Characterization of *Mycobacterium tuberculosis* NAD Kinase: Functional Analysis of the Full-Length Enzyme by Site-Directed Mutagenesis[†]

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ABSTRACT: NAD kinase is the only known enzyme catalyzing the formation of NADP, a coenzyme implicated in most reductive biosynthetic reactions and in many antioxidant defense systems. Despite its importance, nothing is known regarding its structure or mechanism of catalysis. *Mycobacterium tuberculosis* NAD kinase has been overexpressed in *Escherichia coli* and purified to homogeneity. The molecular and kinetic properties of the enzyme resulted in significant differences from those reported by others on a proteolytically degraded form of the protein. Indeed the full-length enzyme displays an allosteric behavior and shows a strict preference for inorganic polyphosphate as the phosphate donor. It is inhibited by the reaction product NADP and by both NADH and NADPH. The mycobacterial enzyme shares with all other known NAD kinases a highly conserved region (spanning residues 189−210), particularly rich in glycines, which differs from the primary sequences of all previously identified nucleotide-binding sites. Alanine-scanning mutagenesis performed on 11 conserved residues within this domain revealed its importance in catalysis. A total of 6 of 11 mutated proteins completely lost the enzymatic activity while retaining the same oligomeric state of the wild-type protein, as demonstrated by gel-filtration analysis. Substitutions of S199 and G208 with alanine rendered enzyme versions with reduced activity. Their kinetic characterization, performed on purified proteins, revealed kinetic parameters toward ATP and polyphosphate similar to those of the wild-type enzyme. On the contrary, when the kinetic analysis was performed by using NAD as the variable substrate, significant differences were observed with respect to both the allosteric behavior and the catalytic efficiency, suggesting that the mutated region is likely involved in NAD binding.

In all organisms, the only known reaction leading to NADP synthesis is catalyzed by the enzyme NAD kinase (EC 2.7.1.23), which phosphorylates NAD at the 2′ position of the ribose linked to the adenine moiety, in the presence of ATP. The importance of this enzyme is related to the crucial role played by NADP as a coenzyme in most reductive biosynthetic reactions and in many antioxidant defense systems (*1*). In eubacteria, the concentration of intracellular NADP is tightly regulated and its level is guaranteed even when NAD is lowered upon stringent conditions, like niacin starvation (*2*). Several evidences indicate that NAD kinase plays a key role in the control of NADP metabolic turnover. For example, both *Bacillus licheniformis* and *Bacillus subtilis* NAD kinases show a marked positive cooperativity toward the substrate ATP, and both are inhibited by the product NADP (*3*, *4*); in addition, quinolinate, a central metabolite in NADP biosynthesis, has been demonstrated to be a strong allosteric activator of the *B. subtilis* enzyme (*4*). Likewise, NADH and NADPH are potent allosteric negative modulators of *Escherichia coli* NAD kinase (*5*, *6*), and both *Mycobacterium tuberculosis* and *Micrococcus flavus* NAD kinases are inhibited by NADP and NADPH (*7*). The essentiality of the enzyme has been demonstrated in several microorganisms, including *B. subtilis*, *M. tuberculosis*, and *E. coli*, supporting the proposal that NAD kinase might represent an interesting novel antimicrobial drug target (*8*−*10*). This is reinforced by the observation that, despite the protein being highly conserved in all organisms, significant functional differences exist between the eubacterial and human enzymes. In particular, a peculiar feature of NAD kinases from *M. tuberculosis*, *M. flavus*, and *B. subtilis* is their ability to phosphorylate NAD by utilizing both ATP and the polymer poly(P)[1] as the phosphate donors (*4*, *7*), whereas the eukaryotic enzymes so far characterized are strictly nucleoside triphosphate-dependent (*11*, *12*). This distinctive property could be exploited in the designing of effective enzyme inhibitors as selective drugs. Drug design requires a profound knowledge of both the enzyme structure and catalytic properties. However, the only study on the molecular and kinetic properties of *M. tuberculosis* NAD kinase has been performed on a truncated form of the recombinant protein (*7*).

Recently, in all NAD kinases, a short motif has been identified (83GGDG86 in *M. tuberculosis* NAD kinase), which in other kinases, including diacylglyceride kinase,

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[1] Abbreviations: poly(P), inorganic polyphosphate; PCR, polymerase chain reaction; LB, Luria−Bertani; IPTG, isopropyl β-D-1-thiogalactopyranoside; DTT, dithiothreitol; PMSF, phenylmethanesulfonylfluoride; EDTA, ethilenediaminetetracetic acid; FPLC, fast protein liquid chromatography; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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sphingosine kinase, and 6-phosphofructokinase, has been demonstrated to be involved in ATP binding (*13*). Indeed, it has been reported that site-directed mutagenesis experiments performed on *M. tuberculosis* NAD kinase resulted in the enzyme inactivation, thus confirming the essentiality of this region in catalysis (*13*).

To obtain a full-length enzyme preparation suitable for a reliable kinetic characterization, we have set up a new protocol dealing with the cloning and expression of the mycobacterial gene in *E. coli*, as well as the purification of the corresponding protein. Here, we report on the kinetic characterization of the pure full-length enzyme, which revealed significant differences with respect to those described for the degraded form. Furthermore, to delineate functionally relevant residues, we have extended the mutational analysis outside of the above-mentioned GGDG motif. A comparison of the kinetic behaviors of the different mutated enzyme forms allowed us to identify a new region, well-conserved in all NAD kinases, as a part of the putative catalytic site.

MATERIALS AND METHODS

Materials. All reagents were supplied from Sigma. The source of poly(P) was sodium hexametaphosphate (Phosphate glass) containing 13 to 18 phosphoryl residues with an estimated molecular weight of 1643. MTCI125 cosmid was kindly provided by Dr. S. T. Cole, Institute Pasteur, Paris, France.

Cloning and Expression of M. tuberculosis NAD Kinase Gene. The synthetic oligonucleotide primers 5′-CTAGAATTCCAGTGACCGCTCATCGCAGT-3′ and 5′-ATAGGATCCCTACTTTCCGCGCCAACCGGT-3′ were used in a PCR to amplify the *M. tuberculosis* Rv1695 gene (GenBank accession number 885660) and to insert the *Eco*RI and *Bam*HI restriction sites at its 5′ and 3′ ends, respectively. PCR was performed with 50 ng of MTCI125 cosmid, as the template, with 25 pmol of each primer in a final volume of 50 μL. Each cycle was set for 30 s of denaturation at 94 °C, 30 s of annealing at 55 °C, and 1 min of elongation at 72 °C, and 30 reaction cycles were carried out in a DNA thermal cycler. The 944-bp product was purified from an agarose gel, digested with *Eco*RI and *Bam*HI, and ligated into similarly digested pT7-7 plasmid vector (*14*). The ligation mixture was used to transform *E. coli* TOP10 F′ cells, and growth was selected on LB-medium-containing ampicillin (100 μg/mL). A representative recombinant plasmid (referred to as pT7-7-*ppnK*) was sequenced completely to confirm the absence of PCR errors. For protein expression, pT7-7-*ppnK* was transformed into *E. coli* BL21 (DE3) cells, and single colonies were inoculated into 400 mL of LB-medium-containing ampicillin and grown at 37 °C. Expression was induced with 1 mM IPTG at an A_{600} of 0.6, and cells were collected after induction for 2 h.

Purification of Recombinant M. tuberculosis NAD Kinase. All steps were performed at 4 °C. Induced cells were harvested by centrifugation at 5000*g* for 10 min and resuspended in 20 mL of lysis buffer containing 20 mM Tris at pH 8.0, 1 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, and 0.02 mg/mL each of leupeptin, antipain, chymostatin, and pepstatin. The suspension was sonicated for 45 s, with 0.5-s interval, at 50 W and centrifuged at 39000*g* for 20 min. The supernatant (crude extract) was made 3 M NaCl, and after centrifugation as above, it was loaded onto a Phenyl Sepharose column (1.8 $cm^2 \times 11$ cm) equilibrated with 20 mM Tris at pH 8.0, 1 mM $MgCl_2$, 0.2 mM EDTA (buffer A), containing 1 mM DTT and 3 M NaCl. The resin was extensively washed with the equilibration buffer, and the recombinant protein was eluted with a linear gradient of NaCl from 3 to 0 M in 200 mL of buffer A, containing 1 mM DTT and 1 mM PMSF. The active pool was dialyzed against 10 mM Tris at pH 8.0, 1 mM $MgCl_2$, 0.5 mM EDTA (buffer B), 1 mM DTT, and 1 mM PMSF. After concentration to 1 mg/mL by ultrafiltration on a YM30 membrane, the pool was applied in 1-mL aliquots to a FPLC column of TSK (diethylamino)ethyl (DEAE), equilibrated with buffer B, containing 1 mM DTT. After washing with the same buffer, an elution was performed with a gradient of increasing NaCl concentration (0−0.3 M) in buffer B, containing 1 mM DTT and 1 mM PMSF. Active fractions were pooled and concentrated by ultrafiltration. The final preparation was stored at −20 °C.

Site-Directed Mutagenesis. Site-directed mutagenesis to change the selected residues to Ala was carried out using QuikChange (Stratagene). Mutagenic primers were 5′-GCGTTTGGCTGCGACGCGGTGTTGGTGTCCACG-3′, its complement for G190A, 5′-GCTGCGACGGGGTGGCG-GTGTCCACGCCGAC-3′, its complement for L192A, 5′-GGGGTGTTGGTGTCCGCGCCGACCGGATCAACC-3′, its complement for T195A, 5′-GTTGGTGTCCACGGC-GACCGGATCAACCGCC-3′, its complement for P196A, 5′-GGTGTCCACGCCGGCCGGATCAACCGCCTAT-3′, its complement for T197A, 5′-GTGTCCACGCCGACCGCAT-CAACCGCCTATGCA-3′, its complement G198A, 5′-TC-CACGCCGACCGGAGCAACCGCCTATGCATTC-3′, its complement for S199A, 5′-ACGCCGACCGGATCAGC-CGCCTATGCATTCTCG-3′, its complement for T200A, 5′-CCGGATCAACCGCCGCTGCATTCTCGGCGG-3′, its complement for Y202A, 5′-GCATTCTCGGCGGCAGGC-CCGGTGCTGTGGC-3′, its complement for G207A, 5′-GCATTCTCGGCGGGAGCCCCGGTGCTGTGGC-3′, and its complement for G208A (substituted nucleotides are underlined). DNA encoding wild-type NAD kinase cloned in pT7-7 (pT7-7-*ppnK*) was used as a template for the PCR mutagenesis reaction. Briefly, 25 ng of template DNA was incubated with the appropriate mutagenic primers, dNTPs, and *Pfu* DNA polymerase using the cycling parameters recommended in the manual of the supplier. After the temperature cycling step, the parental template was digested by the restriction enzyme *Dpn*I, which cuts only dam-methylated DNA. The mutagenized plasmids were then transformed into *E. coli* XL1 Blue cells. The mutants were sequenced to verify incorporation of the desired modification and to ensure the absence of random mutations.

Expression of M. tuberculosis NAD Kinase Mutants. For mutant expression, the mutagenized plasmids were transformed into BL21 (DE3) cells and expression was performed as for the wild-type protein. After IPTG induction for 2 h, cells from the 20-mL culture were harvested by centrifugation (5000*g* for 10 min), resuspended in 1 mL of the lysis buffer, and sonicated as described above. After centrifugation at 39000*g* for 20 min, the supernatants representing the soluble fractions and the corresponding pellets were analyzed

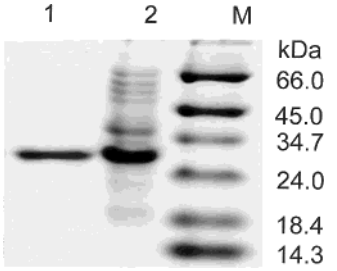

FIGURE 1: Purification of recombinant *M. tuberculosis* NAD kinase. Tricine SDS−PAGE analysis of Phenyl Sepharose (lane 2) and TSK DEAE (lane 1) fractions. Lane M contains the molecular mass markers.

Table 1: Purification of Recombinant *M. tuberculosis* NAD Kinase[a]

step	total protein (mg)	total activity (units)	specific activity (units/mg)	yield (%)	purification (-fold)
crude extract	60	2.72	0.045	100	
Phenyl Sepharose	5.7	1.62	0.28	59	6.3
TSK DEAE	1.0	0.50	0.47	18	10.4

[a] The enzymatic activity was assayed under standard conditions, as reported in the Materials and Methods, in the presence of ATP as the phosphate donor.

by SDS−PAGE. The soluble fractions containing the recombinant proteins were assayed for the enzymatic activity and were subjected to gel-filtration chromatography.

Determination of Oligomeric Structure. The oligomeric structures of wild-type and mutated NAD kinases were determined by gel filtration on a Superose 12 HR 10/30 column (Pharmacia). For elution, a buffer containing 50 mM Tris at pH 8.0, 0.5 M NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, and 1 mM DTT was used. Standard proteins were β-amylase (200 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (30 kDa). Fractions obtained at a flow rate of 0.5 mL/min were analyzed by SDS−PAGE and assayed for enzyme activity.

Assay of NAD Kinase Activity. NAD kinase was assayed spectrophotometrically, by using a coupled assay, according to Lerner et al. (*12*). Varying amounts of enzyme were incubated at 37 °C with 4 mM ATP and 2.5 mM NAD in a GPDH buffer, consisting of 100 mM Tris at pH 8.0, 100 mM KCl, 100 mM NaCl, 20 mM $MgCl_2$, 1 mM glucose-6-phosphate, and 0.6 units of glucose-6-phosphate dehydrogenase. For kinetic analyses, NAD kinase was assayed by means of a two-step method. The reaction mixture (150 μL) consisted of 50 mM Tris at pH 8.0, 20 mM $MgCl_2$, and various amounts of the substrates; the reaction was initiated by the addition of an appropriate quantity of enzyme, and after 20 min at 37 °C, the NADP formed was determined spectrophotometrically in 1 mL of the GPDH buffer. When poly(P) was used as the phosphate donor, the buffer of the reaction mixture consisted of 100 mM Pipes at pH 6.5 and 20 mM $MgCl_2$, At any substrate concentration used, reactions were linear at least for up to 60 min and substrate consumption was less than 5%. V_{max}, $S_{0.5}$, and n_H values were determined by fitting experimental data to the Hill equation (*15*). The same two-step assay method was used to measure the enzyme activity in the presence of NADP, NADH, and NADPH. However, because of the inhibition exerted by NADPH on glucose-6-phosphate dehydrogenase, when the

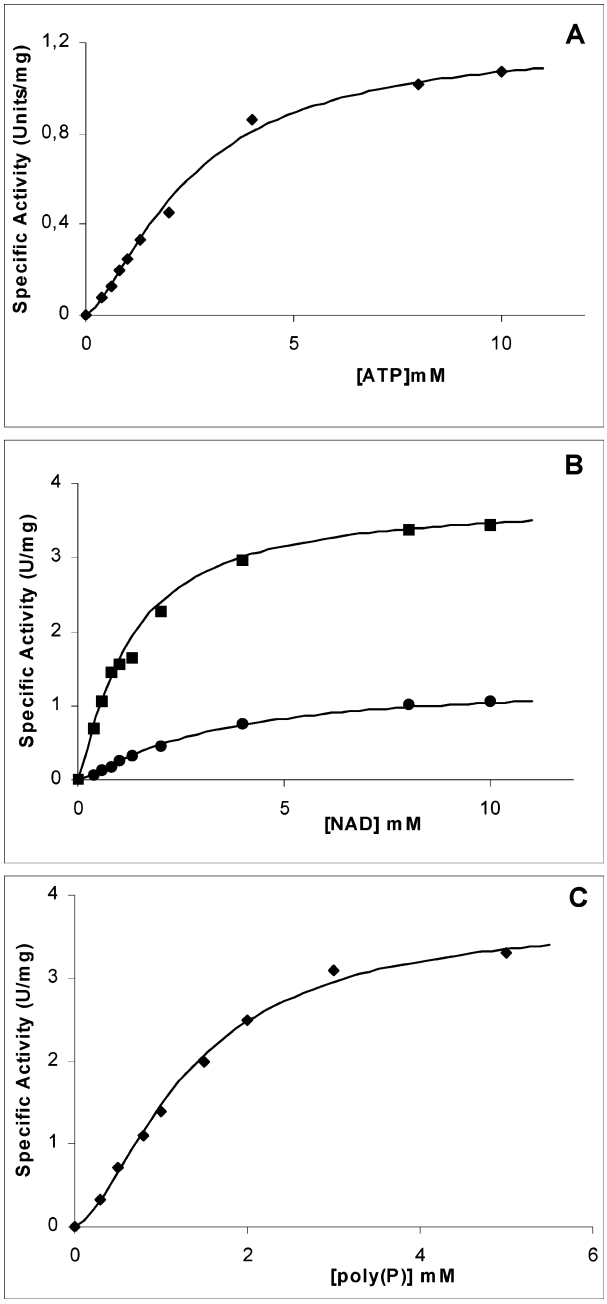

FIGURE 2: Kinetic analysis of recombinant *M. tuberculosis* NAD kinase. The effect of the substrate concentration on the initial velocity of the reaction at (A) 10 mM NAD, (B) 5 mM poly(P) (■) and 10 mM ATP (●), and (C) 10 mM NAD. Enzyme activity was assayed as described in the Materials and Methods. Data are the means of triplicate measurements.

effect of NADPH on the NAD kinase activity was studied, the NADP formed was determined spectrophotometrically in 150 mM Tris at pH 7.5, 10 mM isocitrate, 20 mM $MgCl_2$, and 0.08 units of isocitrate dehydrogenase.

One unit of enzyme is defined as the amount of NAD kinase catalyzing the formation of 1 μmol of NADP per min at 37 °C.

Other Methods. SDS−PAGE was carried out according to refs *16* or *17*. Protein concentration was determined by the method of Bradford (*18*).

RESULTS

Purification and Molecular Properties of Recombinant M. tuberculosis NAD Kinase. M. tuberculosis NAD kinase was

Table 2: Kinetic Properties of *E. coli*-expressed Wild-Type and Mutant *M. tuberculosis* NAD Kinases[a]

		wild type	T195A	S199A	G208A
NAD (+ ATP)	$S_{0.5}$ (mM)	3.3 ± 0.10	2.7 ± 0.20	3.3 ± 0.10	6.5 ± 0.10
	V_{max} (units/mg)	1.2 ± 0.16	1.1 ± 0.10	0.7 ± 0.05	0.5 ± 0.02
	n_H	1.2 ± 0.05	1.5 ± 0.03	1.5 ± 0.02	1.5 ± 0.03
	$V_{max}/S_{0.5}$	0.36 ± 0.04	0.41 ± 0.06	0.21 ± 0.02	0.08 ± 0.04
NAD (+ poly(P))	$S_{0.5}$ (mM)	1.2 ± 0.05	1.4 ± 0.10	2.1 ± 0.05	3.8 ± 0.02
	V_{max} (units/mg)	3.8 ± 0.10	3.5 ± 0.20	2.5 ± 0.05	2.2 ± 0.02
	n_H	1.2 ± 0.02	1.5 ± 0.02	1.4 ± 0.02	1.5 ± 0.01
	$V_{max}/S_{0.5}$	3.16 ± 0.19	2.49 ± 0.03	1.19 ± 0.05	0.58 ± 0.02
ATP	$S_{0.5}$ (mM)	2.5 ± 0.10	2.2 ± 0.05	2.2 ± 0.05	2.7 ± 0.02
	V_{max} (units/mg)	1.2 ± 0.05	1.1 ± 0.10	0.7 ± 0.08	0.5 ± 0.04
	n_H	1.5 ± 0.04	1.4 ± 0.02	1.5 ± 0.02	1.5 ± 0.03
	$V_{max}/S_{0.5}$	0.48 ± 0.04	0.50 ± 0.06	0.32 ± 0.03	0.18 ± 0.03
poly(P)	$S_{0.5}$ (mM)	1.3 ± 0.10	1.3 ± 0.05	1.5 ± 0.05	1.5 ± 0.05
	V_{max} (units/mg)	3.8 ± 0.02	3.5 ± 0.02	2.5 ± 0.02	2.0 ± 0.02
	n_H	1.4 ± 0.02	1.4 ± 0.05	1.4 ± 0.02	1.4 ± 0.02
	$V_{max}/S_{0.5}$	2.93 ± 0.21	2.70 ± 0.12	1.67 ± 0.07	1.33 ± 0.05

[a] Values are given as the means ± standard deviation of triplicate measurements.

overexpressed in *E. coli*, using an expression vector endowing the protein with four additional residues at its N terminus. When the purification protocol is adopted as described in the Materials and Methods, the recombinant enzyme was purified to homogeneity (Figure 1). The results of the purification procedure are summarized in Table 1.

In a previous paper dealing with the characterization of the recombinant enzyme from the same microorganism, it has been reported that during purification the protein was subjected to proteolytic degradation, resulting in the final preparation composed of two major bands of 28 and 4.6 kDa, respectively (*7*). In our case, however, by using a different protocol, the purified enzyme migrated as a single band of about 33 kDa, which is exactly the expected size for the recombinant protein (Figure 1). In addition, the full-length enzyme was stable for several weeks when stored at −20 °C. The integrity of the recombinant NAD kinase can account for the lower specific activity value (0.47 unit/mg), when compared with that reported for the degraded enzyme (1.9 units/mg).

Likewise, a different oligomerization state has been estimated by gel filtration chromatography: the pure full-length enzyme was eluted as a single peak of about 82 000 Da (not shown), which is consistent with both a dimer and trimer, in contrast with the tetrameric organization reported for the truncated form (*7*).

Catalytic Properties of M. tuberculosis NAD Kinase. Kinetic analysis was performed on the enzyme-catalyzed reaction by using both ATP and poly(P) as the phosphate donors. In contrast with the results obtained with the degraded enzyme showing a linear kinetic behavior with all substrates (*7*), the full-length enzyme resulted to be allosteric (Figure 2). Kinetic parameters, determined as described in the Materials and Methods, are summarized in Table 2. n_H values of 1.5 and 1.4 were calculated for ATP and poly(P), respectively, indicating a significant positive cooperativity. The enzyme exhibited a sigmoidal kinetic also toward NAD, even though with a lower n_H value, both in the presence of ATP and poly(P). Interestingly, the mycobacterial enzyme showed a higher affinity toward poly(P) rather than ATP, and the reaction was significantly faster when poly(P) was the phosphate donor, resulting in a catalytic efficiency 6 times higher in the presence of the polymer. This feature was not reported for the degraded enzyme, probably because of both the different enzyme preparation and the lower Mg^{2+} concentration used in the activity assay. In fact, we found that when ATP was replaced by poly(P), a minimum concentration of 20 mM $MgCl_2$ was required to avoid an underestimation of the enzyme activity, because of the strong metals chelating capacity possessed by poly(P) (*19*). Notably, the presence of poly(P) not only increased the V_{max} value of the reaction, but also lowered the $S_{0.5}$ value toward NAD.

Table 3: Effects of NADP, NADH, and NADPH on NAD Kinase Activity[a]

	concentration (mM)	relative activity (%)
NADP	0.05	83
	0.10	64
	0.20	40
	0.40	22
NADPH	0.05	93
	0.10	82
	0.20	63
	0.40	43
NADH	0.05	92
	0.10	87
	0.20	70
	0.40	50

[a] Assays were performed as described in the Materials and Methods, in the presence of 0.6 mM NAD and 4 mM ATP.

Quinolinate, a metabolic intermediate in the de novo NAD biosynthesis, NADPH, NADH, and NADP have been demonstrated to be allosteric effectors of several eubacterial NAD kinases, indicating that NAD kinase is a key regulatory enzyme in NAD(P) biosynthesis. To identify possible allosteric modulators of *M. tuberculosis* NAD kinase, the effect of several metabolites has been tested on the mycobacterial enzyme activity. We found that quinolinate, nicotinic acid, nicotinamide, nicotinamide riboside, nicotinamide mononucleotide, nicotinic acid mononucleotide, and nicotinic acid adenine dinucleotide, present at a 1 mM concentration in the reaction mixture, were ineffective. In contrast, a significant inhibition was exerted by NADP, NADPH, and NADH, at concentrations lower than 0.5 mM (Table 3). In the presence of the reduced dinucleotides, both the $S_{0.5}$ and the V_{max} for NAD were affected, while the n_H value did not change (data not shown).

Sequence-Alignment-Guided Site-Directed Mutagenesis. Multiple sequence alignment of representative NAD kinases

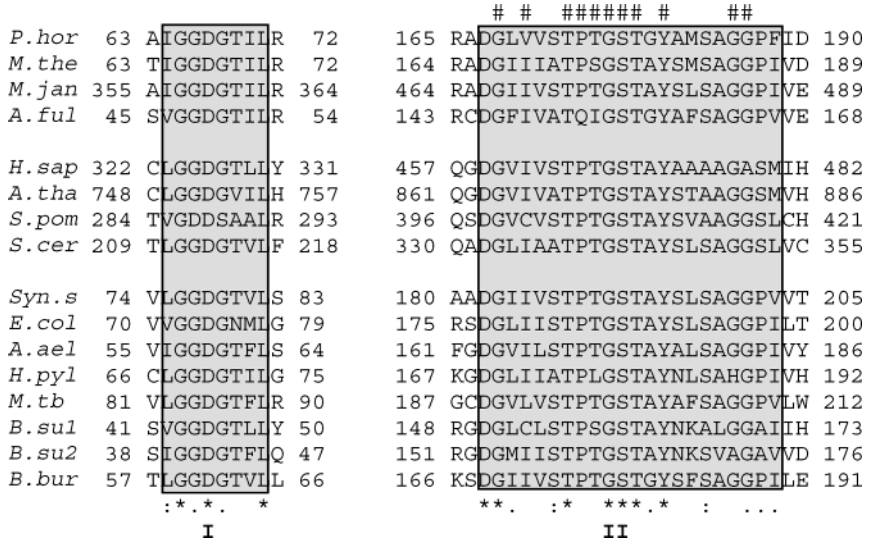

FIGURE 3: Multiple sequence alignment of representative sequences of NAD kinases. Archaeal, bacterial, and eukaryotic NAD kinase sequences were aligned to maximize homology using Clustal V. The highly conserved regions (I and II) are highlighted in shaded boxes. Residues that were subjected to mutational analysis in the *M. tuberculosis* enzyme are indicated by pound signs (#).The first and last residue numbers of the sequences in each block are indicated. The species name abbreviations and the NCBI identification numbers of the NAD kinase genes are as follows: *P.hor*, *Pyrococcus horikoshii* (gi8480213); *M.the*, *Methanothermobacter thermautotrophicus* (gi8480107); *M.jan*, *Methanococcus jannaschii* (gi13959439); *A.ful*, *Archaeoglobus fulgidus* (gi8480649); *H.sap*, *Homo sapiens* (gi20070326); *A.tha*, *Arabidopsis thaliana* (gi18395013); *S.pom*, *Schizosaccharomyces pombe* (gi8928480); *S.cer*, *Saccharomices cerevisiae* (gi729534); *Syn.s*, *Synechocystis* sp. (gi8928463); *E.coli*, *Escherichia coli* (gi8489010); *A.ael*, *Aquifex aeolicus* (gi8480160); *H.pyl*, *Haelicobacter pylori* (gi8928483); *M.tb*, *Mycobacterium tuberculosis* (gi8480539); *B.su1*, *Bacillus subtilis* (gi8928487); *B.su2*, *Bacillus subtilis* (gi24418604); and *B.bur*, *Borrelia burgdorferi* (gi13959692).

from the three domains of life allowed the identification of two highly conserved regions spanning residues 82−89 (region I) and 189−210 (region II) of *M. tubeculosis* NAD kinase (Figure 3). Region I contains the motif GGDG, which has been recently described to be common to several apparently unrelated kinase subfamilies, including diacylglyceride kinase, 6-phosphofructokinase, and sphingosine kinase (in the latter, however, the first glycine of the motif is replaced by serine) (*13*). The GGDG signature has been demonstrated to be part of the ATP-binding site in 6-phosphofructokinase by three-dimensional structure analysis, and mutagenesis studies have indicated its involvement in nucleotide binding also in diacylglyceride kinase and sphingosine kinase (*20*−*23*). The presence of this motif in the NAD kinase subfamily provided an opportunity to define the ATP-binding site of this enzyme, because the conservation of the motif reasonably arises from the ability of all kinases to utilize ATP. Indeed, site-directed mutagenesis experiments performed on the *M. tuberculosis* NAD kinase GGDG sequence led to enzyme inactivation, confirming its importance in catalysis (*13*). We found that region II is unique and specific to all NAD kinases so far identified. Like region I, it is particularly rich in glycine residues, suggesting the presence of loops coordinating nucleotide binding, as found in many nucleotide-binding enzymes (*24*, *25*). To examine the role of this domain on NAD kinase activity, residues highly conserved in such a region were selected for mutation in the *M. tuberculosis* enzyme. G190, L192, T195, P196, T197, G198, S199, T200, Y202, G207, and G208 were replaced individually with alanine using site-directed mutagenesis as described in the Materials and Methods (Figure 4a).

Expression of NAD Kinase Mutants in E. coli Cells. Selected mutants were overexpressed in *E. coli* cells, as described in the Materials and Methods. SDS−PAGE of the cell extracts after IPTG induction showed that all of the mutants, with the exception of G190A and G207A, were expressed as soluble proteins, migrating at the same position (33 kDa) as the wild type (Figure 4b). Densitometric analysis of the gel showed that all recombinant proteins were present at similar levels. G190A and G207A were expressed in the form of inclusion bodies, as revealed by their presence in the cellular insoluble fraction (lanes 14 and 15 of Figure 4b). Conversely, the insoluble fractions of all other *E. coli* cell extracts were devoid of the recombinant protein (not shown). Analysis of the NAD kinase activity of the nine soluble mutants in the cell extracts showed that only three of them were still able to catalyze the reaction by using both phosphate donors (Figure 4c). However, S199A and G208A mutants exhibited a catalytic activity significantly lower than that of the wild-type enzyme, both in the presence of ATP and poly(P) (Figure 4c). To determine whether the loss of activity because of site-directed mutagenesis might be attributed to a disturbed oligomerization process, the extracts from *E. coli* cells expressing the soluble mutants were subjected to gel-filtration chromatography, as described in the Materials and Methods. Figure 5 represents the results obtained with active G208A and inactive L192A mutants. Both of them were eluted from the column as a single peak with an apparent molecular mass of about 82 000 Da, exactly resembling the behavior of the wild-type protein. The same results have been obtained with all other soluble mutants (not shown). In none of the cases were the mutated proteins eluted at volumes corresponding to different molecular weights, suggesting that they did not change their oligomerization state. Therefore, the loss of catalytic activity in these mutants indicates that region II is critical for catalysis.

Kinetic Properties of NAD Kinases Mutants. Mutants T195A, S199A, and G208A were purified to homogeneity, as described for the wild-type enzyme; all of them showed

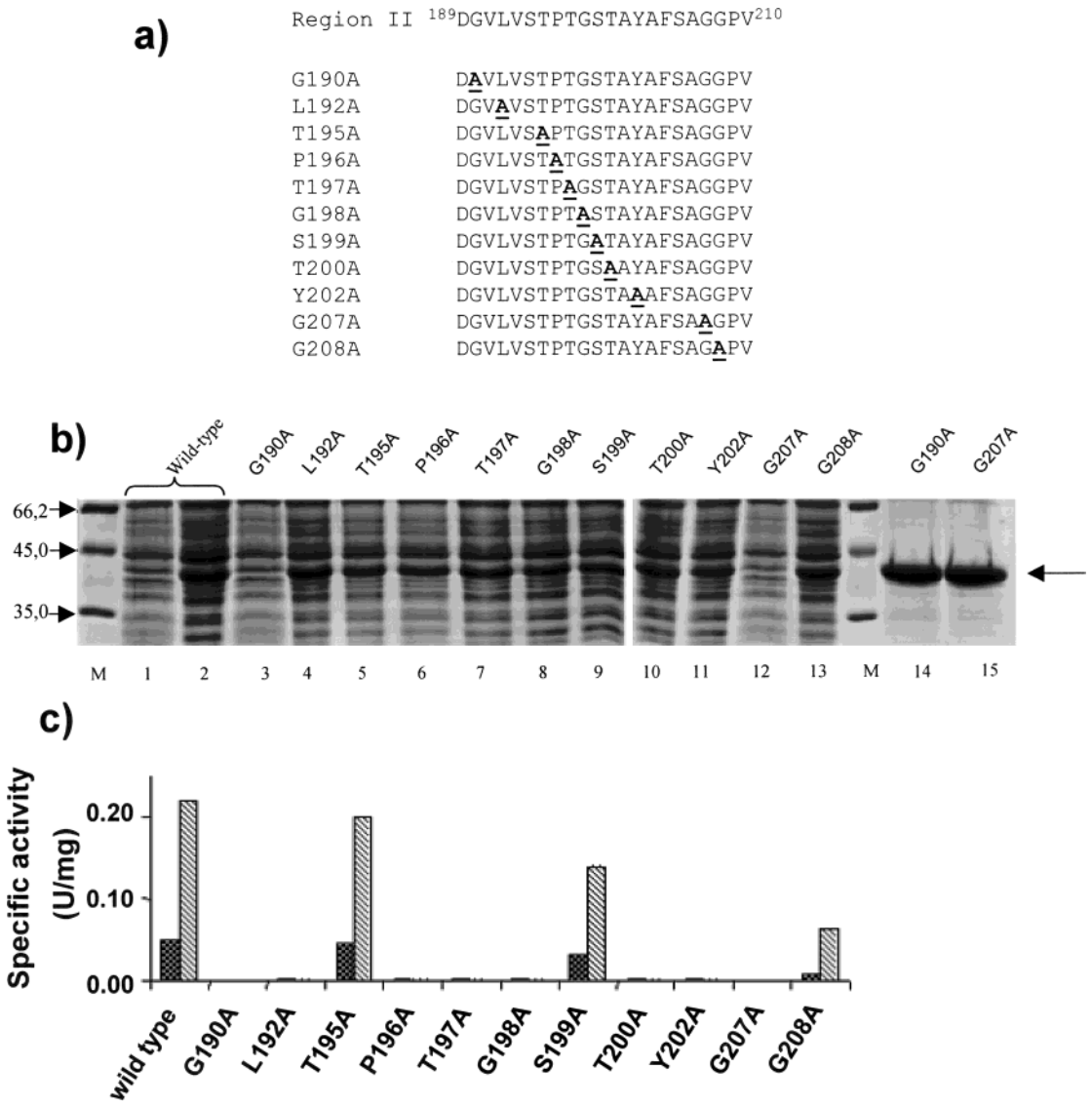

FIGURE 4: Effects of the mutations in region II of *M. tuberculosis* NAD kinase on enzyme activity. (a) Region II mutant constructs of NAD kinase. The mutated residues are underlined. (b) SDS−PAGE (15% polyacrylamide gel) of soluble fractions of extracts from *E. coli* cells expressing the wild-type protein before (lane 1) and after IPTG induction (lane 2) and mutated NAD kinases after IPTG induction (lanes 3−13). Each line was loaded with a similar amount of the total protein (about 20 μg). Lanes 14 and 15 represent the insoluble fractions of the cells expressing mutants G190A and G207A, respectively. Lanes M contain the molecular mass markers. (c) Effects of the mutations on NAD kinase activity. The enzymatic activity was measured with the two-step assay reported in the Materials and Methods, using 4 mM ATP (black- and white-dotted bars) and 3 mM poly(P) (diagonal line bars), in the presence of 2.5 mM NAD.

the same elution profile when purified on Phenyl Sepharose and TSK DEAE columns (not shown). Kinetic properties of the pure mutants were determined and compared with those of the wild-type enzyme (Table 2). Like the wild-type protein, they exhibited an allosteric behavior toward all substrates and showed a higher catalytic efficiency toward poly(P), as well as an increase in the affinity toward NAD when the polymer was used as the phosphate donor. In the presence of ATP, kinetic parameters of mutant T195A were similar to those exhibited by wild-type NAD kinase, with the exception of the n_H value for NAD, which was higher in the mutant, suggesting that substitution of Thr195 to Ala promotes stronger allosteric interactions. On the other hand, mutations of Ser199 and Gly208 to Ala had a clear effect on both the n_H value for NAD and the catalytic activity, in which the V_{max} values of S199A and G208A decreased by about 42 and 58%, respectively. Furthermore, G208A showed a 2-fold increase in the $S_{0.5}$ value for NAD, resulting in a catalytic efficiency 4.5-times lower. On the contrary S199A and G203A exhibited $S_{0.5}$ and n_H values for ATP similar to those calculated for the wild type.

Also, when assayed in the in the presence of poly(P), the three mutants exhibited a higher n_H value for NAD and G208A showed a significant increase in the $S_{0.5}$ value for the dinucleotide. In addition, all mutants showed n_H and $S_{0.5}$ values for poly(P) similar to those exhibited by the wild-type enzyme.

All together, these results indicate that NAD binding is altered in these mutants and provide further evidence for the importance of region II in catalysis.

DISCUSSION

M. tuberculosis NAD kinase has been purified to homogeneity by means of a procedure yielding a high level of pure enzyme. As reported by others, the recombinant protein

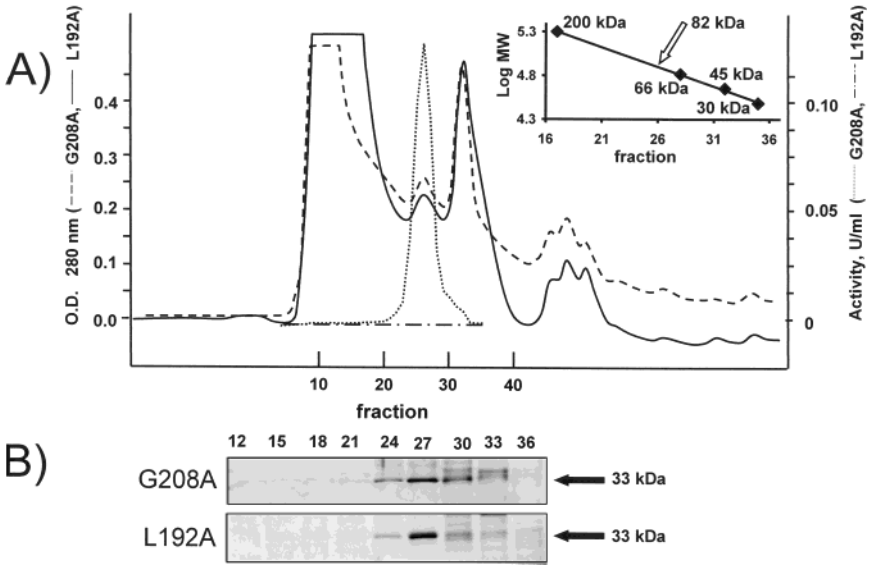

FIGURE 5: Gel-filtration analysis of *E. coli*-expressed NAD kinase mutants. (A) Gel-filtration chromatography of extracts from *E. coli* cells expressing the L192A and G208A mutants. Gel filtration was performed as described in the Materials and Methods. The inset shows the interpolation curve for the estimation of the molecular mass. The open arrow indicates the native molecular mass of the mutants. (B) SDS−PAGE (15% acryl amide gel) of the gel-filtration fractions. Solid arrows indicate the subunit molecular mass of the mutants.

was very prone to proteolytic degradation, which has been overcome by both the use of a mixture of the protease inhibitors in the lysis buffer and the presence of PMSF during the purification. In addition, the combination of hydrophobic and ion-exchange chromatography yielded a final preparation only consisting of a full-length enzyme. *M. tuberculosis* NAD kinase exhibited catalytic and molecular properties significantly different from those reported for the degraded form. The molecular weight of the native protein, estimated by gel filtration, resulted in 82 000, consistent with both a dimeric and trimeric structure. The oligomeric organization of the mycobacterial enzyme is common to all NAD kinases so far characterized, which possess different structures ranging from dimers (*B. subtilis*) (*4*), to tetramers (the human enzyme) (*12*), to hexamers (*E. coli* and *S. cerevisiae*) (*6*, *11*).

Kinetic analysis of the enzyme-catalyzed reaction revealed that *M. tuberculosis* NAD kinase is an allosteric enzyme. Among all NAD kinases studied to date, the mycobacterial enzyme is the only one exhibiting a sigmoidal kinetic with respect to all substrates. In fact, even though both *B. subtilis* and *B. licheniformis* NAD kinases have been reported to show a marked positive cooperativity for ATP and poly(P), they display a hyperbolic NAD saturation curve (*3*, *4*). In addition, *E. coli*, *S. cerevisiae*, and human enzymes show a linear kinetic behavior (*4*−*6*, *11*). The study of the mycobacterial enzyme kinetic properties has given evidence of a significantly higher catalytic efficiency in the presence of poly(P) as the phosphate donor, because of both an increase in the V_{max} of the reaction and higher affinities toward poly(P) rather than ATP and toward NAD when ATP is replaced by poly(P). These results suggest that the polymer, rather than ATP, acts as the major phosphate donor for NAD kinase in *M. tuberculosis*. In view of the observation that eukaryotic NAD kinases are strictly ATP-dependent, we believe that the mycobacterial protein is a good target for the design of selective inhibitors to be used as new antibacterial drugs. In our paper, we have identified in the *M. tuberculosis* NAD kinase a region that is highly conserved in all known NAD kinases. This domain is particularly rich in conserved glycine residues, suggesting its possible involvement in nucleotide binding, as demonstrated for other enzymes (*24*, *25*). To elucidate the function of this highly conserved domain, site-directed mutagenesis was performed on 11 conserved residues within the *M. tuberculosis* NAD kinase domain. All selected residues were individually changed to alanine, and the effect of the mutations on NAD kinase activity was determined in *E. coli*-expressed mutant enzymes. A total of 6 of the 11 mutants completely lost the enzymatic activity when assayed both with ATP and poly(P), and 2 mutants were significantly less-active than the wild-type enzyme. The loss of activity was not due to a perturbation of the protein oligomerization process, because all mutants were still able to assume the same oligomeric state of the wild-type enzyme, as demonstrated by gel-filtration analyses. This suggests a specific involvement of the mutated residues in catalysis. Notably, although mutation of glycine to alanine is relatively subtle in terms of the structure and charge, substitutions of the glycine residues within the domain had a profound effect on the enzyme activity and folding. In fact, G190A and G207A mutants were expressed as insoluble proteins, G198A was inactive, and G208A possessed only 30% of the activity with respect to the wild-type enzyme. The kinetic characterization of mutant G208A revealed that the mutation, while affecting the catalytic efficiency, did not alter the binding affinity for ATP and poly(P). On the other hand, it caused a decrease in the affinity for NAD and altered the allosteric interactions mediated by the dinucleotide, both in the presence of poly(P) and ATP. Likewise, in mutant S199A, a lower catalytic efficiency and a perturbation of the allosteric interactions have been observed when NAD was used as the variable substrate. Finally, the substitution of Thr195 with alanine only affected the n_H value for NAD. When taken together, these results suggest that this domain is likely involved in NAD binding. Our study provides the first experimental evidence demonstrating the identification of specific amino acids as the catalytic determinants of *M. tuberculosis* NAD kinase. The three-dimensional structure

solution of the protein, currently in progress, will be instrumental for both verifying our data and determining the overall architecture of the active site.

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