DNA ligases utilize either ATP or NAD$^+$ as cofactors to catalyze the formation of phosphodiester bonds in nicked DNA. Those utilizing NAD$^+$ are attractive drug targets because of the unique cofactor requirement for ligase activity. We report here the crystal structure of the adenylation domain of the Mycobacterium tuberculosis NAD$^+$-dependent ligase with bound AMP. The adenosine nucleoside moiety of AMP adopts a syn-conformation. The structure also captures a new spatial disposition between the two subdomains of the adenylation domain. Based on the crystal structure and an in-house compound library, we have identified a novel class of inhibitors for the enzyme using in silico docking calculations. The glycosyl ureide-based inhibitors were able to distinguish between NAD$^+$- and ATP-dependent ligases as evidenced by in vitro assays using T4 ligase and human DNA ligase I. Moreover, assays involving an Escherichia coli strain harboring a temperature-sensitive ligase mutant and a ligase-deficient Salmonella typhimurium strain suggested that the bacterial activity of the inhibitors is due to inhibition of the essential ligase enzyme. The results can be used as the basis for rational design of novel antibacterial agents.

DNA ligases are vital enzymes in replication and repair and catalyze the formation of phosphodiester linkage between adjacent termini in double-stranded DNA through similar mechanisms (1). These enzymes can be divided into two classes, viz. NAD$^+$- and ATP-dependent ligases, based on the cofactor specificities (2). NAD$^+$-dependent DNA ligases, commonly called LigA, are found in bacteria and entomopoxviruses (3, 20), whereas ATP-dependent ligases are ubiqui-tous (3). Although there is little sequence homology between the eubacterial and eukaryotic enzymes, they exhibit some structural homology in specific domains (4, 5). The mechanistic steps involved in enzymatic action are also broadly conserved. Briefly, in the first step, the mode of action involves an attack on the α-phosphorus of ATP or NAD$^+$ by the enzyme, releasing pyrophosphate or NMN and forming a ligase-adenylate intermediate. In the second step, the bound AMP is transferred to the 5’-end of DNA to form a DNA-adenylate intermediate. AMP is released in the third step, where the protein catalyzes the joining of the 3’-nicked DNA to the DNA-adenylate intermediate. These steps involve large conformational changes also and encircling and partial unwinding of the nicked DNA substrate (6–8).

Some bacteria code for both NAD$^+$- and ATP-dependent DNA ligases (3, 9). Mycobacterium tuberculosis codes for at least three different types of ATP-dependent ligases and a NAD$^+$-dependent ligase (10, 11). Gene knockout and other studies have shown LigA to be indispensable in several bacte-ria, including Escherichia coli, Staphylococcus aureus, Bacillus subtilis, and M. tuberculosis (10, 12–15).

No LigA structure from mycobacterial sources is available to date. However, the crystal structure of the full-length protein is available for the Thermus filiformis enzyme (Tfiliga)\textsuperscript{1}, whereas structures of the adenylation domain are available for the Bacillus stearothermophilus and Enterococcus faecalis (EfaLigA) enzymes (7, 8, 16). The structures have shown that the enzyme has a modular architecture consisting of distinct domains. The adenylation domain contains the cofactor-binding site and can be divided further into two subdomains. Subdomain 1α contains the NNH-binding pocket, whereas subdomain 1b contains the AMP-binding site. The EfaLigA structures show that the NAD$^+$-binding site is generated by a specific spatial disposition of the two subdomains where subdomain 1α is in close proximity to the AMP-binding site in subdomain 1b (7). Structure-based mutagenesis experiments have also led to the identification of residues important for NAD$^+$ recognition and support systematic active-site remodeling in different reaction steps (17).

With the problem of multiple drug resistance spreading across the world, it is important to find inhibitors from different chemical classes with new modes of action. In this context, specific inhibitors for NAD$^+$-dependent ligases are being identified, as no drug is known to act against this enzyme so far. Other groups have very recently identified compounds belonging to arylamino and pyridochromanone classes as specific inhibitors of NAD$^+$-dependent DNA ligases (18, 19). New inhibitors can also be potentially used as broad bactericidal

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\textsuperscript{1} The abbreviations used are: Tfiliga, T. filiformis LigA; EfaLigA, E. faecalis LigA; MtuLigA, M. tuberculosis LigA; MICs, minimum inhibitory concentrations; EcoLigA, E. coli LigA.
agents, as NAD\(^+\)-specific enzymes have not been identified in eukaryotic genomes and are exclusively found in eubacteria (3) and some viruses (20).

In this work, we report the crystal structure of the adenyl-ation domain of the \textit{M. tuberculosis} NAD\(^+\)-dependent DNA ligase (\textit{MtuLigA}) bound to AMP. The structure captures a new spatial disposition of the two subdomains in the protein. The AMP conformation in the crystal structure is different from that observed in the \textit{TfiLigA} structure, but is similar to the AMP part of NAD\(^+\) in its co-crystal structure with \textit{Efa}-LigA. Based on the crystal structure and in \textit{silico} docking studies, we have identified glycosyl ureides as a new class of DNA ligase inhibitors. In \textit{vitro} assays and bactericidal activities as-sayed using specific \textit{E. coli} and \textit{Salmonella typhimurium} strains demonstrated that the compounds are able to distinguish between NAD\(^+\)- and ATP-dependent ligases. Although the \textit{M. tuberculosis} enzyme was inhibited in the low micromolar range, human DNA ligase I was inhibited only at much higher concentrations.

**MATERIALS AND METHODS**

Cloning, Expression, and Purification—The adenylation domain of \textit{MtuLigA} (Res 301-440) consists of residues 1–328. The DNA sequence encoding this domain was PCR-amplified from genomic DNA of \textit{M. tuberculosis} H37Rv using forward primer 5'-GAATTCCTAGG-CTTCCAGACGGCC-3' and reverse primer 5'-ATCGGATCCCTCGG-GCGGTTACTGGTAG-3' containing Ncol and BamHI restriction sites (underlined), respectively. The amplified PCR product was digested and ligated into pQE80 (Qiagen Inc.) digested at same site. Incorporation of Ncol into the forward primer leads to replacement of the first two amino acids in the sequence: valine and serine to methionine and glycine, respectively. The integrity of the insert was verified by sequencing. The construct was transformed into \textit{E. coli} BL21(DE3) cells (Novagen) and grown in LB medium containing 0.1 mg/ml ampicillin to \textit{A}\textsubscript{\text{max}} ~ 0.5. Protein expression was induced by addition of 0.8 mM isopropyl \(\beta\)-d-thiogalactopyranoside at 28 °C for 8 h. Cells were harvested by centrifugation; resuspended in 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 10 mM imidazole (buffer A); and lysed by sonication. The crude lysate was centrifuged at 27,000 \(\times\) g for 30 min. The supernatant was applied to a nickel-iminodiacetic acid column (Amersham Biosciences) equilibrated with buffer A, and protein was eluted using a 10–500 mM imidazole gradient. Purified fractions were pooled, precipitated using ammonium sulfate (45% saturation), redissolved in a minimum volume of buffer B (50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, and 2 mM dithiothreitol), and loaded onto a Superdex S-200 gel filtration column (Amersham Biosciences) equilibrated with buffer B. Purified protein was pooled and concentrated to 15 mg/ml using a Centricon concentrator (10-kDa cutoff; Amicon, Inc.). Protein concentrations were determined with Bradford reagent (21) using bovine serum albumin as a standard.

Crystalization and Data Collection—Crystals of the \textit{MtuLigA} adenyl-ation domain were grown by vapor diffusion using the hanging drop method. A drop containing 2 \mu l each of 12 mg/ml protein solution in buffer B containing 4 mM NAD\(^+\) and reservoir solution containing 0.1 mM NaCl, 0.1 mM Na-HEPES (pH 7.6), and 1.5 mM \(\text{NH}_4\)_2SO\(_4\) kept for 1 week at 24 °C yielded crystals of typical dimensions (0.7 \(\times\) 0.5 \(\times\) 0.2 mm). These were mounted on capillaries, and x-ray data were collected at room temperature on a MAR imaging plate mounted on a Rigaku rotating anode generator. The crystals diffracted weakly to 3.15 Å, and the data were overall complete to 99.4% with an average redundancy of 8.7. Data integration, reduction, and scaling were performed using the DENZO/SCALEPACK suite of programs (22). The data collection sta-tistics are summarized in Table I.

Structure Solution and Refinement—The structure was solved using CCP4 (23) and AMoRE (24) with models derived from the \textit{T. filiformis} structure (Protein Data Bank code 1V9P) after stripping it of cofactors and solvent molecules. The two subdomains of the adenyl-ation domain were placed independently in the asymmetric unit. Refinements were carried out using XPLOR (25), whereas model building was carried out using Turbo-Frodo (26). Refined model building and simulated annealing refinements were continued until \(R\) and \(R\_\text{free}\) values converged to 25.3 and 31.4%, respectively. The final model consists of residues 8–328, AMP, and 4 water molecules. More than 91% of all residues are in the core regions of the Ramachandran map (27). The geometric parameters are also well within acceptable values for a model at this resolution. The refinement and model statistics are summarized in Table I. The coordinates have been submitted to the Protein Data Bank with code 1ZAU (28).

In \textit{Silico} Docking—The current crystal structure and also that of NAD\(^+\)-bound \textit{EfaLigA} (Protein Data Bank code 1TAE) were used as models in \textit{in silico} ligand docking calculations using the programs AutoDock Version 3.0 (29) and Gold Version 2.2 (30). We used a Perl/Python-based script to add the capability of automated docking against a ligand data base to AutoDock. A computer cluster consisting of Silicon Graphics Origin 350 servers and Silicon Graphics Octane workstations was used for the computation and analysis of docked complexes. The NAD\(^+\)-binding site in \textit{MtuLigA} was generated by superposing subdomain 1a onto the orientation observed in the NAD\(^+\)-bound structure of \textit{EfaLigA} (Protein Data Bank code 1TAE). The docked ligands form part of an in-house collection of ~15,000 compounds whose synthesis expertise is also available. It can be filtered for activity against tuberculosis, etc., based on prior in-house experiments. Control docking runs to optimize the docking parameters were carried out using AMP and NAD\(^+\), whose co-crystal structures with LigA were available in the Protein Data Bank for comparison. Selected compounds from the best 10% docked complexes (as observed from the AutoDock scoring and Gold fitness scores) were taken up further for \textit{in vitro} and \textit{in vivo} ligase assays.

In \textit{Vitro} Activity—In \textit{vitro} assays for ligase activity were performed using a 40-\(\mu\)l double-stranded DNA substrate carrying a single-strand nick between bases 22 and 23 (31). This substrate was created in \textit{T4} T7A buffer by annealing a 22-mer (5'-CCT GGA CAT AGA CTC GTA CCT T-3') and a 18-mer (5'-AGC TGG ATC ACT GGA CAT-3'). The 18-mer was radiolabeled at the 5'-end by incubating 10 \mu g of the oligonucleotide with 100 \mu Ci of \[^{32P}\]ATP (3000 Ci/mmol; Board of Radiation and Isotope Technology) and 30 units of \textit{T4} polynucleotide kinase for 1 h. The unincorporated label was removed using a Sephadex G-25 column. The labeled 40-\(\mu\)l nicked DNA substrate was used to assay the \textit{in vitro} inhibitory activity of different compounds against \textit{MtuLigA}, \textit{bacilli} and \textit{human} DNA ligase I.

The full-length \textit{MtuLigA} protein was cloned into the NdeI/Ncol-digested \textit{pET41a} vector (Novagen). After expression in \textit{E. coli} BL21(DE3) cells, the C-terminally His-tagged protein was purified according to standard procedures. The assays were done with 2 ng of the purified protein. Reaction mixtures (15 \mu l) containing 50 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol, 10 mM MgCl\(_2\), 10% Me\(_2\)SO, 2 \muM NAD\(^+\), 2 pmol of \[^{32P}\]-labeled nicked duplex DNA substrate, and different concentrations of compounds were incubated for 1 h at 25 °C.

Reactions were quenched with formamide and EDTA. The reaction products were resolved electrophoretically on 15% polyacrylamide gel containing 8 \% urea in 90 mM Tris borate. The autoradiograms of the gels were developed, and the extent of ligation was measured by scanning the gel using ImageMaster 1D Elite software (Amersham Biosciences). All the compounds were dissolved in 100% Me\(_2\)SO. The compound solutions comprised 0.1 volume of the ligation reaction mixture; thus, 10% Me\(_2\)SO was included in all the control reactions. The activity assay was performed in the same way for \textit{T4}
ligase in a volume of 15 μl containing 0.05 unit of enzyme (Amersham Biosciences), 2 pmol of labeled template, and 66 μM Tris·HCl (pH 7.6), 6.6 mM MgCl₂, 10 mM dithiothreitol, and 10% Me₂SO.

The human DNA ligase I expression plasmid was transformed into E. coli BL21(DE3) cells and purified as described previously (33). Purified protein was concentrated to 2 mg/ml. 2 μg protein was used for assay in 50 mM Tris·HCl (pH 8.0), 10 mM MgCl₂, 5 mM dithiothreitol, 50 μg/ml bovine serum albumin, and 1 mM ATP as described above.

The IC₅₀ values were determined by plotting the relative ligation activity versus inhibitor concentration and fitting to equation $V/V_c = IC_{50}/IC_{50} + [I]$ using GraphPad Prism®. $V_c$ and $V$ represent the rates of ligation in the absence and presence of inhibitor, respectively, and [I] refers to the inhibitor concentration.

**Antimicrobial Activity and Inhibition of Ligase in Vivo**—The recombinant plasmid pRBL (34) containing the gene for T4 DNA ligase in pter99A was transformed into the E. coli GR501 ligA mutant (35). To have the same genetic background, the M. tuberculosis ligA gene was amplified from genomic DNA using primers containing sites for NcoI and HindIII, cloned into NcoI- and HindIII-digested pTrc99A (36), and transformed into E. coli GR501. In growth experiments, the strains expressing MtuLigA or T4 DNA ligase were compared with a control GR501 strain carrying empty pTrc99A without any gene insertions at 37 °C. As reported previously (19) and reproduced by us, the E. coli GR501 ligA mutant strain grows well at 30 °C, whereas it is strongly delayed at 37 °C. Complementation with either MtuLigA or T4 ligase restores the growth of the mutant strain.

Minimum inhibitory concentrations (MICs) of the inhibitors were determined for MtuLigA and T4 DNA ligase in the E. coli GR501 ligA mutant and in S. typhimurium LT2 (37) and its DNA ligase-null mutant derivative, which had been rescued with a plasmid (pBR313/598/81b) encoding the T4 DNA ligase gene (38), to check the specificity of compounds for NAD⁺-dependent ligases from other sources as well. Antimicrobial activity was monitored in microtiter plates using a microdilution assay technique using a microplate reader for determination of ligation in the absence and presence of inhibitor, respectively, and [I] refers to the inhibitor concentration.

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**Growth Inhibition Studies**—To investigate the specificity and sensitivity of the compounds to NAD⁺-dependent ligase, exponentially growing cultures of S. typhimurium LT2 and its DNA ligase-null mutant derivative in nutrient broth were treated at $A_{560} = 0.4$ with increasing compound concentrations. The effect on the growth and viability of both the strains was compared by monitoring $A_{560}$ and the number of colony-forming units for 4–5 h after addition of the compound. Serially diluted culture aliquots of both strains in phosphate-buffered saline were plated on nutrient agar, and visible colonies were counted after incubation for 15 h at 37 °C.

**DNA-Inhibitor Interaction**—In this assay, the DNA intercalating properties of the inhibitors were measured by the ability to compete with ethidium bromide for DNA binding. Detection of ethidium bromide displacement from DNA, if any, is based on the strong loss of fluorescence that should occur upon its detachment from DNA (39). The assay mixture contained, in a volume of 100 μl, 5 μg of calf thymus DNA, 5 μM ethidium bromide, 25 mM Tris·HCl (pH 8.0), 50 mM NaCl, and 1 mM EDTA. Upon addition of the inhibitor at increasing concentrations, ethidium bromide fluorescence was immediately detected at an excitation wavelength of 485 nm and an emission wavelength of 612 nm.

**RESULTS**

The adenylation domain of LigA contains all the residues necessary for AMP/NAD⁺ binding. This domain (consisting of residues 1–328 in MtuLigA) was cloned, expressed, and purified as described. The adenylation domain itself consists of two subdomains. Subdomain 1a is known to be flexible and adopts different spatial dispositions relative to subdomain 1b (7, 8). We therefore carried out independent molecular replacement calculations for the subdomains. The cofactor was not used in the calculations (Fig. 1). Clear connectivity was observed in the initial electron density maps between the two subdomains. We added NAD⁺ under the crystallization conditions, but well defined density only for noncovalently bound AMP was observed in the initial electron density map itself (Fig. 2). The data collection and refinement statistics are summarized in Table I.

Subdomain 1a consists of residues 1–76 and contains residues involved in NMN recognition. This subdomain consists mainly of two helical stretches (Figs. 1 and 3A). Subdomain 1b contains bound AMP in the crystal structure and consists of residues 77–328. The two domains adopt a novel relative spatial disposition in the structure (Fig. 3). Subdomain 1a in the E. steaerothermophilus LigA structure is at one end of the conformation spectrum, whereas in TtLigA, it is at the other end (Fig. 3B). In E6aLigA, this domain comes in close proximity to the AMP-binding site and generates the complete NAD⁺-binding site. This subdomain in MtuLigA adopts a new spatial disposition between the above two extremes.

**Adenylation Site and AMP Conformation**—The adenylation domain contains five of six conserved sequence motifs in NAD⁺-dependent ligases (40). These mainly line the AMP/NAD⁺-binding pocket. The active-site lysine (Lys₁⁸⁴ in MtuLigA), which covalently binds AMP to form the ligase-adenylate intermediate in the first step of the reaction, is part of the conserved motif I, whereas a Glu residue (Glu₁⁸⁴ in MtuLigA), which apparently discriminates between AMP conformations, is part of motif III.
Intriguingly, in the present structure, AMP is not observed to form the phosphoamide adduct with the motif I lysine. In fact, the side chain of this residue appears to be more mobile as evidenced by weaker electron density. The only other AMP-bound LigA structure is that of TfiLigA, whereas an NAD$^+$-bound structure is available for EfaLigA. Although the model used for molecular replacement calculations was derived from TfiLigA, the AMP molecule adopts a conformation similar to that of the AMP part in the NAD$^+$-bound EfaLigA structure (Protein Data Bank code 1TAE). In the TfiLigA structure, the adenylation domain is in an “open” state (Fig. 3B), and the adenosine nucleoside moiety of the covalently bound AMP adopts an anti-conformation. In the NAD$^+$-bound EfaLigA structure, the adenylation domain is observed to be in a “closed” state, and the adenosine nucleoside moiety adopts a syn-conformation (41).

In the present structure, although the adenylation domain adopts an open conformation, the adenosine nucleoside moiety adopts a syn-conformation. Residues that are <4 Å from the AMP moiety in MtuLigA are Leu$^{90}$, Ser$^{91}$, Leu$^{92}$, Asn$^{94}$, Glu$^{121}$, Leu$^{122}$, Lys$^{123}$, Ala$^{124}$, Ala$^{128}$, Arg$^{144}$, Glu$^{184}$, His$^{236}$, Val$^{298}$, and Lys$^{300}$. The possible hydrogen bonds with AMP are indicated in Fig. 2. Lys$^{123}$ is the residue that should covalently bind to AMP to form the adenylyte intermediate, although it is not covalently bound in the present structure. A stacking interaction with the adenosine nucleoside moiety is provided by His$^{236}$ in MtuLigA. In the EfaLigA and TfiLigA structures, this interaction is provided by a tyrosine. Other interactions include those with Lys$^{300}$ and Val$^{298}$. Previous mutational studies on E. coli LigA (EcoLigA) (42) have shown that the lysine is essential for activity, whereas mutations to the valine result in reduced activities. Another interaction is with Glu$^{184}$, a conserved residue essential for NAD$^+$ recognition (17). This interaction involving the corresponding Glu$^{174}$ is not observed in TfiLigA because of differences in the AMP conformations (Fig. 4A) (41).

**NAD$^+$-binding Site in MtuLigA**—To structurally identify corresponding residues involved in NAD$^+$ recognition on the basis of the EfaLigA structure and also for use in ligand docking calculations, we superposed the individual subdomains of MtuLigA onto the corresponding ones in the NAD$^+$-bound EfaLigA structure (Protein Data Bank code 1TAE). As expected, the NAD$^+$ molecule fits well into the generated model (Fig. 4B). The interactions with the AMP part are conserved because of

![Fig. 3. A, schematic of the MtuLigA adenylation domain crystal structure. Individual subdomains 1a and 1b are shown in dark blue and cyan, respectively. The bound cofactor is also indicated. The figure were made using MolScript (44). B, superposition of the adenylations domains from B. stearothermophilus LigA (B. st; Protein Data Bank code 1BO4), TfiLigA (T. f; code 1V9P), and EfaLigA (E. f; code 1TAE) onto the MtuLigA (M. tb) structure. Subdomain 1b is shown in cyan, whereas subdomains 1a from B. stearothermophilus LigA (pink), TfiLigA (light blue), EfaLigA (violet), and MtuLigA (dark blue) are color-coded and indicated separately for clarity. The bound NAD$^+$ cofactor in the EfaLigA structure is shown in ball-and-stick representation.](image-url)
their similar conformations as described above. The nicotinamide moiety is stacking interactions with Tyr29 and Tyr42 in the EfaLigA structure. The corresponding interactions are provided by Tyr31 and Phe44, respectively. Mutating the former tyrosine (which is also highly conserved) in EcoLigA is known to abolish activity (43), whereas mutating the latter tyrosine (Phe44 in MtuLigA) is known to reduce activity.

In Silico Screening—In silico ligand docking was used to shortlist compounds from an in-house compound collection for evaluation as inhibitors of MtuLigA. This forms part of a long-term goal to identify novel inhibitors for developing antibacterial agents. The crystal structure in this study, i.e. the model generated after superposing subdomain 1a onto the NAD$^+$-bound EfaLigA structure, and also the structure of the latter were used as the docking targets (Table II). Conformations of interacting residues in subdomain 1a of MtuLigA were adjusted to those observed in EfaLigA after superposition for use in in silico docking experiments. Two popular programs (AutoDock and Gold) were used to generate the docked complexes. The complexes were sorted based on the scoring function and fitness scores as implemented in the two programs, respectively. Control docking experiments were able to reproduce the AMP-NAD$^+$ complexes and were used to optimize the docking parameters. The docking energy of chloroquine was much less than that of NAD$^+$ (Table II), in line with a previous report that chloroquine does not interact with the cofactor-binding site in LigA (18). The other selected compounds had higher predicted binding affinities compared with NAD$^+$. An analysis of the binding modes of the glycosyl ureides, which were selected for further assays, showed that they appear to bind to LigA by mimicking the binding modes of AMP/NAD$^+$ in the respective co-crystal structures (Fig. 4B). The compounds were then taken up to evaluate their inhibitory efficacies.

In Vitro Assays and Mode of Inhibition—We cloned and expressed full-length MtuLigA for evaluation of compounds. We were interested in compounds that could distinguish between NAD$^+$ and ATP-dependent ligases, as such inhibitors could potentially be useful as novel antibiotics. We therefore simultaneously evaluated the inhibitory efficacy of the compounds against human DNA ligase I and bacteriophage T4 ligase. The results are summarized in Table III. Although the glycosyl ureide-derived compounds inhibited MtuLigA with IC$_{50}$ values in the low micromolar range, human DNA ligase I was much less sensitive to the compounds and was inhibited with IC$_{50}$ values in the higher 100–200 µM range. It was observed previously that doxorubicin and chloroquine are also able to inhibit LigA (18). We therefore used them as control inhibitors.

To check whether glycosyl ureides generally interact with DNA and thereby influence the inhibitory behavior, we carried out ethidium bromide displacement assays. Compounds were added to a maximum concentration of 250 µM. Even at this high concentration (representing a 50-fold excess over ethidium bromide), no loss of fluorescence was observed. We also carried out gel shift assays (data not shown) in which the electrophoretic mobility of DNA was checked in the presence of increasing inhibitor concentrations. The experiments did not support any general interaction of glycosyl ureides with DNA.

In silico docking analysis suggested an overlap of the binding sites of NAD$^+$ and glycosyl ureides. We therefore evaluated by standard kinetics whether the compounds act competitively with NAD$^+$ in the overall nick-sealing reaction in vitro. In the absence of the inhibitor, we determined a $K_m$ of 1.56 µM for NAD$^+$ in the presence of 10% MeSO in the assay mixture, which agrees well with previously reported data (10). In our inhibition studies, when the amount of NAD$^+$ was increased up to 50 µM in the presence of increasing concentrations of compound 2 (0–20 µM) and a saturating DNA concentration (0.85 µmol), the kinetics clearly indicated competitive inhibition of NAD$^+$ by the compound (Fig. 5A), as also visualized in a double-reciprocal plot (Fig. 5B). Linear regression using the apparent $K_i$ values leads to a $K_i$ of 4.9 µM (Fig. 5C). These results strongly suggest that the binding sites of glycosyl ureides and NAD$^+$ overlap with each other.
In Vivo Ligase Inhibition and Antibacterial Activities—To check the selectivity and specificity of the glycosyl ureides for NAD\(^+\) ligase in vivo, we chose pTrc99A-based systems in *E. coli* GR501 involving *Mtu* LigA and T4 DNA ligase (34). This strain is known to harbor a temperature-sensitive lig251 mutation in LigA (35). It grows well at 30 °C, but growth is strongly delayed at 37 °C. This deficiency can be overcome, however, by complementing it with NAD\(^+\) or ATP-dependent ligase (11, 19, 35), which restores the growth of *E. coli* GR501 at elevated temperatures. This strain has therefore been useful in demonstrating the LigA specificity of inhibitors in vivo (19).

We also reproduced earlier results (11, 35) that *Mtu* LigA and T4 ligase complement the growth of the mutant strain at elevated temperatures for use in the in vivo assays. NAD\(^+\) ligase has also been reported to be essential for survival in the prominent human pathogen *S. typhimurium* LT2 strain (37). As yet another system for testing the specificity of the inhibitors in vivo and to determine whether the compounds act also against other NAD\(^+\)-dependent ligases, we used the *S. typhimurium* LT2 strain and its DNA ligase-null derivative (TT15151) (38), which had been rescued with T4 ligase.

The much higher sensitivity of the *E. coli* GR501 strain harboring only the pTrc99A plasmid (Table IV) to the compounds compared with the corresponding ligase-rescued strains is attributed to the low residual ligase activity in the mutant strain compared with the growth-rescued strains, which possess a high copy number of the overexpressed ligase used to rescue them. This was also observed in the case of pyridochromanones (19). Consistent with the in vitro results, the MICs of the compounds (Table IV) were less for the strain rescued by *Mtu* LigA and higher for the strain rescued by T4 ligase. The trend that the tested compounds were more selective for NAD\(^+\) ligase compared with the ATP-dependent ligase in *E. coli* GR501 was also seen in the case of the *S. typhimurium* system. The compounds exhibited more sensitivity to the *Salmonella* wild-type strain harboring the NAD\(^+\)-dependent ligase compared with its ligase-deficient variant, rescued by the ATP-dependent ligase.

The growth inhibition studies performed using compound 2 (Table III) supported the above results that increasing the inhibitor concentration led to more bactericidal activity against the *S. typhimurium* LT2 strain (harboring its NAD\(^+\) ligase) (Fig. 6A) compared with its ligase-null derivative (rescued by T4 ligase) at the same compound concentration. The cell viability tested (Fig. 6B) using the compound also supports this result and shows that the *S. typhimurium* wild-type LT2 strain was less viable at same compound concentrations compared with the ligase-deficient variant, rescued with the ATP-de-
pendent T4 ligase. This further supports the higher specificity of the inhibitors for NAD$^+$-dependent ligases and also suggests that their antibacterial activities are due to in vivo inhibition of the ligase.

**DISCUSSION**

Subdomain 1a is known to be mobile and undergoes large conformational changes during the time course of enzymatic action. The present structure captures a new spatial disposition of the two subdomains composing the adenylation domain.

Although we added NAD$^+$ under the crystallization conditions, we observed density only for noncovalently bound AMP. The conformation of the cofactor in the current crystal structure mimics that of the AMP moiety in the NAD$^+$-bound EfaLigA structure. The only other corresponding cofactor-bound LigA structure is that of AMP-bound TfiLigA, where AMP is reported to be covalently linked to the motif I lysine in a different conformation (8). It was suggested previously that a syn- to anti-conformational switch around the adenosine nucleoside of AMP is linked to the progression of the ligase reaction and that the active site is "serially remodeled" in the interactions with NAD$^+$ and AMP (17, 41). Based on mutational analysis of the motif III Glu residue in EcoLigA (Glu$^{184}$ in MtuLigA), it was observed that contact with this Glu residue is essential in the third step of the reaction, but not in the second step. The syn-conformation exhibited by the moiety in the EfaLigA-NAD$^+$ complex in the first step is replaced by the anti-conformation around the moiety in the AMP-TfiLigA complex in the second step of the reaction. In this step, contact with the motif III Glu residue is lost, and subsequently, the adenosine nucleoside moiety in AMP must undergo a conformational change again to syn to interact with this Glu residue in the third step (41). We would therefore expect to find AMP in at least two conformations when subdomain 1a is in the open state, viz. one in which it is covalently attached and the other in which it interacts with the motif III Glu residue. The present structure appears to have captured a snapshot of the syn-switched conformation of AMP after the covalent bond with the motif I lysine is broken in LigA. More structures of wild-type and mutant enzymes, especially with bound DNA substrate, will be very interesting in this context.

With multiple drug resistance spreading across the world, the identification of new classes of inhibitors with novel mechanisms is essential to keep pace with the adaptability of bacterial populations. NAD$^+$ ligases are now proven novel targets, and additionally, no drug is as yet known to target them. We were therefore interested in identifying novel inhibitors for the enzyme.

**FIG. 5.** Competitive inhibition of MtuLigA with respect to NAD$^+$ by glycosyl ureides. A and B, activity of MtuLigA measured in the presence of increasing concentrations of compound 2 (0–20 μM) and NAD$^+$ (0–50 μM). The double-reciprocal plot in B clearly indicates competitive binding between NAD$^+$ and glycosyl ureides. C, linear regression plot of the inhibitor concentration versus $K_m$($_{\text{app}}$). The $K_m$ value is marked with an arrow.

**TABLE IV**

Antibacterial activity of glycosyl ureides

| Compound | MIC E. coli GR501 | MIC E. coli GR501 + pTrc99A | MIC E. coli GR501 + MtuLigA | MIC S. typhimurium LT2 | MIC S. typhimurium TT15151 |
|----------|----------------|-----------------|---------------|----------------|-----------------|
| 1        | 0.2            | 7               | 32            | 10             | 35              |
| 2        | 0.1            | 4.5             | 24            | 8              | 30              |
| 3        | 0.4            | 8               | 45            | 14             | 40              |

MICs were determined by broth microdilution for *E. coli* GR501 and *S. typhimurium* LT2 (which contains its NAD$^+$ ligase) and its DNA ligase null derivative (TT15151, lig-2::Mu dJ/pBR313/598/8/1b (T4 Lig$^+$) AMPr (38)), rescued with the T4 Lig$^+$ plasmid. *E. coli* GR501 ligA$^+$ is a strain containing a temperature-sensitive ligase mutant (35), a defect that is restored by overexpression of MtuLigA (this study and Ref. 11) or T4 ATP-dependent ligase (35). Polymyxin B nonapeptide (20 μg/ml) was added to the growth medium to facilitate passage of the inhibitors across the outer membrane of the cell.
manones, as expected, as indeed are NAD30280 to the growth medium. The cells were plated at dilution ratios of 10^-2 to compound 2 at 4–20g/ml, representing 0.5–2.5 times the MIC. The effectiveness of the compounds against them was also reported that the inhibitory effect of the compounds is not due to binding to the cofactor-binding site. The inhibitory effect of the compounds, which were previously reported to inhibit EcoLigA with IC50 values of 1.3 and 53µM, respectively (18). Although doxorubicin could not distinguish between NAD+ and ATP ligases, chloroquine exhibited exquisite specificity. Doxorubicin and chloroquine inhibited MtuLigA with IC50 values of 5 and 46µM, respectively (Table III). Analogous to the previous report, our results with MtuLigA also confirm that doxorubicin cannot distinguish between the ligase classes, whereas chloroquine is more specific for MtuLigA.

It is possible that compounds that inhibit the protein in vitro may affect bacterial growth through completely unrelated mechanisms such as nonspecific interference with a variety of essential cellular functions and disturbance of membrane integrity, etc. To exclude such other effects, we carried out assays against specific ligase-deficient E. coli and S. typhimurium strains (Table IV). Incidentally, the latter is also a major human pathogen. Our assays suggested that the observed antibacterial activities of glycosyl ureides are based on inhibition of the ligase enzyme. The ligase-deficient E. coli and S. typhimurium strains harboring MtuLigA, S. typhimurium LigA, temperature-sensitive EcoLigA, and T4 ligase, respectively, were used. The effectiveness of the compounds against them shows that these compounds can inhibit a variety of ligases, as observed in the case of pyridochromanones, most likely due to the conserved nature of the binding site in this class of enzyme.

In summary, we report the x-ray structure of the MtuLigA adenylation domain with bound AMP. We subsequently used structure-based computational approaches to identify glycosyl ureides as novel inhibitors of LigA. Our assay results demonstrate that these compounds represent a new class of inhibitors that can distinguish between NAD+ and ATP-dependent ligases and that can potentially be used to develop novel antibacterial therapies.

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