Conserved Residues in Domain Ia Are Required for the Reaction of *Escherichia coli* DNA Ligase with NAD$^{+,*}$

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NAD$^{+}$-dependent DNA ligases are present in all bacteria and are essential for growth. Their unique substrate specificity compared with ATP-dependent human DNA ligases recommends the NAD$^{+}$ ligases as targets for the development of new broad-spectrum antibiotics. A plausible strategy for drug discovery is to identify the structural components of bacterial DNA ligase that interact with NAD$^{+}$ and then to isolate small molecules that recognize these components and thereby block the binding of NAD$^{+}$ to the ligase. The limitation to this strategy is that the structural determinants of NAD$^{+}$ specificity are not known. Here we show that reactivity of *Escherichia coli* DNA ligase (LigA) with NAD$^{+}$ requires N-terminal domain Ia, which is unique to, and conserved among, NAD$^{+}$ ligases but absent from ATP-dependent ligases. Deletion of domain Ia abolished the sealing of 3′-OH/5′-PO$_4$ nicks and the reaction with NAD$^{+}$ to form ligase-adenylate but had no effect on phosphodiester formation at a preadenylated nick. Alanine substitutions at conserved residues within domain Ia either reduced (His-23, Tyr-35) or abolished (Tyr-22, Asp-32, Asp-36) sealing of a 5′-PO$_4$ nick and adenylylation transfer from NAD$^{+}$ without affecting ligation of pre-formed DNA-adenylate. We suggest that these five side chains comprise a binding site for the nicotinamide mononucleotide moiety of NAD$^{+}$. Structure-activity relationships were clarified by conservative substitutions.

DNA ligases are grouped into two families, ATP-dependent ligases and NAD$^{+}$-dependent ligases, according to the cofactor required for ligase-adenylate formation (1). Both types of DNA ligases catalyze the sealing of 5′-phosphate and 3′-hydroxyl termini at nicks in duplex DNA by means of three sequential nucleotidyl transfer reactions. In the first step, attack on the α-phosphorus of ATP or NAD$^{+}$ by ligase results in release of pyrophosphate or nicotinamide mononucleotide (NNM)$^1$ and formation of a covalent intermediate (ligase-adenylate) in which AMP is linked via a phosphoamide bond to 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 two polynucleotides and release AMP.

At least one NAD$^{+}$-dependent DNA ligase (referred to as LigA) is found in every bacterial species (2). The bacterial LigA enzymes are of fairly uniform size (647–841 amino acids), and they display extensive amino acid sequence conservation throughout the entire lengths of the polypeptides. The atomic structures of the LigA enzymes from *Bacillus stearothermophilus* and *Thermus filiformis* have been determined by x-ray crystallography (3, 4). TjLigA contains a catalytic core composed of nucleotidyl transferase and oligomer-binding (OB)-fold domains, flanked by a 73-amino acid N-terminal domain (Ia) and three C-terminal domains: a tetracysteine domain that binds a single zinc atom, a helix-hairpin-helix domain (HhH), and a BRCT domain (named after the C terminus of the breast cancer gene product BRCA1) (Fig. 1A). *Escherichia coli* encodes a second NAD$^{+}$-dependent DNA ligase isozyme (LigB) in addition to LigA. The LigB protein contains the Ia, nucleotidyl transferase, OB-fold, and HhH domains, but lacks the tetracysteine zinc finger and the BRCT structural domains found in LigA (5). The genomes of *Tersinia pestis* and *Salmonella typhi* also encode two NAD$^{+}$-dependent ligases corresponding to LigA and LigB. The abbreviated domain structure of the bacterial LigB enzymes resembles that of the recently identified NAD$^{+}$-dependent ligase of *Amsacta moorei* entomopoxivirus (*AmEPV*), a eukaryotic poxvirus (6).

Although there is scant primary structure similarity between NAD$^{+}$- and ATP-dependent ligases, the tertiary structures of the nucleotidyl transferase and OB-fold domains are conserved (3, 4, 7, 8). The adenylylation binding pockets of the NAD$^{+}$- and ATP-dependent ligases are composed of five motifs (I, III, IIIa, IV, and V) that define the DNA ligase/mRNA capping enzyme superfamily of conserved nucleotidyl transferases (9–11). Motif I (KKXDG) contains the lysine nucleophile to which AMP becomes covalently linked in the first step of the ligase reaction (4, 8). Motifs I, III, IIIa, IV, and V include side chains that contact AMP and are essential for enzyme activity *in vitro* and *in vivo* (8, 12–14, 25, 26). The OB-fold domain consists of a five-stranded antiparallel β barrel and an α helix. The OB-fold domain of ATP-dependent ligases and GTP-dependent capping enzymes includes at its C terminus nucleotidyl transferase motif VI (RXDK), which contacts the β and γ phosphates of the NTP substrate and is required for the reactions with ATP or GTP to form the enzyme-NTP intermediate (10, 13, 15, 16). The NAD$^{+}$ ligases lack a recognizable counterpart of motif VI within their OB-fold domains, which is sensible given that the NAD$^{+}$ substrate does not contain a γ phosphate.

Early genetic studies showed that the ligA gene is essential for growth of *E. coli* (17, 18). Genes encoding NAD$^{+}$-dependent LigA are also essential in *Salmonella typhimurium*, *Bacillus subtilis*, and *Staphylococcus aureus* (19–21). It is reasonable to think that LigA will be essential for all bacteria. To date, there have been no reports of an NAD$^{+}$-dependent DNA ligase in a eukaryotic species. Therefore, bacterial LigA presents an-
tractive target for broad-spectrum antibiotic therapy predicated on blocking the reaction of DNA ligase with NAD⁺. A rational strategy for drug discovery would entail the identification of the structural components of LigA that interact with NAD⁺ and the isolation of small molecules that recognize these components and thereby block the binding of NAD⁺ to bacterial ligase. The drug-binding site on the NAD⁺ ligase would ideally be unique to, and conserved among, NAD⁺ ligases but absent from ATP-dependent ligases and other essential NAD⁺-requiring enzymes. The limitation to this strategy is that the structural components of bacterial LigA that interact specifically with NAD⁺ are not known.

N-terminal fragments of *B. stearothermophilus*, *S. aureus*, and *Aquifex pyrophilus* LigA composed solely of the Ia and nucleotidyl transferase domains retained full ligase adenylate activity although they were no longer active in the composite nick joining reaction (21–23). Similarly, an N-terminal fragment of entomopoxvirus ligase containing the Ia and nucleotidyl transferase domains sufficed for the reaction with NAD⁺ to form the ligase-AMP intermediate; said fragment was unable to catalyze phosphodiester formation at a standard 5'-PO₄ nick or at a preadenylated nick (6). An instructive finding was that deletion of domain Ia of entomopoxvirus DNA ligase abolished the reaction with NAD⁺ to form ligase-adenylate but had no effect on phosphodiester bond formation at a preadenylated nick (6); these results implicated domain Ia of AmEPV ligase in recognition of the NAD⁺ substrate.

Here we show that domain Ia is essential for the reaction of *E. coli* DNA ligase with NAD⁺. We identify individual amino acids within domain Ia that are required for ligase adenylate but not for phosphodiester bond formation. The essential residues, which are conserved in all known NAD⁺-dependent DNA ligases, are located on the surface of the LigA protein where we posit that they interact with the NMN moiety of the NAD⁺ substrate. We discuss the likely role of protein conformational changes in orchestrating the adenylate transferase reaction.

**EXPERIMENTAL PROCEDURES**

*Ligase Mutants—Missense mutations of domain Ia of EcoLigA were introduced into the pET-EcoLig expression plasmid using the PCR-based two-stage overlap extension method as described previously (14). The entire ligA gene was sequenced in every case to confirm the desired mutation and exclude the acquisition of unwanted changes during PCR amplification and cloning. The expression plasmids were transformed into *E. coli* BL21(DE3). Mutant and wild-type ligases were purified from the soluble lysates of isopropyl-1-thio-β-D-galactopyranoside-induced BL21(DE3) cells by nickel-agarose chromatography as described (14). The protein concentrations of the phosphocellulose enzyme preparations were determined using the Bio-Rad dye reagent with bovine serum albumin as a standard. N-terminal deletion mutants NA38 and NΔ78 were purified as described previously (14).

**Assay of Nick Joining—** Reaction mixtures (20 µl) containing 50 mM Tris-HCl (pH 7.5), 10 mM (NH₄)₂SO₄, 5 mM DTT, 5 mM MgCl₂, 20 µM NAD⁺, 1 pmol of 5’-³²P-labeled nicked duplex DNA substrate (shown in Fig. 2C), and aliquots of serial 2-fold dilutions of wild-type or mutant ligases were incubated at 22°C for 10 min. The products were resolved by denaturing PAGE, and the extents of ligation were determined by scanning the gel with a FujiX PhosphorImager. The specific activities of wild-type and mutant ligases were determined from the slopes of the titration curves in the linear range of enzyme dependence.

**Ligation at a Preadenylated Nick—** The nicked DNA-adenylate sub-strate is shown in Fig. 3. The 5’-adenylated ³²P-labeled 18-mer strand was synthesized and gel-purified as described (24). The DNA-adenylate ligation reaction mixtures (20 µl) contained 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 5 mM MgCl₂, nicked DNA-adenylate substrate, and wild-type or mutant EcoLigA ligase proteins as specified. The mixtures were incubated for 60 min at 22°C. The products were resolved by denaturing PAGE, and the extents of ligation were determined by scanning the gel with a PhosphorImager. For kinetic analysis, reaction mixtures containing (per 20 µl) 200 fmol of nicked DNA-adenylate substrate, 2 pmol of ligase, and other components as specified above were incubated at 22°C. The sealing reactions were initiated by adding ligase. Aliquots (20 µl) were withdrawn at the times specified and quenched immediately with EDTA and formamide (12).

**RESULTS**

**Domain Ia of *E. coli* DNA Ligase Is Required for Reaction with NAD⁺—** Prior studies had shown that N-terminal deletions NΔ78 and NΔ38 of *E. coli* DNA ligase (EcoLigA), which eliminate all or part of domain Ia, resulted in complete loss of nick joining activity (14). To probe the essential role of domain Ia in the ligase reaction, we examined the effects of the NΔ78 and NΔ38 mutations on individual steps in the reaction pathway. The first step in DNA ligation involves formation of a covalent enzyme-adenylate intermediate. Whereas incubation of wild-type EcoLigA with [³²P]AMP-labeled NAD⁺ and magnesium resulted in the formation of a ³²P-labeled covalent nucleotidyl protein adduct that comigrated with the full-sized ligase polypeptide during SDS-PAGE, the NΔ78 and NΔ38 mutants were inert in ligase adenylation (Fig. 2B).

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 preadenylated nicked DNA substrate labeled with ³²P on the 5’-PO₄ of the DNA-adenylate strand (Fig. 3). 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. NΔ78 and NΔ38 were also capable of forming a phosphodiester bond at the preadenylated nick (Fig. 3). The latter finding underscores that the abrogation of the overall nick joining reaction by the NΔ78 and NΔ38 deletions cannot be ascribed to a global folding defect but instead reflects a specific requirement for domain Ia in the reaction of *E. coli* ligase with NAD⁺.

**Single Alanine Mutations in Domain Ia of EcoLigA Affect Binding and Nick Ligation—** To further probe the role of domain Ia in NAD⁺ recognition and nucleotidyl transfer, single alanine substitutions were introduced at six positions in the Ia domain of EcoLigA. The six mutated residues, Glu-10, Tyr-22, His-23, Asp-32, Tyr-51, and Asp-52, are denoted by dots in Fig. 1B. Five of the six positions (Tyr-22, His-23, Asp-32, Tyr-51, and Asp-52) are conserved in the NAD⁺-dependent ligases (LigA homologs) from 30 other bacterial species and in entomopoxvirus NAD⁺-dependent DNA ligase. The recombinant *Eco*LigA mutants E10A, Y22A, H23A, D32A, Y51A, and D56A were purified by nickel-agarose chromatography in parallel with wild-type ligase (Fig. 2A). The extent of ligation of singly nicked 3’-OH/5’-PO₄ DNA by wild-type *Eco*LigA was proportional to input protein, and ~80% of the input nicked substrate was sealed at saturating levels of enzyme (Fig. 2C and data not shown). The specific activity of the E10A protein was 90% that of the wild-type ligase; however, the other alanine mutations elicited significant defects in nick joining (Fig. 2C). The specific activities of the mutants relative to wild-type ligase were as follows: Y22A (0.1%), H23A (10%), D32A (0.2%), Y51A (2%), and D56A (0.2%) (Table I). The defects in nick sealing were accompanied by defects in the reactions of the mutant proteins with NAD⁺ to form the covalent ligase-adenylate intermediate (Fig. 2B). In particular, the Y22A, D32A, and D56A mutants were virtually inert in both nick ligation and ligase adenylate. H23A and Y51A, which were less active than wild-type ligase in nick joining, were also less active in ligase-AMP formation. The E10A mutation, which had no effect on nick joining, also did not affect the yield of ligase-AMP adduct (Fig. 2B and data not shown).

Control experiments confirmed that all of the domain Ia mutants (E10A, Y22A, H23A, D32A, Y51A, and D56A) were catalytically active in phosphodiester formation with the
FIG. 1. Domain Ia is conserved in NAD⁺-dependent DNA ligases. A, bacterial NAD⁺-dependent DNA ligase is depicted as a linear array of conserved structural domains (Ia, Nucleotidyl Transferase, OB-fold, Zn-binding, HhH, and BRCT). B, the amino acid sequence of domain Ia of E. coli LigA (Eco) from residues 9–68 is aligned to the domain Ia sequences of NAD⁺ ligases from 30 other species of bacteria plus the entomopoxvirus AmEPV. Domain Ia of E. coli LigB (EcoLigB) is also included in the alignment. The secondary structure of Tfi ligase domain Ia is shown below the aligned sequences with helices depicted as horizontal bars. The six positions of EcoLigA that were targeted for mutational analysis in the present study are denoted by dots. The five amino acids that are conserved in all of the NAD⁺ ligases and are defined by the mutational analysis as important for the reaction of E. coli ligase with NAD⁺ are highlighted by shaded boxes. The other bacterial ligases included in the alignment are from Aquifex aeolicus (Aae), Agrobacterium tumefaciens (Atu), Borrelia burgdorferi (Bbu), Bordetella pertussis (Bpe), Campylobacter jejuni (Cje), Chlamydia pneumoniae (Cpn), Chlamydia trachomatis (Ctr), Deinococcus radiodurans (Dra), Hemophilus influenzae (Hin), Geobacter sulfurreducens (Gsu), Lactococcus lactis (Lla), Legionella pneumophila (Lpn), Mycoplasma genitalia (Mge), Mycoplasma pneumoniae (Mpn), Mycobacterium leprae (Mle), Mycobacterium tuberculosis (Mtu), Neisseria meningitidis (Nme), Pasteurella multocida (Pma), Pseudomonas aeruginosa (Pae), Pseudomonas putida (Ppu), Pseudomonas stutzer (Pse), Rhodobacter sphaeroides (Rba), Rickettsia prowazekii (Rpr), S. aureus (Sau), Streptococcus mutans (Smu), Thiobacillus ferrooxidans (Tfe), Vibrio cholerae (Vch), Y. pestis (Ype), Zymomonas mobilis (Zmo), B. stearothermophilus (Bst), and T. filiformis (Tfi).

FIG. 2. Effects of deletion and alanine mutations in domain Ia of E. coli DNA ligase. A, aliquots (5 μg) of wild-type (WT) E. coli ligase, N-terminal deletion mutants N38 and N78, and the full-length ligase proteins containing the indicated alanine mutations in domain Ia were analyzed by SDS-PAGE. The Coomassie Blue-stained gel is shown. The positions and sizes (in kDa) of marker proteins are indicated on the left. B, ligase adenylation reaction mixtures (20 μl) containing 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 5 mM MgCl₂, 1 μM [³²P]AMP-labeled NAD⁺ (NEN Life Science Products) and 8 pmol of WT ligase, N38, N78, or the indicated alanine mutants were incubated for 5 min at 37 °C. The reaction products were resolved by SDS-PAGE and visualized by autoradiography. C, nick joining reaction mixtures (20 μl) containing 1 pmol of [³²P]-labeled nicked DNA (as shown) and increasing amounts of WT ligase or the indicated alanine mutants were incubated for 10 min at 22 °C. The extents of ligation are plotted as a function of input protein.
that most NAD-tyrosine restored activity to near-wild-type level (88%). Note that most NAD-dependent enzymes (Fig. 1B). These experiments show that specific functional groups within domain Ia of E. coli DNA ligase are important for the reaction of ligase with NAD+ but are not required for catalysis when the AMP pocket of the nucleotidyl transferase domain is filled by the adenylated DNA intermediate.

**Effects of Conservative Mutations in Domain Ia.**—To evaluate the roles of charge, hydrogen bonding potential, and steric constraints in the functions of the domain Ia residues implicated in the step 1 reaction with NAD+, we tested the effects of conservative substitutions. Tyr-22 and Tyr-35 were replaced by Phe and Ser, His-23 was changed to Tyr, and Asp-32 and Asp-36 were mutated to Glu and Asn. The recombinant mutant ligases were purified from soluble bacterial extracts by nickel-agarose chromatography (Fig. 5A). The specific activity of each mutant was determined under steady-state conditions by protein titration and normalized to the specific activity of wild-type (WT) E. coli LigA mutant Nick joining activity

| LigA mutant | Nick joining activity % of WT |
|-------------|-----------------------------|
| E10A        | 90                          |
| Y22A        | 0.1                         |
| Y22F        | 9                           |
| Y22S        | 0.2                         |
| H23A        | 10                          |
| H23Y        | 88                          |
| D32A        | 0.2                         |
| D32E        | 0.4                         |
| D32N        | 9                           |
| Y35A        | 2                           |
| Y35F        | 23                          |
| Y35S        | <0.1                        |
| D36A        | 0.2                         |
| D36E        | 4                           |
| D36N        | 12                          |

optimal strand joining activity, the isosteric amide functional groups are tolerated with a significant, but not catastrophic, activity decrement. We infer that hydrogen-bonding interactions of the Asp-32 and Asp-36 functional groups are critical for activity and that the ligase does not tolerate lengthening of the distance from the main chain to the carboxylates, presumably because of steric clashes. Positions 32 and 36 are strictly conserved as aspartate in all NAD+-dependent ligases. (Fig. 1B).

**Fig. 3. Domain Ia is dispensable for phosphodiester synthesis at a preadenylated nick.** Reaction mixtures containing 1 pmol of [32P]-labeled nicked DNA-adenylate (AppDNA) and either 8 pmol of wild-type (WT) E. coli ligase, deletion mutants NΔ38 and NΔ78, or the full-length ligase proteins containing the indicated alanine mutations in domain Ia were incubated for 60 min at 22 °C. The reaction products were resolved by denaturing PAGE. An autoradiograph of the gel is shown. Control reaction mixtures containing either [32P]-labeled nicked DNA-adenylate (AppDNA) or nicked DNA (pDNA) substrates and no ligase are shown in lanes -. The position of the pDNA, AppDNA, and ligated 36-mer DNA strands are indicated by arrows on the right. The position of the radiolabeled phosphate of AppDNA is denoted by a dot. The nicked DNA-adenylate substrate used in the ligation reactions is illustrated at the bottom.

**Fig. 4. Kinetics of phosphodiester formation at a preadenylated nick.** Assays were performed as described under “Experimental Procedures.” The extent of strand joining is plotted as a function of reaction time.

**Effects of Conservative Domain Ia Mutations on Ligase-AMP Formation.**—The effects of the conservative domain Ia mutations on the reactions of the recombinant ligases with NAD+ to form the covalent ligase-adenylate intermediate paralleled the effects on the composite nick joining reaction (Fig. 5B). The Y22F mutant was weakly active in ligase adenylation, whereas the Y22S protein was virtually inert. Y35F restored adenylation activity, but the Y35S mutant was apparently unreactive with NAD+. The H23Y change restored the yield of ligase-AMP adduct to the wild-type level. The D32N and D36N proteins were partially active, while the D32E and D36E ligases were more severely affected (Fig. 5B).

A kinetic analysis of the reaction of EcoLigA with 1 μM [32P]AMP-labeled NAD+ is shown in Fig. 6. Wild-type ligase attained its reaction end point in ≈ 15 s (the earliest time tested) with -24% of the input ligase molecules being labeled with [32P]AMP. Mutational effects on the rates of ligase adenylation were generally consistent with the hierarchy of effects on the steady-state nick joining reaction. H23Y, which had the highest nick joining activity (88%) of the conservative domain
Ia mutants, displayed a kinetic pattern similar to wild-type ligase, whereas catalytically impaired mutants H23A and Y22F (9–10% activity) reacted slowly (Fig. 6A). Y35F was adenylated faster than Y35A (Fig. 6A); D32N was faster than D32E, and D36N was faster than D36E (Fig. 6B). We infer from these results that the conserved Tyr-22, His-23, Asp-32, Tyr-35, and Asp-36 side chains are constituents of the NAD⁺ binding site of bacterial DNA ligase.

**DISCUSSION**

The selective effects of deletions and mutations in domain Ia of *EcoLigA* on the nucleotidyl transferase reaction with NAD⁺ extend our findings for entomopoxvirus DNA ligase (6) and provide evidence for common structural determinants of substrate specificity for the NAD⁺ ligase family, which are located within domain Ia. Domain Ia is unique to NAD⁺-dependent ligases, and there is no discernable counterpart in ATP-dependent ligases. Thus, it is sensible that domain Ia is involved in NAD⁺ recognition.

Our results suggest a model whereby ligase substrate specificity at the step of ligase-adenylate formation is determined by the interactions of domain Ia of the NAD⁺-dependent enzymes with the NMM moiety of NAD⁺ (Fig. 7). The crystal structures of NAD⁺ ligase, ATP ligases, and mRNA capping enzyme in various functional states all indicate that contacts of the enzymes with the AMP or GMP moieties are confined to the nucleotidyl transferase domain (3, 4, 7, 8, 10). The nucleoside portion is buried within a pocket while the α-phosphate is exposed on the surface of the domain. The first step in ligation and capping entails the attack of the motif I lysine on the nucleotide triphosphate or NAD⁺ substrates to form enzyme-adenylate or enzyme-guanulate. The reaction is believed to proceed through a pentacoordinate phosphorane transition state in which the attacking lysine nucleophile is apical to the pyrophosphate or NMN leaving group. We propose that the proper orientation of NAD⁺ is achieved by closure of domain Ia over the nucleotide binding pocket, resulting in contacts between the side chains of domain Ia and the nicotinamide nucleoside (Fig. 7). The breaking of the α-β phosphoanhydride bond of NAD⁺ upon enzyme-adenylate formation would release the tether of domain Ia to the nucleotidyl transferase domain and allow the domains to spring apart to adopt the conformation observed in the crystal structure of the *Tfi* ligase-adenylate intermediate (4). The domain movement would then allow the binding of the nicked DNA substrate immediately above the AMP phosphate on the surface of the nucleotidyl transferase domain. Domain Ia is apparently dispensable once the ligase-adenylation reaction is completed or when it can be bypassed, i.e., deletions and point mutations of domain Ia do not affect recognition of the nicked DNA-adenylate intermediate and the chemical step of phosphodiester bond formation.

There is as yet no reported crystal structure of an NAD⁺ ligase bound to NAD⁺. However, the analysis of the effects of single alanine mutations in domain Ia of AmEPV ligase and *EcoLigA* ligases identifies five residues (Y22A, H23A, D32A, Y35A, and D36A in *EcoLigA*) that are involved specifically in adenylate transfer from NAD⁺. These five residues are con-
served in the NAD⁺ ligases from 30 other bacterial species (shown in Fig. 1B) and in additional bacterial NAD⁺ ligases that are not shown. Indeed, the five side chains are tightly clustered on the same surface of domain Ia in the Tfi ligase and Bst ligase crystal structures (3, 4). Accordingly, we speculate that these residues are constituents of an NMN binding site.

The structures of domain Ia of Tfi and Bst ligases consist principally of two antiparallel α helices and an intervening loop (Fig. 1B). In the Tfi and Bst ligases, the essential aspartates (Asp-34 and Asp-38, corresponding to EcoLigA residues Asp-32 and Asp-36) are located on the enzyme surface with their O6 atoms separated by 3.5–5.6 Å (3, 4). We speculate that this pair of surface Asp residues may coordinate the vicinal ribose oxygens of the nicotinamide nucleoside via hydrogen bonding. Alternatively, the aspartates may interact with the nicotinamide base. A role for the surface aspartates in coordinating a divalent cation seems less attractive, insofar as their replacement by Phe results in a near-total loss of ligase activity. Loss of the aromatic ring of Tyr-22 in EcoLigA is positioned with its phenolic hydroxyl 3.2–4.0 Å from O6 of Asp-34 (essential Asp-32 in EcoLigA). The Asp-Tyr contact may be functionally important, insofar as the Tyr is invariant and its replacement by Phe results in an order of magnitude decrement in ligase activity. Loss of the aromatic ring of Tyr-22 in EcoLigA abolishes ligase function, which could reflect an interaction of the tyrosine with the nicotinamide ring. Tyr-25 of Tfi and Bst ligases (His-23 in EcoLigA) is located on the enzyme surface, and its phenolic hydroxyl is within hydrogen-bonding distance of other constituents of the protein. We speculate that the tyrosine/histidine side chain interacts via a hydrogen bond with NAD⁺.

Inhibitors of bacterial NAD⁺-dependent DNA ligases would, in principle, be outstanding candidates for antibiotic development because of the following reasons. (i) NAD⁺-dependent ligases are present in all bacteria and are essential for bacterial growth. (ii) They are structurally conserved among bacteria but display unique substrate specificity compared with the ATP-dependent ligases of humans and other mammals. The present discoveries concerning the function of domain Ia in E. coli LigA raise the prospects for identifying small molecules that either compete for the predicted NMM site on domain Ia (said site being absent from ATP ligases) or else interfere with the conformational movements of domain Ia that are postulated to orchestrate the adenylate transfer reaction from NAD⁺. Candidate ligands can be screened for binding to the conserved and functionally important surface of domain Ia of bacterial LigA, using as specificity controls the mutated versions of domain Ia that are defective in the adenylate reaction.

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