Bifunctional NMN Adenylyltransferase/ADP-Ribose Pyrophosphatase: Structure and Function in Bacterial NAD Metabolism

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SUMMARY

Bacterial NadM-Nudix is a bifunctional enzyme containing a nicotinamide mononucleotide (NMN) adenylyltransferase and an ADP-ribose (ADPR) pyrophosphatase domain. While most members of this enzyme family, such as that from a model cyanobacterium Synechocystis sp., are involved primarily in nicotinamide adenine dinucleotide (NAD) salvage/recycling pathways, its close homolog in a category-A biodefense pathogen, Francisella tularensis, likely plays a central role in a recently discovered novel pathway of NAD de novo synthesis. The crystal structures of NadM-Nudix from both species, including their complexes with various ligands and catalytic metal ions, revealed detailed configurations of the substrate binding and catalytic sites in both domains. The structure of the N-terminal NadM domain may be exploited for designing new antitularemia therapeutics. The ADPR binding site in the C-terminal Nudix domain is substantially different from that of Escherichia coli ADPR pyrophosphatase, and is more similar to human NUDT9. The latter observation provided new insights into the ligand binding mode of ADPR-gated Ca2+ channel TRPM2.

INTRODUCTION

Nicotinamide adenine dinucleotides (NAD and NADP) are ubiquitous cofactors in hundreds of redox reactions in the cell and are thus essential for all living organisms. In addition to its fundamental redox functions, NAD also serves as a cosubstrate in a number of reactions that deplete its cellular pool, such as NAD-dependent protein deacetylation by the members of the broadly conserved CobB/Sirtuin family (Denu, 2005; Landry et al., 2000; Mian et al., 1999, Penfound and Foster, 1996). The de novo NAD biosynthesis routes generate NAD from either aspartate (in prokaryotes) or tryptophan (mostly in eukaryotes), while nicotinamide (Nm), Niacin (vitamin B3), or Nm riboside (NmR, or V-factor) can be salvaged from either external sources or recycled intracellularly via NAD degradation and some of the NAD-consuming reactions mentioned above. As most bacterial species can not salvage NAD(P) or its phosphorylated precursors from the medium, the last steps of NAD biosynthesis have been recognized as potential targets for developing novel anti-infectious therapeutics (Gerdes et al., 2002; Osterman and Begley, 2007; Velu et al., 2003).

All routes that lead to NAD synthesis require an adenylation step that generates the adenylated dinucleotides (NAD or deamido-NAD (NaAD)) from Nm or nicotinic acid mononucleotide (NMN or NaMN; see Figure 1A). Adenylyltransases responsible for this central step of NAD biosynthesis, termed NmnATase (Enzyme Commission [EC] number 2.7.7.1) or NaMNATase (EC 2.7.7.18) depending on their substrate specificity, occur in at least four distinct families within a common fold that belong to the “(H/T)IGH motif containing nucleotidyltransferase superfamily.” (Bork et al., 1995; Magni et al., 2004). Members of the NadD family, conserved in most bacteria, have a strong preference for NaMN over NMN substrate, in keeping with their essential role in the conventional two-step synthesis of NAD from the NaMN precursor via NaAD intermediate (Figure 1 and Begley et al., 2001). Distant NadD homologs present in all eukaryotes form a separate PNAT family, which displays a dual specificity with almost equal catalytic efficiency for both physiological substrates NaMN and NMN (Magni et al., 2004). Both activities play important roles in NAD metabolism in eukaryotic cells, the former in de novo NAD synthesis via NaMN, and the latter in the salvage and recycling of amidated precursors (e.g., Nm and NmR, via NMN (Berger et al., 2005; Sorci et al., 2007). A distantly related NadR family with a strong preference for NMN over NaMN is present in a limited group of bacteria (mostly Enterobacteriaceae). Most members of this family contain at least two domains with NMMNATase and NmR kinase activities, which, together with PnuC transporter, form an NmR salvage pathway (Foster et al., 1990; Kurnasov et al., 2002; Raffaelli et al., 1999b). Members of the last, so-called NadM family, are present in all archaea, where they are likely involved in the main NAD synthesis pathways (Raffaelli et al., 1994, 1997). Close NadM homologs are present in a limited...
number of diverse bacteria, where they are almost always fused with a C-terminal Nudix hydrolyase domain. Historically, the first, and until recently, the only characterized member of bacterial NadM-Nudix subfamily was the enzyme from *Synechocystis* sp. (slr0878), which displays a strong preference for NMN over NaMN in the adenylyltransferase reaction, and an ADP-ribose (ADPR) pyrophosphatase (ADPRase) activity via its Nudix domain (Raffaelli et al., 1999a). In *Synechocystis* sp., as well as in most other NadM-Nudix-harboring bacteria, the main housekeeping NadM enzymes are also present. In most of these species, the NadM-Nudix enzymes, together with Nm phosphoribosyltransferase (NMPRT, EC 2.4.2.12, encoded by *nadV* gene) comprise an Nm salvage/recycling pathway (Gerdes et al., 2006). A remarkable exception to this rule has recently been established in *Francisella tularensis*, where, due to the absence of the gene, *nadD*, the NMNATase domain of NadM-Nudix is proposed to play the dispensable central role in NAD synthesis (Figure 1B, and L.S. and A.L.O., unpublished data). In contrast to most other bacteria, *F. tularensis* is shown to implement a nonconventional NAD synthesis pathway, where the NaMN precursor is first amidated to NMN by a distant homolog of NAD synthetase and then converted to NAD by NMNATase of NadM (Figure 1B, and L.S. and A.L.O., unpublished data). These observations indicate that the closely related bacterial NadM-Nudix proteins, represented by those from *Synechocystis* spp. and *F. tularensis*, play drastically different cellular functions. A comparative biochemical and structural characterization of these two proteins will not only enable a better understanding of their respective functions, but also provide a starting point for the development of NMNATase inhibitors as potential new therapeutics against tularemia.

Currently, the crystal structures of several NaMNATase and NMNATase enzymes, including their complexes with substrates or products, have been reported. These include representatives of bacterial NadD (Han et al., 2006; Olland et al., 2002; Yoon et al., 2005; Zhang et al., 2002), eukaryotic PNAT (Garavaglia et al., 2002; Werner et al., 2002; Zhang et al., 2003; Zhou et al., 2002), NadR (Singh et al., 2002), and archaeal NadM families
(D’Angelo et al., 2000; Saridakis et al., 2001, 2003). Prior to the current study, the bacterial NadM-Nudix subfamily remained the last group of NMMATases to be structurally characterized.

The C-terminal domain of bacterial NadM-Nudix protein belongs to a large superfamily of pyrophosphohydrolases that are generally involved in the hydrolysis of physiological nucleotide metabolites and potentially toxic nucleoside diphosphate derivatives (Bessman et al., 1996; Koonin, 1993). Members of Nudix hydrolase superfamily occur in a broad range of species and as multiple paralogs with various substrate specificities. For example, at least 13 Nudix proteins are present in Escherichia coli, 22 in human genome (McLennan, 2006), and 21 Nudix hydrolases were systematically characterized in the radiation-resistant organism, Deinococcus radiodurans (Xu et al., 2001). So far, structures of several members of Nudix superfamily with ADPRase activity have been reported, including a monofunctional NudF enzyme from several bacterial species (Gabelli et al., 2001, 2002; Kang et al., 2003; Yoshita et al., 2004), and human NUDT9 and NUDT5 (Shen et al., 2003; Zha et al., 2006). Catalytic mechanisms of several representative Nudix enzymes, including ADPRase, were studied in detail (Mildvan et al., 2005). Interestingly, the C-terminal domain of bacterial NadM-Nudix enzyme appears to be more closely related to its archaeal homologs and mammalian NUDT9 than to bacterial NudF.

Here we report the crystal structures of NadM-Nudix proteins from a model cyanobacterium, Synechocystis sp., and a category-A biodefense pathogen, F. tularensis (syNadM-Nudix and ftNadM-Nudix, respectively). These structures, combined with preliminary mutagenesis results, suggest a potentially complex mechanism underlying the observed stringent substrate preference for NMN over NaMN in the NadM domain, which may involve several structural elements surrounding the pyridine binding site. They also revealed detailed configurations and substrate binding mode in the active site of the ADPRase domain, providing new insight to the catalytic mechanism of this subfamily of Nudix hydrolases.

RESULTS

Kinetic Characterization

Steady-state kinetic parameters were determined for both enzymatic activities of purified recombinant ftNadM-Nudix and syNadM-Nudix—the adenylyltransferase activity toward NMN and NaMN, and the pyrophosphohydrolase activity toward ADPR substrate. In contrast to members of archael NadF family, which display dual specificity toward NMN and NaMN substrate (L.S. and A.L.O., unpublished data), both ftNadM and syNadM show a strong preference for NMN versus deamidated NaMN substrate (L.S. and A.L.O., unpublished data). A much higher catalytic NMMATase efficiency was observed for F. tularensis enzyme compared with Synechocystis sp. (Kcat/Km of 82.6 versus 5.2 s⁻¹ mM⁻¹), consistent with its unique, essential role in NAD biosynthesis. The pyrophosphohydrolase activities of ftNadM-Nudix investigated in this study are similar to those reported previously for syNadM-Nudix (Raffaelli et al., 1999a). Like the Synechocystis sp. enzyme, ftNadM-Nudix strongly prefers ADPR over other tested potential substrates of Nudix hydrolases, including NAD⁺, NaAD⁺, NADH, NADP⁺, NADPH, ADPR, ADP-glucose, ATP, and dNTPs. Compared to ADPR, no more than 0.5% conversion was observed under tested conditions for other substrates. The steady-state kinetic constants of ftNadM-Nudix for ADPR are Kcat = 3.6 ± 0.4 s⁻¹ and Km = 41 ± 10 μM.

Overall Structure of Bacterial NadM-Nudix

We have determined the crystal structures of NadM-Nudix from both F. tularensis and Synechocystis sp. The syNadM-Nudix structure was solved first by the selenomethionyl multiwavelength anomalous dispersion (MAD) phasing method. This structure was refined to 2.6 Å resolution and was found to be complexed with the copurified NAD and pyrophosphate in the NadM domain active site, and with ADPR substrate in the Nudix domain (Figures 2A and 2B). The structure of ftNadM-Nudix was subsequently solved at 2.3 Å resolution by the molecular

Figure 2. Fo-Fc Difference Electron Density Maps of the Bound Substrate and Product in syNadM-Nudix and ftNadM-Nudix Active Sites

(A) NAD and pyrophosphate in NMNATase active site of syNadM-Nudix.

(B) ADP-ribose in ADPRase active site of syNadM-Nudix.

(C) AMP and Mn⁺⁺ ions in ADPRase active site of ftNadM-Nudix Mn-AMP complex. The anomalous difference Fourier map (colored red) from the same complex crystal is also shown. All maps are contoured at 3σ level.
replacement method, with syNadM-Nudix structure as the search model. Because ftNadM-Nudix crystals were grown in the presence of 0.2 M MgCl₂, a metal ion cluster was observed in the active site of its Nudix-domain. In contrast to syNadM-Nudix, no other ligand was found in either NadM or Nudix active site of the native ftNadM-Nudix crystal structure. We then determined the cocrystal structure of ftNadM-Nudix complexed with the product AMP and Mn²⁺ ions in its Nudix active site, and were able to accurately determine the metal ion positions (Figure 2C).

The overall structures of ftNadM-Nudix and syNadM-Nudix are very similar, with an average root-mean-square deviation (rmsd) between Cα positions of 1.44 Å over the entire length of the protein. As expected, these proteins contain both NMNATase and ADPRase domains (Figure 3A). Their NMNATase domain belongs to the large superfamily of (H/T)IGH motif containing nucleotidyltransferases, and is most closely related to archaeal NadM, with a sequence identity of ~25% and an rmsd of 2.1 Å to Methanococcus jannaschii NadM (D’Angelo et al., 2000). The overall structure of the ADPRase domain is similar to other Nudix enzymes with known structures. However, there are significant conformational differences between the ADPRase Nudix domain of NadM-Nudix and bacterial monofunctional ADPRase, which will subsequently be discussed here in more detail. The two domains are connected by a long α helix (α8), with ADPRase active site facing away from the NMNATase domain, while the NMNATase active site opens partially toward the ADPRase domain. A large cleft remains between the two domains that should allow the entry of the substrates to the NMNATase active site and the release of the reaction products. Since the relative positioning of the two domains in NadM-Nudix of different ligand-bound states are very similar, it would suggest that each domain functions independently. Indeed, substrate and products of ADPRase activity (ADPR, AMP, and ribose-5-P) do not exert any effect on the NMNATase activity of ftNadM-Nudix (data not shown). These data, along with the results of earlier biochemical experiments, indicate that there is no interaction between the two catalytic sites (Raffaelli et al., 1999a).

syNadM-Nudix forms hexamer in both crystal and solution (data not shown). Two types of dimer interfaces are observed in syNadM-Nudix crystal structures, one of which is also conserved in ftNadM-Nudix, which exists as dimer in both crystal and solution (data not shown). This dimer interface is formed primarily through ADPRase domain of each monomer (Figure 3B), and has a surprisingly small contact area of only 6.4% of the total surface area of the protein (1036 Å²). In this dimer, residues from the second monomer also contribute to the binding of substrate ADPR to the first monomer. We believe that this shared dimeric organization must be prevalent among bacterial NadM-Nudix proteins.

NMNATase Active Site
The NMNATase domain of bacterial NadM-Nudix protein contains a central five-strand parallel β sheet and several surrounding helices, a fold typical of the (H/T)IGH motif containing nucleotidyltransferases (Bork et al., 1995). The sequence and structure of bacterial NadM-NMNATase domain are more similar to archaeal NadM (~25% sequence identity) than to the bacterial NaMNATase (~15% identity) encoded by the gene, nadD (Raffaelli et al., 1999a; Singh et al., 2002), suggesting that it is probably acquired from the ancient archaeal NadM family through horizontal gene transfer. The overall structures, as well as the active site configurations between bacterial and archaeal NadM,
Figure 4. Comparison of Bacterial and Archaeal NadM

(A) Superposition of syNadM (cyan) and mtNadM (gray). The bound NAD in both structures and the pyrophosphate (PPi) in syNadM are shown as sticks.

(B) Comparison of the pyridine binding sites in syNadM (cyan) and mtNadM (gray). The hydrogen bonds are shown as dashed lines. This superposition was achieved by superimposing the entire NMNATase domains of syNadM and mtNadM.

are very similar, indicating that the two enzymes share similar substrate binding and catalytic mechanisms (Figure 4A). Here, the [H/T]IGH motif and the preceding glycine-rich loop located between strand β1 and helix α1 are involved in binding ATP phosphates with the second histidine residue of the motif coordinating the transferring χ-phosphate. The second nucleotidyltransferase signature motif, “ISSTxxR,” is located at the N-terminus of helix α6 and is involved in binding the β and γ phosphates and stabilizing the leaving pyrophosphate group (Saridakis and Pai, 2003).

There are, however, notable differences in the pyridine nucleotide binding sites in archaeal and bacterial NadM that may account for their different substrate specificities. First, substantial conformational differences are observed in the “pyridine recognition loop,” as well as in helices α4, α5, and the loop connecting α5 and β4 (Figure 4A). Most of these regions are in the proximity of the bound NAD molecule. Despite these conformational differences, examining the detailed protein-Nm interactions revealed nearly identical binding modes in the archaeal and bacterial NadM enzymes (Figure 4B). In both proteins, the Nm ring forms a stacking interaction with the side chain of a tryptophan residue (Trp88 of syNadM and Trp97 of mtNadM, respectively); its carboxyamide groups are hydrogen bonded to the main chain amide and carbonyl groups of a residue from the “pyridine recognition loop” (Trp81 of syNadM and I81 of mtNadM, respectively. Figure 4B). The most noticeable difference between the pyridine binding sites in the archaeal and bacterial NadM appears to be in the position of residue 85 of syNadM, where the amino group of Nm is hydrogen bonded to the side chain of Asp85 (Figure 4B). While an Asp or Glu residue is nearly universally conserved at this position in bacterial NadM, the corresponding residue in archaeal NadM proteins is Asn (or Ser and Thr in few cases; see Figure S1 in the Supplemental Data available with this article online). It is thus tempting to speculate that a change of this residue to an acidic Asp or Glu would render the active site of bacterial NadM less favorable for binding the more acidic NaMN due to electrostatic repulsion. Preliminary results from a Glu84→Gln mutant of ftNadM showed that the mutant did not have an improved catalytic efficiency toward NaMN substrate (data not shown). Apparently, other structural elements, in particular the backbone of Trp81 that interacts with the carboxyamide group of the substrate, may play an important role in defining the NNM specificity of the enzyme. An analogous case can be found in human dual specificity PNAT enzymes, where the exocyclic carboxyamide or carboxylate group also forms hydrogen bonds with protein main chain groups. The dual specificity for both NNM and NaMN in human PNAT is believed to be achieved via slight movements of protein backbones and an active site water molecule (Zhou, et al., 2002). In such cases, the protein structural flexibility or plasticity around the pyridine binding site may be essential for a more relaxed substrate specificity. Inspection of protein residues around the pyridine binding site in syNadM showed that the “pyridine recognition loop,” where Trp81 is located, is in close contact with residues from the C-terminal Nudix domain (Figure 3A; see also Figure S2). The movement of this loop may be limited due to these contacts. On the other hand, the pyridine nucleotide binding site in syNadM appears to be more confined than that in mtNadM (Figure 4B); thus, the movement of the pyridine nucleotide substrate may be more restricted. All these observations point to a more rigid pyridine nucleotide binding site in bacterial NadM, which may render the enzyme less adaptive to the deamidated substrate NaMN. Further mutagenesis, biochemical, and structural analysis of both archaeal and bacterial NadM enzymes will be needed to test the above hypothesis and identify all the important structural determinants for their distinct substrate specificities.

Similar to what has been observed in mtNadM crystal structure, syNadM-Nudix was also copurified and cocryrstallized with the reaction products, NAD and pyrophosphate (Figure 2A). In comparison, no product was retained in the active site of the NMNATase domain of ftNadM-Nudix. Two regions of the NMNATase domain, residues 110–120 between strand β4 and β5 encompassing short helix α5 and residues 131–137 between strand β5 and helix α6, are highly flexible and adopt conformations that deviate from the ligand bound form of syNadM. In the structure of syNadM complexed with NAD, several residues from these
regions interact with the bound ligand directly and form part of the active site of the enzyme. Because of the lack of substrate or product complex structures for ftNadM, at this point it is difficult to rationalize the large difference in the catalytic efficiencies between the two enzymes from Synecocystis sp. and F. tularensis. These differences are manifested at both overall turnover rate and substrate affinity levels as reflected in the different $k_{cat}$ and $K_m$ values of the two enzymes. The observed catalytic efficiency of ftNadM is comparable to the efficiency of other housekeeping NaMN adenylyltransferases and, therefore, it is consistent with its proposed cellular function as a housekeeping enzyme in de novo NAD synthesis.

Structure of the ADPRase Domain

The ADPRase domain of bacterial NadM-Nudix adopts a fold that is unique for the Nudix protein superfamily. It contains a mixed five-strand β sheet (comprised of β10, β11, β6, β7, and β12) that is split in the middle between β6 and β7 with the insertion of two additional short strands, β8 and β9 (Figure 3A). Two α helices are packed on each side of the sheet. The substrate ADPR binds near the split of the central β sheet, between strands β8, β9, helix α9, and the C-terminal end of helix α8 that connects the two domains (Figure 3A). The Nudix signature motif, or “Nudix-box,” encompasses the structural elements [β9-loop-α9 and intimately interacts with the bound substrate.

While the overall structure of the ADPRase domain of NadM-Nudix is similar to the Nudix core domain of the monofunctional bacterial ADPRase exemplified by E. coli ADPRase (ecADPRase, Orf 209), with an average rmsd of 2.8 Å over 124 superimposed Cα atoms, NadM-ADPRase lacks the N-terminal three-strand β sheet domain that is conserved in the monofunctional bacterial ADPRases (Gabelli et al., 2002; Kang et al., 2003; Yoshida et al., 2004; Figure 5). Instead, the connector helix α8 of NadM-Nudix partially occupies the position corresponding to this β sheet domain. Because this β sheet domain of ecADPRase contributes to the dimerization of the enzyme through extensive domain swapping, lack of this domain in NadM-Nudix leads to a very different dimer interface (Figures 3B and 5). The primary contact between the two subunits of bacterial NadM-Nudix dimer is formed through their ADPRase domains, between the C-terminal end of connector helix α8 and the succeeding loop from each subunit (Figure 3B). This loop, connecting α8 and strand β6, protrudes into the substrate binding site of the second subunit of the dimer and specifically interacts with the bound substrate in the second active site (see below).

ADPR Binding

Surprisingly, syNadM-Nudix is copurified and cocrystallized with a bound ADPR substrate as revealed in the unambiguous electron density for ADPR (Figure 2B). Understandably, the enzyme might not be active during the purification process, since the required divalent metal ion was not present in buffers. However, it is not clear why ADPR is not hydrolyzed by the enzyme in the cell. The intrinsic high affinity of syNadM-Nudix for ADPR, the low ADPRase activity of the enzyme (Raffaelli, et al., 1999a), and the presence of a substantial ADPR pool in E. coli (data not shown) may contribute to the retention of the substrate in its active site.

The bound ADPR molecule is shaped like a saddle and interacts with protein residues extensively, especially through the terminal ribose and the adenine base (Figure 6A). Remarkably, every functional group of the terminal ribose is involved in specific hydrogen bond interactions with the enzyme (Figure 6A): the O4’ of the ribose interacts with the side chain of Arg280, O1’ with Arg277 and Glu326, O2’ with His 328, Arg 277, and Asp205, and O3’ with Asp 205. On the other end of the substrate, the adenine ring is tightly sandwiched between Phe234 and Tyr197B from the second monomer of the dimer. A specific hydrogen bond is formed between the side chain of Tyr188 and N7 of the adenine, while the N1 and N6 groups interact with the enzyme indirectly through two water molecules (data not shown). The direct interactions between the enzyme and the diphosphate moiety of ADPR are less extensive in this complex. The β phosphate interacts with Arg219 directly, and with Arg280 and Glu252 indirectly through two water molecules (Figure 6A). The adenylyl phosphate (α phosphate) is surrounded by several acidic residues from the Nudix-motif, including Glu248, Glu251, Glu252, as well as Arg 247. It is possible that some of the acidic glutamate side chains may be protonated. Specific hydrogen bonds are formed between α phosphate oxygens with main chain amide of Phe234 (also part of the Nudix motif), and with two water molecules that are coordinated by Arg 247, Glu248, and Glu 251 (Figure 6A). There are essentially no specific direct interactions between protein and the adenosyl ribose group. Hence, ADPR derivatives modified at this ribose, such as the NADP degradation product, 2’-phospho-ADPR, is also a good substrate for the enzyme (Raffaelli et al., 1999a).

The substrate binding mode of NadM-ADPRase turns out to be significantly different from that of ecADPRase, especially in the adenosine and terminal ribose portions of the substrate. (Figure 6B). When the active site residues of the two enzymes are superimposed, the bound ADPR substrates in the two
sets of protein residues (Figure 6B). In particular, residues from the swapped N-terminal three-strand β sheet domain of ecADPRase are involved in substrate binding, while this domain is absent in NadM-ADPRase and is partially replaced by a helical segment of the same subunit. Nevertheless, the protein residues surrounding the diphosphate moiety, mostly residues from the Nudix motif, remain conserved in sequence and structure, suggesting that these two enzymes still share substantial common features in catalytic mechanism (discussed subsequently here).

In contrast to ecADPRase, the substrate binding site of NadM-ADPRase exhibits significant similarity to human NUDT9 protein, a human Nudix enzyme highly specific for ADPR (Perraud et al., 2003; Shen et al., 2003). The structure of human NUDT9 in complex with product ribose 5-phosphate (R5P) is available (Shen et al., 2003). The position and orientation of the bound R5P in NUDT9 superimpose relatively well with the corresponding portion of the bound ADPR in syNadM-Nudix (Figure 6C). The protein conformations surrounding the substrate in the two structures are also very similar. Four out of five residues that specifically interact with the terminal ribose are conserved in both structures. These include Asp172, Arg204, Arg 273, and His324 of NUDT9, corresponding to Asp205, Arg219, Arg280, and His328 of syNadM-Nudix, respectively (Figure 6C). Only Arg277 of syNadM-Nudix is not conserved in NUDT9. Currently, no experimentally determined structure of NUDT9-substrate complex is available. Superposition of syNadM-ADPRase domain with NUDT9 brings the syNadM-Nudix bound ADPR into NUDT9 active site without any steric clashes, thus affording a model for substrate binding in NUDT9 (Figure 6C). In this model, the adenine ring would be sandwiched between Trp110 and Met216 of NUDT9, and the N6 group of adenine would interact with the side chain of Asn168. Remarkably, this model, obtained through superposition with NadM-ADPRase domain, appears to be very similar to a previously described model obtained by manual docking and energy minimization, particularly in the conformation of the adenine portion of the substrate (Shen et al., 2003). The described protein-substrate interactions in the previous model are essentially the same as those observed in the present work.

**Structure of ftNadM-Nudix Complexed with AMP and Metal Ions**

The difference anomalous electron density map calculated from a Mn²⁺-soaked ftNadM-Nudix crystal revealed unambiguously the positions of three Mn²⁺ ions (Figure 2C). They are coordinated directly to the Nudix motif residues Gly234, Glu250, and Glu254 (F. tularensis enzyme numbering), to Asp308 from the so-called “L9 loop” (Gabelli et al., 2001, 2002), and to the two oxygen atoms from AMP phosphate (Figure 7A). While Glu250 is ligated to both Mn1 and Mn2, Glu254 and Asp308 each also coordinates two metal ions, Mn2 and Mn3. A total of seven water molecules complete the rest of the coordination spheres for the three metal ions. Overall, all three metal ions have good octahedral coordination configurations. The metal coordinating water molecules are further hydrogen bonded to Glu238, Arg249, and Glu253 of the Nudix motif, to Asp307 of L9 loop, and to Arg221 from strand β7. In particular, one water molecule (w1), or, more likely, a hydroxide ion bridging Mn1 and Mn2, is further ligated to Asp307 and strategically located about 3.2 Å away from the 2 phosphate of the bound nucleotide, ideally suited for a nucleophilic attack on
The structure of NadM-Nudix should have a similar catalytic mechanism as that of ADPRase despite a very different substrate recognition mode. Here, Asp307 may perform a similar role as Glu162 in catalytic ADPRase. However, the L4 loop of ADPRase also contains an N-terminal three-strand β sheet domain that contributes to substrate binding and formation of a similar dimer to that of ecADPRase. In addition to the NadM-Nudix domain, ttADPRase also contains an N-terminal three-strand β sheet domain that contributes to substrate binding and formation of a similar dimer to that of ecADPRase. In contrast to that observed in NadM-ADPRase, the substrate ADPR binds to ttADPRase active site in a conformation that is similar to the ecADPRase bound substrate in the adenosine and terminal ribose moieties, but substantially different in the central diphosphate group. The metal ion positions in ttADPRase are also

The similar catalytic mechanism and different substrate recognition mode between NadM-ADPRase and ecADPRase forms an interesting contrast to that of ADPRase from *T. thermophilus* (ttADPRase) (Yoshida et al., 2004). ttADPRase is a member of bacterial monofunctional ADPRase subfamily, and its structure closely resembles ecADPRase. In addition to the Nudix core domain, ttADPRase also contains an N-terminal three-strand β sheet domain that contributes to substrate binding and formation of a similar dimer to that of ecADPRase. However, the L4 loop of ttADPRase, corresponding to the L9 loop of ecADPRase, adopts a very different conformation and does not undergo conformational changes upon metal/substrate binding. Although the two Glu residues at the tip of this loop in ecADPRase, Glu162 and Glu164, have been shown to be critical catalytic residues, with Glu164 coordinating the catalytic metal ions and Glu162 acting as a catalytic base (Gabelli et al., 2002). In human NUDT9, a double mutation of Asp304 and Asp305, corresponding to Asp307 and Asp308 of ttNadM-ADPRase, to alanines resulted in a reduction of catalytic efficiency to about 1/100 of the wild-type enzyme (Shen et al., 2003), suggesting that these two Asp residues are indeed important for catalysis, though they might not be absolutely indispensable for NUDT9.

**Proposed Catalytic Mechanisms for NadM-ADPRase**

Superposition of ttNadM-Nudix-Mn-AMP with syNadM-Nudix-ADPR complex structures revealed that the product AMP bound to ttNadM-Nudix overlaps closely with the adenosyl moiety of the bound substrate ADPR in syNadM-Nudix (Figure 7B). It is likely that metal ions in the substrate bound NadM-Nudix complex would occupy the same positions as in the product complex structure. More significantly, when superimposed with the structure of ecADPRase complexed with AMPCPR, a nonhydrolyzable ADPR analog, the three metal ions and the bridging water, w1, in ttNadM-Nudix overlay nearly perfectly with the corresponding atoms in the ecADPRase complex (Figure 6B). Most NadM-Nudix motif residues, G234, E238, R249, E250, E253, and E254 (F. tularensis enzyme numbering) also superimpose well between the two enzymes (Figure 6B). The other two catalytic residues, E162 and E164 from ecADPRase, are positioned differently from the corresponding residues, D307 and D308 of ttNadM-ADPRase, and yet they appear to perform similar roles in coordinating metal ions and the catalytic water molecule, w1. The similarity in the catalytic site configuration immediately suggests that ADPRase of NadM-Nudix should have a similar catalytic mechanism as that of ecADPRase despite a very different substrate recognition mode. Here, Asp307 may perform a similar role as Glu162 in ecADPRase (i.e., to function as a catalytic base activating the metal bound water [w1] for nucleophilic attack on the adenosyl phosphate.

**Figure 7. Metal Ion Binding in ADPRase Domain of NadM-Nudix**

(A) Stereo diagram of ttNadM-Nudix ADPRase domain active site with bound AMP and three Mn²⁺ ions. The carbons of protein residues are colored green (or cyan for Phe199 of the second monomer), AMP colored yellow, Mn²⁺ ions purple, and water molecules red. The thin solid lines represent metal coordination, while dashed lines represent hydrogen bonds.

(B) Superposition of the metal-free ADP-ribose bound syNadM-Nudix (green) with ttNadM-Nudix Mn-AMP complex showing the large conformational changes in the L9 loop region. The three Mn²⁺ ions are shown as small spheres. The side chains of the two catalytically important Asp residues at the tip of L9 loop are also shown.

Comparing the metal-free substrate complex of syNadM-Nudix with metal bound ttNadM-Nudix, the most notable structural difference is in the conformation of the L9 loop (residues 302–314, ttNadM-Nudix numbering), which contains two catalytically important Asp residues (Asp307 and Asp308) (Figure 7B). In the metal-free syNadM-Nudix structure, this loop is highly flexible and is disordered in two of the three monomers in the asymmetric unit. In the third monomer, this loop adopts a more open conformation compared to that in the metal bound ttNadM-Nudix structure (Figure 7B). It appears that metal binding causes the re-orientation and stabilization of this loop through the two Asp residues at the tip of the loop that are now intimately involved in metal binding and likely participate in catalysis. The metal and substrate binding-induced conformational change of L9 loop has also been observed in the structures of ADPRase from *E. coli* and *Mycobacterium tuberculosis*, but not in *Thermus thermophilus* ADPRase (Gabelli et al., 2002; Kang et al., 2003; Yosh-
drastically different from those in ecADPRase. All these structural observations support a different catalytic mechanism for ttADPRase (Yoshiba et al., 2004).

**DISCUSSION**

**Function of NadM-Nudix in Bacterial NAD Metabolism**

The present study fills in the last gap in the structural coverage of the divergent pyridine adenylyltransferase family involved in the last steps of NAD synthesis from its mononucleotide precursors, NaMN and/or NMN (Figure 1). Comparative genomic reconstruction of NAD metabolism in /C24 40 completely sequenced bacterial genomes integrated within the SEED database (Overbeek et al., 2005) ("NAD and NADP cofactor biosynthesis global" subsystem spreadsheet available at http://anno-3.nmpdr.org/anno/Fig/subsys.cgi) indicates that the major role of bacterial NadM homologs is likely in a two-step, nondeamidating pathway of recycling Nm released by a glycohydrolytic degradation of NAD or/and NADP (Figure 1B). This conjecture is supported by the following observations: (1) in contrast to the biosynthetic NaMNATase of NadD family conserved in the overwhelming majority of bacterial genomes, NadM homologs are present only in /C24 20 genomes of diverse bacteria (see Table S1); (2) in most of these genomes (with the exception of /F. tularensis/ and two species of /Deinococcus/), NadM is present in addition to (and not instead of) the housekeeping NadD; (3) in this subset of genomes (with the single exception of /Gloebacter violaceus/), NadM homologs are encoded within a conserved operon with genes encoding Nm phosphoribosyltransferase (NadV), suggesting their participation in a two-step conversion of Nm to NAD via NMN intermediate; (4) all bacterial NadM homologs (with the single exception of /Acinetobacter sp./) occur as fusion proteins with a Nudix domain that hydrolyzes ADPR, the second product of NAD glycohydrolytic cleavage reactions releasing Nm (Figure 1); (5) both products of ADPRase reaction, AMP and ribose-5-phosphate, can be further converted to ATP and phosphoribose pyrophosphate (PRPP), metabolites required for NAD synthesis as well as many other metabolic pathways; (6) a noted presence of both genes encoding NadV and NadM-Nudix in the genomes of several T4-like bacteriophages (Miller et al., 2003) alludes to the importance of NAD recycling for bacteriophage development and supports a likely role of horizontal gene transfer in the propagation of these genes in bacterial genomes (S. Gerdes, personal communication). More importantly, results from the gene inactivation experiments done with /Synechocystis sp. nadV-nadM (slr0788-slr0787)/ (Gerdes et al., 2006) are in full agreement with the above proposed role for bacterial NadM (Yoshida et al., 2004). It should be noted that, although the processes of NAD degradation that generate Nm and ADPR have been known for many years (Pollack et al., 2007), current knowledge on enzymes involved in these processes is rather sparse. Genes encoding such enzymes are missing in many bacterial species, including /Synechocystis sp./ and /F. tularensis/.

Although, in principle, a two-step conversion of Nm to NAD may contribute to both recycling of endogenous Nm and salvage of exogenous Nm, the latter pathway was ruled out by the experiments in the Synechocystis sp. model (Gerdes et al., 2006). So far, all known bacterial genomes harboring both NadM-Nudix...
and NadD contain a full complement of genes for de novo NAD biosynthesis from aspartate (as in some Burkholderiales and cyanobacteria) or from tryptophan (as in some Xanthomonadales), and some of them carry out additional nicotinate and Nm riboside salvage routes (see Table S1). This observation, together with other considerations listed above, argues in favor of the main role of Nadv- NadM-Nudix pathway in NAD recycling.

Reconstruction of NAD metabolism in F. tularensis reveals a remarkable example of adaptive evolution where the NMNATase activity of a peripheral recycling/salvage enzyme (NadM) was recruited to compensate for the lack of NadD in the main route of NAD cofactor biosynthesis. This adaptation included evolution of a novel enzymatic activity, NMN synthetase (EC 6.3.1.--)., which amidates NaMN to NMN prior to its adenylation by NadM (Figure 1B, and L.S. and A.L.O., unpublished data). This novel activity is performed by a divergent member of NadE family of NAD synthetases (EC 6.3.1.5), the role of which in most other species is to amitate NaAD intermediate generated by NaMNAzase.

Another special case is observed in Deinococcus geothermalis and D. radiodurans, the only genomes, other than F. tularensis, in which NadM-Nudix homologs have replaced NadD enzyme. Interestingly, each of these genomes contains two NadM-Nudix paralogs. The multiple sequence alignment analysis shows that one of these paralogs (e.g., DR2428 in D. radiodurans) is closely related to other bacterial enzymes of NadM-Nudix subfamily, whereas the second paralog (DRA0273) is substantially more divergent (Figure S1). Moreover, in the second paralog, the “pyridine recognition loop” region appears to be more similar to archael NadM and contains a one amino acid deletion compared to bacterial NadM (Figure S1). Therefore, DRA0273 may have a similar substrate preference to archael NadM and be able to accept a deamidated mononucleotide substrate. This speculation is additionally supported by the chromosomal clustering of DRA0273 (as well as its ortholog in D. geothermalis) with nicotinate phosphoribosyltransferase (EC 2.4.2.11), the enzyme converting nicotinic acid to NaMN in the regular salvage pathway. These observations, along with the apparent presence of a regular Nad synthetase (rather than NMN synthetase, as in F. tularensis) suggest that, in contrast to all other bacterial NadM-Nudix enzymes, DRA0273 may play a role of NMNATase in the conventional NaMN → NMN → NAD pathway associated with the salvage of nicotinic acid. The first paralog, DR2428, on the other hand, is likely involved in the nondeamidating recycling/salvage of Nm, together with NadV homolog (DR0294). The role of the respective NadM-Nudix paralog in D. geothermalis is less clear, as this genome does not contain NadV, whereas, unlike D. radiodurans, it contains an entire set of genes for the de novo NAD biosynthesis from aspartate (Table S1).

**Structure**

**Structure of Bifunctional NadM-Nudix**

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**Implications for Human NUDT9 and the ADPR-Gating Domain of TRPM2 Channel**

Structure and sequence comparison of the ADPRase domain of bacterial NadM-Nudix with other members of the Nadx superfamily showed that NadM-ADPRase is more closely related to archael ADPRase and human NUDT9 than to the monofunctional bacterial ADPRases (Figure 8). Given that the NMNATase domain of bacterial NadM-Nudix also has an archael origin, it is not surprising that its ADPRase domain should be of archael origin as well. Results from sequence alignment and structure superposi-

**EXPERIMENTAL PROCEDURES**

Expression and Purification of syNadM-Nudix and ftfNadM-Nudix

Expression vector containing open reading frame sin0787 encoding syNadM-Nudix was previously constructed by Raffaelli et al. (1999a). For structural analysis, the insert was subcloned into a pPROEX vector (Invitrogen, Carlsbad, CA) and transformed into E. coli strain BL21(DE3) for protein expression. The cells were induced by adding 0.8 mM IPTG when OD600 reached 0.6, and harvested after shaking at 20°C overnight. The clarified cell lysate was mixed with the appropriate amount of nickel-nitrilotriacetic acid (NTA)-agarose beads (QIAGEN, Valencia, CA) and incubated at 4°C for 1 hr. After extensive washing, the bound protein was eluted with 50 mM Tris, pH 8.0, 300 mM NaCl, and 250 mM imidazole. The protein was then treated with TEV protease at 4°C overnight to cleave the 6× His tag. The tag-free protein was further purified with a Mono Q ion exchange column (GE Healthcare Bio-Sciences, Piscataway, NJ) and eluted with a gradient of NaCl. Gel filtration column Superdex 75 (GE Healthcare Bio-Sciences, Piscataway, NJ) was used to further purify the protein. The final protein was concentrated in a Centricon-1000 centrifugal filter device (Millipore). The purity of the protein was confirmed by SDS-PAGE analysis.
Healthcare Bio-Sciences) was used as the last purification step with a buffer containing 20 mM Tris, pH 8.0, 150 mM NaCl, and 1 mM DTT. The selenomethionyl syNadM-Nudix was expressed in minimal medium supplemented with selenomethionine and other nutrients as described by Doublie (1997), and purified by the same procedure as the native protein.

The gene encoding ftNadM-Nudix was originally cloned into the vector pODC29 (L.S. and A.L.O., unpublished data). In order to increase the yield of protein for crystallographic analysis, the insert was subcloned into the vector pGST-parallel1 (Sheffield et al., 1999). Transformation and expression of ftNadM-Nudix followed the same procedure as syNadM-Nudix. Cell pellets were suspended in the phosphate-buffered saline (PBS) (Sigma, St. Louis, MO) and lysed by sonication. The clarified cell lysate was mixed with an appropriate amount of Glutathione Sepharose 4B beads (GE Healthcare Life Sciences) and incubated at 4°C for 1 hr. After washing thoroughly with PBS buffer, the protein-bound beads were treated with TEV protease at 4°C overnight, and the tag-cleaved protein was eluted with PBS buffer. The protein was then loaded on a Mono Q ion exchange column buffered with 50 mM Tris, pH 8.0, and eluted with a gradient of NaCl.

Enzyme Activity Assays
NaMNAT and NMNAT activities were measured with coupled spectrophotometric assays, as previously described (Kurnasov et al., 2002). The Nudix hydrolase activity was measured by an HPLC-based assay. The reaction, in a mixture of 50 mM HEPES, pH 8.2, 5 mM MgCl₂, 10–40 ng recombinant NadM in 100 μl volume, was initiated by addition of ADPR to 0.01–2.0 mM range and incubated for 15 min at 37°C. Other biochemicals that were tested as potential substrates at 1 mM are: NAD+, NAD, NADH, NADP+, NADPH, ADPR, cyclic-ADPR (cADPR), ADP-glucose, diadenosine diphosphate (Ap₂A), ATP, ADP, and dNTPs.

Due to a substantial metal-ion-catalyzed decomposition of nucleoside diphosphate sugars (Nunez and Barker, 1976), measurements of AMP product were adjusted by parallel processing of controls without the enzyme. When testing reduced pyridine nucleotides as the substrates, reactions were stopped by boiling for 3 min. For the other nucleoside diphosphate derivatives, reactions were terminated and products analyzed by HPLC using a Shimadzu Prominence HPLC system on a Shimadzu C18 column (4.6 x 50 mm, 5 μm pore size). The elution conditions were 2 min in 95% buffer A (8 mM tetrabutylammonium bromide in 0.1 M potassium phosphate, pH 6.0), 3 min in up to 100% buffer B

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Note: a Rsym = Σhkli/Σhkli - < I >/Σhkli, Bijvoet pairs were treated as equivalent reflections for calculating Rsym.

b Rwork = Σhkli(Fo - Fc)/Σhkli(Fo), where Fo and Fc are the observed and calculated structure factors, respectively.

c Five percent randomly selected reflections were excluded from refinement and used in the calculation of Rfree.
buffer B (30% methanol), and holding in 100% buffer B for 2.5 min. After returning the gradient to 100% buffer A in 0.5 min, the column was flushed with buffer A for 1.5 min prior to the next run. K_m and V_max values were calculated by fitting initial rates to the integrated Michaelis-Menten equation with Prism 4 software (GraphPad).

**Structure of Bifunctional NadM-Nudix**

Crystallization and Data Collection

Crystallization of syNadM-Nudix was performed by the hanging drop vapor diffusion method; 1.5 μl of syNadM-Nudix protein (15 mg/ml) was mixed with 1.5 μl of reservoir solution containing 100 mM Tris, pH 7.5, and 1.5 M LiSO_4, and equilibrated against the reservoir at 20 °C. Single crystals usually appeared within 3 days and grew to a final size of about 0.5 x 0.3 x 0.3 mm³ within 2 weeks.

FtNadM-Nudix crystals were grown at 20 °C by the sitting drop vapor diffusion method; 1 μl of FtNadM-Nudix protein (15 mg/ml) was mixed with an equal volume of the reservoir solution containing 0.1 M Tris, pH 7.5, 200 mM MgCl_2, and 19% PEG3350, and equilibrated against the reservoir. To obtain the FtNadM-Nudix-AMP complex crystals, FtNadM-Nudix was mixed with 30 mM (final concentration) nucleotide before crystallization.

For data collection, crystals were transferred stepwise to a cryoprotection solution containing the original reservoir solution and an additional 30% glycerol. For Mn_2+-soaked FtNadM-Nudix-AMP complex crystal, cryoprotection solution included 200 mM MnCl_2 instead of 200 mM MgCl_2. Native and SeMet MAD data sets from syNadM-Nudix crystals were collected at beamline 19BM of Advance Photon Source, Argonne National Laboratory (Argonne, IL). FtNadM-Nudix data sets were collected in-house on a RAXIX-ISF™ image plate detector equipped with a Rigaku FR-E SuperBright X-ray generator and Varimax HF mirrors.

Diffraction data were processed with DENZO and SCALEPACK (Otwinowski and Minor, 1997). The syNadM-Nudix crystals belong to space group P2_1_2_1 with unit cell parameters a = b = 201.5 Å, c = 85.7 Å, V = 125.6 Å³. The statistics of all data sets are listed in Table 1.

Phasing and Refinement

The initial phases of syNadM-Nudix structure were determined by the MAD phasing method with data collected at three different wavelengths from a SeMet syNadM-Nudix crystal. The Se site determination, phasing, and density modification were performed with the program SHELX/C/E/D (Schneider and Sheldrick, 2002; Sheldrick, 2002) modules in the CCP4 package (CCP4, 1994), which resulted in a clearly interpretable electron density map. The model of syNadM-Nudix was built with Arp/warp (Perakhis et al., 1999, 1997) and COOT (Emsley and Cowtan, 2004). Refinement of the model was performed with simulated annealing refinement protocol in the PHENIX suite (Adams et al., 2000) and Refmac (Murshudov et al., 1997) monitoring R_mol and R_free. The final model includes three syNadM-Nudix monomers, each containing 344 residues, one NAD molecule, and one ADPR molecule. A total of 313 water molecules are also included in the model.

The structure of ftNadM-Nudix was determined by the molecular replacement method with the program Phaser (McCoy et al., 2005), with syNadM-Nudix monomer as the searching model. Model building and refinement was done following procedures similar to that for syNadM-Nudix. The final model for ftNadM-Nudix contains two protein monomers, each associated with three Mg ions. The ftNadM-Nudix-AMP complex model also contains two AMP molecules in addition to the metal ions.

Supplemental Data

Supplemental Data include two additional figures and one table and are available online at http://www.structure.org/cgi/content/full/16/2/196/DC1/.

**ACKNOWLEDGMENTS**

We thank Darek Martynowski for help with crystallography experiments, and Jimin Pei and Lisa Kinch for help with protein sequence analysis. F. tularensis subsp. Holarctica LVS genomic DNA was a kind gift from Karl E. Klose (University of Texas, San Antonio). This work was supported by National Institutes of Health grants GM58243 to H.Z and AI059146 to A.O., and Welch Foundation grant 1-F505 (to N.V.G.). Use of Argonne National Laboratory Structure Biology Center beamline at Advanced Photon Source was supported by the U.S. Department of Energy, Office of Biological and Environmental Research, under contract W-31-109-ENG-38.

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Structure of Bifunctional NadM-Nudix


Accession Numbers

The coordinates and structure factors for ftNadM-Nudix and syNadM-Nudix have been deposited in the Protein Data Bank with accession codes 2QJO for syNadM-Nudix complex, 2R5W for ftNadM-Nudix-Mg complex, and 2QJT for ftNadM-Nudix-AMP-Mn$^{2+}$ complex.


