A Novel Fold Revealed by \textit{Mycobacterium tuberculosis} NAD Kinase, a Key Allosteric Enzyme in NADP Biosynthesis*

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NAD kinase catalyzes the magnesium-dependent phosphorylation of NAD, representing the sole source of freshly synthesized NADP in all organisms. The enzyme is essential for the growth of the deadly multidrug-resistant pathogen \textit{Mycobacterium tuberculosis} and is an attractive target for novel antitubercular agents. The crystal structure of NAD kinase has been solved by multiwavelength anomalous dispersion at a resolution of 2.3 Å in its T state. Two crystal forms have been obtained revealing either a dimer or a tetramer. The enzyme architecture discloses a novel molecular arrangement, with each subunit consisting of an αβ N-terminal domain and a C-terminal 12-stranded β sandwich domain, connected by swapped β strands. The C-terminal domain shows a striking internal approximate 222 symmetry and an unprecedented topology, revealing a novel fold within the family of all β structures. The catalytic site is located in the long crevice that defines the interface between the domains. The conserved GGDG structural fingerprint of the catalytic site is reminiscent of the related region in 6-phosphofructokinase, supporting the hypothesis that NAD kinase belongs to a newly reported superfamily of kinases.

NAD(P) is today fully recognized as a vital dual function cofactor that is intimately involved in both energy and signal transduction (1–3). The phosphorylated form of the cofactor NAD is a key molecule in most reductive biosynthetic reactions and is an important constituent of cellular defense mechanisms against oxidative stress (4–6). The pathways resulting in NADP biosynthesis have been extensively studied in both prokaryotes (7, 8) and eukaryotes (8, 9). In all organisms, de novo NADP synthesis occurs exclusively via NAD phosphorylation through a reaction catalyzed by NAD kinase (EC 2.7.1.23). The enzyme phosphorylates NAD, yielding NADP in the presence of ATP in a magnesium-dependent reaction; and this being the only biochemical event leading to de novo synthesis of NADP, NAD kinase is of crucial importance for NADP-dependent anabolic/biosynthetic pathways in the cell. The enzyme was indeed recently reported to be essential in \textit{Bacillus subtilis} (10) and \textit{Mycobacterium tuberculosis} (11), confirming the early proposal of its relevance as a novel antibacterial drug target (12). Although human NAD kinase has been observed to be strictly ATP-dependent (13), the \textit{M. tuberculosis} enzyme has also been shown to use inorganic polyphosphate (poly(P)) as the phosphate donor (14). NAD kinase from both \textit{Bacillus licheniformis} (15) and \textit{B. subtilis} (16) were reported to have a marked positive cooperativity toward the substrate ATP and to be strongly inhibited by the product NADP. On the other hand, the human enzyme was shown to follow perfect hyperbolic kinetics (13). We recently published an extensive biochemical and enzymatic characterization of \textit{M. tuberculosis} NAD kinase, observing both a strong cooperativity for all substrates (Hill coefficients of 1.2, 1.5, and 1.4 for NAD, ATP, and poly(P), respectively) and a remarkable inhibition exerted by the product NADP (14). These findings led to the proposal of a relevant role for NAD kinase in the regulation of the NADP metabolic flux in \textit{M. tuberculosis} (14), as earlier hypothesized for the \textit{B. subtilis} enzyme (16). Based on the conservation of a common signature, NAD kinase was recently proposed to be a member of a new superfamily of kinases, including 6-phosphofructokinases (PFKs), diacylglyceride kinases, and sphingosine kinases (17). These enzymes were suggested to adopt a common fold and to have a similar phosphate-donor binding site (17). Indeed, the strictly conserved GGDG sequence fingerprint has been demonstrated to be essential for the involvement of catalysis in ATP binding (17). A second highly conserved glycine-rich region, a peculiar feature of NAD kinases, was recently identified (14). Site-directed mutagenesis of this region in \textit{M. tuberculosis} NAD kinase demonstrated its essentiality in catalysis and pointed to its involvement in NAD binding (14). Therefore, a robust picture of the catalytic mechanism carried out by the \textit{M. tuberculosis} enzyme is available. Within the framework of our structural studies on NAD biosynthetic enzymes (18), we report here the crystal structure of \textit{M. tuberculosis} NAD kinase in its inactive T state, which represents the first structure of a NAD kinase ever reported. The structure of each subunit consists of an αβ N-terminal domain and an antiparallel 12-stranded β sandwich C-terminal domain, with the catalytic site located in the long crevice that defines the interface between the domains. Being an essential enzyme in \textit{M. tuberculosis} and showing significant functional differences with its human counterpart, NAD kinase can be regarded as a

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‡ The abbreviations used are: PFK, 6-phosphofructokinase; MES, 4-morpholineethanesulfonic acid; r.m.s.d., root mean square deviation; FHA, fork-head-associated.
promising target for the treatment of tuberculosis, a re-emerging worldwide leading cause of morbidity and mortality, which has a devastating impact in developing countries (19).

**EXPERIMENTAL PROCEDURES**

**Crystalization and Data Collection**—The recombinant protein used in the crystallization experiments was purified from *Escherichia coli* described previously (14). Two different crystal forms were obtained (Table I). Crystals of form I were grown by the hanging drop vapor-diffusion method by mixing 2 μl of a protein solution at 8 mg/ml with an equal volume of a reservoir solution containing 1 M Li2SO4, 0.5 M (NH4)2SO4, 0.1 M sodium citrate, pH 5.6, and equilibrating against 1000 l of the reservoir solution. Crystals grew to a maximum dimension of 0.1 × 0.1 × 0.2 mm at 20 °C in about 7 days. For data collection, the crystals were flash-cooled at 100 K under a stream of liquid nitrogen. Prior to flash cooling, the crystals were stepwise transferred from the crystallization solution to the cryoprotectant solution, which had the same composition as the reservoir solution, with the addition of 20% glycerol as the cryoprotectant. A complete 2.8-Å resolution data set was collected on a native crystal using synchrotron radiation at the ID14 EH4 beam line, European Synchrotron Radiation Facility, Grenoble, France. The analysis of the collected diffraction data sets allowed us to assign these crystals to the orthorhombic space group P21212 with cell dimensions a = 64.0 Å, b = 68.0 Å, and c = 145.0 Å, containing two molecules per asymmetric unit with a corresponding solvent content of 72%. For all data collections, diffraction intensities were evaluated and integrated using the program MOSFLM (21), whereas the CCP4 suite of programs (22) was used for data reduction. Table I gives a summary of all data collections.

| TABLE I Data collection, phasing, and refinement statistics |
|------------------------------------------------------------|
| **Native** | **MAD** |
| P2₁2₁2 | P2₁2₁2 | Peak | Inflection | Remote |
| Wavelength (Å) | 0.979 | 0.979 | 0.9793 | 0.9795 | 0.9392 |
| Max resolution (Å) | 245,485 | 246,875 | 182,388 |
| Observation | 195,235 | 225,138 |
| Unique reflections | 29,110 | 44,644 |
| Rmerge (%) | 4.3 | 4.2 |
| Multiplicity | 10.0 | 12.3 |
| Completeness (%) | 98.3 | 99.8 |
| Anomalous completeness (%) | 98.6 | 98.6 |
| Phasing power (centric/acentric) | 0.28/0.320 | 0.28/0.341 |
| FOM | 0.14 | 0.14 |

FOM, figure of merit.

The structure of crystal form I was solved by using the three-wavelength anomalous diffraction data and the SHELXD program (23). SHELXD identified ten selenium sites, which were refined using the program SHARP (24) giving the phasing statistics reported in Table I. Inspection of the electron density maps unambiguously indicated P4₂₁2 as the correct space group. The initial electron density map was of good quality despite the limited resolution and allowed us to trace about 80% of the tetramer present in the asymmetric unit. Model building was carried out with the program O (25) and was guided by the position of the selenium sites, which were of great help for a correct sequence assignment. The crystallographic refinement was carried out at 2.8 Å by using the program REFMAC (26). A random sample containing 2% of the total reflections (900 reflections) was excluded from the refinement and used for the calculation of the free R-factor (27). Tight noncrystallographic symmetry restraints were maintained for the C-terminal domain throughout all the different stages of refinement. The initial model was subjected to iterative cycles of crystallographic refinement alternated with graphic sessions for model building using the program O (25). Solvent molecules were manually added at positions with density >4σ in the Fo – Fc map, considering only peaks engaged in at least one hydrogen bond with a protein atom or a solvent atom. The procedure converged to an R-factor and free R-factor of 22.8 and 28.9%, respectively. The current model contains 1125 amino acids and 303 solvent molecules. No or very poor electron density was visible between residues 1A–3A, 14A–17A, 44A–60A, 306–307A, 1B–6B, 12B–17B, 33B–65B, 306–307B, 1C–2C, 47C–63C, 306C–307C, 1D, 15D–17D, and 306D–307D, which were therefore not included in the model. The average B-factors for the 8488 protein atoms and for the 303 solvent molecules were 62.5 and 63.4 Å² (60.4 and 63.4 Å² for the main chain and side chain, respectively).

The structure of the most complete monomer of crystal form I was mixed with an equal volume of the reservoir solution containing 1.8% polyethylene glycol 4000 and 0.1 M MES, and the drop equilibrated against 1000 μl of the reservoir solution. For data collection, these crystals were quickly washed in a solution containing the crystallization buffer and 35% glycerol as the cryoprotectant and flash-frozen under a stream of liquid nitrogen. A complete data set was collected at a temperature of 100 K up to 2.3 Å resolution using synchrotron radiation at the ID14 EH4 beam line, European Synchrotron Radiation Facility, Grenoble, France. The analysis of the collected diffraction data sets allowed us to assign these crystals to the orthorhombic space group P2₁2₁2, with cell dimensions a = 64.0 Å, b = 68.0 Å, and c = 145.0 Å, containing two molecules per asymmetric unit with a corresponding solvent content of 72%. For all data collections, diffraction intensities were evaluated and integrated using the program MOSFLM (21), whereas the CCP4 suite of programs (22) was used for data reduction. Table I gives a summary of all data collections.
Crystal Structure of M. tuberculosis NAD Kinase

FIG. 1. Stereo ribbon representation of the NAD kinase subunit, oriented with the N- and C-terminal domains in the upper and lower part of the molecule, respectively. The crevice hosting the enzyme active site can be seen running horizontally between the two domains. Nt, N terminus; Ct, C terminus.

FIG. 2. Stereo ribbon representation of the C-terminal domain of M. tuberculosis NAD kinase. Each of the four structural repeats of the three-stranded unit, related by internal 222 symmetry, is colored differently.

RESULTS AND DISCUSSION

Overall Quality of the Model—Two different crystal forms were obtained. Crystals of form I diffract to 2.8 Å and belong to the tetragonal space group P4_3212 with four molecules in the asymmetric unit; crystals of form II diffract to 2.3 Å and belong to the orthorhombic space group P2_1212 with two molecules in the asymmetric unit (see “Experimental Procedures”). The tetragonal crystal form was employed for structure determination by means of the multiple wavelength anomalous dispersion method, using the anomalous signal of selenium. A monomer of the tetragonal crystal form was then used for structure determination of the orthorhombic crystal through molecular replacement. The stereochemistry of the refined models have been assessed with the program PROCHECK (30). Crystal form I shows 84% of the residues in the most favored regions of the Ramachandran plot, and no outliers are present. Crystal form II shows 90% of the residues in the most favored regions of the Ramachandran plot and no outliers.

Overall Structure—M. tuberculosis NAD kinase folds into 7 α-helices, 18 β strands, and the connecting loops organized in two domains: an N- and a C-terminal domain (Fig. 1). The N-terminal domain (residues 1–137 and 279–284) consists of a classical α/β dinucleotide binding domain (31), in which the core is a twisted six-stranded parallel open β-sheet flanked on both sides by α-helices. Interestingly, the sixth parallel β strand is contributed by strand 18 of the C-terminal portion of the protein, revealing a swapping of secondary structure elements between the two domains (Fig. 1). The N-terminal domain also contains the N-terminal portion of the long β6 strand, which runs antiparallel to β18 and extends, with its C-terminal half, into the C-terminal domain, completing the observed swapping of secondary structure elements between the two domains (Fig. 1). Remarkably, the domain connection is therefore based upon a motif made of parts of the antiparallel β6 and β18 strands, the two swapped secondary structural elements. The C-terminal domain (residues 140–277) consists of 12 β strands and 1 α-helix arranged in an antiparallel 12-stranded β sandwich, with a short α-helical insertion (helix α6) between strands β11 and β12 (Fig. 2). The two β-sheets of the C-terminal domain β sandwich can be described as consisting of four structural repeats of a three-stranded unit, which...
appear to be related by an internal ~222 symmetry (Fig. 2). Such a striking feature suggests that the C-terminal domain has probably resulted from duplication of this simple tetramerization unit. The molecular architecture of *M. tuberculosis* NAD kinase is completed by a C-terminal tail (residues 285–307) containing helix α7 and a long C-terminal extension located on the external face of the protein, roughly half-way between the two domains (Fig. 1).

**Quaternary Structure**—Gel filtration experiments showed that *M. tuberculosis* NAD kinase exists in solution either as a tetramer (32) or a dimer (14). Structural analysis revealed the presence of a tetramer and a dimer in the asymmetric unit of crystal form I and II, respectively. Within the tetramer, the four subunits associate with 222 symmetry (Fig. 3a). Although the C-terminal domains strictly follow the 222 symmetry, the N-terminal domains deviate from perfect symmetry showing a variation in the orientation of up to a 7° rotation, as calculated by the program DynDom (33) for the N-terminal domains of subunits C and D. This hinge-bending rotation has its pivotal point in the linker region between the N- and C-terminal domains. The inter-subunit contacts are entirely contributed by residues of the C-terminal domains, and no contacts involving residues of the N-terminal domains are present. Two major inter-subunit interfaces can be distinguished: one between the A-B subunits and the other between the A-D subunits (Fig. 3a). 1050 Å² of the accessible surface area are buried in each monomer upon formation of the AB dimer. Several intermolecular contacts, including 57 polar interactions, occur across the dyad axis relating the A and B monomers. The second and most prominent subunit interface is established between monomers A and D, with 1800 Å² of the accessible surface area buried in each monomer upon dimer formation. In this case, 68 polar interactions and many hydrophobic contacts participate in dimer stabilization. In particular, the regions encompassing residues 207–212 and 231–236 (β13) of one monomer meet their equivalent in the facing subunit. The resulting arrange-
ment creates a parallel β-sheet at the A-D interface (Fig. 3b) characterized by a high number of hydrogen bonds involving backbone atoms. The second remarkable structural trait of the A-D interface is represented by the hooking of the C-terminal tail of one monomer onto a structural motif made by the anti-A-D interface is represented by the hooking of the C-terminal backbone atoms. The second remarkable structural trait of the A-D interface is represented by the hooking of the C-terminal tail of one monomer onto a structural motif made by the antiparallel β strands β9 and β10 of the other, tightly anchoring the two monomers to each other (Fig. 3b).

The asymmetric unit of orthorhombic crystal form contains a dimer with subunits related by a local dyad, where χ 1980 Å 2 of the accessible surface area for each subunit is buried upon dimer formation. The dimer observed in the orthorhombic crystal form corresponds to the AD dimer of the tetramer described above, with a remarkable conservation of all the contacts established between subunits. Such an observation suggests that the dimer is the minimal functional unit for the allosteric NAD kinase, in agreement with the studies carried out in solution (14).

Similarity with Other Structures—A structural similarity search using the program DALI (34) has been performed for the whole enzyme as well as for the two separated domains. No structural similarity encompassing the whole enzyme could be identified when the entire monomer was employed. Not surprisingly, however, when the isolated α/β N-terminal domain was subjected to a DALI search, a number of Protein Data Bank entries showing high structural similarity were detected. The best score (Z value of 8.5) was produced by E. coli MurG, a membrane-associated glycotransferase (Protein Data Bank code 1fkP) (35), which shares 18% sequence identity and can be superimposed onto the N-terminal domain of M. tuberculosis NAD kinase, with an r.m.s.d. of 3.1 Å for 113 C-α pairs. NAD kinase was recently proposed to belong to a new superfamily of kinases, which includes 6-PFKs, diacylglyceride kinases, and sphingosine kinases (17). The members of this new superfamily share a conserved phosphate-binding GGDG(T/S) sequence fingerprint and were proposed to adopt a common structure based on the PFK fold (17). Although the DALI search did not identify any PFK structures in the top list, we performed a manual superimposition and discovered that the N-terminal domains of M. tuberculosis NAD kinase and members of the PFK family adopt a common fold. In fact, the N-terminal domains of M. tuberculosis NAD kinase and E. coli PFK, in its unliganded form (Protein Data Bank code 2fkP) (36) cannot be superimposed with a r.m.s.d. of 2.0 Å for 72 C-α pairs, leading to a remarkable structural similarity of the GGDG phosphate-binding motif. Therefore, our observation confirms the earlier hypothesis that PFK and NAD kinases are members of a new family of kinases (17). On the other hand, when only the M. tuberculosis NAD kinase C-terminal domain was subject to a structural similarity search, fewer Protein Data Bank entries were detected, and all showed limited structural similarity, as judged by the low Z score and poor r.m.s.d. parameters. The highest score (Z value of 4.6) was produced with the human Ki67 fork-head-associated (FHA) domain (Protein Data Bank code 1r21) (37). The 100-residue human Ki67 shares 11% sequence identity with the C-terminal domain of M. tuberculosis NAD kinase and can be superimposed onto this domain with a r.m.s.d. of 2.9 Å for 76 C-α pairs. The FHA domains are small signaling protein modules found in both prokaryotes and eukaryotes that act by binding to phosphoserine/threonine epitopes, mediating the assembly of protein complexes (38). Although the packing of a set of secondary structure elements is similar in the two structures and led DALI to detect a reasonable degree of structural similarity, the topology shown by the C-terminal domain of NAD kinase is very different from that of the FHA domains. In fact, the 11-stranded β sandwich featuring the FHA fold is built with strands 1, 2, 7, 8, 10, and 11 forming one β-sheet and strands 3, 4, 5, 6, and 9 forming the second, where the strands 3 and 4 are parallel (39). On the other hand, the M. tuberculosis NAD kinase C-terminal domain folds into an all antiparallel 12-stranded β sandwich with strands 1, 2, 4, 5, 9, and 12 forming one β-sheet and strands 3, 6, 7, 8, 10, and 11 building the second. Moreover, to the best of our knowledge, the observed topology is distinct from those previously reported in any protein within the family of all-β structures (40). Therefore, M. tuberculosis NAD kinase has revealed an unprecedented molecular arrangement for its two domains and a novel fold for its C-terminal domain.

Although topologically unrelated, the structural arrangement of the loops connecting the β strands in M. tuberculosis NAD kinase C-terminal domain is reminiscent of those seen in the loops responsible for the phosphopeptide binding in FHA domains (39) and in the loops presented to target antibodies on antibody VH and VL chains (41). Human and plant NAD kinases have been reported to bind calmodulin (42, 43), although the molecular details of the interaction are unknown. Moreover, a number of calmodulin-like proteins have been identified in bacteria (44), and plant NAD kinase has been shown to be activated by a bacterial calmodulin-like protein from sporulating cells of B. subtilis (45). We are therefore tempted to speculate on a possible role of the NAD kinase C-terminal domain in binding either a M. tuberculosis calmodulin-like protein or an, as yet, unidentified protein partner.

The Catalytic Site and Implications for Catalysis—The reaction catalyzed by M. tuberculosis NAD kinase proceeds through the transfer of a phosphate group from either ATP or inorganic poly(P) to the 2′ position of NAD, yielding NADP in a magnesium-dependent reaction (14). An exhaustive biochemical investigation on M. tuberculosis NAD kinase has recently been pub-
lished, revealing a marked allosteric behavior with Hill coefficients of 1.2 and 1.5 for NAD and ATP, respectively (14). We cannot presently obtain diffracting crystals of the enzyme complexed with any of its substrates. Repeated attempts at crystal soaking and co-crystallization with ATP, inorganic pyrophosphate (PPi), NAD, and NADP invariably yielded either no crystals or crystals of the inactive T form. We infer that our failure in obtaining crystals of the enzyme in the R form is probably because of conformational changes accompanying the T→R transition, resulting in an active R state incompatible with efficient crystal packing.

Inspection of the structure revealed the presence of a long crevice housing the enzyme active site on each monomer, located at the domain interface (Figs. 1 and 4). The upper and lower parts of the crevice are delimited by the N- and C-terminal domains respectively, whereas its bottom is defined by the structural motif linking the two domains (Figs. 1 and 4). Each active site does not reveal any direct connection with any of the others, and the residues surrounding each active site are entirely provided by the same subunit (Fig. 3, a and b). The highly conserved GGDG sequence motif (residues 83–86 in the M. tuberculosis enzyme) has been demonstrated by site-directed mutagenesis to be essential for catalysis in M. tuberculosis NAD kinase (17). This signature was proposed to define a common phosphatase donor binding site in all members of a new superfamily of kinases (17) in which the prototype is represented by PFK. Indeed, three-dimensional structural analysis revealed that this signature is part of the ATP binding site in PFK (46), and its involvement in ATP binding has recently been assessed also in diacylglycerol and sphingosine kinases (47, 48), two other members of the superfamily (17). By inspection of the N-terminal domain structure, we identified the GGDG motif in the lower part of the crevice hosting the active site (Fig. 4). In particular, the GGDG residues are part of the “P-loop” (Fig. 4), a peculiar region for nucleotide binding in α/β dinucleotide binding domains (49, 50). Because of the observed structural similarity of the M. tuberculosis NAD kinase N-terminal domain with the PFK nucleotide binding domain, we modeled an ADP molecule into the enzyme active site by optimal structural superimposition with the liganded form of the enzyme (Fig. 4). The result of this structure demonstrates the T state of the enzyme, we are not in a position to precisely describe either the ADP binding mode or the exact extent of its interactions with the surrounding residues. However, the modeling procedure demonstrates the involvement of M. tuberculosis NAD kinase 60GGDG residues in ATP binding.

A second highly conserved region spanning a 22-residue glycine-rich sequence (residues 189–210 in M. tuberculosis NAD kinase) was identified in NAD kinases, and its role in catalysis was investigated through alanine scanning mutagenesis in the mycobacterial enzyme (14). Overall, 11 strictly conserved residues within the region were mutated, with eight mutations (G190A, L192A, P196A, T197A, G198A, T200A, and G207A) resulting in a complete loss of enzymatic activity (14). On the other hand, the mutants T195A, S199A, and G208A resulted in a less active enzyme showing strongly altered kinetic parameters only for the substrate NAD (14). All of the mutated residues belong to the C-terminal domain and are located in the upper part of the long crevice hosting the catalytic site at the domain interface (Fig. 4). Although the present structure does not allow precise assignment of the roles of pure NAD or NAD and ATP binding in catalysis, we noticed that some of the mutation, yielding a completely inactive enzyme, are located in key points for the structural integrity of the C-terminal domain. In particular, the mutations G190A, L192A, and P196A, which are located in the core of the β11 strand, are likely to perturb the structure of this domain (Fig. 4). On the contrary, the other mutated residues within this conserved region are located on the exterior of helix α6 and the following loop and do not appear to have any structural role (Fig. 4). We therefore suggest, for this set of mutants, either a direct involvement in NAD binding or a role in the still unknown, molecular mechanism for the observed cooperativity, in agreement with the reported kinetic behavior of mutants T195A, S199A, and G208A (14).

A striking difference between M. tuberculosis and human NAD kinase is that only the mycobacterial enzyme shows allosteric behavior (13, 14). We are aware that a major concern for the design of antitubercular agents targeting NAD kinase is represented by the issue of selectivity. In this respect, we consider the structure of M. tuberculosis NAD kinase in its T state a valuable target for the rational design of inhibitors that will lock the enzyme in its inactive state and that have the potential to be highly selective for the allosteric mycobacterial NAD kinase.

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