Structure-guided Mutational Analysis of the Nucleotidyltransferase Domain of Escherichia coli NAD\textsuperscript{+}-dependent DNA Ligase (LigA)

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NAD\textsuperscript{+}-dependent DNA ligase (LigA) is essential for bacterial growth and a potential target for antimicrobial drug discovery. Here we queried the role of 14 conserved amino acids of Escherichia coli LigA by alanine scanning and thereby identified five new residues within the nucleotidyltransferase domain as being essential for LigA function in vitro and in vivo. Structure activity relationships were determined by conservative mutagenesis for the Glu-173, Arg-200, Arg-208, and Arg-277 side chains, as well as four other essential side chains that had been identified previously (Lys-115, Asp-277, and Lys-314). In addition, we identified Lys-290 as important for LigA activity. Essential to the structure of Enterococcus faecalis LigA allowed us to discriminate three classes of essential/important side chains that: (i) contact NAD\textsuperscript{+} directly (Lys-115, Glu-173, Lys-290, and Lys-314); (ii) comprise the interface between the NMMN-binding domain (domain Ia) and the nucleotidyltransferase domain or comprise part of a nick-binding site on the surface of the nucleotidyltransferase domain (Arg-200 and Arg-208); or (iii) stabilize the active site fold of the nucleotidyltransferase domain (Arg-277). Analysis of mutational effects on the isolated ligase adenylylation and phosphodiester formation reactions revealed different functions for essential side chains at different steps of the DNA ligase pathway, consistent with the proposal that the active site is serially remodeled as the reaction proceeds.

DNA ligases are grouped into two families, ATP-dependent ligases and NAD\textsuperscript{+}-dependent ligases, according to their nucleotide substrate requirement. DNA ligases seal 5’-phosphate and 3’-hydroxyl termini by means of three nucleotidyl transfer reactions (1). In the first step, attack on the α-phosphorus of ATP or NAD\textsuperscript{+} by ligase results in the release of pyrophosphate or NMMN and the formation of a covalent ligase-adenylate intermediate. In the second step, the AMP is transferred to the 5’ end of the 5’-phosphate-terminated DNA strand to form a DNA-adenylate intermediate. In the third step, ligase catalyzes attack by the 3’-OH of the nick on DNA-adenylate to join the two polynucleotides and release AMP.

The ATP-dependent DNA ligases are found in bacteria, Archaea, and eukarya, whereas the NAD\textsuperscript{+}-dependent enzymes are present only in bacteria and entomopox viruses (2, 3). All known bacterial species encode at least one highly conserved NAD\textsuperscript{+}-dependent DNA ligase (LigA),\textsuperscript{1} which is essential for growth, even when the bacterium encodes additional NAD\textsuperscript{+}-dependent or ATP-dependent ligase enzymes (4, 5). Therefore, LigA presents an attractive target for broad spectrum antimicrobial therapy predicated on blocking the reaction of LigA with NAD\textsuperscript{+} (6).

Crystal structures of a LigA apoenzyme and a covalent LigA-adenylate intermediate (7, 8) revealed that the AMP-binding pocket of bacterial NAD\textsuperscript{+}-dependent ligases is located within a nucleotidyltransferase domain shared with ATP-dependent DNA ligases, certain ATP-dependent RNA ligases, and GTP-dependent mRNA capping enzymes (9–13). The polynucleotide ligases and capping enzymes comprise a covalent nucleotidyltransferase superfamily, members of which catalyze transfer of a nucleoside monophosphate to the 5’ phosphorolyalted end of a polynucleotide via a covalent enzyme-lysyl-nucleoside monophosphate intermediate. Five signature motifs of the nucleotidyltransferase domain (motifs I, III, IIIa, IV, and V) form the nucleotide-bridging pocket (14). Extensive mutational analyses of ATP-dependent DNA ligases, RNA ligases, and RNA capping enzymes have identified essential catalytic side chains within these motifs and begun to clarify their distinctive contributions at different steps along their respective reaction pathways (15–19). In contrast, mutational analyses of NAD\textsuperscript{+}-dependent ligases are less advanced, at least with respect to the nucleotidyltransferase domain.

Previous studies of NAD\textsuperscript{+}-dependent ligases have ascribed functions to individual domains of the LigA protein. All LigA enzymes consist of a central “ligase module” composed of an adenylyltransferase domain and an OB fold domain, which is flanked by a N-terminal domain (domain Ia) and several C-terminal modules, including a tetracysteine zinc-binding motif, a helix-hairpin-helix domain, and the BRCT domain (7, 8). An N-terminal fragment composed of the Ia and nucleotidyltransferase domains retains full ligase adenylylation activity with NAD\textsuperscript{+} but is unable to catalyze phosphodiester formation at a standard 5’-PO\textsubscript{4} nick or at a pre-adenylated nick (3, 20–22). An instructive finding was that deletion of domain Ia abolished the reaction of LigA with NAD\textsuperscript{+} to form ligase-adenylate but had no effect on phosphodiester bond formation at a pre-adenylated nick (3, 23); these results implicated domain Ia in recognition of the NAD\textsuperscript{+} substrate. Mutations of five individual amino acids within domain Ia either reduced or abolished sealing of a 5’-PO\textsubscript{4} nick and adenylyl transfer from NAD\textsuperscript{+}, without affecting ligation of pre-formed DNA-adenylate. It was suggested that these five side chains comprise a binding site for the NMMN moiety of NAD\textsuperscript{+} (3, 23), a prediction borne out by the recently reported structure of the N-terminal domain of LigA bound to NMMN (24).

Mutational analysis of the nucleotidyltransferase domain of LigA (25, 26) has highlighted essential roles for five amino acids: the motif I lysine (Lys-115 in Escherichia coli LigA), which is the site of covalent attachment of AMP to the enzyme (27); the motif I aspartate (Asp-117 in EcoLigA); the motif IV aspartate (Asp-285 in EcoLigA); and the motif V lysine (Lys-
314 in EcoLigA. The essential residues above are denoted by ‘+’ over the sequence alignment in Fig. 1. Several residues, found by alanine scanning to be nonessential for LigA function, are indicated by ‘−’ above the sequence alignment in Fig. 1. Here we performed a new alanine scan of 14 residues of the nucleotidyltransferase domain of EcoLigA indicated by ‘+’ in Fig. 1. The choice of positions to mutate was guided by side chain conservation and, in some cases, proximity to the covalently bound AMP in the crystal structure of the Thermus filiformis LigA-AMP adduct. Specific residues were targeted to test models of metal-dependent catalysis proposed by Lee et al. (8) on the basis of the LigA-AMP structure. We report the identification of five new essential residues in EcoLigA, clarify structure-activity relationships at these and other essential positions by conservative mutagenesis, and analyze mutational effects on individual steps of the ligation pathway. We discuss our findings in light of the recently reported structure of Enterooccus faecalis LigA bound to NAD+ (24).

EXPERIMENTAL PROCEDURES

Ligase Mutants—Missense mutations were introduced into the pET-EcoLIG expression plasmid using the PCR-based two-stage overlap extension method as described previously (23). 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 fraction of the induced cells by nickel-agarose chromatography, as described previously (23). The protein concentrations were determined using the Bio-Rad dye reagent and quantified by scanning the gel with a Fujix imager.

Results

Alanine Scanning Identifies LigA Residues Essential for Nick Joining in Vitro—Single alanine substitutions were introduced at 14 conserved positions in the nucleotidyltransferase domain of EcoLigA (Fig. 1). His-tagged G118A, L119A, D138A, E143A, G172A, E173A, N198A, R200A, R208A, R277A, D283A, G286A, V288A, and K290A mutants were produced in E. coli and purified by nickel-agarose chromatography in parallel with wild-type EcoLigA (Fig. 2A). The extent of ligation of singly nicked 3′-OH/5′-PO4 DNA by wild-type EcoLigA and each mutant was gauged as a function of the input enzyme, and the specific activities were normalized to the wild-type value (defined as 100%). Our operational definition of an essential amino acid was one at which alanine substitution reduced nick-sealing activity to <10% of the wild-type level. By this criterion, Gly-118, Glu-173, Arg-200, Arg-208, and Arg-277 were deemed essential for ligation (Fig. 2B).

Eight residues were judged to be nonessential for ligation in vitro (Fig. 2B). The L119A change flanking motif I had no impact on nick-joining activity. This leucine side chain was initially reported to line the AMP-binding pocket of TfiLigA and to make multiple van der Waals contacts with the ribose sugar of the covalently bound adenylate (8). It has since been determined that the amino acid sequence used to build the TfiLigA structure contained numerous errors (including incorrect side chains, deleted amino acids, and extra amino acids in the nucleotidyltransferase domain), which the authors have recently rectified. In the revised version of the TfiLigA structure (Protein Data Bank 1V9P), the leucine corresponding to Leu-119 of EcoLigA makes no contact with the bound adenylate. (Henceforth, all references to the TfiLigA structure will be to the revised version.) The equivalent leucine side chain makes no contacts with NAD+ in the EfaLigA structure (24).

The D138A change caused a modest reduction of ligation activity to 37% of wild-type LigA. The conserved Asp side chain is located on the surface of TfiLigA—15 Å from the bound AMP. The E143A mutation had little effect on ligation (52% of wild-type activity); this conserved glutamate is located on the surface of TfiLigA—17 Å away from the bound adenylate. The modest decrement in activity elicited by the G172A change in motif III (36% of wild-type nick joining) might reflect a subtle effect on the position of the vicinal Glu-173 side chain, which lines the AMP-binding pocket and is essential for EcoLigA catalysis. The TfiN198A change reduced nick-joining activity to one-fourth of wild-type LigA. This conserved Asn side chain is located —9 Å away from the AMP phosphate in TfiLigA-adenylate and is 7–8 Å away from the NNM moiety of NAD+ in the EfaLigA structure.

The D283A mutation had little impact on ligation activity (57% of wild-type activity), a finding that vitiated the model of Lee et al. (8), which proposes that this carboxylic acid side chain is required to coordinate an essential divalent cation cofactor during the step of phosphodiester formation. The G286A mutation in nucleotidyltransferase motif IV lowers nick-joining activity to 14% of wild-
type; we suspect that the loss of activity reflects steric effects of the alanine methyl group on the conformation of the motif IV beta strand, especially the position of the vicinal Asp285 side chain, which is essential for EcoLigA activity (26). The V288A change in motif IV was benign (74% of wild-type activity). This conserved aliphatic side chain makes van der Waals contacts with the adenine base of AMP in TfiLigA and the adenine of NAD in EfaLigA (24). We conclude that such contacts are not important for EcoLigA function. The K290A change reduced nick-joining activity to 13% of the wild-type level. This conserved lysine side chain lines the adenosine-binding pocket of TfiLigA and makes van der Waals contacts with the N1 and C2 atoms of the adenine base. Although Lys-290 does not meet our criterion for essentiality, it probably does play a role in substrate binding (see "Discussion").

Effects of Alanine Mutations on EcoLigA Function in Vivo in Yeast—A deletion of the essential Saccharomyces cerevisiae ATP-dependent DNA ligase Cdc9 can be complemented by expression of EcoLigA (26). Viability of the yeast cdc9/H9004 strain is contingent on maintenance of an extrachromosomal CDC9 gene on a CEN URA3 plasmid. Hence, cdc9/H9004 cells cannot grow on medium containing 5-FOA (a drug that selects against the URA3 CDC9 plasmid), but they can grow on 5-FOA if the cells have been transformed with a CEN TRP1 plasmid expressing wild-type EcoLigA under the control of the constitutive yeast TPI1 promoter. Here we tested, by the plasmid shuffle assay (26), whether the EcoLigA-Ala mutants were functional in cdc9/H9004 complementation in vivo. We found that mutations of the five residues defined as essential for EcoLigA function in vitro (Gly-118, Glu-173, Arg-200, Arg-208, Arg-277) were all lethal in vivo, i.e., they were unable to support growth of cdc9Δ on 5-FOA at either 18, 25, 30, or 37 °C (Fig. 2B). Mutations of seven of the residues defined as nonessential for nick-joining activity in vitro were functional in cdc9Δ complementation in vivo. Only the least

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**Fig. 1. Nucleotidyltransferase domain of bacterial LigA.** The amino acid sequence of the nucleotidyltransferase domain of E. coli (Eco) LigA is aligned to the sequences of LigA from M. tuberculosis (Mtu), Bacillus stearothermophilus (Bst), T. filiformis (Tfi), and E. faecalis (Efa). Nucleotidyltransferase motifs I, III, IIIa, IV, and V are highlighted in shaded boxes. Gaps in the alignment are indicated by dashes. Conserved residues are indicated by •. Amino acids shown previously to be essential for EcoLigA function are denoted by /H20841. Residues shown previously to be nonessential are denoted by /H11001. The fourteen amino acids of EcoLigA selected for alanine scanning in the present study are indicated by ?.

**Fig. 2. Effects of alanine mutations on EcoLigA function.** A, recombinant LigA proteins. Aliquots (5 μg) of the nickel-agarose preparations of wild-type (WT) EcoLigA and the indicated Ala mutants were analyzed by SDS-PAGE. Polypeptides were visualized by staining the gel with Coomassie Blue dye. The positions and sizes (in kDa) of marker proteins are indicated on the left. B, LigA activity. Nick-joining activity of the LigA-Ala mutants was gauged by enzyme titration as described under "Experimental Procedures" and expressed as the percent of the wild-type value. LigA complementation of a yeast cdc9/H9004 strain was tested by plasmid shuffle (26). Growth was scored as described under "Experimental Procedures."
active of the nonessential mutants, K290A, with 13% of wild-type activity in vitro, was unable to complement growth of cdc9Δ. The viable cdc9Δ ligA-Ala strains L119A, D138A, E143A, G172A, N198A, D283A, and V288A grew as well as the wild-type cdc9Δ ligA strain on YPD agar medium at 18, 25, 30, and 37 °C, as gauged by colony size (not shown). The G288A strain grew as well as a wild-type ligA strain at 25 and 30 °C but formed smaller colonies at 37 °C (not shown).

**Structure-Activity Relationships at Essential Residues of EcoLigA**—The present results, together with previous mutational data (26), highlight eight individual non-glycine amino acids within the nucleotidyltransferase domain that are essential for EcoLigA activity in vitro: Lys-115, Asp-117, Glu-173, Arg-200, Arg-208, Arg-277, Asp-285, and Lys-314. To better evaluate the contributions of these residues to the DNA ligase reaction, we tested the effects of conservative substitutions whereby arginine was replaced by lysine and glutamine, glutamate by glutamine and aspartate, asparagine and glutamate, and lysine by arginine and glutamine. Sixteen conservative EcoLigA mutants were produced in E. coli and purified from soluble bacterial extracts by nickel-agarose chromatography. The LigA polypeptide was the predominant species detected by SDS-PAGE, and the extents of purification were comparable for mutant and wild-type EcoLigA (Fig. 3A).

The nick-joining and ligase adenylylation activities of the mutants were determined and normalized to the wild-type value (Fig. 3B). Conservative replacement of the motif I Lys-115 by either arginine or glutamine abolished nick-joining activity and the reaction with \[^{32}P\text{NAD}^+\] to form the ligase-adenylate intermediate. These results highlight a strict requirement for lysine as the active site nucleophile. Replacing Asp-117 with asparagine abolished nick-joining activity (<0.1% of wild-type), and a glutamate substitution resulted in a minimal restoration of function (to 3% of wild-type). These data establish the requirement for a carboxylate residue at position 117, and we infer that there is a tight steric constraint that precludes accommodation of the extra methylene group of a glutamate. Previous studies showed that replacing the motif I Asp with alanine does not prevent the ligase adenylylation reaction with NAD\(^+\) (25). Here we find that the conservative D117N and D117E mutants retained 35 and 53% of wild-type adenylyltransferase activity, respectively. These findings point to an essential role of the conserved motif I aspartate during either step 2 or step 3 of the ligase pathway (25).

LigA nick-joining activity was abolished when the motif III Glu-173 side chain was replaced with either aspartate or glutamine. Thus, activity requires a carboxylate and a minimum distance from the main chain to the carboxylate that is met by glutamate but not aspartate. The E173D and E173Q mutants retained 13 and 54% of wild-type adenylyltransferase activity, respectively, signifying that an amide group suffices for the reaction of LigA with NAD\(^+\) to form ligase-adenylate, and the stringent requirement for a carboxylate is probably exerted at a downstream step of the ligation pathway.

Conservative replacement of Arg-200 and Arg-208 with either lysine or glutamine failed to restore nick-joining activity. Changing Arg-277 to glutamine reduced nick joining to 1.8% of the wild-type level, similar to the R277A mutant, and there was only marginal restoration of activity with a lysine substitution (to 5.6% of wild-type). Thus a positive charge is not sufficient at positions 200, 208, or 277; rather, we posit that these side chains engage in bidentate hydrogen bonding or ionic contacts that are sustained uniquely by arginine. Arg-208 is apparently essential for the reaction of LigA with NAD\(^+\), insofar as the adenylyltransferase activity of the R208Q and R208K proteins was 0.1–2% of the wild-type activity (Fig. 3B). In contrast, Arg-200 plays a more complex role whereby adenylyltransferase activity is partially restored by a lysine (14% of wild-type) but not a glutamine (1% activity); but overall ligation requires an arginine. The effects of the R277Q and R277K changes on ligase adenylylation closely paralleled their impact on overall ligation.

Replacing the essential motif IV Asp-285 side chain with asparagine abolished nick-joining activity (<0.1% of wild-type), and introducing a glutamate had minimal salutary effect (4% of wild-type). Thus, a carboxylate is required at position 285, and the extra methylene group of a glutamate side chain is not easily accommodated. The D285E and D285N mutants retained 6 and 23% of wild-type adenylyltransferase activity, respectively, signifying that, although an amide group at the proper distance from the main chain partially restores reactivity of LigA with NAD\(^+\), a carboxylate is required at a downstream step.

Conservative substitution of the motif V Lys-314 side chain with glutamine abolished nick-joining activity and reduced...
adenylyltransferase to 3% of wild-type. Introducing an arginine increased ligation and adenylyltransferase activities to 5 and 13% of wild-type, respectively.

Conservative Mutational Effects on EcoLigA Function in Vivo in Yeast—The conservative mutants were tested by plasmid shuffle for cdc9/H9004 complementation (Fig. 3B). The results underscore the theme that LigA mutations that abolish or severely depress nick-joining activity in vitro are lethal in vivo in yeast. The R227K mutant, which was the most active of the proteins in this collection (with 5.6% of wild-type ligase function), was able to sustain yeast growth on YPD medium at 25 and 30 °C, but not at 37 °C (scored as ts in Fig. 3B). The classical ligA-ts7 mutant strain of E. coli has a similar low residual level of strand-joining activity at the permissive temperature (28).

Effects of Lys-115, Glu-173, Arg-200, Arg-208, and Arg-277 Mutations on the Isolated Step of Phosphodiester Formation at a Pre-adenylated 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 of the ligation reaction using a pre-adenylated nicked DNA substrate labeled with32P at the 5′-PO4 of the DNA-adenylate strand (Fig. 4). Reaction of wild-type EcoLigA with the nicked DNA-adenylate in the presence of magnesium without added NAD+ resulted in strand closure, evinced by formation of a radiolabeled 36-mer product. A kinetic analysis of the sealing of nicked DNA-adenylate by wild-type ligase and mutants of residues Lys-115, Glu-173, Arg-200, Arg-208, and Arg-277 is presented in Fig. 4. As reported previously (23), the wild-type step 3 reaction of wild-type LigA attained an end point in 5 min (Fig. 4A). This result shows that a positive charge at this motif position is necessary and sufficient for step 3 catalysis and underscores how the motif I lysine plays different roles at different steps of the ligation pathway.

Mutations of Glu-173 in motif III have instructive effects on sealing at a pre-adenylated nick. Although alanine and glutamine substitutions slow the initial rate to 3 and 2% of the wild-type, respectively, the aspartate substitution elicits a gain of step 3 function to 14% of the wild-type rate (Fig. 4B). The hierarchy of conservative effects on step 3 activity, whereby E174D is more active than E173Q, is opposite to that on step 1 ligase adenylylation, where E173Q is more active than E173D (Fig. 3B). Thus, the motif III side chain acts differently at different steps along the reaction pathway, with an amide functional group sufficing for step 1 but not step 3 and a carboxylate being required for step 3.

Changing Arg-208 to either alanine or lysine abolished overall ligation and precluded the ligase adenylylation step (Fig. 3B); thus it is remarkable that these same mutations had relatively little impact on the kinetics of phosphodiester formation at a pre-adenylated nick (Fig. 4C). The rate of step 3 by R208A and R208K was about one-half that of wild-type LigA, signifying that Arg-208 is not required for catalysis of the sealing step. Nonetheless, the R208Q change was apparently deleterious to step 3 catalysis, insofar as the step 3 rate of R208Q was ~7-fold slower than that of either R208A or R208K (Fig. 4C).

Mutations R277A, R277Q, and R277K slowed the rate of the isolated sealing step to 20, 10, and 4% of the wild-type LigA, respectively (Fig. 4D). Although the lysine change had a greater impact on step 3 than did glutamine, the opposite was seen for the nick ligation and ligase adenylylation reactions, for which the R277K mutant was more active than R277Q.
All of the Arg-200 mutations strongly suppressed sealing at a pre-adenylated nick (Fig. 4E). The step 3 rates of the R200K, R200Q, and R200A proteins were 7, 3, and 2% of the wild-type, respectively. Thus, although a lysine could partially substitute for arginine in ligase-adenylylation, an arginine was strictly required for step 3 and overall nick joining.

DISCUSSION

The LigA reaction is initiated by the attack of the motif I lysine on the NAD\(^+\) substrate to form ligase-adenylate. The reaction likely proceeds through a tetra-coordinate phosphorane transition state in which the attacking lysine nucleophile is apical to the NMN leaving group. The structure of \textit{E. faecalis} ligase revealed that coordination of the NMN moiety of NAD\(^+\) is achieved by the closure of domain Ia over the AMP-binding pocket of the nucleotidyltransferase domain. Breaking of the \(\alpha\)-phosphoanhydride bond of NAD\(^+\) upon enzyme-adenylate formation releases the tether of domain Ia to the nucleotidyltransferase domain and allows LigA to adopt an open conformation in which the AMP phosphate is exposed on the now free surface of the nucleotidyltransferase domain, to which the DNA nick must bind for catalysis of steps 2 and 3. The nucleotidyltransferase domain of LigA is not able to bind DNA by itself; rather the DNA-binding step and catalysis of phosphodiester formation require contribution from the flanking C-terminal domain modules (3, 20–22, 25).

Here we performed an alanine scan of 14 conserved amino acids of the nucleotidyltransferase domain of \textit{EcoLigA} and thereby identified five essential residues (Gly-118, Glu-173, Arg-200, Arg-208, and Arg-277). Structure-activity relationships were determined for Glu-173, Arg-200, Arg-208, and Arg-277, as well as four other essential side chains (Lys-115, Asp-117, Asp-285, and Lys-314). In addition, we identified Lys-290 as important for LigA activity, although not essential by our cut-off criterion. Reference to the \textit{EfaLigA} crystal structure (24) allows us to discriminate three classes of essential/important side chains: (i) those that contact NAD\(^+\) directly (e.g., Lys-115, Glu-173, Lys-290, and Lys-314), (ii) those that either comprise the interface between domain Ia and the nucleotidyltransferase domain in the closed conformation or comprise part of the DNA docking site on the surface of the nucleotidyltransferase domain in the open conformation (e.g., Arg-200 and Arg-208), and (iii) those that stabilize the fold of the nucleotidyltransferase domain (e.g., Arg-277). Although not directly implicated as such from available LigA structures, we suggest that Asp-285 and perhaps Asp-117 comprise a metal-binding site. The several classes of essential residues are considered individually below.

\textit{NAD}^+-binding Residues—The role of the motif I lysine nucleophile in the ligase adenylylation step is established biochemically and structurally (8, 25) (Fig. 5). Here we show that arginine is unable to replace lysine in forming the ligase-AMP intermediate of \textit{EcoLigA}; similar findings were reported previously for \textit{Thermus thermophilus} LigA (25). We presume that the higher pK\(_{a}\) of arginine hinders the necessary deprotonation of the nucleophilic nitrogen atom during catalysis of step 1. Yet, arginine is fully able to perform the essential role of the motif I lysine during the step of phosphodiester bond formation by EcoLigA. A role for the motif I lysine in step 3 of the NAD\(^-\) dependent ligase reaction had not been demonstrated previously. Our results suggest that the positively charged side chain of the motif I lysine provides an essential contact with the AMP leaving group during the attack of the 3'-OH of the nick on the DNA 5'-phosphate of the AppDNA intermediate.

The essential motif III glutamate coordinates the adenosine ribose O2' atom in the NAD\(^+\)-bound substrate complex of \textit{Efa-}

\begin{figure}[h]
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\caption{Conformational switch at the AMP-binding pocket of LigA. Left panel, the structure of \textit{E. faecalis} DNA ligase bound to NAD\(^+\) shows that the adenosine nucleoside adopts a \textit{syn} conformation in which contacts are observed between the AMP ribose O2' and the motif III glutamate and between the motif I lysine nucleophile and the AMP phosphate. Right panel, the structure of the \textit{T. filiformis} ligase-AMP covalent intermediate shows an \textit{anti} conformation of the adenosine nucleoside. The rotation about the glycosidic bond disrupts the contacts to the motif III Glu side chain seen in the left panel.}
\end{figure}
The essential and conserved motif V lysine (Lys-314 in EcoLigA) makes a bifurcated contact to both the AMP phosphate of NAD\(^+\) and the ribose O\(^2\) atom of the nicotinamide nucleoside (24). The phosphate interaction of the motif V lysine is a common thread for the ligases and capping enzymes that comprise the covalent nucleotidyltransferase superfamily. Previous studies implicated Lys-314 as a catalyst of both steps 1 and 3 of the ligation pathway (26). Here we showed that Lys-314 could not be replaced by glutamine and that arginine restored only marginal activity overall.

**Residues at the Domain Interface and Putative Nick-binding Surface**—Two conserved and essential arginine residues (Arg-200 and Arg-208 in EcoLigA) project from a helix out to the exposed surface of the open conformation of the nucleotidyltransferase domain of EfaLigA (24). In the closed NAD\(^+\)-bound conformation, the Arg-208 equivalent makes bidentate contacts to two sites in domain Ia: one from Arg-NH\(1\) to the backbone carbonyl of a valine corresponding to Val-24 of EcoLigA and one from Arg-Ne to the O\(\delta\) atom of an aspartate side chain equivalent to Asp-26 of EcoLigA. Our mutational findings show that Arg-208 is critical for overall nick joining and for ligase adenylylation but not for catalysis of step 3. To a first approximation, the R208A and R208K mutations phenocopy either a complete deletion of domain Ia or missense mutations in the residues of domain Ia that form the NMM-binding site (23). We surmise that the cross-domain contacts of Arg-208 are critical to trigger domain Ia closure and achieve proper orientation of the NAD\(^+\) substrate for step 1 catalysis. The use of a pre-adenylated nicked substrate (which bypasses step 1) relieves the requirement for Arg-208 just as it relieves the requirement for domain Ia. It is noteworthy that Arg-208 also forms an internal salt bridge from NH\(1\) and NH\(2\) to a glutamate within the nucleotidyltransferase domain (equivalent to Glu-143 of EcoLigA); yet, because the Glu-143 side chain is itself not important (Fig. 2B), we infer that the intradomain salt bridge is not the critical feature of Arg-208 function.

The other essential arginine (Arg-200) also makes a cross-domain contact from Arg-NH\(1\) to the Asp-NH\(1\) of the ATP-dependent Chlorella virus DNA ligase-AMP intermediate coordinates a single lutetium atom implicates this side chain as a component of the divalent cation-binding site of DNA ligase (11). However, none of the available crystal structures of NAD\(^+\)-dependent ligases directly illuminate the function of this essential acidic residue or the function of the conserved Asp residue in motif I (KXDG). The motif I and IV aspartates are located close to one another in the EfaLigA and TrfLigA structures, although neither makes contact with NAD\(^+\) or AMP. Lee et al. (8) propose a model in which these two side chains, plus the acidic residue two amino acids upstream of motif IV (Asp-283 in EcoLigA), comprise a binding site for two magnesium ions that are poised to directly promote catalysis of step 3. Our finding that Asp-283 is not important for EcoLigA activity in vitro or in vivo effectively disproves a requirement for three carboxylates as metal-binding ligands but does not speak to the issue of one-metal versus two-metal mechanisms or the nature of the metal-binding site. Asp-285 in motif IV is a plausible candidate for metal binding insofar as its replacement by alanine abolishes overall ligation (26). The finding here that a conservative asparagine mutation partially restores ligase adenylylation (to 23% of wild-type activity) suggests that an amide functional group can provide the putative metal-binding function for step 1 but not for subsequent steps (i.e. D285N was inactive in overall nick joining). If the motif I aspartate plays a role in metal binding, then it is likely to do so only subsequent to the ligase adenylylation step, because we show that the D117E and D117N mutants retain step 1 function despite the loss of overall nick-joining activity in vitro and in vivo. Studies of Chlorella virus DNA ligase suggest that the metal-binding site is remodeled as the ligation reaction proceeds (15). An evaluation of whether this is true of NAD\(^+\)-dependent ligases will hinge on capturing structural snapshots of LigA bound to nucleotide and DNA substrates in the presence of metals, which will, in turn, hinge on methods to maintain the ligand-binding properties of LigA while precluding the completion of the ligation reaction during the time frame of the crystalization experiment.

**Roles of the Motif IV and Motif I Aspartates**—The finding that the motif IV Asp of the ATP-dependent Chlorella virus DNA ligase-AMP intermediate coordinates a single lutetium atom implicates this side chain as a component of the divalent cation-binding site of DNA ligase (11). However, none of the available crystal structures of NAD\(^+\)-dependent ligases directly illuminate the function of this essential acidic residue or the function of the conserved Asp residue in motif I (KXDG). The motif I and IV aspartates are located close to one another in the EfaLigA and TrfLigA structures, although neither makes contact with NAD\(^+\) or AMP. Lee et al. (8) propose a model in which these two side chains, plus the acidic residue two amino acids upstream of motif IV (Asp-283 in EcoLigA), comprise a binding site for two magnesium ions that are poised to directly promote catalysis of step 3. Our finding that Asp-283 is not important for EcoLigA activity in vitro or in vivo effectively disproves a requirement for three carboxylates as metal-binding ligands but does not speak to the issue of one-metal versus two-metal mechanisms or the nature of the metal-binding site. Asp-285 in motif IV is a plausible candidate for metal binding insofar as its replacement by alanine abolishes overall ligation (26). The finding here that a conservative asparagine mutation partially restores ligase adenylylation (to 23% of wild-type activity) suggests that an amide functional group can provide the putative metal-binding function for step 1 but not for subsequent steps (i.e. D285N was inactive in overall nick joining). If the motif I aspartate plays a role in metal binding, then it is likely to do so only subsequent to the ligase adenylylation step, because we show that the D117E and D117N mutants retain step 1 function despite the loss of overall nick-joining activity in vitro and in vivo. Studies of Chlorella virus DNA ligase suggest that the metal-binding site is remodeled as the ligation reaction proceeds (15). An evaluation of whether this is true of NAD\(^+\)-dependent ligases will hinge on capturing structural snapshots of LigA bound to nucleotide and DNA substrates in the presence of metals, which will, in turn, hinge on methods to maintain the ligand-binding properties of LigA while precluding the completion of the ligation reaction during the time frame of the crystalization experiment.

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