Insights into Ligand Binding and Catalysis of a Central Step in NAD\(^+\) Synthesis

STRUCTURES OF METHANOBACTERIUM THERMOAUTOTROPHICUM NMN ADENYLTRANSFERASE COMPLEXES\(^a\)

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Nicotinamide mononucleotide adenylyltransferase (NMNATase) catalyzes the linking of MN\(^{\#}\) or NaMN\(^*\) with ATP, which in all organisms is one of the common steps in the synthesis of the ubiquitous coenzyme NAD\(^+\), via both de novo and salvage biosynthetic pathways. The structure of Methanobacterium thermooautotrophicum NMNATase determined using multiwavelength anomalous dispersion phasing revealed a nucleotide-binding fold common to nucleotidyltransferase proteins. An NAD\(^+\) molecule and a sulfate ion were bound in the active site allowing the identification of residues involved in product binding. In addition, the role of the conserved \(^ {16}\)HXGH\(^ {18}\) active site motif in catalysis was probed by mutagenic, enzymatic and crystallographic techniques, including the characterization of an MN\(^{\#}\)/SO\(^{4}\)\(^-\) complex of mutant H19A NMNATase.

Nicotinamide mononucleotide adenylyltransferase (EC 2.7.7.1) catalyzes the synthesis of nicotinamide adenine dinucleotide (NAD\(^+\)) or nicotinic acid dinucleotide (NaAD\(^+\)) from nicotinamide mononucleotide (MN\(^{\#}\)) or nicotinic acid mononucleotide (NaMN\(^+\)), respectively, by transferring the adenylyl part of ATP and concomitantly releasing pyrophosphate (PP\(_i\)) (Fig. 1A). The reaction product, NAD\(^+\), plays a central role in many cellular processes; it functions as a coenzyme in reduction-oxidation reactions and as a substrate in DNA ligation and protein ADP-ribosylation reactions (1). There is also considerable medical interest in this enzyme as it is implicated in the metabolism of the antitumor drug tiazofurin (2). In vivo, tiazofurin is phosphorylated to tiazofurin monophosphate and then converted by NMNATase to the actual pharmacophore thiazole-4-carboxamide adenine dinucleotide, an analog of NAD\(^+\). Thiazole-4-carboxamide adenine dinucleotide is a potent inhibitor of inosine monophosphate dehydrogenase causing arrest of guanylate biosynthesis and thus inhibition of tumor cell proliferation. Consistent with this interpretation, low NMNATase activity is observed in cancer patients showing resistance to tiazofurin therapy (2).

Two mechanisms for the NMNATase-catalyzed synthesis of NAD\(^+\) have been postulated; the first one assumes a double displacement reaction that involves the formation of an adenylyl enzyme covalent intermediate upon release of pyrophosphate followed by transfer of the adenylyl group to MN\(^{\#}\) to form NAD\(^+\), whereas the second one describes a nucleophilic attack of the 5'-phosphate of MN\(^{\#}\) on the ω-phosphate of ATP to form NAD\(^+\) and releasing PP\(_i\). The latter mechanism is supported by \(^ {17}\)O NMR studies of NAD\(^+\) synthesis (3), but a complete understanding of the catalytic chemistry awaited more detailed structural information.

NMNATase proteins have been identified, purified, and characterized from archaea, bacteria, and eukarya. All of these proteins are oligomeric; trimeric, tetrameric, and hexameric forms have been observed (1). Several NMNATase genes have been sequenced from a variety of sources (Fig. 1B). Although these gene products remain annotated in the GenBank\(^{\text{TM}}\) data base as of unknown function, related sequences from Methanococcus jannaschii, Escherichia coli, Synechocystis sp., and Sulfolobus solfataricus have been overexpressed as recombinant proteins in E. coli and shown to exhibit NMNATase activity (4–8).

Recently, the crystal structure of NMNATase from M. jannaschii in complex with ATP and Mg\(^2+\) was reported (9). Our results on the NAD\(^+\) and NMN\(^+\) complexes of the Methanobacterium thermooautotrophicum enzyme complement this result, especially when interpreting the catalytic mechanism of NMNATase. We describe the crystal structures of the NAD\(^+\) complex of NMNATase and of the NMN\(^+\) complex of an active site mutant (H19A) of NMNATase at 1.9 and 2.5 Å resolution, respectively. These structural results, combined with mutagenesis and enzymatic experiments, define the spatial geometry of the ligand binding sites, identify residues with potential roles in substrate binding and catalysis, and suggest aspects of the product release mechanism.
EXPERIMENTAL PROCEDURES

Cloning, Protein Expression, and Purification—The NMNATase gene (GenBank™ accession number AE000803) was amplified by polymerase chain reaction using M. thermoautotrophicum genomic DNA and cloned into the pET15b (Novagen) expression vector at the NdeI and BglII sites. Recombinant NMNATase was overexpressed in E. coli BL21 Gold (DE3) cells (Stratagene) harboring a plasmid encoding rare E. coli tRNA genes. The cells were grown at 37 °C in Luria-Bertoni broth with carbenicillin (50 μg/ml) and kanamycin (50 μg/ml) to an A600 nm of 0.7 and induced overnight with 0.5 mM isopropyl-β-D-thiogalactopyranoside at 24 °C. The bacteria were harvested by centrifugation and resuspended in binding buffer (50 mM Tris, 500 mM NaCl, 5% glycerol, and 5 mM imidazole) supplemented with 2 mM phenylmethylsulfonyl fluoride. Bacteria were lysed by several passages through a French pressure cell at 1.4 x 10⁸ pascals, and DNA was sheared by sonication. Cell debris was removed through centrifugation for 30 min at 20,000 x g. Contaminating E. coli proteins were removed by heating for 20 min at 55 °C followed by centrifugation at 5000 x g for 30 min. The supernatant was applied by gravity to a DE52 column (Whatman) immediately coupled to a Ni²⁺ column (Qiagen). The Ni²⁺ column was washed with 50 volumes of binding buffer containing 30 mM imidazole. The bound NMNATase was eluted from the Ni²⁺ column with 500 mM imidazole in binding buffer. The hexahistidine tag was cleaved by digesting for 16 h with thrombin (1 μg of thrombin per mg of recombinant NMNATase) resulting in the synthesis of NAD⁺. B, amino acid sequence alignment of archaeal and bacterial NMNATase proteins from M. thermoautotrophicum (Mth), M. jannaschi (Mja), Methanolobus tindarius (Mti), Archaeoglobus fulgidus (Afu), Pyrococcus abyssi (Pab), Aquifex purnix (Ape), E. coli (Eco), Salmonella typhymurium (Sty), Hemophilus influenza (Hin), Streptomyces viridochromogenes (Svi), and Synechocystis sp. (Ssp). Residues that are conserved between bacterial as well as archaeal organisms are highlighted in black. Residues that are only conserved between archaeal organisms and are involved in ligand binding are highlighted in gray.

FIG. 1. A, reaction catalyzed by NMNATase resulting in the synthesis of NAD⁺. B, amino acid sequence alignment of archaeal and bacterial NMNATase proteins from M. thermoautotrophicum (Mth), M. jannaschi (Mja), Methanolobus tindarius (Mti), Archaeoglobus fulgidus (Afu), Pyrococcus abyssi (Pab), Aquifex purnix (Ape), E. coli (Eco), Salmonella typhymurium (Sty), Hemophilus influenza (Hin), Streptomyces viridochromogenes (Svi), and Synechocystis sp. (Ssp).
Crystal Structures of NMN Adenylyltransferase Complexes

Numbers in parentheses refer to the highest resolution shell, 1.97–1.90 Å for the native data, 3.01–2.9 Å for the MAD data, and 2.59–2.50 Å for the mutant data.

### Table I

**Summary of data collection statistics**

| X-ray data | Native | Peak | Edge | Remote | H19A |
|------------|--------|------|------|--------|------|
| Space group | P6₁2₂ | P6₁2₂ | P6₁2₂ | P6₁2₂ | P6₁2₂ |
| Unit cell (Å³) | 89.0 × 89.0 × 109.9 | 89.2 × 89.2 × 110.3 | 89.2 × 89.2 × 110.3 | 89.7 × 89.7 × 109.7 | 89.7 × 89.7 × 109.7 |
| Resolution (Å) | 1.9 | 2.9 | 2.9 | 2.9 | 2.5 |
| Wavelength (Å) | 1.0000 | 0.97954 | 0.97930 | 0.95373 | 1.0000 |
| Se sites (no.) | 4 | 4 | 4 | 4 | 4 |
| Total observations (no.) | 492526 | 125251 | 126325 | 122548 | 60748 |
| Unique reflections (no.) | 24524 | 9311 | 9311 | 9479 | 9311 |
| Intensity (I/σ(I)) | 37 (5) | 33 (10) | 30 (8) | 27 (6) | 30.2 (5.4) |
| Completeness (%) | 99.4 (99.0) | 98.9 (99.5) | 98.3 (97.6) | 97.1 (97.3) | 99.3 (97.1) |
| R̃sym (%) | 0.041 (0.351) | 0.079 (0.238) | 0.082 (0.249) | 0.076 (0.285) | 0.043 (0.273) |
| Figure of merit (%) | 40.76 | 40.76 | 40.76 | 40.76 | 40.76 |

### Table II

**Summary of refinement statistics**

| | WT | H19A |
|---|------|------|
| R<sub>cryst</sub> | 0.212 | 0.236 |
| R<sub>free</sub> | 0.242 | 0.294 |
| Protein atoms (no.) | 1340 | 1275 |
| Water molecules (no.) | 119 | 10 |
| NAD atoms (no.) | 44 | |
| NMN atoms (no.) | |
| Sodium ions (no.) | 1 | 22 |
| Sulfate ions (no.) | 1 | 1 |
| r.m.s.d. bond lengths (Å) | 0.016 | 0.008 |
| r.m.s.d. bond angles (°) | 1.8 | 1.3 |
| r.m.s.d. dihedrals (°) | 23.9 | 22.0 |
| Average main chain B-factor (Å²) | 33.7 | 42.7 |
| Average side chain B-factor (Å²) | 36.0 | 47.6 |
| Average ligand B-factor (Å²) | 38.3 | 59.1 |

### Results and Discussion

#### Enzymatic Assay

WT NMNATase activity was measured in a coupled assay according to Raffaelli et al. (6). Varying amounts of NMNATase (1–1000 ng) were incubated with 2 mM NAD, 2 mM ATP, 10 mM MgCl₂, and 50 mM HEPES (pH 7.5) at 65 °C for 20 min. The amount of NAD⁺ formed was measured spectrophotometrically at 340 nm using alcohol dehydrogenase to convert NAD⁺ to NADH. The enzymatic activity of H19A NMNATase was measured the same way but varying the amount of enzyme in each assay from 1 to 5000 μg.

#### Structure Determination

The structure of the NAD⁺ complex of NMNATase has been determined by the MAD method using selenium as the anomalous scatterer. The resulting electron density is of high quality except for the loop consisting of residues 124–129, in which the main chain density is continuous but increased mobility has compromised the clarity of side chain densities. In addition, 12 C-terminal and 3 N-terminal amino acids are not visible in the electron density map. The final model contains 167 amino acids (residues 4–171), with 57% in a cis-conformation, 119 water molecules; one molecule of NAD⁺, 1 molecule of sulfate ion (Fig. 2A). Refinement at 1.9 Å resolution resulted in an R<sub>cryst</sub> of 0.212 and an R<sub>free</sub> of 0.242. According to PROCHECK (20), 92% of the residues are in the most favored regions, and no residue is in the disallowed regions of the Ramachandran plot.

The structure of the NMN⁺ complex of H19A NMNATase was determined by molecular replacement techniques. Its elec-
Subunit consists of two domains (Fig. 2A). The second domain (residues 131–170) is made up from three \(\alpha\)-helices (helices 5–7) and is the major contributor to intratrimer subunit interactions.

Subunit Interactions—The intratrimer interactions are almost exclusively electrostatic and occur preferentially between helix 5 of subunit A and helix 7 of subunit B. Salt bridges are formed by Arg-110A and Glu-164B; Glu-114A with His-44B and Arg-165B; and Gln-109A with His-168B. These contacts are repeated between subunits B and C as well as subunits C and A (Fig. 2B). In each case, 1457 Å\(^2\) of a total of 16,938 Å\(^2\) of molecular surface are buried upon oligomerization.

In contrast to the intratrimer interactions, the dominant intertrimer contacts are nonpolar and hydrophobic. The side chains of \(\beta\)-strand 3 (Ile-75, Ile-76, and Val-78) of subunit A pack against their counterparts of \(\beta\)-strand 3 of subunit D with the same interactions repeated between subunits B and E, as well as C and F. In each case, 2796 Å\(^2\) of a total of 15,609 Å\(^2\) of molecular surface are buried (Fig. 2B).

**NAD\(^+\) and Sulfate Binding to WT NMNATase—**The active site is located in a deep cleft facing a narrow channel running along the 3-fold symmetry axis of the hexamer. The site is open to solvent, possibly reflecting its readiness to release the product NAD\(^+\), which, together with a sulfate ion, could easily be identified in the electron density after the first round of refinement (Fig. 3A). As no NAD\(^+\) was added during enzyme purification and crystallization, thermophilic NMNATase must have trapped its product. NAD\(^+\) binds in an extended conformation with its adenine ring adopting an anti orientation and both the adenylyl and the nicotinamide ribose rings showing 3'-endo puckering (Fig. 3C). In contrast, the adenylyl ribose ring seems to be in the 2'-endo conformation in the *M. jannaschii* NMNATase structure (9). The exocyclic nitrogen of adenine is H-bonded to the main chain carbonyls of Phe-125 and Tyr-130, two aromatic amino acids the side chains of which interact closely. N1 binds to the main chain amide of Phe-125 and N7 to a water molecule. The adenylyl ribose forms an H-bond (3.1 Å long) between its 3'-hydroxyl and the backbone amide of Gly-104, in contrast to what has been found in NAD(P)\(^+\)-dependent dehydrogenases, in which the adenylyl ribose ring is held in place by a conserved aspartate located at the C terminus of the second \(\beta\)-strand (21). In NMNATase, it is the nicotinamide ribose in which one finds H-bonds between its 2'-hydroxyl and the carboxyl side chain of Asp-80; the 3'-hydroxyl interacts with the side chain of Ser-39. The nicotinamide ring stacks with the aromatic ring of Trp-87 and its amide substituent links to the main chain of Ile-81, the oxygen to its amino and the nitrogen to its carbonyl atoms. In addition, the side chain of Asn-84 holds the amide NH\(_2\) of the substituent in place (Fig. 3, C and D). As the enzyme has to accept both NMM\(^+\)\(^+\) and NaMN\(^+\) as substrates, an amide side chain is ideal for this purpose. A single bond rotation will provide an H-bonding partner for the second oxygen atom in the carboxylate of NaMN\(^+\). Such a change in backbone conformation would not be difficult to achieve as this region of the chain runs along the surface of the protein molecule and seems unconstrained.

The “NMM-phosphate” in the pyrophosphate linkage forms H-bonds to Asn-105. The oxygen atom bridging the two phosphorous atoms of the pyrophosphate group contacts the main chain amide of Arg-11. The “AMP-phosphate” binds to His-19, the main chain of Met-12, two water molecules and a rather high (3\(\sigma\)) spherical electron density that we interpret as a Na\(^+\) ion. This Na\(^+\) ion could contribute to balancing the negative charges of the pyrophosphate and of another electron-dense

![Image](image_url)

**Fig. 2.** A, ribbon and ball-and-stick diagram of monomeric NMNATase with helices shown in purple, strands in green, and ball-and-stick representations of NAD\(^+\) and SO\(_4\)\(^-\) highlighted. B, ribbon diagram of hexameric NMNATase viewed along its 3-fold axis. Notice that all NAD\(^+\) molecules face the interior channel.
feature, the center of which is located 5.3 Å from the phosphorous atom of the AMP-phosphate; the shape and position of this feature indicate a sulfate ion, an excellent mimic of a phosphate group. Both sodium and sulfate ions could have been provided by the crystallization buffer (0.5 M NaCl, 1.5 M Li₂SO₄). The two waters mentioned above and the metal ion are located so they can bridge oxygen atoms from the AMP-phosphate and the SO₄²⁻ ion. The sulfate oxygens are bound to the guanidinium groups of Arg-11 and Arg-136 (Fig. 3, C and D), two residues absolutely conserved among the known archaean and bacterial NMNATases (Fig. 1B), and also to the guanidinium group of Arg-47 and the backbone of Thr-133. The interaction with Arg-47 seems to be accidental as the corresponding residue in the *M. jannaschii* structure is a glutamate (9). The location of the sulfate ion is consistent with it occupying the binding site of the γ-phosphate of the substrate ATP and is reminiscent of the sulfate ion bound at the γ-phosphate position in the active site of glutaminyl tRNA synthetase complexed with AMP (22). This assignment gains further credibility by interpreting the figures portraying the binding of Mg²⁺-ATP to *M. jannaschii* NMNATase (9).

**Functional and Structural Comparisons**—Analysis of the structure of NMNATase using the program DALI (23) identified several proteins all of them belonging to the nucleotidyltransferase superfamily of dinucleotide-binding fold containing α/β phosphodiesterases (24). Presently known members of this
family are CTP:glycerol-3-phosphate cytidyltransferase (CTP:G3PCase) (25), glutaminyl tRNA synthetase (26), tyrosyl tRNA synthetase (27) and phosphopantetheine adenyltransferase (PPATase) (24). Their overall structures are remarkably similar (r.m.s.d. of 116, 137, 124, and 143 Cα atoms, equal to 2.5, 3.5, 3.9, and 2.3 Å, respectively). PPATase not only closely resembles the overall fold of NMNATase but even forms a hexamer as the functional unit. Nevertheless, the arrangement of subunits relative to each other is quite different. All of these related proteins contain a nucleotide-binding domain, present the active site sequence motif (T/H)XGH, and catalyze a nucleotidyltransfer reaction that is similar to that of NMNATase. This reaction involves the attack of a nucleophilic group of one substrate at the α-phosphate of the nucleoside triphosphate (the second substrate), thereby forming a new phosphodiester bond and releasing a pyrophosphate molecule.

Originally, identification of NAD\(^+\) in the active site cleft and the DALI results indicating nucleotidyltransferase function led us to propose that protein MT0150, annotated as “conserved” in the \textit{M. thermoautotrophicum} genome sequence data base (28), was in fact an NMNATase. Preliminary enzymatic assays confirmed that the enzyme catalyzed the biosynthesis of NAD\(^+\) from NMN\(^-\) and ATP (Fig. 4B). The validity of this assignment was established when a literature search revealed that the homologous enzyme from \textit{M. jannaschii} had NMNATase activity (4, 6).

In addition to the common nucleotide-binding fold, all of these enzymes contain an active site sequence motif, (T/H)XGH, that is absolutely conserved in all known archaeal and bacterial NMNATases (\textsuperscript{16}HRGH\textsuperscript{19} in \textit{M. thermoautotrophicum} NMNATase; Fig. 1B). This active site sequence motif has also been identified in other nucleotide binding enzymes catalyzing adenyltransferase reactions such as the ATP sulfurylase domain of human phosphoadenosine phosphosulfate synthase (\textsuperscript{425}HNGH\textsuperscript{428}), \textit{A. thaliana} ATP sulfurylase, and \textit{E. coli} flavin adenine dinucleotide synthetase (\textsuperscript{29}HRGH\textsuperscript{32}) (29–31). For several of these enzymes, the roles of the two conserved histidine residues in this motif have been investigated using crystallographic and site-directed mutagenesis techniques (22, 24–27, 32). Although their mutation to alanine residues in CTP:G3PCase, phosphosulfate synthase, and tyrosyl tRNA synthetase demonstrated that they were essential for catalysis (28, 32, 33), structural studies of glutaminyl tRNA synthetase were more specific, implicating them in the binding of the β- and γ-phosphates of ATP and the stabilization of the postulated pentacoordinate transition state (22). Mutagenesis of each of the two His residues (His-425 and His-428) to alanine in phosphosulfate synthase and measurement of catalytic activities in crude extracts of transformed \textit{E. coli} cells confirmed that the mutant proteins exhibited only background levels of adenyltransferase activity (29).

Whereas the binding sites of the nucleoside triphosphate substrates are well characterized and overlap quite closely for all structurally known members of the nucleotidyltransferase superfamily, PPATase is the only one for which the interactions with the product have been described (24). This enzyme is also the one with the highest degree of structural similarity to NMNATase. When NMNATase and PPATase are superimposed using their (T/H)XGH motifs, it is evident that not only do the adenyl moieties line up closely but the remaining chemically dissimilar halves of the products (NMN\(^+\) versus 4′-phosphopantetheine) occupy overlapping parts of the respective active sites, too (Fig. 4C). It is interesting to note that space that is filled by the terminal β-mercaptoethylamine moiety of dephospho-coenzyme A in PPATase is taken up by the side chain of Trp-87 in NMNATase, which, through its large...
aromatic ring, provides the nicotinamide ring with the opportunity to undergo a stacking interaction.

**Mutagenic, Enzymatic, and Crystallographic Studies of His-19 in the THRGH Motif—**Superimposing the (T/H)XGH structural motif of CTP-G3PCase, glutaminyl tRNA synthetase, and PPATase on that of NMNATase places the respective histidines in comparable positions, from which they bind to the β- and γ-phosphate oxygens of ATP (Fig. 4A).

The importance of His-19 in NMNATase activity was investigated further using site-directed mutagenesis. Preliminary enzymatic analysis shows that the H19A mutant retains less than 1% of the specific activity of WT NMNATase, indicating a strong involvement in catalysis for this residue (Fig. 4B). The results are also in good agreement with previous studies of phosphosulfate synthase and CTP-G3PCase, in which mutagenesis of the His residues to Ala resulted in enzymes with activities lowered by at least 4 orders of magnitude (29, 34). To confirm that the H19A protein was stable and properly folded and that its lack of NMNATase activity was not caused by partial denaturation or misfolding, its crystal structure was determined.

The mutant enzyme crystallized in the same crystal form as WT NMNATase; its polypeptide structure was quite similar to that of WT NMNATase (r.m.s.d. of all Cα positions is 0.21 Å). Instead of the product NAD⁺, however, a molecule of the substrate NMN⁺ was trapped in its active site (Fig. 3B). This finding supports the kinetic experiments that showed a lack of NMNATase activity for this mutant. At this point, however, we cannot distinguish between a drastic loss of affinity for ATP and an incompatibility to undergo the chemistry necessary for catalysis. The variation in ligands seems also to be responsible for the only remarkable structural difference evident between the protein parts of the native and mutant complexes. In WT NMNATase, although the loop region between residues 123 and 130 shows increased B-factors of both the main and side chains indicating flexibility, there is clear density to follow the main chain. For residues 124–129 in the H19A mutant, however, there is no continuous electron density visible at all. Obviously, the presence of the adenine ring of ATP or NAD⁺ is necessary to at least partially stabilize this stretch of peptide chain.

**Proposed Reaction Mechanism—**Several conserved and positively charged residues (Arg-11, His-16, His-19, and Arg-136) line the active site of NMNATase, and their interactions with ATP have recently been determined. The absence of any covalent adenyl-NMNATase intermediate has also been established (9). When the position of ATP is considered together with the results of the crystal structure of H19A NMNATase complexed with NMN⁺, it becomes clear that NMN⁺ is bound in such a way that its phosphate group is positioned close to the α-phosphate of ATP, ready to attack from the side opposite of the pyrophosphate leaving group, in line with proposed direct attack adenyltransferase mechanisms (22). A small shift in position accompanies the transition of the α-phosphate of ATP to the AMP-phosphate of NAD⁺. This seems to be reflected in a switch of the pucker of the adenosine ribose from 3'-endo to 2'-endo. The crystallographic results are fully consistent with 15O NMR studies of NAD⁺ that support direct attack of ATP by NMN⁺ (3). As the phosphate group of NMN⁺ is a reasonable nucelophile and pyrophosphate a good leaving group, residues from the active site of NMNATase may not be required to directly participate in the chemistry of the reaction by acid/base or covalent catalysis (22, 29). Locking the reaction partners into the proper geometry and electrostatic support might be all that is needed for catalysis to occur. An equivalent adenyltransferase mechanism was first reported for tRNA aminoacylation by glutaminyl tRNA synthetase (22). We believe that this is the mechanism used by the archaeal and bacterial NMNATases because the HRGH active site motif is conserved at an equivalent position among all prokaryotic organisms for which the corresponding gene has been sequenced (Fig. 1B). The existence of Mg²⁺-ATP (9) and NMN⁺ complexes (see above) shows that archaeal NMNATases have the capacity to bind the two substrates independently from each other, whereas eukaryotic enzymes follow an ordered bi-bi mechanism (1).

**Proposed Product Release Mechanism—**When *M. thermoautotrophicum* NMNATase is expressed in *E. coli*, NAD⁺ molecules are trapped within the active site of the protein. Full catalytic function of this enzyme is only observed at 65 °C, the optimal growth temperature of the archaeal thermophilic source. NAD⁺ bound to WT NMNATase could only be released if the protein sample was heated to 65 °C (data not shown). These results point to an obligatory conformational change in this protein before the release of product can occur.

Before the binding of the ATP substrate, the loop surrounding the adenine ring (residues 123–130) is quite mobile, as indicated by the absence of reasonable corresponding electron density in the NMN⁺ complex of H19A NMNATase. D'Angelo et al. (9) showed that Arg-121 in *M. jannaschii* NMNATase, which corresponds to Arg-127 in the *M. thermoautotrophicum* enzyme, is bound to one of the oxygens of the β-phosphate of ATP. Its alphatic chain undergoes hydrophobic interactions with the adenine ring moiety (9). Once NAD⁺ is generated, however, this tighter interaction seems to loosen up again, resulting in higher B-factors for main chain atoms and weaker density at the positions of the side chains as seen in our structure of the NAD⁺-WT NMNATase complex. Despite this increased mobility, it is clear that in the NAD⁺-WT NMNATase complex Arg-127 is pointing away from the adenine ring moiety (Fig. 3C). Its guanidinium group is now −15 Å from where it is positioned in the ATP-Mg²⁺ NMNATase complex. We presume that at room temperature the thermal energy is not sufficient to break the main chain away from the adenine ring keeping NAD⁺ bound at the active site. At elevated temperatures, however, the loop could separate from the adenine ring moiety as the first step in product release.

*M. thermoautotrophicum* NMNATase is only one example of a surprising number of proteins from thermophilic organisms seen to carry ligands with them when purified from overexpressing *E. coli* cells (35, 36). If this effect can be verified for more cases it could convey additional advantages to the use of thermophilic proteins in the challenging task of assigning function to hitherto unknown parts of proteomes.

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