Identification, Characterization, and Crystal Structure of Bacillus subtilis Nicotinic Acid Mononucleotide Adenylyltransferase

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The nadD gene, encoding the enzyme nicotinic acid mononucleotide (NaMN) adenylyltransferase (AT), is essential for the synthesis of NAD and subsequent viability of the cell. The nadD gene in Bacillus subtilis (yqej) was identified by sequence homology with other bacterial nadD genes and by biochemical characterization of the gene product. NaMN AT catalyzes the reversible adenylation of both NaMN and the nicotinamide mononucleotide (NMN) but shows specificity for the nicotinate. In contrast to other known NMN ATs, biophysical characterizations reveal it to be a dimer. The NaMN AT crystal structure was determined for both the apo enzyme and product-bound form, to 2.1 and 3.2 Å, respectively. The structures reveal a “functional” dimer conserved in both crystal forms and a monomer fold common to members of the nucleotidyl-transferase α/β phosphodiesterase superfamily. A structural comparison with family members suggests a new conserved motif (SXXXX[R/K]) at the N terminus of an α-helix, which is not part of the shared fold. Interactions of the nicotinic acid with backbone atoms indicate the structural basis for specificity.

NAD is an essential molecule in all living cells. In addition to its role in oxidation reduction reactions, in which NAD(H) and its phosphorylated form, NADP(H), act as hydride donors and acceptors, NAD is also important for other cellular processes, such as the activity of NAD-dependent DNA ligases, mono- and poly(A)DP-riboylation of proteins, and production of the intracellular calcium-mobilizing molecules cADPR and NaADP (1, 2).

NAD is synthesized via a multi-step de novo pathway or via a pyridine salvage pathway. The enzyme nicotinic acid mononucleotide (NaMN)1 adenylyltransferase (AT, EC 2.7.7.18) sits at the convergence of these two pathways. NaMN AT catalyzes the conversion of ATP and NaMN to nicotinic acid adenine dinucleotide (NaAD) (Fig. 1) that is directly processed to NAD by NaAD synthetase. The nadD gene, encoding NaMN AT, was the first enzyme demonstrated to be essential for NAD biosynthesis and bacterial cell survival by both the de novo and salvage pathways (3). A number of enzymes demonstrating in vitro adenylyltransferase activity for NaMN and NMN have been identified in eukarya, archaea, and bacteria (4–11). Along with sequence homology, the specificity of these enzymes for NMN versus NaMN provides a useful method for classifying new genes within this family.

Although there is sequence conservation between the eubacterial nadD genes (Fig. 2), sequence alignment of nadD NaMN ATs to the eukaryotic enzymes or archeal enzymes is difficult outside of the region surrounding the (H/T)XGH nucleotidyl transferase consensus sequence. Adenylyltransferases encoded by the nadD gene prefer the nicotinic acid containing NaMN over NMN as a substrate by a factor that ranges from 6:1 to 2000:1 (4, 12, 13). Eubacteria also contain enzymes that demonstrate higher specificity for the nicotinamide-containing NMN. This group includes the products of the nadR gene, which in addition to its regulatory role in NAD biosynthesis also displays NMN AT activity (14). The eukaryotic and archeal NMN AT (EC 2.7.7.1), such as those from human (15), Methanosarcina jannaschii (16), and Methanobacterium thermoautotrophicum (17), either demonstrate higher specificity for NMN as a substrate as compared with NaMN, or show little preference for either substrate (4).

Primary sequence studies indicate that NaMN AT belongs to the nucleotidyl-transferase α/β phosphodiesterases superfamily of enzymes that contain the (H/T)XGH signature motif. Members of this family share the same basic catalytic mechanism, involving direct nucleophilic attack upon an α-phosphate followed by the release of pyrophosphate, whereas the enzyme provides stabilization of the transition state prior to the formation of a new phosphodiester bond. The recent structure determination of NMN ATs from M. jannaschii and M. thermoautotrophicum has allowed this sequence and functional homology to be extended to the structural conservation of residues involved in substrate binding and catalysis (16, 17).

Genes that have been identified to be essential for bacterial cell survival are currently being evaluated for their potential as targets for anti-microbial chemotherapy. Understanding the biochemical, physical, and structural properties of these essential enzymes and placing them in a larger biological context are the first steps in exploring this potential. To this end, we report here the identification of an unassigned reading frame in Bacillus subtilis (yqej) as a NaMN AT. We have expressed the recombinant enzyme in Escherichia coli and show that it pre-
fers NaMN as a substrate to NMN, allowing us to assign it as the nadD gene of *B. subtilis*. It differs from the NMN ATs from *M. jannaschii* and *M. thermoautotrophicum* both in its substrate specificity and oligomeric state. It is dimeric as opposed to hexameric (16, 17). We have determined the three-dimensional structure of NaMN AT from *B. subtilis* to 2.2 Å and 3.2 Å with the NaAD bound. This has allowed us to identify key residues in substrate binding and catalysis. These structures will provide invaluable information in the ongoing development of anti-microbial agents targeting NAD biosynthesis.

**EXPERIMENTAL PROCEDURES**

**Cloning and Expression of B. subtilis NaMN AT**—The *B. subtilis* *yqej* gene was PCR-cloned into a modified version of pET16b to yield pML208. This *E. coli* expression vector has the *yqej* coding sequence downstream of the T7 RNA polymerase promoter. The expressed protein contains the peptide MGHHHHHHHHHHHSHGERHMPGGS fused to Lys of the native sequence. This provides a purification tag and contains the cleavage site for Factor Xa between Arg and His of the peptide, resulting in the cleaved protein having six extra amino acids on its N terminus. To produce selenomethionine-labeled *yqej*, the protein was expressed in BL21(DE3) *E. coli* at 25 °C. The cultures were grown in shake flasks in LeMaster’s medium and induced at log phase with 0.5 mM isopropyl-1-thio-galactopyranoside (IPTG). The cultures were harvested 4 h post-induction. Unlabeled *yqej* was also expressed in BL21(DE3) *E. coli*, but at 37 °C. High density expression was carried out in a Binstat C-10 bioreactor (B. Braun Biotech). The culture was induced with 1.0 mM isopropyl-1-thio-β-D-galactopyranoside (final) at 4.6 Acell. The cells were harvested 4 h post-induction at 9.0 Acell.

**Purification and Cleavage of B. subtilis NaMN AT**—The purification, unless otherwise stated, was performed at 4 °C. Bacteria were resuspended in buffer (50 mM Hepes, pH 7.5, 500 mM NaCl) and lysed by passage through a Microfluidics microfluidizer. The lysate was collected and centrifuged at 20,000 × g for 30 min. The supernatant, containing 40% of the expressed *yqej* of NaMN AT (the remainder being insoluble), was applied to a Poros PI column (Applied Biosystems) that was coupled to a nickel-nitrilotriacetic acid column (Qiagen). The nickel-nitrilotriacetic acid column was washed with 50 mM imidazole, and the protein was eluted with a 50–800 mM imidazole gradient. 10 mM EDTA was added to the fraction containing *yqej* NaMN AT, for 6 h, followed by dialysis against 50 mM Tris, pH 8.0, 50 mM NaCl, 2 mM CaCl2. The His tag was removed from *yqej* NaMN AT by a 6-h digestion with Factor Xa (New England Biolabs) at room temperature. The reaction was applied to a Poros HQ 50 column, and the bound protein was eluted with a 0–1 M NaCl gradient. The peak fraction containing *yqej* NaMN AT was dialyzed and applied to a Poros S column. The flow through, containing *yqej* NaMN AT, was applied to TSK gel G3000 SW column ( Tosohaas), equilibrated with 50 mM Hepes, pH 7.5, 50 mM NaCl. Protein purity was >95%.

**Adenylyltransferase Assays**—Discontinuous HPLC assay. The discontinuous HPLC assay is based upon the assays published by Mehl et al. (12) and Balducci et al. (18). The reaction conditions were 20 mM Hepes, pH 7.4, 10 mM MgCl2, and 0.36 or 0.18 mM *yqej* protein incubated at 37 °C. For the forward reaction, the incubations contained 1 mM ATP and 1 mM NaMN or NMN. For the reverse reaction, the incubations contained 1 mM sodium pyrophosphate and 0.5 mM NaAD or NAD. The reactants and products were separated by chromatography on a 3.9 × 150-mm C18 column (Novapack 5 μm; Waters Inc.). Buffer A was 100 mM potassium phosphate, pH 7.5. Buffer B was 100 mM potassium phosphate, pH 7.5, in 25% MeOH. The elution conditions were: 0–3 min in 100% buffer B, 3.0–3.1 min to 100% buffer B, and 3.1–7 min in 100% buffer B. The absorbance of reactants and products was detected at 254 nm. Under these conditions NaMN eluted at 1.16 min, NMN eluted at 1.29 min, ATP eluted at 2.12 min, NaAD eluted at 5.37 min, and NAD eluted at 5.47 min.

A continuous assay to monitor the reaction in the forward direction was based upon the EnzChek pyrophosphate assay from Molecular Probes (Eugene, OR). In this assay inorganic pyrophosphate produced in the forward reaction of *yqej* is cleaved by inorganic pyrophosphatase to phosphate, which is used by the second coupling enzyme, purine nucleoside phosphorylase, to convert the chromogenic substrate 2-aminoo-6-mercaptop-7-methylpyridine ribonucleoside to ribose-1-phosphate and 2-amino-6-mercaptop-7-methylpyridine (Km = 11,000 m–1 cm–1). The reaction conditions were 20 mM Hepes, pH 7.5, 10 mM MgCl2, 0.2 mM 2-amino-6-mercaptop-7-methylpyridine ribonucleoside, 1 unit of purine nucleoside phosphorylase, 0.01 unit of inorganic pyrophosphatase, and 0.03 μg/ml *yqej*. The reaction volume was 125 μl and was carried out in a 96-well plate at room temperature using a Spectomax 384 Plus plate reader recording continuously at 360 nm (Molecular Devices, Sunnyvale, CA). For the KM determinations, NaMN was varied from 25 to 500 μM with ATP held at 2 mM, and ATP was varied from 50 to 1000 μM with NaMN held constant at 1 mM. All kinetic constants were determined from nonlinear fits of the experimental data using the enzyme kinetic module of Sigmaplot 7.0 (SPSS, Inc., San Rafael, CA). The reaction conditions were the same for the KM determinations for NMN except the MgCl2 concentration was increased to 50 mM, and the *yqej* concentration was increased to 75 μg/ml because of the lower activity against this substrate. Substrate inhibition was seen in assays using NMN as a substrate, with the double reciprocal plots curving sharply upwards above 5 mM. The data were fit to a model of substrate inhibition using the equation: v = Vmax(1 + KM(S + S/K) ). The KM determined for NaMN was 17 ± 3 mM. NMN was varied from 0.5 to 10 mM with ATP held constant at 0.6 mM. We also determined the kinetic constants of *yqej* using NMN and ATP substrates with a second coupled assay system, one using NAD production to reduce NADP, and the other using NADPH. This assay was described by Balducci et al. (5) and has been used to characterize other NMN ATase. Using this assay we obtained values of KM and Vmax for NMN very similar to those determined with the purine nucleoside phosphorylase-coupled assay described above. The alcohol dehydrogenase-coupled assay was not suitable to assay NaMN as a substrate, presumably because NaAD is not a good substrate for alcohol dehydrogenase.

The back reaction for *yqej* NaMN AT was monitored using the coupled enzyme assay of hexokinase and glucose-6-phosphate dehydrogenase (from yeast). *yqej* NaMN AT converts pyrophosphate and NaAD (or NAD) to NaMN (or NMN) and ATP. The ATP is then used by hexokinase to phosphorylate glucose to give glucose-6-phosphate and ADP. Glucose-6-phosphate is oxidized to 6-phospho-glucono-6-lactone by glucose-6-phosphate dehydrogenase, and NADP is reduced to NADPH. The assay is followed by the absorbance of NADPH at 340 nm. We used glucose-6-phosphate dehydrogenase from bakers’ yeast because this enzyme prefers NADP to NAD as a co-substrate. The reaction conditions were: 20 mM Hepes, pH 7.5, 50 mM MgCl2, 1 mM NaPi, 10 mM KCl, 5 units of hexokinase, 5 units of glucose-6-phosphate dehydrogenase, and NADP is reduced to NADPH. The assay is followed by the absorbance of NADPH at 340 nm. We used glucose-6-phosphate dehydrogenase from bakers’ yeast because this enzyme causes an enhancement of NaMN AT activity as a co-substrate. The reaction conditions were: 20 mM Hepes, pH 7.5, 50 mM MgCl2, 1 mM NaPi, 10 mM KCl, 5 units of hexokinase, 5 units of glucose-6-phosphate dehydrogenase, and NADP is reduced to NADPH. The assay is followed by the absorbance of NADPH at 340 nm.
FIG. 2. Sequence alignment of \textit{B. subtilis} yqeJ with known eubacterial \textit{nadD} genes. \textit{E. coli}, \textit{Bacillus halodurans}, \textit{Lactococcus lactis}, \textit{Mycobacterium leprae}, \textit{Streptomyces coelicolor}, \textit{Mycobacterium tuberculosis}, \textit{Treponema pallidum}, \textit{Borrelia burgdorferi}, \textit{Neisseria meningitidis}, and \textit{Pseudomonas aeruginosa} are used. Residues highlighted in red are identical, residues boxed in blue designate the nucleotidyl transferase consensus sequence, and residues boxed in red designate the SXXXX(R/K) motif. The secondary structure elements of \textit{B. subtilis} NaMN AT are overlaid on the sequence, with the blue arrows and green cylinders representing the \(\beta\)-strands and \(\alpha\)-helices, respectively.
Crystal Structure of B. subtilis NaMN Adenylyltransferase 3701

mg/ml protein, 50 mm Hepes, pH 7.5, 50 mm NaCl) mixed with 1.5 μl of well solution (8% PEG 3350, 100 mm MgCl2) and equilibrated against 1 ml of well solution. Block shaped crystals grew in 3 weeks, measuring ~50 μm across. NaAD-NaMN AT complex co-crystals were grown at 18 °C in drops containing 1.0 μl of protein stock solution (14 mg/ml protein, 50 mm NaCl, 50 mm NaAD) and 1.0 ml of well solution (20% PEG 3350, 100 mm sodium acetate) and 0.3 μl of xylitol (30% w/v). Plate-like crystals grew in 1–3 weeks to ~200 × 50 × 20 μm.

Data Collection and Processing—Crystals of the apo-form belong to the space group P21, with unit cell parameters a = 43.98 Å, b = 126.10 Å, c = 70.58 Å, and β = 92.73° and contain four molecules of NaMN AT in the asymmetric unit, implying a solvent content of 58.5%. Crystals of the NaAD crystals, an equal volume of a solution of 35% PEG 3350, 100 mM MgCl2 was added to drops, and after equilibration for several minutes the crystals were swiped through another drop of this solution and cooled rapidly in liquid nitrogen. The data collection statistics are shown in Table II. Multiple wavelength anomalous dispersion (MAD) data were recorded at the 5.0.2 beam line of the Advanced Light Source (ALS) at the Advanced Light Source at Lawrence Berkeley National Laboratory. Experiments using the following equation.

\[ C_r = C_0 \exp(\frac{M_1 - \rho_0 D}{2}) + \text{base} \] (Eq. 1)

Where \( C_r \) is the absorbance at radius \( r \); \( C_0 \) is the absorbance at reference radius \( r_0 \); \( M \) is the molar mass of the macromolecule; \( \rho \) is the partial specific volume of the macromolecule (ml/g); \( \rho_0 \) is the density of the solvent; \( \omega \) is the angular velocity of the rotor; \( B \) is the gas constant; \( T \) is the temperature; and \( v \) is the baseline offset. The molecular weight of \( yqeJ \) NaMN AT was obtained from sedimentation velocity experiments using Equation 2.

\[ M = \frac{(s - 1) \rho D}{(s - 2)(s - 1)} \] (Eq. 2)

where \( s \) is the sedimentation coefficient and \( D \) is the diffusion coefficient obtained by fitting the data to the program SVEDBERG (26).

RESULTS AND DISCUSSION

The B. subtilis yqeJ Gene Identified as the nadD Gene Encoding NaMN AT—The Streptococcus pneumoniae genome was sequenced and searched for essential genes that may be suitable targets for the development of anti-microbial agents. Those genes identified were then tested in B. subtilis and E. coli. An unassigned open reading frame, yqeJ, that was essential in all three organisms was identified in B. subtilis. Comparisons of its amino acid sequence with those in GenBank™ using the program Blast (27) revealed that it has homology to a number of putative adenylyltransferases including the recently assigned nadD gene of E. coli (12). yqeJ contains the signature nucleotidyl transferase consensus sequence (H/T)XGH. As can be seen in Fig. 2, B. subtilis yqeJ is closely related to E. coli nadD and other putative eubacterial NaMN ATs. The B. subtilis enzyme is more distantly related to E. coli nadR and other eukaryotic and archeal NNATs. Alignment of the B. subtilis NaMN AT to these species was difficult because of little homology outside of the region around the (H/T)XGH consensus sequence. This later group of enzymes includes the NNAT from M. jannaschii and M. thermotrophicum which have three-dimensional structures that have recently been determined (16, 17). Theoretical analysis at least 1 h prior to the sequence of the B. subtilis enzyme is more closely related to the group of eubacterial enzymes that prefer NaMN as a substrate than to the archeal or eukaryotic enzymes that show little preference among the substrates or preferring NNN over NaMN (4, 12, 13). To confirm that yqeJ was indeed a NaMN AT recombinant protein was expressed in E. coli and purified (see "Ex-
Adenylyltransferase assays were performed to determine the activity and substrate specificity of yqeJ NaMN AT. Incubation of the enzyme with NaMN and ATP led to the formation of a new peak as detected by HPLC that eluted with the NaAD standard, the expected product of the forward reaction of NaMN AT (Fig. 3A). Similarly, the enzyme was able to convert NMN and ATP to a new peak that eluted with the NaAD standard, the expected product of the reverse reaction of NaMN AT (Fig. 3B). Using the same assay system, we were able to demonstrate that yqeJ protein was also able to catalyze the reverse reaction. Fig. 3C shows NaAD being converted to NMN and ATP, and Fig. 3D shows NAD being converted to NMN and ATP. No product was formed under any of these reaction conditions in the absence of yqeJ protein.

To better understand the substrate specificity of B. subtilis yqeJ, we determined the catalytic constants for substrates in the forward and reverse reactions using coupled enzyme assays as described under “Experimental Procedures.” The data are summarized in Table I and shown in graph form in Fig. 4. Comparing the $V_{\text{max}}/K_m$ ratio for the different substrates in the forward and reverse reactions, we see that the B. subtilis enzyme has a clear preference for nicotinic acid containing substrates (NaMN and NaAD) over nicotinamide containing ones (NMN and NAD) by factors of 12,900 to 1 for the forward reaction and 2,000 to 1 for the reverse reaction. This same substrate preference is shared with enzymes from other eubacterial sources (12, 13) and allows us to assign yqeJ as the nadD gene in B. subtilis.

**Structure Determination—**NaMN AT was crystallized both empty and with NaAD-bound. For the apo structure good data were collected to 2.1 Å, whereas anisotropic data of the NaAD-bound form of NaMN AT were collected to 3.2 Å (Table II). The apo structure of NaMN AT was determined using experimental phases derived from selenium substituted methionine protein crystals by the MAD method (see “Experimental Procedures”). The final model of NaMN AT contains four molecules in the asymmetric unit of which residues 42–52 are disordered in all molecules, and residues 118–126 are disordered in molecules A and C. The NaAD-bound structure of NaMN AT was determined using experimental MAD phases combined with phases from molecular replacement using fragments of the apo structure. The crystal structure of NaAD-bound NaMN AT contains six molecules in the asymmetric unit (two are predominately disordered), all of which show clear density for the bound NaAD (see Fig. 8A).

**Overall Architecture of NaMN AT—**NaMN AT contains six $\beta$-strands and six $\alpha$-helices that form a single large domain with a smaller C-terminal lobe domain (Fig. 5). The core of the large domain is a twisted, parallel $\beta$-sheet formed by six $\beta$-strands (of order: 321456); a classic nucleotide-binding Rossman fold (28). Two $\alpha$-helices ($\alpha$-helix C and $\alpha$-helix D) above and two ($\alpha$-helix A and $\alpha$-helix B) below the central $\beta$-sheet form characteristic right-handed $\beta/\alpha/\beta$ super secondary structure elements. The C-terminal lobe domain is formed by the last 33 amino acids and comprises two $\alpha$-helices (E and F). Significant structural similarities place NaMN AT in the superfamily of nucleotidyl-transferase $a/\beta$ phosphodiesterases (29) (Fig. 6).

The prototypical member of the nucleotidyltransferase superfamily is the class I aminooacyl-tRNA synthetase, other members include phosphopantetheine adenylyltransferase (PPAT) (29), glycerol-3-phosphate cytidylyltransferase (GCT) (30), and nicotinamide mononucleotide adenylyltransferases from M. jannaschii (NaMN ATmj) (16) and M. thermotoleratum (NaMN ATmt) (17). The root mean square deviation of NaMN AT to PPAT is 2.4 Å, that to NaMN ATmj it is 2.9 Å, and that to GCT is 3.0 Å for all C$_{\text{N}}$ atoms (31). All these enzymes catalyze the same chemical reaction: the formation of a phosphodiester bond by the nucleophilic attack on the $\alpha$-phosphate of ATP or CTP (for GCT) resulting in the loss of pyrophosphate and the adenylation (cytidylation) of a unique moiety (an amino acid in the case of aminooacyl-tRNA synthetases, a phosphopantoate for PPAT, glycerol-3-phosphate for GCT, and a nicotinamide for the NaMN ATs).

In comparison with other members of this superfamily, a striking difference of NaMN AT is the presence of a sixth strand. Because the central interactions of an important dimerization interface occur directly after $\beta$-strand 6 (see below), it is possible that the additional $\beta$-strand allows the loops following $\beta$-strands 4 and 5 the necessary flexibility to participate in substrate/product coordination. Other differences mimic the main differences between empty and NaAD-bound structures of NaMN AT and center around a reordering of residues after $\beta$-strand 4 and before $\alpha$-helix E. This suggests that the conformation of this region is dependent on occupation of the active site.

| Substrate | $K_m$ | $V_{\text{max}}$ | $V_{\text{max}}/K_m$ |
|------------|-------|-----------------|-------------------|
| NaMN$^a$ | 0.043 ± 0.009 | 11.1 ± 0.7 | 258 |
| NMN$^b$ | 2.3 ± 0.2 | 0.054 ± 0.003 | 0.02 |
| ATP$^c$ | 0.135 ± 0.013 | 10.1 ± 0.4 | 0.02 |
| NaAD$^d$ | 0.0068 ± 0.0012 | 2.8 ± 0.2 | 412 |
| NAD$^e$ | 1.7 ± 0.2 | 0.34 ± 0.01 | 0.2 |
| PPI$^f$ | 4.2 ± 0.6 | 11.5 ± 1.3 | |

$^a$ Kinetic constants for forward reaction.

$^b$ Data for NMN was fit to the equation for substrate inhibition: $v = V_{\text{max}}(1 + K_s/S) / (K_s + S)$, $K_s = 17 ± 3$ mM.

$^c$ At saturating concentrations of NaMN.

$^d$ Kinetic constants for back reaction.

$^e$ At subsaturating concentrations of PPI.

$^f$ At saturating concentrations of NaAD.

**TABLE I**

Kinetic constants for NaMN AT

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Fig. 3. HPLC analysis of yqeJ NaMN AT activity. 0.36 mg/ml yqeJ NaMN AT was incubated with 1 mM ATP and 1 mM NaMN (A) or 1 mM NMN (B) for 30 min. The reverse reaction with 0.18 mg/ml yqeJ NaMN AT was assayed with 1 mM sodium pyrophosphate and either 0.5 mM NaAD (C) or 0.5 mM NAD (D) for 30 min. Under these conditions NaMN eluted at 1.16 min, NMN eluted at 1.29 min, ATP eluted at 2.12 min, NaAD eluted at 5.37, and NAD eluted at 5.47 min.
**B. subtilis NaMN AT Functions as a Dimer**—The NaAD-NaMN AT complex crystal structure reveals that NaAD binds in an extended conformation to a 20 Å long channel, almost perpendicular to the β-sheet. A large cleft at the C termini of β-strands 1 and 4 accommodates the diphosphate moiety of NaAD. In the apo-NaMN AT crystal this channel is occupied, and its conformation is distorted, by noncrystallographic interactions of one of the two dimers present in the asymmetric unit (Fig. 7A). This “handshake” dimer buries a total surface area of 2034 Å²/monomer with interactions that involve residues 102–146 and results in the burying of an extensive hydrophobic surface and the formation of three salt bridges. Based on the extent of the surface area and shape complementarity ($S_c = 0.71$ (32)) and in the absence of other structural data, this dimer would likely be interpreted as being physiological relevant. It is, however, the second (“functional”) dimer, which buries only 848 Å²/monomer ($S_c = 0.64$), that is seen to be conserved in the NaAD-bound NaMN AT crystal (Fig. 7B). The independent crystal packing of the complex crystal structure of NaAD-NaMN AT environment presents three functional dimers, one of which is partially disordered. The functional dimer is formed by a pseudo-2-fold symmetry operation centered around an anti-parallel β-sheet interaction from residues 151–153 (β-bridge at residue 152). In addition, the aromatic ring of Phe$^{152}$ stacks with Phe$^{152}$ from the other monomer, and the side chain of Glu$^{153}$ interacts with its dimer equivalent backbone amide group and Pro$^{150}$. An analysis of nadD sequences reveals the conservation of Pro$^{150}$ and a hydrophobic residue at position 152. The functional dimer interface is further composed of interactions of residues from a loop at the C terminus of β-strand 5, the loop between α-helices E and F (in the lobe domain) and α-helix A. Residues that are sequentially proximal to the dimer interface form part of the ATP-binding pocket (see below), suggesting a possible role for dimer formation prior to productive substrate binding.

Temperature-sensitive lethal mutations in nadD from Sal-
the multimeric state of the enzyme has been investigated. Two of the enzymes, those from *M. fannashii* (16) and *M. thermoautotrophicum* (17), have been shown to crystallize as homohexamers, and a third from *Synechocystis* was identified as a homohexamer by size exclusion chromatography (33). The remaining five, *E. coli nadR* (14), and the NMN ATs from *Sulfolobus solfataricus* (11), *Saccharomyces cerevisiae* (7, 8), bull testes (5), and human (9, 10) appear to be trimers or tetramers by size exclusion chromatography. If the two crystal structures presented here reflect the physiological oligomeric state of NaMN AT, then the functional dimer, which is conserved in the independent crystal environments of the apo and NaAD-bound form, is proposed to be the dimer observed in solution. This is a surprising finding, given the large, intimate interface of the handshake dimer. It clearly provides a caveat to the overinterpretation of oligomeric states observed by crystallography and emphasizes the necessity of independent determination of quaternary structures.

**NaAD Binding**—NaAD is seen bound in an extended conformation (the adenine C8 to nicotinate C2 distance is 16 Å (34)) to NaMN AT. The NaAD is buried in a large enclosed cavity (Fig. 8A) with solvent channels to the diphosphate and nicotinic acid moieties, such that release of product would require a rearrangement of the protein. The adenylate ribose and diphosphate moiety of NaAD bind across a groove formed by the C-terminal splitting of β-strand 1 and β-strand 4. Comparing the apo and NaAD-bound NaMN AT crystal structures reveals a conformational flexibility of regions that interact with the upper binding surface of the adenylate ribose and diphosphate moiety of NaAD (Fig. 8A). However, interpretation of these conformational changes must be tempered by the extensive noncrystallographic contacts made by the formation of the handshake dimer in the apo crystal structure.

The classic alternating β-strand-α-helix motif of the Rossman fold unit is repeated in NaMN AT such that β-strands 4, 5, and 6 compose a modified second binding motif (Fig. 8A). It is this structural unit that is seen to have the greatest mobility upon binding of NaAD. At the C-terminal end of β-strand 4, Gly<sup>106</sup> and Asp<sup>108</sup> are highly conserved in *nadD* proteins. Gly<sup>106</sup> interacts with the adenyl ribose ring, and the side chain of Asp<sup>108</sup> is within hydrogen bonding distance with the guanadinium group of *Arg<sup>132</sup>*, another conserved residue (Fig. 8B). The aliphatic portion of the side chain of *Arg<sup>132</sup>* stacks on top of the adenine ring but are positioned 3 amino acids before a conformational flexibility of these conserved arginines are positioned to play a role in stabilizing the β-phosphate of the tri-phosphate substrates (36). However, Met<sup>118</sup> and Leu<sup>124</sup> in NMN AT<sub>m</sub> and NMN AT<sub>n</sub>, respectively, replace the hydrophobic stacking on the adenine ring but are positioned 3 amino acids before a conserved arginine (Arg<sup>121</sup> and Arg<sup>127</sup>, respectively), which retains the proposed charge-charge interaction to the ATP.

More striking differences between NaMN AT and the NMN AT structures are present in the loop following β-strand 4. The NaAD-bound structure of NaMN AT has a small α-helical turn prior to α-helix D. The α-helical turn starts after Asp<sup>108</sup> and finishes before Trp<sup>116</sup>, both of which are conserved in *nadD* proteins (Fig. 2). The indole ring of Trp<sup>116</sup> stacks in a parallel fashion against the lower (i.e. class B hydride transfer) surface of the pyridinic ring. A similar interaction of the pyridinic ring is seen in complexes of NMN AT<sub>m</sub> (17). Here the bound NAD and NMN π-stack with the indole ring of Trp<sup>87</sup>, positioned on α-helix C. In NaMN AT, the dipole moment of α-helix C is positioned to complement the negative charge of the nicotinic acid. Because these critical tryptophan residues occur in different parts of the structure, the NaAD in NaMN AT has consid-
The nicotinic group is disordered.

Comparison of enzymes of the nucleotidyl-transferase α/β phosphodiesterases. A, *B. subtilis* NaMN AT; B, *E. coli* phosphopantetheine adenyltransferase; C, *M. jannaschii* NMN AT; D, *M. thermoautotrophicum* NMN AT; E, *B. subtilis* glycerol-3-phosphate cytidylyltransferase; F, *E. coli* glytaminyl tRNA synthetase. Strands of the central parallel β-sheet are shown in green, the α4GH motif is shown in magenta, and the SXXXX(R/K) motif is shown in orange.

The loop from β-strand 2 to α-helix B becomes ordered by its interactions with NaAD. This loop contains a partially conserved Pro^12-Pro^14-His^15-Lys^16 motif that interacts with both the nicotinic ring, through Pro^14 and His^15, and the modeled γ-phosphate of ATP through His^15 and Lys^16. Residues that form the floor of this pocket remain invariant in the apo and NaAD-bound structures. These include Gly^9, Thr^10, and Phe^11, at the end of β-strand 1 that interact with the diphosphate group. The main chain oxygens of Phe^15 and Ile^15, immobilized at the dimerization interface, are directed toward the adenine ring. The aromatic ring of Phe^2 fixes the orientation of imidazole group of His^15 in an edge-on interaction. The adenine then rests on a platform comprising His^15, His^18, and Gly^17 of the α4GH signature motif on one face and with Arg^18 layered on the other.

**Conserved Motifs in the Nucleotidyl-transferase α/β Phosphodiesterases**—The signature sequence motif αXGH, first seen in class I aminoacyl-tRNA synthetases, has been identified as being conserved in all nucleotidyl-transferase α/β phosphodiesterases (17). In *B. subtilis* NaMN AT this conserved motif is His^15-Asn^18-Gly^17-His^16. The apo crystal structure of NaMN AT shows this loop to be unchanged in its conformation to that of NaAD-bound. The two imidazole rings of His^15 and His^16 stack on each other in a parallel (π-π) fashion, and the backbone amide of His^15 hydrogen bonds to the hydroxyl of His^16. The arrangement of this loop results in a similar conformation seen in all other adenylyltransferase structures. The side chain of His^15 makes extensive interactions with the adenyl ribose, and the side chain of His^16 is positioned to interact with the modeled β-phosphate of ATP. Site-directed mutagenesis studies of NMN ATmt and other adenylyltransferases have

![Ribbon diagram of NaMN AT](image)
implicated His\(^{15}\) in the loss of enzymatic activity and in the stabilization of the transition state (17).

In addition to the HXGH motif, class I aminoacyl-tRNA synthetases are characterized by a KMSKS region. The core functionality of this region can now be extended to the whole superfamily by the observation that the serine stabilizes a loop at the N-terminal end of an \(\alpha\)-helix proximal to the ATP phosphate-binding site for all known members. In NaMN AT, the tri-phosphate of ATP would be stabilized by the N-terminal dipole of \(\alpha\)-helix E. It has been previously noted that this second conserved sequence motif seen in tRNA synthetases (230KFGKT in tyrosyl-tRNA synthetase and 267VMSKR in glutaminyl-tRNA synthetase) has a structurally equivalent 113RTXGISTT motif in the GCT family (30). Both of these sequences lie at the N terminus of an \(\alpha\)-helix and make direct interactions to the \(\beta\)- and \(\gamma\)-phosphates of bound ATP. Comparison of the current complex structure with the two published NMN AT structures shows a similar set of structural interactions from conservatively mutated sequences and suggests an equivalent sequence motif (Fig. 6). The key residues of this motif for cytidylyl transferase and adenylyltransferases appear to be the initial serine and the terminal arginine/lysine and suggest a S\(^{n}X^{n+1}X^{n+2}\)(R/K)\(^{n+5}\) minimal consensus sequence (Table IV). The main chain carbonyl preceding the seminal serine is within hydrogen bonding distance to the exocyclic nitrogen of adenine, and the O\(^{n}\) of this serine (at position \(n\)) is within hydrogen bonding distance to the \(n+3\) main chain nitrogen. This appears to cap the N terminus of an \(\alpha\)-helix whose dipole moment stabilizes the tri-phosphate binding pocket. Further, this \(\alpha\)-helix is not part of the \(\beta\)/\(\alpha\)/\(\beta\) dinucleotide binding fold but occurs in a distinct domain. The positive charge at position \(n+5\) of this motif appears to coordinate the \(\gamma\)-phosphate of ATP in NaMN AT\(\text{mj}\), consistent with modeling studies for Arg\(^{160}\) of NaMN AT. In GCT however, Lys\(^{123}\) is built pointing away from the catalytic active site, but the higher temperature factor for the side chain may indicate a high degree mobility. In pantothenate synthetase, this motif occurs in a region that is believed to show dramatic structural rearrangement (38). The crystal structure of glutaminyl-tRNA synthetase complexed with tRNA and ATP shows that the positively charged residue is on the same face of the \(\alpha\)-helix but is positioned only a half-turn from the seminal serine. Further studies should reveal whether the composition of this \(\alpha\)-helix plays a role in catalysis.

Although these residues are structurally conserved among known structures in this superfamily, other contributors to the active site vary. An arginine from \(\beta\)-strand 1 in NMN AT\(\text{mj}\) and NMN AT\(\text{mt}\) (Arg\(^{8}\) and Arg\(^{11}\), respectively) contributes to stabilization of the tri-phosphate group of ATP. In contrast PPAT, GCT, and NaMN AT lack this residue, but the conserved Lys\(^{42}\) in PPAT (Lys\(^{46}\) of GCT and Lys\(^{45}\) of NaMN AT) is thought to play a similar role.

In conclusion, the \(B.\ subtilis\) yqeJ gene encoding NaMN AT has been identified as an essential part of the NAD synthesis pathway exhibiting structural homology to other known adenylyltransferases. The biochemical and structural characteriza-

| Protein Family                  | Consensus | Ser | X   | X   | X   | X   | Arg/Lys |
|--------------------------------|-----------|-----|-----|-----|-----|-----|---------|
| **B. subtilis NaMN AT**         | Ser        |     |     |     |     |     |         |
| NMN AT\(\text{mj}\)             | Ser\(^{125}\) | Ser\(^{155}\) | Ser\(^{156}\) |     |     |     |         |
| NMN AT\(\text{mt}\)             | Ser        |     |     |     |     |     |         |
| PPAT                           | Ser\(^{128}\) | Ser\(^{152}\) | Ser\(^{153}\) |     |     |     |         |
| GCT                            | Ser\(^{114}\) | Thr\(^{119}\) |     |     |     |     |         |
| Glutaminyl-tRNA synthetase     | Ser\(^{269}\) | Lys\(^{370}\) |     |     |     |     |         |
| Pantothenate synthetase        | Ser\(^{187}\) | Ser\(^{188}\) |     |     |     |     |         |

**TABLE IV**

\(SXXXX/(R/K)\) motif in cytidylyl transferase and adenylyl transferase families
tion of substrate specificity (NaMN versus NMN) may provide an exciting new target for anti-microbial therapies. Further studies are needed to elucidate the function of NaMN AT and the role of the conserved sequence and structural motifs in catalysis.

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Identification, Characterization, and Crystal Structure of *Bacillus subtilis* Nicotinic Acid Mononucleotide Adenylyltransferase

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