guided mutational analysis of the NTase domain, surveying the effects of 35 mutations in 19 residues on ChVLig activity in vivo and in vitro, including biochemical tests of the composite nick sealing reaction and of the three component steps of the ligation pathway (ligase adenyllylation, DNA adenyllylation, and phosphodiester synthesis). The results highlight (i) key contacts by Thr-84 and Lys-173 to the template DNA strand phosphates at the outer margins of the DNA ligase footprint; (ii) essential contacts of Ser-41, Arg-42, Met-83, and Phe-75 with the 3'-OH strand at the nick; (iii) Arg-176 phosphate contacts at the nick and with ATP during ligase adenyllylation; (iv) the role of Phe-44 in forming the protein clamp around the nicked DNA substrate; and (v) the importance of adenine-binding residue Phe-98 in all three steps of ligation. Kinetic analysis of single-turnover nick sealing by ChVLig-AMP underscored the importance of Phe-75-mediated distortion of the nick 3'-OH nucleoside in the catalysis of DNA 5'-adenyllylation (step 2) and phosphodiester synthesis (step 3). Induced fit of the nicked DNA into a distorted conformation when bound within the ligase clamp may account for the nick-sensing capacity of ChVLig.

DNA ligases are ubiquitous enzymes essential for DNA replication, recombination, and repair. They seal breaks in the phosphodiester backbone by joining the 3'-OH and 5'-PO$_4$ termini. Ligation entails three sequential nucleotidyl transfer reactions (1, 2). In the first step, nucleophilic attack on the α-phosphorus of ATP or NAD$^+$ by ligase results in the release of PP$_i$ or NMN and the formation of a covalent ligase-adenylate intermediate in which AMP is linked via a P–N bond to the Nε of a lysine. In the second step, the AMP is transferred to the 5'-end of the 5'-phosphate-terminated DNA strand to form DNA-adenylate (AppDNA). In the third step, ligase catalyzes attack by the 3'-OH of the nick on DNA-adenylate to join the polynucleotides and liberate AMP.

DNA ligases function in Okazaki fragment joining, nucleotide and base excision repair, homologous recombination, and nonhomologous end joining. In eukaryota, these chores are divided among multiple ATP-dependent ligase isozymes: LigI, LigIII, and LigIV in mammals; or LigI (Cdc9) and LigIV in yeast (1). Eukaryal cellular ligases are large polypeptides consisting of a core catalytic unit, composed of a nucleotidyltransferase (NTase) domain and an OB domain and embellished by a large globular DNA-binding domain (DBD), plus additional isozyme-specific structural modules (3, 4). Homologous ATP-dependent ligases composed of core plus DBD are present in all known archaeal taxa (2), consistent with a common ancestry for the archaeal/eukaryal DNA replication machinery. Several families of eukaryal DNA viruses (such as poxviruses and baculoviruses) also encode ATP-dependent ligases composed of core plus DBD.

The minimal essential features of the ATP-dependent ligase clade are exemplified by Chlorella virus DNA ligase (ChVLig) (5–15). ChVLig is the smallest eukaryal ligase known (298 amino acids); it lacks the large globular DBD and accessory modules found in cellular DNA ligases. Notwithstanding its compact size, ChVLig has an intrinsic nick-sensing function whereby it binds stably and with high affinity to duplex DNA containing a single 3'-OH/5'-PO$_4$ nick but not to intact duplex DNA (the ligation reaction product) (6). Nick recognition depends on the nick 5'-PO$_4$ and covalent adenyllylation of the ligase (6, 9). The most impressive feature of ChVLig is that it is able to sustain mitotic growth, excision repair, and nonhomologous end joining in budding yeast when it is the only source of ligase in the cell (13, 16). Thus, ChVLig ligase represents a stripped down “pluripotent” eukaryal ligase that can recapitulate most of the biological functions performed by the two yeast ligases. Recent studies show that ChVLig can even fulfill the essential functions of mammalian DNA ligase III in mitochondri al physiology (17).

The basis for nick sensing by ChVLig is illuminated by the crystal structure of the ligase-AMP intermediate bound to a nicked duplex DNA, which shows that ChVLig encircles the
DNA as a C-shaped protein clamp (14). The NTase domain (amino acids 1–189) binds to the broken and intact DNA strands in the major groove flanking the nick and also in the minor groove on the 3’-OH side of the nick (Fig. 1). The OB domain, composed of a five-stand antiparallel β-barrel and an α-helix, binds across the minor groove on the face of the duplex behind the nick (Fig. 1A). A distinctive “latch” module, consisting of a β-hairpin loop (amino acids 203–231) that emanates from the OB domain, occupies the major groove flanking the nick and completes the circumferential clamp via contacts between the tip of the loop and the surface of the NTase domain (Fig. 1, A and C). The latch is a key determinant of nick sensing (14).

The NTase domain performs the chemical steps of nick sealing. The active site is composed of a cage of β-strands and interstrand loops that includes the six peptide motifs (I, Ia, III, IIa, IV, and V) that define the covalent nucleotidyltransferase superfamily. The superfamily embraces ATP-dependent DNA ligases, NAD+-dependent DNA ligases, ATP-dependent RNA ligases, and GTP-dependent RNA-capping enzymes, all of which are polynucleotide 5’-endo-modifying enzymes that act through a lysyl-N7-NMP intermediate (18). Motif I (25TPKID-GIR32 in ChVLig) contains the lysine to which AMP becomes covalently linked in the first step of the DNA ligase reaction. The motif I lysine nucleophile is located in a loop between the two antiparallel β-sheets that form the adenylate-binding pocket. Other amino acids in motifs I, Ia, III, IIa, IV, and V contact AMP or the DNA nick and are thought to play essential roles in one or more steps of the ligation pathway, as surmised from the mutational effects on ChVLig activity in vitro or in vivo (6, 8–12, 16).

Initial mutational analyses of the NTase domain of ChVLig focused on amino acids within the conserved peptide motifs. Ten individual residues of the NTase domain were identified as critical for ChVLig function: Lys-27 (the site of covalent AMP attachment), Asp-28, Gly-30, and Arg-32 in motif I; Arg-42 in motif Ia; Asp-65 and Glu-67 in motif III; Phe-98 in motif IIa; Glu-161 in motif IV; and Lys-186 and Lys-188 in motif V (6, 8, 10–12). Reference to the crystal structures of free and nick-bound ChVLig-adenylate (10, 14) revealed atomic contacts with AMP or other critical amino acids that could plausibly account for the essentiality of these side chains (Fig. 1A).

Here, we used the ChVLig-AMP-DNA crystal structure to guide a new round of mutational analysis of the NTase domain, focusing on the side chains that make atomic contacts to the nicked DNA duplex. We also interrogated the roles of residues that interact with the adenine of lysyl-AMP and of the side chains comprising the kissing interface of the NTase domain with the latch. We thereby identified 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**

**Recombinant ChVLig**—Missense mutations were introduced by PCR into the pET-ChVLig expression plasmid as described previously (6). The entire ChVLig insert 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 *Escherichia 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 and phosphocellulose chromatography as described (6).

**ChVLig Purity and Concentration**—Total protein concentrations for the recombinant WT and mutant ChVLig preparations were determined by using the Bio-Rad protein reagent with bovine serum albumin as a standard. Aliquots (nominally 6 μg as measured by dye binding) of the ChVLig preparations were analyzed by SDS-PAGE (12% polyacrylamide), and polypeptides were visualized by staining with Coomassie Blue dye (supplemental Fig. S1). The gels were then scanned with a Kodak ImagePro 4000R instrument. Image Gauge software was then used to obtain a densitometric profile of each ChVLig electropherogram as follows: (i) full-length scans of individual lanes were collected through the centers of the lanes using a bandwidth of ~60% of the total lane width; (ii) the intensity profile for each lane was base line-corrected; and (iii) the areas under the peaks corresponding to ChVLig and any contaminating peptides were determined. The percent purity of each preparation was then calculated as the ratio of the ChVLig peak to the sum of all peak intensities in the lane. The results are as follows: WT, 83%; K5A, 79%; T25A, 84%; S41A, 86%; R42A, 83%; R42K, 82%; R42Q, 89%; T43A, 80%; F44A, 87%; F44L, 83%; T43A/K45A, 82%; F75A, 80%; F75L, 94%; M83A, 91%; M83K, 83%; M83Q, 70%; T84A, 94%; T84S, 94%; T84V, 95%; F98A, 94%; F98L, 83%; R166A, 94%; R166K, 92%; R166Q, 94%; K173A, 94%; K173Q, 95%; K173R, 95%; R176A, 93%; R176K, 96%; and R176Q, 93%; T178A, 87%. The WT purity value was then used to determine the amount of WT ChVLig protein corresponding to the integrated area under the ChVLig polypeptide peak. The amounts of the mutant ChVLig protein were calculated by scaling the integrated areas under the mutant ChVLig polypeptide peaks to that of WT ChVLig.

**Assays of Nick Ligation**—Reaction mixtures (10 μl) containing 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 10 mM MgCl2, 1 mM ATP, 1 pmol of singly nicked 36-bp duplex DNA substrate (5’33P-labeled at the nick, prepared as described (19)), and wild-type or mutant ChVLig as specified were incubated for 10 min at 22 °C. Single-turnover nick ligation mixtures lacked exogenous ATP. The reactions were initiated by the addition of ChVLig and quenched by the addition of 10 μl of 90% formamide, 40 mM EDTA. The samples were heated at 95 °C for 5 min. The products were resolved by electrophoresis through a 15-cm 18% polyacrylamide gel containing 7 M urea in 45 mM Tris borate, 1.25 mM EDTA. The extent of ligation was determined by scanning the gel with a Fujiix BAS2500 imager. Ligation was plotted as a function of input ChVLig, with each datum being the average of three separate enzyme titration experiments. The specific activities of the wild-type and mutant ligases were determined from the slopes of the titration curves. The activities of the mutant ligases were normalized to the specific activity of wild-type ChVLig protein that was purified in parallel with that mutant and assayed in parallel using the same preparation of radiolabeled DNA substrate.
DNA Ligase Catalysis

Assay of ChVLig Activity in Vivo by Complementation of Yeast cdc9Δ—NdeI-BamHI restriction fragments containing the wild-type and mutated ChVLig ORFs were excised from their respective pET-ChVLig plasmids and inserted into the yeast expression plasmid pYX1 (CEN TRP1), wherein the ChVLIG gene is driven by the yeast TPI1 promoter. The pYX1-ChVLig plasmids were transformed into a yeast cdc9Δ/h9004 strain bearing a CEN URA3 CDC9 plasmid (16). Trp+ isolates were streaked on agar plates containing 0.75 mg/ml 5-fluoroorotic acid (FOA). Lethal mutations were those that formed no FOA-resistant colonies at 18, 30, or 37 °C (scored as minus (−) at all temperatures in Table 1). The viable cdc9Δ ChVLIG yeast strains were tested for growth on YPD agar at 18, 30, and 37 °C.

In Table 1, +++ indicates colony size indistinguishable from a cdc9Δ strain expressing wild-type ChV ligase, ++ indicates smaller colony size, and − indicates no growth.

RESULTS

Mutagenesis Strategy—The ChVLig NTase domain binds along the face of the DNA helix overlying the nick. An interstrand loop, 173KFGGRST178, engages the major groove on the 5'-PO4 side of the nick and makes contacts to the phosphates and nucleoside sugars. The DNA contacts of the motif Ia loop (175FQDTSAvMTG185) that inserts into the minor groove on the 3'-OH side of the nick and makes contacts to the phosphates and nucleoside sugars. The DNA contacts of the motif Ia loop (175FQDTSAvMTG185) are also shown. Atomic contacts are depicted as dashed blue lines (hydrogen bonds) or dashed magenta lines (van der Waals contacts).

An α-helix of the NTase domain (75FQDTTSAVMTG85) inserts into the minor groove on the 3'-OH side of the nick, making contacts to the phosphates and nucleoside sugars (van der Waals contacts). At the proximal end of the α-helix, Phe-75 makes van der Waals contacts to the 3'-terminal sugar that impart an...
A-like conformation to the terminal base pair. Gln-76, Thr-79, Met-83, and Thr-84 also make van der Waals interactions with the sugar-phosphate backbone of the strands across the minor groove (Fig. 1B). The α-helix donates hydrogen bonds from Ser-80 Oγ and the Gly-85 amide to two adjacent phosphates of the template DNA strand remote from the nick (Fig. 1, A and B). The Arg-48 side chain (motif Ia) is situated near the distal end of the α-helix; Arg-48 projects into the minor groove and makes water-mediated contacts to a sugar and an adjacent nucleobase of the 3′-OH strand (Fig. 1, A and B). Here we probed the roles of residues Arg-48, Phe-75, Gln-76, Thr-79, Ser-80, Met-83, and Thr-84 by replacing them with alanine singly or in pairs.

The rest of the "SRTFKPIR" motif Ia surface loop comprises a major component of the DNA-binding site. Residues Ser-41, Thr-43, and Lys-45 donate hydrogen bonds to the second and third phosphates of the broken 3′-OH strand flanking the nick; the phosphate of the terminal nucleotide on the 3′-OH side of the nick is engaged by Arg-42 in the major groove (Fig. 1, B and C). Here we introduced mutations at Ser-41, Arg-42, Thr-43, and Lys-45.

The clamp-closing interface between the tip of the latch loop and the NTase domain is shown in Fig. 1C. It entails a network of van der Waals contacts from Phe-215 and Tyr-217 of the latch and Phe-44 (in motif Ia) and Lys-5 of the NTase domain. Here we mutated Lys-5 and Phe-44 to alanine.

We also extended the mutational analysis to three residues of the adenylate-binding pocket that engage the adenine nucleobase: Phe-98 (motif IIIa), which forms a π-stack on the purine ring; Thr-25 (motif I), which makes a hydrogen bond to adenine-N6; and a water-mediated contact to adenine-N1; and Arg-166 (motif IV), which coordinates the same bridging water to adenine-N1 as does Thr-25 and which also tethers the motif IV β-strand to the nearby motif IIIa β-strand via a salt bridge to Asp-99 (Fig. 1C).

Effects of Alanine Mutations on ChVLig 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 ChVLig (16). Viability of the yeast cdc9Δ strain is contingent on maintenance of an extrachromosomal CDC9 gene on a CEN URA3 plasmid. Hence, cdc9Δ 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 ChVLIG under the control of the constitutive yeast TRP1 promoter. Here we tested by the plasmid shuffle assay (16) whether the 20 ChVLig-Ala mutants were functional in yeast. We found that eight single-alanine mutations were lethal in vivo, i.e., the ChVLIG-Ala alleles were unable to support growth of cdc9Δ on FOA at 18, 30, or 37 °C (scored as minus (−) in Table 1). The lethal changes were S41A, R42A, F44A, F75A, M83A, T84A, K173A, and R176A, of which all but F44A entailed subtraction of atomic contacts between ChVLig and the nicked DNA.

Nine of the ChVLIG-Ala alleles supported growth of cdc9Δ cells on FOA at one or more of the selection temperatures. Six of the viable cdc9Δ ChVLIG-Ala strains grew as well as the "wild-type" cdc9Δ ChVLIG strain on YPD agar medium at 18, 30, and 37 °C as gauged by colony size (scored as +++ in Table 1). The fully functional single-Ala mutants were K5A (at the latch-NTase interface), T25A (in the adenine pocket), and T43A, K45A, R48A, and S80A (at the DNA interface). Double-Ala mutants Q76A/T79A and T79/S80A were also fully active in yeast as gauged by growth on rich medium at 18–37 °C (Table 1). By contrast, combining the benign mutations R166Q and K173Q resulted in lethality at all temperatures tested (Table 1). We surmise that Thr-43 and Lys-45, which contact adjacent phosphates of the 3′-OH strand, are functionally redundant in vivo. Three of the viable mutants displayed hypomorphic growth phenotypes of varying severity. The mildest was ChVLIG-T178A, which formed smaller colonies than wild-type ChVLIG yeast at all temperatures (scored as ++ in Table 1). Severe conditional lethality was seen with strains ChVLIG-F98A and ChVLIG-R166A, which displayed +++ growth at 18 °C but failed to grow at 30 or 37 °C (Table 1).

Structure-Activity Relationships in Vivo at Essential Residues of the NTase Domain—We tested the effects of conservative substitutions for nine of the 10 amino acids identified above in the alanine scan as essential or conditionally essential per se for ChVLig function in vivo: Arg-42, Phe-44, Phe-75, Met-83, Thr-84, Phe-98, Arg-166, Lys-173, and Arg-176. (We eschewed further mutation of the Ser-41, insofar as it is obvious that subtraction of the hydroxyl group accounts for the lethality of S41A.) Arg-42 and Arg-176 were replaced with lysine and glutamine; Phe-44, Phe-75, and Phe-98 with leucine; Met-83 with glutap.

### Table 1

| Mutant | Nick sealing | 18 °C | 30 °C | 37 °C |
|--------|--------------|-------|-------|-------|
| K5A    | ++           | +++   | +++   | +++   |
| T25A   | ++           | +++   | +++   | +++   |
| S41A   | ++           | +++   | +++   | +++   |
| R42A   | ++           | +++   | +++   | +++   |
| R42K   | ++           | +++   | +++   | +++   |
| R42Q   | ++           | +++   | +++   | +++   |
| T43A   | ++           | +++   | +++   | +++   |
| F44A   | ++           | +++   | +++   | +++   |
| F44L   | ++           | +++   | +++   | +++   |
| K45A   | ++           | +++   | +++   | +++   |
| T43A/K45A | ++ | +++   | +++   | +++   |
| R48A   | ++           | +++   | +++   | +++   |
| F75A   | ++           | +++   | +++   | +++   |
| F75L   | ++           | +++   | +++   | +++   |
| Q76A/T79A | ++ | +++   | +++   | +++   |
| S80A   | ++           | +++   | +++   | +++   |
| T79A/S80A | ++ | +++   | +++   | +++   |
| M83A   | ++           | +++   | +++   | +++   |
| M83Q   | ++           | +++   | +++   | +++   |
| M83K   | ++           | +++   | +++   | +++   |
| T84A   | ++           | +++   | +++   | +++   |
| T84S   | ++           | +++   | +++   | +++   |
| T84V   | ++           | +++   | +++   | +++   |
| F98A   | ++           | +++   | +++   | +++   |
| F98L   | ++           | +++   | +++   | +++   |
| R166A  | ++           | +++   | +++   | +++   |
| R166K  | ++           | +++   | +++   | +++   |
| R166Q  | ++           | +++   | +++   | +++   |
| K173A  | ++           | +++   | +++   | +++   |
| K173R  | ++           | +++   | +++   | +++   |
| K173Q  | ++           | +++   | +++   | +++   |
| R176A  | ++           | +++   | +++   | +++   |
| R176K  | ++           | +++   | +++   | +++   |
| R176Q  | ++           | +++   | +++   | +++   |
| T178A  | ++           | +++   | +++   | +++   |

**Note:** + + + indicates colony size indistinguishable from a cdc9Δ strain expressing wild-type ChVLig; ++ indicates smaller colony size; – indicates no growth.
DNA Ligase Catalysis

mine and lysine; Thr-84 with serine and valine; and Lys-173 with arginine and glutamine. The conservative mutants were tested for activity in yeast cdc9Δ complementation. The results are compiled in Table 1.

Distinct structure-activity relationships were observed at the three essential arginines. At Arg-42, full cdc9Δ complementation activity was restored by lysine (+++ growth at all temperatures), but glutamine was lethal, implying that the ionic interaction of this side chain with the first phosphodiester of the 3‘-OH strand seen in the crystal structure is the pertinent property, which can be fulfilled by either arginine or lysine. By contrast, Arg-166 could not be replaced functionally by either lysine or glutamine. Indeed, the R166K and R166Q alleles were less active in vivo than R166A, insofar as they did not support the growth of cdc9Δ cells at 18 °C (Table 1). Introducing glutamine in lieu of Arg-176 restored in vivo activity, seen as ++ growth of ChVLig-R176Q cells at 18–37 °C, whereas the conservative R176K change was lethal (Table 1). We surmised that neutral hydrogen bond donation from this residue to the nick 5′-PO4 was the relevant property.

Distinct structure-activity relationships were also seen at the three essential phenylalanines. Full activity in vivo was restored when Phe-44 was replaced by leucine (Table 1), signifying that the aromatic ring is not critical. The aliphatic γ-branched leucine is a partial isostere of Phe and should be capable of sustaining the van der Waals contacts to its Cγ and Cδ atoms (from leucine residue Phe-215) that apparently stabilize the DNA ligase clamp (Fig. 1C). By contrast, leucine was inactive in lieu of Phe-98 (Table 1), which attests to the essentiality of the aromatic π-stacking interaction with the adenine base of the lysyl-AMP adduct (Fig. 1C). Leucine was also inactive in place of Phe-75, thereby underscoring the importance of the van der Waals contacts of the Phe-75 Cγ atom with the 3′-terminal nucleoside sugar at the nick (Fig. 1B).

Lys-173 was strictly essential for ChVLig activity in vivo, i.e. the K173R and K173Q alleles were lethal (Table 1). These results stress the importance of the ionic contacts of Lys-173 with the DNA phosphodiester backbone and the prospect that the bulkier arginine side chain imposes sterically hindered van der Waals contacts of the 3′-OH strand (Fig. 1C). This effect is manifested at the DNA interface, where valine (an apolar isostere) was lethal (Table 1). Finally, replacing Met-83 with glutamine restored ligase activity, whereas lysine did not (Table 1). We speculate that Met and Gln, which have similar side chain lengths, can sustain pertinent van der Waals contacts to DNA (Fig. 1B), whereas the longer lysine side chain creates a steric clash at the DNA interface.

Mutational Effects on Nick Sealing Activity in Vitro—The wild-type and mutated ChVLig proteins were produced as N-terminal His10 fusions and purified from soluble bacterial extracts by nickel-agarose and phosphocellulose chromatography (supplemental Fig. S1). The extent of ligation of a singly nicked 36-bp DNA substrate (labeled with 32P at the 5′-PO4 of a centrally placed 3′-OH/5′ PO4 nick) by wild-type ChVLig and each mutant was gauged as a function of input enzyme. To determine specific activities, the nick sealing data from three independent enzyme titration experiments were averaged, and the mean values for ligation (fmol of nicks sealed) were plotted versus fmol of input ChVLig. The specific activities were calculated (in Prism) from the slopes of the titration curves in the linear range of enzyme dependence and then normalized to the wild-type specific activity of 70 fmol of nicks sealed/fmol of ChVLig (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 <25% of wild-type ChVLig. An essential residue is one at which alanine mutation reduced activity to <10%. By these criteria, five of the targeted residues were deemed important (Ser-41, Phe-44, Met-83, Thr-84, and Lys-173), and five were deemed essential (Arg-42, Phe-75, Phe-98, Arg-166, and Arg-176) (Table 1). Note that the single-alanine changes that defined importance for nick sealing under standard conditions in vitro were well correlated with those that were lethal in vivo in yeast (Table 1). The T43A/K45A double mutant, which was lethal in vivo, displayed 28% of wild-type nick sealing in vitro, just missing our cutoff for importance. The rest of the ChVLig-Ala mutants retained nick sealing activity in vitro at levels within a factor of 2 of the wild-type enzyme; each mutant in this category was active in vivo in yeast (Table 1).

Structure-activity relationships for nick sealing in vitro were gauged from the specific activities of conservative mutants (Table 1). In many cases, the in vitro data correlated nicely with the in vivo effects on ChVLig function. For example, the substantial gain of sealing activity by R42K (to 60% of wild type compared with 0.7% for R42A) accounted for the revival of cdc9Δ complementation; the virtual inertness of R42Q in vitro (0.5% activity) agreed with its lethality in vivo. Similarly, a gain of function of F44L in vitro (to 71% of wild-type activity versus 20% for F44A) tracked with its restoration of activity in yeast (Table 1). The similarly low nick sealing activities of K173A (13% of wild type), K173R (8%), and K173Q (11%) explained why neither of the conservative changes was beneficial in vivo. That said, it was not always the case that in vitro and in vivo activities tracked together, e.g. F75L elicited a significant gain of function in vitro (to 61% of wild type versus 4% for F75A) but did not restore activity in yeast, notwithstanding that a lesser gain of function in M83Q (to 28% of wild-type nick sealing versus 20% for M83A) did confer cdc9Δ complementation (Table 1).

Possible reasons for these discrepancies are considered below.

Assay of Nick Sealing under More Stringent Reaction Conditions Exacerbates Functional Deficits of ChVLig Mutants—Our standard reaction mixture for nick sealing contained 50 mM Tris-HCl buffer, 10 mM MgCl2, 1 mM ATP, and no added salt above the 1–2 mM NaCl contributed by the enzyme solution. We considered that the use of such relatively nonstringent hypotonic conditions might mask the effects of mutations at the DNA ligase interface on sealing in vitro, i.e. the loss of one of many protein DNA contacts might have little impact at low ionic strength in vitro but still exert significant effects in vivo in yeast, where the intracellular monovalent cation concentration during log phase growth on YPD medium is reported to be 330 mM (20). To explore this issue, we gauged the effects of increasing salt concentrations (50, 100, and 200 mM) on the extent of nick sealing by wild-type ChVLig and selected ChVLig mutants
that were inactive in yeast despite retaining substantial ligase activity in vitro under standard conditions. The amounts of each enzyme added were varied to attain comparable levels of nick sealing in the control reactions lacking added salt or phosphate. The extent of nick sealing by each ChVLig preparation is plotted as a function of added NaCl or Na$_3$PO$_4$ concentrations. Each datum is the average of three separate salt or phosphate titration experiments ± S.E.

Because many of the DNA contacts probed here by mutagenesis were to the phosphodiester backbone, we also gauged the effects of inorganic phosphate on the nick sealing activities of the wild-type and mutant ChVLig proteins. A recent study showed that 50 mM phosphate elicits specific NMR chemical shift perturbations in the ChVLig NTase domain, as well as local and global changes in domain dynamics, suggesting that phosphate anion mimics conformational transitions that occur when ChV ligase-adenylate interacts with the nicked DNA substrate (21). The intracellular concentration of orthophosphate in yeast is reported to be ~25 mM (22). In light of these results, we included 0, 50, 100, 150, and 200 mM phosphate in the ligase reaction mixtures. The results are shown in Fig. 2B. Wild-type ChVLig activity was reduced by 11, 19, 63, and 88% at 50, 100, 150, and 200 mM phosphate, respectively. The phosphate inhibition curves of mutants T84V, K173A, and K173R were similar to that of wild-type ChVLig (Fig. 2B, left panel). By contrast, mutants T84V, K173Q, S41A, F75L, T43A/K45A, M83A, and F44A were hypersensitive to inhibition by phosphate concentrations of 100 to 200 mM (Fig. 2B). Taken together, the salt and phosphate effects showed that increasing the stringency of the in vitro assay conditions did indeed exacerbate the functional impact of ChVLig mutations at the DNA interface and helped account for their lethality in yeast.

**Mutational Effects on Ligase Adenylylation**—Reaction of ChVLig with 100 μM [α$^{32}$P]ATP and magnesium in the absence of DNA leads to the formation of a covalent ChVLig-[α$^{32}$P]adenylate adduct. The autoadenylylation activities of the wild-type and mutant ChVLig preparations were assayed in parallel; the results are depicted in Fig. 3 and expressed in Table 2 as the percent of total input enzyme that was labeled in vitro with [α$^{32}$P]AMP. In the case of wild-type ChVLig, 41% of the available enzyme was labeled. Recombinant ChVLig purified from bacteria comprises a mixture of ligase apoenzyme and preformed enzyme-AMP intermediate; only the apoenzyme is available to react in vitro with [α$^{32}$P]ATP. The level of preformed Lig-AMP can be determined by assaying nick sealing in the absence of exogenous ATP, such that there is a 1:1 correspondence between the molar yield of ligated DNA and the molar amount of catalytically active Lig-AMP in the reaction. Here the extent of ligation of...
singly nicked 36-bp DNA substrate by wild-type ChVLig and each mutant was gauged as a function of input enzyme in the absence of ATP. The nick sealing data from three independent enzyme titration experiments were averaged, and the mean values for ligation (fmol nicks sealed) were plotted versus fmol of input ChVLig. The concentration of active Lig-AMP were calculated (in Prism) from the slopes of the titration curves in the linear range of enzyme dependence and then expressed in Table 2 as the percent of the total enzyme comprising preformed Lig-AMP. In the case of wild-type ChVLig, 53% of the available enzyme was preadenylylated and active in single-turnover nick sealing. Summing 53% preformed Lig-AMP and 41% reactive apoenzyme, we conclude that >90% of the wild-type preparation was catalytically active in one or more steps of the ligation pathway.

As one might expect, the ChVLig-Ala mutants that were active in vivo displayed wild-type or near wild-type summed levels of reactive preformed Lig-AMP plus apoenzyme as follows: K5A, 80%; T25A, 76%; T43A, 99%; K45A, 95%; R48A, 92%; Q76A/T79A, 92%; S80A, 98%; and T178A, 89%. At the opposite end of the spectrum were the in vivo lethal mutants R166A and R166Q, which were grossly defective in adenylylation in vitro (2% AMP labeling) and for which active preformed Lig-AMP comprised only 3 and 0.1% of the respective enzyme preparations (Table 2). Arg-166 lines the adenine-binding pocket and, with Thr-25, contacts adenine-N1 via a water. Because the severity of the R166A mutation contrasts with the benign effects of T25A, we conclude that the water-mediated adenine contact is not the basis for the loss of function attendant on R166A and R166Q. Rather, we surmise that the Arg-166—Asp-99 salt bridge is essential to maintain proper conformation of the ligase active site. It is noteworthy that the conservative R166K change, which restores activity in yeast and partially restored nick sealing in vitro (Table 1), elicited a large gain of function in ligase adenylylation during protein production in bacteria (43% preformed Lig-AMP), even though the adenylyltransferase activity of the apoenzyme fraction of R166K remained weak (4% AMP labeling). This pattern, reprised with other ChVLig variants (Table 2, also and see below), attests to the ability of some mutant ligases to adenylylate better during their prolonged exposure to relatively high intracellular con-

| ChVLig | Preformed Lig-AMP in vivo | Lig-[32P]AMP in vitro |
|-------|---------------------------|----------------------|
| WT    | 53                        | 41                   |
| K5A   | 38                        | 42                   |
| T25A  | 47                        | 29                   |
| S41A  | 23                        | 6                    |
| R42A  | 39                        | 2                    |
| R42K  | 46                        | 24                   |
| R42Q  | 23                        | 7                    |
| T43A  | 49                        | 50                   |
| F44A  | 28                        | 36                   |
| F44L  | 32                        | 26                   |
| K45A  | 61                        | 34                   |
| T43A/K45A | 34                   | 17                   |
| R48A  | 63                        | 29                   |
| F75A  | 29                        | 8                    |
| F75L  | 71                        | 20                   |
| Q76A/T79A | 63                   | 29                   |
| S80A  | 66                        | 32                   |
| T79A/S80A | 76                   | 20                   |
| M83A  | 44                        | 1                    |
| M83K  | 53                        | 13                   |
| M83Q  | 26                        | 2                    |
| M83R  | 10                        | 1                    |
| T84A  | 50                        | 12                   |
| T84S  | 35                        | 12                   |
| T84V  | 32                        | 15                   |
| F98A  | 29                        | 1                    |
| F98L  | 86                        | 2                    |
| R166A | 3                         | 2                    |
| R166K | 43                        | 4                    |
| R166Q | 0.1                       | 2                    |
| K173A | 30                        | 14                   |
| K173R | 22                        | 10                   |
| K173Q | 31                        | 16                   |
| R176A | 9                         | 7                    |
| R176K | 15                        | 7                    |
| R176Q | 39                        | 15                   |
| T178A | 61                        | 28                   |

FIGURE 3. Ligase adenylylation in vitro. Reaction mixtures (20 µl) containing 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 5 mM MgCl2, 100 µM [α-32P]ATP, and 2 pmol of wild-type or mutant ChVLig were incubated at 37 °C for 10 min. The reactions were quenched by adding SDS to 1% final concentration. The reaction products were analyzed by SDS-PAGE. The ChVLig-[32P]AMP adduct was visualized by autoradiography of the dried gels and quantified by scanning the gels with a Fujix BAS2500 imager. Each datum is the average of three separate adenylylation experiments ± S.E.
centrations of ATP in vivo (9.6 mM in exponentially growing E. coli (23)) compared with the 10-min reactions in vitro with 100 μM ATP.

This scenario was applicable to lethal mutants R42A and R42Q, for which Lig-AMP comprised 39 and 23% of the preparations, yet only 2 and 7% of the apoenzymes were AMP-labeled in vitro (Table 2). These results suggest a role for motif Ia residue Arg-42 during step 1 of the ligation reaction; indeed, although we do not have a crystal structure of ChVLig bound to ATP (prior to step 1 chemistry), the structures of other covalent nucleotidyltransferases complexed with NTP indicate that the motif Ia arginine (or lysine) coordinates the γ-phosphate of the NTP substrate (24–26). Nonetheless, the severe defects of the R42A and R42Q mutants in overall nick sealing in the presence of 1 mM ATP (0.7 and 0.5% of wild-type specific activity (Table 1)) were out of proportion to their levels of preformed Lig-AMP, suggesting that Arg-42 is also important for subsequent steps of the ligation pathway (an issue addressed below). The conservative R42K change that restored activity in vivo and nick sealing in vitro (Table 1) also revived step 1 adenylylation activity (46% preformed Lig-AMP plus 24% AMP labeling in vitro (Table 2)). We conclude that an ionic interaction of the motif Ia Arg-42 (or Lys) with ATP is the key factor. We suspect that the modest adenylylation defect caused by the nearby S41A change (23% preformed Lig-AMP plus 6% AMP labeling in vitro) might reflect the role of Ser-41 in stabilizing the conformation of the motif Ia loop via trifurcated hydrogen bonding of Ser-41 Oγ to Thr-43 Oγ and the Thr-43 and Lys-45 main chain amides.

Lethal mutants R176A and R176K also evinced defects in step 1 adenylylation in vitro (7% AMP labeling) and during protein production in bacteria (9 and 15% preformed Lig-AMP), which, at least for R176A, were consonant with the impact on overall nick sealing in vitro. Whereas Arg-176 contacts the nick 5′-PO4 in the cocystal structure (Fig. 1C), its position also suggests contacts with the PPi, leaving group of ATP during the ligase adenylylation reaction. The R176Q change that partially restored activity in yeast had a concomitant salutary effect on nick sealing activity (46% preformed Lig-AMP plus 24% AMP labeling in vitro) also revived step 1 adenylylation (46% preformed Lig-AMP plus 24% AMP labeling in vitro) (Table 2). We conclude that an ionic interaction of the motif Ia Arg-42 (or Lys) with ATP is the key factor. We suspect that the modest adenylylation defect caused by the nearby S41A change (23% preformed Lig-AMP plus 6% AMP labeling in vitro) might reflect the role of Ser-41 in stabilizing the conformation of the motif Ia loop via trifurcated hydrogen bonding of Ser-41 Oγ to Thr-43 Oγ and the Thr-43 and Lys-45 main chain amides.

The conservative F75L mutation, which was lethal in yeast, notwithstanding significant restoration of nick sealing activity (albeit salt- and phosphate-sensitive), substantially revived step 1 adenylylation such that 71% of the enzyme preparation was preformed Lig-AMP and 20% was ATP-reactive apoenzyme (Table 2). The higher than wild type proportion of Lig-AMP hinted that the F75L might impact subsequent AMP transfer steps of the ligation pathway (see below).

The F44A (lethal in yeast) and F44L (active in yeast) mutations did not differentially impact ligase adenylylation (Table 2), implying that the functional defect of F44A is at a downstream step, presumably involving clamp closure with the latch (Fig. 1C). The lethal K173A mutant displayed modest decreases in preformed Lig-AMP (30%) and AMP labeling (14%) that we cannot rationalize based on the available structures (Lys-173 being distant from the active site). As noted above, the sensitivity of nick sealing to ionic strength implicates weakened DNA binding as the main cause of the mutational defects at Lys-173.

Kinetics of Single-turnover Nick Sealing by Lig-AMP and Mutational Effects on Step 2 Catalysis—We subjected wild-type ChVLig and selected mutants to transient state kinetic analysis of single-turnover nick sealing by preformed Lig-AMP in the absence of added ATP. A rapid chemical quench apparatus was used to assay the reaction of nicked DNA with a 10-fold molar excess of preformed ChVLig-AMP in the reaction time range of 0.1 to 5 s. (Longer time points were assayed manually when warranted.) The products were analyzed by denaturing PAGE, and the distributions of 32P-labeled DNAs (as sealed 36-mer DNA product, 18-mer AppDNA intermediate, and residual 18-mer pDNA substrate) were quantified by scanning the gels with a phosphorimaging device. The rate of step 2 catalysis (DNA adenylylation) was gauged by plotting the sum of AppDNA plus sealed DNA as a function of reaction time for the wild-type and mutant ChVLig preparations (Fig. 4). Virtually all of the pDNA substrate was converted to ligated product by wild-type ChVLig; the kinetic profile fit well to a single exponential function by nonlinear regression curve fitting (Fig. 4).

In selecting mutants for kinetic analysis, we focused on a subset that displayed functional defects in yeast and/or nick sealing in vitro and for which preformed Lig-AMP comprised a significant fraction of the enzyme preparation. In each case, nearly all of the pDNA substrate was converted to AppDNA or

DNA Ligase Catalysis
DNA Ligase Catalysis

![Graphs of DNA adenylylation and nick sealing by ChVLig-AMP.](image)

**Figure 4.** Kinetics of single-turnover DNA adenylylation and nick sealing by preadenylylated ChVLig-AMP. A Kinetec RQF3 rapid chemical quench apparatus was used to assay the reaction of nicked DNA with a 10-fold molar excess of preformed ChVLig-AMP at 22 °C in the absence of added ATP, with reaction times in the range of 0.1 to 5 s. Where applicable, longer reaction times were assayed manually. The reaction mixtures contained 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 10 mM MgCl₂, 500 nM wild-type or mutant ChVLig-AMP, and 50 nM 5'-32P-nick-labeled DNA substrate. The rapid kinetic measurements were initiated by mixing two buffer solutions (20 μl each of 50 mM Tris-HCl (pH 7.5), 5 mM DTT, and 10 mM MgCl₂) containing 100 nM nicked DNA substrate and 1 μM ChVLig-AMP, respectively. The reactions were quenched by rapid mixing with 110 μl of 90% formamide, 50 mM EDTA. The products were analyzed by denaturing PAGE, and the distribution of 32P-labeled DNAs (as sealed 36-mer DNA product, 18-mer AppDNA intermediate, and residual 18-mer pDNA substrate) was quantified by scanning the gels with a phosphorimaging device. The rate of step 2 catalysis (DNA adenylylation) was gauged by plotting the sum of AppDNA plus sealed DNA (see the reaction scheme at lower right) as function of reaction time for the wild-type and mutant ChVLig preparations. Each datum in the graphs is the average of three separate kinetic experiments ± S.E. Nonlinear regression curve fitting of the data to a single exponential was performed in Prism; the calculated step 2 rate constants are compiled in Table 3.

**Table 3.** Mutational effects on the rates of single-turnover DNA adenylylation and phosphodiester synthesis

| ChVLig       | Step 2 rate constant a | Step 3 rate constant b |
|--------------|------------------------|------------------------|
| WT           | 2.17 ± 0.16            | 13.6 ± 1.13            |
| R42A         | 0.21 ± 0.02            | 1.73 ± 0.21            |
| T43A/K45A    | 1.12 ± 0.06            | 8.0 ± 0.45             |
| F75A         | 0.025 ± 0.035          | 0.25 ± 0.027           |
| F75L         | 0.42 ± 0.025           | 0.39 ± 0.015           |
| M83A         | 1.76 ± 0.11            | 7.47 ± 0.41            |
| M83Q         | 1.92 ± 0.08            | 18.6 ± 1.69            |
| M83K         | 1.24 ± 0.06            | 8.25 ± 0.59            |
| T46A         | 2.0 ± 0.09             | 15.4 ± 0.98            |
| F98A         | 0.82 ± 0.05            | 2.57 ± 0.24            |
| F98L         | 0.50 ± 0.02            | 2.65 ± 0.12            |
| R166K        | 2.07 ± 0.08            | 14.0 ± 0.12            |
| K173A        | 2.81 ± 0.27            | 10.9 ± 0.74            |

| ChVLig       | Step 2 rate constant a | Step 3 rate constant b |
|--------------|------------------------|------------------------|
| F75A         | 0.74 ± 0.25            | 1.98 ± 0.24            |
| F75L         | 0.41 ± 0.035           | 0.98 ± 0.023           |
| K173A        | 0.05 ± 0.02            | 0.41 ± 0.035           |
| T46A         | 0.20 ± 0.05            | 0.12 ± 0.035           |
| F98A         | 2.07 ± 0.08            | 14.0 ± 0.12            |
| F98L         | 1.24 ± 0.06            | 8.25 ± 0.59            |
| M83A         | 1.76 ± 0.11            | 7.47 ± 0.41            |
| M83Q         | 1.92 ± 0.08            | 18.6 ± 1.69            |
| M83K         | 1.24 ± 0.06            | 8.25 ± 0.59            |
| T46A         | 2.0 ± 0.09             | 15.4 ± 0.98            |
| F98A         | 0.82 ± 0.05            | 2.57 ± 0.24            |
| F98L         | 0.50 ± 0.02            | 2.65 ± 0.12            |
| R166K        | 2.07 ± 0.08            | 14.0 ± 0.12            |
| K173A        | 2.81 ± 0.27            | 10.9 ± 0.74            |

a The step 2 rate constants were derived by fitting the kinetic data in Fig. 4 to a single exponential in Prism.
b The step 3 rate constants were calculated in MATLAB from the kinetic profiles in Figs. 5 and 6 according to the reaction scheme depicted in Fig. 5 (see supplemental Table S1). The rate constants are specified ± the 95% confidence intervals.

The impact of the F98A and F98L mutations in the adenine-3'-OH nucleoside (Fig. 1) (14); this is in keeping with the essential role of the 3'-terminal nucleotide during the chemical step of DNA-adenylate formation (9). In the same vein, The 60-fold step 2 defect of F75A was especially striking and attested to the previously imputed role of this residue in distortion of the available crystal structures and earlier functional studies. For example, the 20-fold step 2 defect of R42A underscores its role in nick sensing via engagement of the 3'-OH terminal nucleotide at the nick (Fig. 1) (10, 14); this is in keeping with the essential role of the 3’-terminal nucleotide during the chemical step of DNA-adenylate formation (9). In the same vein, The 60-fold step 2 defect of F75A was especially striking and attested to the previously imputed role of this residue in distortion of the local DNA conformation on the 3’-OH side of the nick into an RNA-like A helix by packing against the terminal sugar (Fig. 1C) (14). The step 2 defect was alleviated in part by the introduction of leucine at position 75, a maneuver that would be expected, in light of the crystal structure (Fig. 1C), to elicit less of the requisite distortion of the 3’- OH nucleoside. Finally, the impact of the F98A and F98L mutations in the adenine-
binding pocket on step 2 catalysis was intriguing in light of prior suggestions that cis-trans variations in adenosine conformation might accompany the sequential steps of the polynucleotide ligation pathway (18, 27).

Distinctive Mutational Effects on DNA Adenylylation (Step 2) and Phosphodiester Synthesis (Step 3)—Previous studies of ChVLig and vaccinia virus DNA ligase highlight the fleeting nature of the AppDNA intermediate, which was virtually undetectable at the shortest times surveyed by manual assays (11). However, the accumulation of AppDNA could be achieved by manipulating the DNA substrate in ways that severely slowed step 3 chemistry (relative to the step 2 rate) and/or promoted dissociation of ChVLig from the nicked DNA-adenylate intermediate. Such maneuvers included mutations of the enzyme or structural perturbations of the 3'-OH strand of the nick by 3'-base mispairing or introducing a one-nucleotide gap (8, 11, 19). Using a rapid mix-quench protocol, we were able to probe the single-turnover sealing reaction at a higher temporal resolution, which allowed us to visualize flux through AppDNA en route to the ligated end product.

The wild-type and K173A reactions displayed similar kinetic profiles (Figs. 5 and 6) in which the AppDNA intermediate peaked at 0.1 s, at which time AppDNA comprised 12 and 18% of the labeled DNA, respectively, and 15–16% of the labeled nicks had been ligated. AppDNA decayed steadily thereafter as the sealed 36-mer product accumulated. The wild-type and K173A ligation reactions proceeded to completion by 2.5 s. The M83Q, T84A, and R166K proteins behaved much the same; their AppDNA levels peaked at 0.1 to 0.2 s, comprising 8–10% of the labeled DNA (Fig. 5). Our initial attempts to model the data to a simple sequential reaction pathway described by two rate constants, for DNA adenylylation (step 2) and phosphodiester synthesis (step 3), yielded poor fits to the data, especially the experimental AppDNA kinetic profiles, which consistently declined at slower rates than could be simulated by the simple sequential pathway. To better model the experimental scenario, we took into account the evidence that the immediate step 2 product, a Lig AppDNA complex, can partition between two states: (i) it can proceed directly forward with step 3 phosphodiester synthesis, or (ii) it can dissociate prematurely to free ligase apoenzyme and free nicked DNA-adenylate (or alternatively undergo a hypothetical conformational change to Lig†AppDNA, which prevents it from catalysis of step 3 without frank dissociation) (Fig. 5) (8, 15). Ligase can recover from the latter state(s) by reengagement of the adenylylated nick by the ligase apoenzyme (which was present in excess over DNA in our reactions in the absence of added ATP) or via reversion from a hypothetical inactive conformation to an active conformation of the Lig AppDNA complex. Thus, we incorporated a reversible transition of the step 2 product from an active “in pathway” state to an inactive “out of pathway” state (described by rate terms $k_{out}$ and $k_{in}$) into a revised kinetic model (Fig. 5).

The scheme posits that ligation steps 2 and 3 are effectively unidirectional under our assay conditions. These assumptions are consistent with the available data, to wit: (i) reaction of excess ChVLig with preformed singly nicked AppDNA yields exclusively the ligated product, with no detectable reversal of step 2 to form a 5'-PO₄ DNA nick (8, 9); and (ii) reversal of step

**FIGURE 5.** Flux through DNA-adenylate to ligated product during single-turnover nick sealing. The distribution of 18-mer AppDNA intermediate (gray circles) and sealed 36-mer DNA (black squares) during single-turnover nick sealing by the indicated ChVLig-AMP proteins is plotted as a function of reaction time. Each datum in the graphs is the average of three separate kinetic experiments. The data were modeled in MATLAB to the kinetic scheme depicted at bottom right, and the resulting curve fits are shown.
3 phosphodiester synthesis can occur but only in the presence of high concentrations of AMP (15).

The first-order differential equations for the kinetic scheme (supplemental Fig. S2) were solved in MATLAB (version R2010b, The Mathworks, Inc.). Simulations and curve fitting to the experimental data were also performed in MATLAB by inputting the experimentally derived step 2 rate constants (Table 3) and stipulating only that \( k_{\text{step 3}} \), \( k_{\text{out}} \), and \( k_{\text{in}} \) must be greater than zero. The modeled curves are plotted in Figs. 5 and 6 and fit well to the experimental data for flux through the AppDNA intermediate and the formation of the ligated product. The sets of rate constants calculated in MATLAB are compiled in supplemental Table S1. The step 3 rate constants are included in Table 3.

The apparent step 3 rate constant for wild-type ChVLig was 13.6 s\(^{-1}\), and thus the attack of the nick 3'-OH on AppDNA was 6-fold faster than the formation of AppDNA. Several of the mutant ligases displayed step 3 rates similar to the wild type (within a factor of 2), including: T84A, 15.4 s\(^{-1}\); R166K, 14.0 s\(^{-1}\); K173A, 10.9 s\(^{-1}\); M83Q, 18.6 s\(^{-1}\); M83A, 7.5 s\(^{-1}\); M83K, 8.3 s\(^{-1}\); and T43A/K45A, 8.0 s\(^{-1}\) (Table 3 and supplemental Table S1).

The F75A and F75L mutations slowed the rate of phosphodiester synthesis to 0.25 and 0.39 s\(^{-1}\), respectively, i.e. 54- and 35-fold decrements compared with the wild-type \( k_{\text{step 3}} \). Subtraction of Arg-42, which coordinates the 3'-nucleoside phosphate, elicited an 8-fold decrement in \( k_{\text{step 3}} \) (to 1.7 s\(^{-1}\)). These results underscore the key role of the Phe-75- and Arg-42-mediated distortion of the nick 3'-nucleoside in attaining an optimal catalytic conformation for phosphodiester synthesis, wherein the 3'-OH nucleophile is properly oriented with respect to the nick 5'-phosphate and the AMP leaving group. Note also that mutations of Phe-75 and Arg-42 slowed \( k_{\text{step 2}} \) and \( k_{\text{step 3}} \) (Table 3), signifying that the contacts to the 3'-terminal nucleotide are critical for both reactions at the nick. Whereas R42A and F75A caused similar decrements in the two steps such that their \( k_{\text{step 3}}/k_{\text{step 2}} \) ratios (8 and 7, respectively) were similar to wild-type ChVLig, the F75L change exerted a relatively greater impact on the rate of phosphodiester synthesis such that the \( k_{\text{step 3}}/k_{\text{step 2}} \) ratio was near unity. This was evident in the F75L kinetic profile as a higher peak of AppDNA intermediate accumulation, to 34% of total labeled DNA at 2.5 s (Fig. 5). The F98A and F98L mutations in the adenine-binding pocket resulted in a 5-fold decrement in \( k_{\text{step 3}} \) in the same range as their impact on step 2 catalysis.

**DISCUSSION**

Here we conducted a structure-guided mutational analysis of the ChVLig NTase domain, surveying the effects of 35 mutations in 19 residues on DNA ligase activity in vivo and in vitro. Alanine scanning defined 10 individual amino acids as essential (Ser-41, Arg-42, Phe-44, Phe-75, Met-83, Thr-84, Phe-98, Arg-166, Lys-173, and Arg-176), after which structure activity relations were clarified by conservative substitutions. We also identified one essential pair of functionally redundant residues: Thr-43 plus Lys-45. The distinct contributions of these amino acids to ligase function were illuminated by a series of biochemical tests of the composite nick sealing reaction and of each of the three component steps of the ligation pathway. The results highlight (i) key contacts by Thr-84 and Lys-173 to the template DNA strand phosphates at the outer margins of the DNA ligase footprint; (ii) essential contacts of Ser-41, Arg-42, Met-83, and Phe-75 with the 3'-OH strand at the nick; (iii) Arg-176 phosphate contacts at the nick and with ATP during ligase adenylylation; (iv) the key role of Phe-44 in the formation of the ligase protein clamp around the nicked DNA substrate; (v) the importance of adenine-binding residue Phe-98 in all three steps of ligation; and (vi) a structural role for Arg-166 in forming the
adenine-binding pocket. We expand on some of these themes below, as they relate to nick sensing and catalysis.

**DNA Binding at the Margins and the Role of DNA Distortion, Especially at the 3'-OH End**—Binding of ChVLig-AMP to nicked duplex DNA binding induces a 12° bend in the DNA centered at the nick (14). The DNA helix has a typical B-form secondary structure throughout, except at the 2 basepairs on either side of the nick, which adopt an A-like conformation. Here we found that loosening of specific NTase contacts (by Thr-84 and Lys-173) to the phosphodiester backbone at the outer limits of the interface compromises ChVLig function *in vivo* and nick sealing *in vitro*, especially under conditions of increased ionic strength. By contrast, mutating Thr-84 and Lys-173 had little or no impact on the rates of DNA adenylylation and phosphodiester synthesis under single-turnover conditions. These results attest to a noncatalytic role of Thr-84 and Lys-173 residues at the margin, presumably by optimizing the initial binding of ChVLig to nicked DNA and inducing or stabilizing the DNA bend seen in the crystal structure. Two other residues (Thr-178 and Ser-80) that make similar contacts to the template strand are not important for ligase activity *in vivo* or *in vitro*.

Closer to the nick, the NTase domain makes extensive contacts with the 3'-OH strand that emanate from two structural elements, motif Ia loop 45SRTFKPIR48 and the 75FQDTTSAVMTG85 minor groove-binding α-helix (14). The motif Ia residues Thr-43 and Lys-45, which contact the second and third phosphates from the 3'-OH end, respectively, are essential pairwise although functionally redundant. The Thr-43—Lys-45 dyad contributes to DNA binding (surmised from the salt sensitivity of nick sealing by the T43A/K45A mutant) but not to step 2 and step 3 chemistry (as gauged by the step 2 and step 3 rate constants for single turnover ligation). It is noteworthy that Ser-41, which is essential *per se*, contacts the same DNA phosphate as nonessential Thr-43, but they donate hydrogen bonds to different nonbridging phosphate oxygens (Ser-41 to O1P and Thr-43 to O2P).

Arg-42 (in motif Ia) and Phe-75 (in the minor groove helix) had the biggest impact on ChVLig function at steps 2 and 3 of the pathway by virtue of their contacts with the 3'-terminal nucleotide. Phe-75 is of special interest in light of its imputed function in distorting the helical conformation of the 3'-OH strand. The present results implicate this distortion in the catalysis of AMP transfer from ChVLig to the nick 5'-phosphate (a remarkable finding given that the 3'-OH is not chemically reactive during the step 2 reaction) as well as in phosphodiester synthesis (in which the 3'-OH is the attacking nucleophile). In effect, the Phe-75-induced conformational change at the nick prepares the DNA substrate for nick sensing by ChVLig and for the adoption of catalytically proficient Lig-AMP·pDNA and Lig·AppDNA binary complexes. This mechanism, entailing “induced fit” of the nicked DNA into a bent/distorted conformation when bound within the ligase clamp, provides a satisfying explanation for the selective binding of ChVLig-AMP to nicked DNA versus an otherwise identical duplex (6), to wit: the nick allows the DNA conformation freedom to bend, tor, and adapt to the ligase interface, whereas the closed duplex is less free to do so.

**Clamp Closure Is Important for Ligase Activity**—An instructive outcome of the present study was the finding that perturbation of the clamp-closing interface between the NTase domain and the latch module adversely affected ChVLig activity. Of the two NTase side chains that contact the latch, Phe-44 was essential for ChVLig function *in vivo*, but Lys-5 was not. The viability of F44L underscored the contributions of the van der Waals contacts of Phe-44 to the latch seen in the crystal structure. Consistent with its remoteness from the active site, the F44A mutation had little impact on ligase adenylylation *in vitro*, but it affected overall nick sealing and sensitized ChVLig to inhibition by increasing ionic strength. We speculated that the diminished stability of the closed clamp conformation of the F44A mutant underlies these deficits, which are qualitatively similar, although quantitatively less severe, than the effects of deleting the latch module from ChVLig (14).

**Adenine Binding by Phe-98 Affects All Steps of ChVLig Function**—π-Stacking of the motif IIIa aromatic residue on the purine base of the NTP substrate and lysyl-NMP intermediate is a feature shared by DNA ligases, RNA ligases, and mRNA capping enzymes (18). Here we confirmed that the F98A change in ChVLig motif IIIa inhibited steady-state nick sealing and ligase adenylylation *in vitro* (12), and we extended the analysis to show that the F98A and F98L mutations abolished ligase activity *in vivo*. Whereas earlier studies lacked the time resolution to interrogate a possible role for Phe-98 at steps distal to ligase adenylylation, we showed here that the F98A and F98L changes do slow the rates of DNA adenylylation and phosphodiester synthesis. How might this be, given that Phe-98 is part of a hydrophobic pocket and remote from the centers of adenylate transfer at the lysyl-AMP phosphate (in step 2) and the polynucleotide 5'-phosphate of AppDNA (in step 3)? We suggest that the π-stacking on Phe-98 anchors the adenine firmly in the pocket and provides a fixed axis for the conformational movements of the adenosine sugar (abound the glycosidic bond) that are proposed to accompany progression through the sequential steps of the ligation pathway, which can result in local remodeling of the active site contacts with the ribose (18, 27). In this light, we suspect that replacing Phe-98 by alanine or leucine allows some mobility of the adenine in the pocket, which then affects the conformational switches of the adenosine in the lysyl-AMP and AppDNA species during nick sealing.

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