Supplementary Material

Cloning, expression and Purification

Bacterial strains and growth conditions.

Chemically competent *E. coli* strain DH5α (Invitrogen Corp., Carlsbad, CA) was used for routine propagation of recombinant plasmids. Chemically competent *E. coli* strain BL21 (DE3) Star (Invitrogen Corp., Carlsbad, CA) was used for (isopropyl-1-thio-β-D-galactopyranoside) IPTG-induced protein expression. Transformation of *E. coli* cells was performed as described by the manufacturer (Invitrogen Corp., Carlsbad, CA). *E. coli* strains were grown aerobically at 37 ºC (for cloning) or 30 ºC (for protein expression) on Luria-Bertani (LB; BD Diagnostics, Sparks, MD) agar or in broth supplemented with either ampicillin at 100 μg/ml or kanamycin at 25 μg/ml. Ampicillin and kanamycin were obtained from Sigma (St. Louis, Mo.).

Recombinant DNA Methods.

The recombinant DNA methods and reagents used have been described previously (Sambrook *et al.* 1989). Plasmid DNA was prepared by alkaline lysis using the Wizard Plus plasmid miniprep kit according the manufacturer's instructions (Promega Corp., Madison, WI). Oligodeoxynucleotide primers used for Polymerase Chain Reaction (PCR) and DNA sequencing were synthesized by Invitrogen (Carlsbad, CA). High-fidelity PCR Supermix was used to amplify DNA fragments by PCR (Invitrogen Corp., Carlsbad, CA). Restriction endonucleases were obtained from New England Biolabs Inc. (Ipswich, MA). DNA was extracted from agarose gels with the QIAEX II kit (QIAGEN Inc., Valencia, CA). The Rapid DNA ligation kit including T4 ligase was used for DNA cloning (Roche Diagnostics Corp., Indianapolis, IN). SDS–PAGE was performed with precast gels (Novagen). Cloned DNA fragments were sequenced using an ABI Prism genetic analyzer, model 3100, after the preparation of ABI Prism BigDye Terminator (version 3.1) cycle sequencing reactions according to the manufacturer's instructions (Life Technologies Corp., Carlsbad, CA). The resulting DNA sequence chromatographs were assembled and analyzed with Sequencher software (version 4.7; Gene Codes Corporation, Ann Arbor, MI).

Cloning and expression of *H. influenzae* LigA adenylation domain and LigA adenylation domain with domain 1a deletion.

Oligonucleotide primers (*ligA*-F and *ligA*-AD-R; below) were used to PCR-amplify the *ligA* adenylation domain (*ligA*-AD) coding sequence of *H. influenzae* strain Rd KW20 from pHIN-LigA.[6] The amplified DNA fragment was purified after agarose gel electrophoresis, ligated into pGEM-T

(TA cloning vector; Promega, Madison, WI), and transformed into *E. coli* strain DH5α to generate pSM157. The cloned *ligA*-AD in pSM157 was sequenced and analyzed relative to the published *ligA* sequence. The coding region of the cloned *ligA*-AD in pSM157 encoded a polypeptide with 100% identity to the first 324 amino acids of LigA from *H. influenzae* strain Rd KW20 (NCBI Reference Sequence: NP_439257.2). Subcloning of *ligA*-AD was facilitated by incorporating restriction endonuclease sites into the cloning primers; *ligA*-F (*Nde*I) and *ligA-AD*-R (*Eco*RI). Thus, pSM157 and the expression vector pET-30a (EMD4Biosciences, San Diego, CA) were both digested with *Nde*I and *Eco*RI, the *ligA*-AD- and pET30a-containing fragments were gel-purified, and ligated to generate pSM158. The resultant LigA-AD expression plasmid, pSM158, contained a precise translational fusion between the ATG start codons of pET-30a and *ligA*.

Primers:

ligA-F (*Nde*I)	5' CCGAGAAT***CATATG***ACAAATATTCAAACTCAAC 3'
ligA-AD-R (*Eco*RI)	5' AACA***GAATTC***TTACAGGGTTAATTCTTCTTGGGC 3'
ligA-AD-1a-F (*Nde*I)	5' GGG***CATATG***TTTAGCCAAATTCGTCACG 3'

An expression plasmid containing the coding sequence for the LigA adenylation domain without domain 1a (*ligA*-AD-1a) was constructed in a similar fashion. Oligonucleotide primers (*ligA*-AD-1a-F and *ligA*-AD-R; above) were used to amplify the *ligA*-AD-1a coding sequence from pHIN-LigA, and ligate into pGEM-T to make pWY439. The DNA sequence was confirmed to contain the *ligA*-AD coding sequence without amino acids 2-69. The LigA-AD-1a expression plasmid, pWY440, contained a precise translational fusion between the ATG start codons of pET-30a and *ligA*.

The LigA-AD expression plasmid, pSM158, was transformed into chemically competent *E. coli* BL21 (DE3) Star cells and grown shaking overnight at 30°C. The following morning, the culture was diluted 1:200, grown to an OD_{600nm}=0.5, and incubated for 3 h after the addition of 0.5 mM IPTG. Cells were collected and processed for SDS-PAGE analysis prior to protein purification. Likewise, the LigA-AD-1a expression plasmid, pWY440, was transformed into BL21 (DE3) Star cells and grown as described above except that the cells were incubated for 4 h after the addition of 0.5 mM IPTG.

Purification

The frozen cell paste was suspended in 60 ml of Lysis Buffer (25 mM Tris-HCl, pH 8.0, 2 mM EDTA, 5 mM DTT, 10% glycerol, 1 mM PMSF, 1 Protease inhibitor cocktail tablet (Roche Molecular Biochemical). Cells were disrupted by passing them twice through a French press operated at 18,000 psi, and the crude extract was centrifuged at 25,000 rpm (45Ti rotor, Beckman) for 30 min at 4ºC. The supernatant was loaded at a flow rate of 1.5 ml/min onto a 20 ml Q-Sepharose HP (HR16/10)

column (GE Healthcare Life Sciences) pre-equilibrated with Buffer A (25 mM Tris-HCl, pH 8.0, 2 mM EDTA, 5 mM DTT, 10% glycerol). The column was then washed with Buffer A, and the protein was eluted by a linear gradient from 0 to 1 M NaCl in Buffer A. Fractions containing ligase were pooled, and 3 M $(NH_4)_2SO_4$ in 25 mM Tris-HCl, pH 8.0, 2 mM EDTA, 5 mM DTT, 10% glycerol was added to a final concentration of 1 M ammonium sulphate. The sample was applied at a flow rate of 1.5 ml/min to a 20 ml Phenyl Sepharose HP (HR16/10) column pre-equilibrated with Buffer B (25 mM Tris-HCl, pH 8.0, 2 mM EDTA, 5 mM DTT, 10% glycerol, 1M $(NH_4)_2SO_4$). The column was washed with Buffer B, and the protein was eluted by a linear gradient from 1 to 0 M $(NH_4)_2SO_4$ in Buffer A. Fractions containing ligase were pooled, and solid $(NH_4)_2SO_4$ (0.4 gm ml^{-1}) was added to precipitate all the proteins and mixed on ice for 1 hour. The sample was centrifuged at 25,000 rpm for 30 min at 4ºC (45Ti rotor, Beckman), the pellet was then dissolved in 10 ml of Buffer A. The 10 ml sample was applied at a flow rate of 1.5 ml/min to a 320 ml Sephacryl S-100 pre-equilibrated with Buffer C (25 mM Tris-HCl, pH 8.0, 2 mM EDTA, 5 mM DTT, 10% glycerol, 150 mM NaCl). The fractions containing ligase were pooled and dialyzed against 1 L Storage Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 100 mM KCl, 2 mM DTT, 20% glycerol). The protein was characterized by SDS-PAGE analysis and analytical LC-MS.

Deadenylation of ligase adenylation domain

Two different methods were used to remove the covalently bound AMP typically present in bacterially-expressed ligase adenylation domain preparations, the first involving ammonium sulfate precipitation and a second that maintains protein soluble through an extensive dialysis protocol.

Ammonium sulfate precipitation method: A solution 30 mg/ml of purified ligase adenylation domain was incubated with a 400-fold molar excess of NMN (SIGMA) and 50 mM $MgCl_2$ in 0.5 M MOPS, pH 7.5 overnight at 4°C. Ammonium Sulfate (0.3 gm ml^{-1}) was added to precipitate protein. The sample was centrifuged at 14,000 Xg for 10 min. at 4°C, the pellet was washed with ammonium sulfate solution (0.3 g/ml) for 5 more times. The final washed pellet was dissolved in 1 ml of 10 mM Tris-HCl, pH 8.0 with extensive dialysis in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 100 mM KCl, 2 mM DTT, 20% Glycerol. The determined mass of the ligase indicated that the protein was 90% – 95% deadenylated.

Dialysis method: A solution comprising 160 uL of 30 mg/mL (815 μM) purified *H. influenzae* adenylation domain protein in Storage Buffer, 40 μL of 0.5 M NMN in water adjusted to pH 7.5 with sodium hydroxide, 50 μL of 1 M MOPS pH 7.5, 50 μL of 1 M $MgCl_2$, and 700 μL ITC Dialysis Buffer (see isothermal titration calorimetric methods section) was incubated at room temperature for one hour. The sample was then transferred to a 3,500 MW cutoff dialysis cassette (Pierce) and dialyzed for 1.5 hour against 0.5 liter of ITC Dialysis Buffer twice in succession.

Adenylation procedure

For ITC studies, adenylated *H. influenzae* DNA ligase adenylation domain protein was prepared from the deadenylated stocks. A solution comprising 104 uL of 130 uM deadenylated protein in ITC

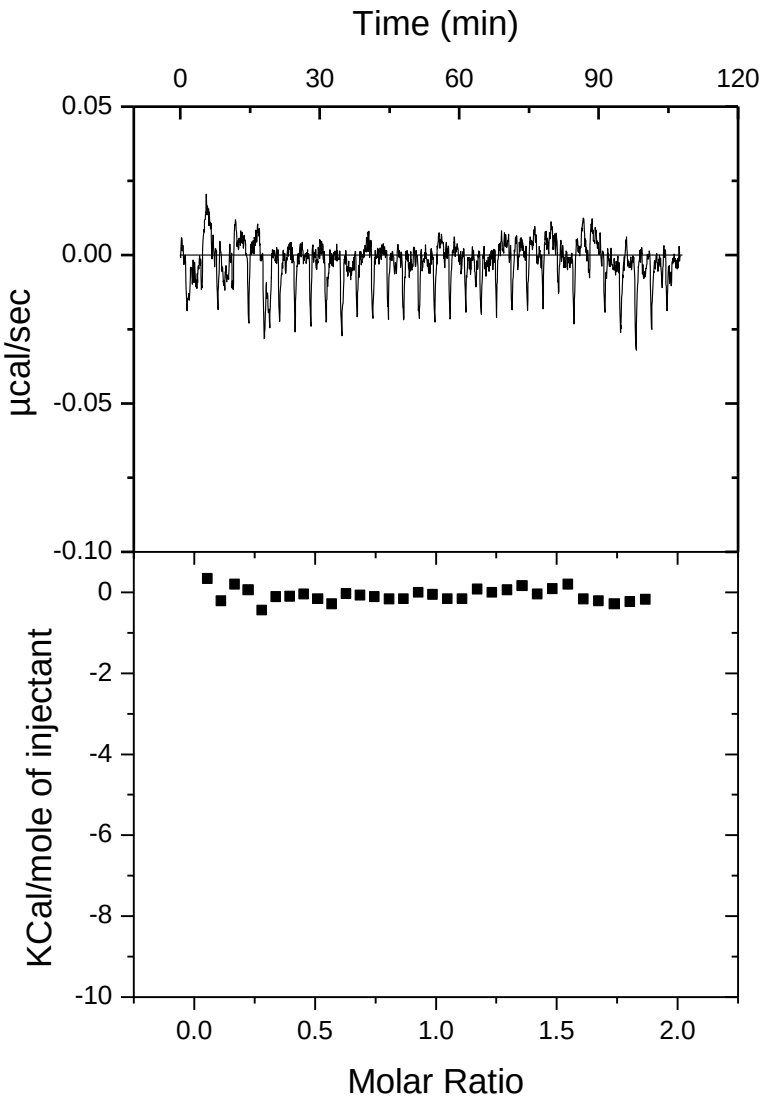
Dialysis Buffer (250 uL of 100 mM NAD^+ in water adjusted to pH 7.5 with NaOH, 25 uL $MgCl_2$, and 121 uL of water) was incubated at room temperature for one hour. The solution was then transferred to a 3500 MW cutoff dialysis cassette and dialyzed with the same protocol as described for the dialysis method of deadenylation. Preparations of adenylated protein were tested by electrospray mass spectroscopy to confirm the extent of adenylation at the end of the procedure, no deadenylated ligase was detectible.

Isothermal Titration Calorimetry

Titration of compound 1 or NAD^+ into *H. influenzae* DNA ligase adenylation domain construct (70-324) was performed with a Microcal VP-ITC instrument (GE Healthcare, Piscataway, NJ). Experiments were conducted using vacuum-degassed solutions at a temperature of 25 °C, a reference power of 10 μcal sec^{-1}, a stirring rate of 270 min^{-1} and a delay between injections of 200 seconds. Experiments were run with the protein receptor in the analysis cell at 50 μM, and ligand in the injector at 500 μM. Injections were programmed with an initial 1 μL addition followed by 32 successive 8 μL additions. The buffer for all titrations consisted of ITC Dialysis Buffer consisting of 50 mM MOPS pH 7.5, 30 mM ammonium sulfate, 30 mM potassium chloride, 1 mM EDTA, 10% v/v glycerol, and 0.002% Brij-35. Data were analyzed using the software supplied by the manufacturer, were fit to a single site binding model when applicable, and the errors listed are the results from the fitting process.

Supplementary Figure 1.

Thermogram for the titration of NAD^+ into *H. influenzae* ligase adenylation domain Δ1a (residues 70-324). This engineered protein contains only the domain 1b binding site residues. The lower panel of the figure has been scaled to compare with Supplementary Figure 2 and Figure 3 in the main text. No binding could be detected for NAD^+ with the protein lacking domain 1a. This result is in contrast to those obtained for NAD^+ binding to the deadenylated full length adenylation domain protein which gave a result of (28 ± 3) μM for a dissociation constant, and an enthalpy of binding of (-28 ± 1) kcal $mole^{-1}$. (See Fig. 3B in the main text.)



Supplementary Figure 2.

Binding of compound 1 to *H. influenzae* ligase adenylation domain Δ1a (residues 70-324). This engineered protein contains only the domain 1b binding site residues. The dissociation constant determined is (6 ± 1) μM with a stoichiometry of (0.96 ± 0.03). This result is comparable with that determined for compound 1 with the deadenylated full length adenylation domain protein which gave a result of (4.4 ± 0.5) μM.