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# Inorganic Polyphosphate/ATP-NAD Kinase of *Micrococcus flavus* and *Mycobacterium tuberculosis* H37Rv

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**An enzyme with both inorganic polyphosphate [poly(P)]- and ATP-dependent NAD kinase activities was isolated from *Micrococcus flavus*. The enzyme was a dimer consisting of 34 kDa subunits, and was named poly(P)/ATP-NAD kinase. Internal amino acid sequences of the enzyme showed homologies with some function-unknown proteins released on the GenBank database. Among such proteins, hypothetical Rv1695 protein (Accession No. Z98268-16), which was encoded by a gene named "Rv1695" on genomic DNA of *Mycobacterium tuberculosis* H37Rv, was proposed to be poly(P)-dependent NAD kinase. By cloning and expression in *Escherichia coli*, Rv1695 was shown to encode poly(P)/ATP-NAD kinase and named *ppnk*. The *ppnk* product, recombinant-poly(P)/ATP-NAD kinase (Ppnk) was purified and characterized. The enzyme was a tetramaer consisting of 35 kDa subunits when expressed in *E. coli*. Poly(P)/ATP-NAD kinases of *M. flavus* and Ppnk of *M. tuberculosis* H37Rv specifically and completely phosphorylated NAD by utilizing commercially available poly(P)s and nucleoside triphosphates as phosphoryl donors.** © 2000 Academic Press

NAD kinase (ATP:NAD phosphotransferase, EC 2.7.1.23) catalyzes the only (known) biochemical reaction leading to the formation of NADP from NAD by the use of ATP, playing a crucial role in the regulation of NAD/NADP level in organism (1). Completely purified NAD kinases have been obtained, only from pigeon liver (2), pigeon heart (3), *Saccharomyces cerevisiae* (4), and *Candida utilis* (5), while the gene encoding the enzyme has not been cloned from any organisms.

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It had been well accepted that NAD kinase utilizes ATP or other nucleoside triphosphates as a sole source for phosphorus, until our report was presented describing the occurrence of poly(P)-dependent NAD kinase activities in some bacteria, especially in genera of *Micrococcus* and *Brevibacterium* (6). Poly(P) is a polymer of inorganic orthophosphate residues linked by high-energy phosphoanhydride bond, which is approximately equivalent to that of ATP (7), and is now regarded as present in nearly all classes of living things, from bacteria to mammals (8). Poly(P) has been considered to function as an ATP substitute and poly(P)-metabolizing enzymes in some organisms are well outlined in reviews (7, 9). These enzymes include poly(P) kinase, poly(P) glucokinase, poly(P):AMP phosphotransferase, poly(P):1, 3-diphosphoglycerate phosphotransferase, and polyphosphatase.

However, an enzyme responsible for poly(P)-dependent NAD kinase activity, as well as a gene coding for the enzyme, have never been isolated hitherto. To elucidate the properties of the enzyme and to obtain the gene coding for the enzyme, we purified NAD kinase from *Micrococcus flavus*, and found that the kinase, which we designated poly(P)/ATP-NAD kinase, could utilize both poly(P) and ATP as phosphoryl donors as the poly(P)/ATP-glucokinases do (10, 11). Furthermore, on the basis of amino acid sequence information of the kinase, we succeeded in identifying a gene coding for poly(P)/ATP-NAD kinase of *Mycobacterium tuberculosis* H37Rv. This is the first reports on isolation of poly(P)/ATP-NAD kinase and of a gene encoding the enzyme.

## MATERIALS AND METHODS

*Strains.* Cultivation conditions of *M. flavus* (IFO 3242) and the derivative strains of *E. coli* BL21(DE3) pLysS (Novagen, Darmstadt, Germany) were described in the text. *M. tuberculosis* H37Rv was grown as previously described (12). *Escherichia coli* DH5α (Toyobo,



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Osaka, Japan) was routinely cultured at 37°C in Luria-Bertani (LB) medium (13) with ampicillin (100 $\mu$g/ml).

*Assays.* Poly(P)- and ATP-dependent NAD kinases were incubated in a reaction mixture (1.0 ml) consisting of 5.0 mM NAD, 5.0 mM $MgCl_2$, 100 mM Tris–HCl (pH 7.0), phosphoryl donor [5.0 mM ATP or poly(P)], and NADP formed was enzymatically determined with isocitrate dehydrogenase (14). Metaphosphate (Wako Pure Chemical Industries, Osaka, Japan) was routinely used at 1.0 mg/ml as poly(P), unless otherwise stated. Phosphoryl acceptor specificity of the enzyme was determined by replacing NAD with other candidates and the products were analyzed with thin-layer chromatography, except for NADPH, which was enzymatically determined after conversion to NADP in acid. One unit of enzyme activity was defined as 1.0 $\mu$mol of NADP produced in 1 h at 37°C, and specific activity was expressed in unit/mg protein. Protein concentration was determined by the method of Bradford (15) with bovine serum albumin as a standard. Phosphatase activity of the enzyme was assayed by measuring orthophosphate released with molybdate method (16) in an above-described reaction mixture without NAD.

*Amino acid sequence analysis.* For internal amino acid sequence of poly(P)/ATP-NAD kinase, purified enzyme from *M. flavus* was digested with trypsin (Nacalai Tesque, Kyoto, Japan), loaded on TSKgel ODS-80Ts QA column (0.2 × 25 cm) (Tosoh, Tokyo, Japan) equipped with Waters 600E HPLC system (Millipore, Bedford, MA), and eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (0–90%, 20 ml) into 200 $\mu$l portions every 1 min. The fractions containing peptides were collected and analyzed with SHIMAZU/PSQ-1 system (Shimazu, Kyoto, Japan). For N-terminal amino acid sequence, the enzyme was directly analyzed with Procise 492 protein sequence system (Applied Biosystems Division of Perkin-Elmer, Foster City, CA). N-terminal amino acid sequence of the protein in polyacrylamide gel was analyzed with Procise 492 protein sequence system, after electroblotting the protein to PVDF membrane (Millipore).

*DNA sequence analysis.* DNA sequences were determined using an automated DNA sequencer (Model 377; Applied Biosystems Division of Perkin-Elmer, Foster City, CA).

*Other analytical methods.* Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described (17). Native–PAGE was done as described (17) without SDS. Native-gradient PAGE was performed using Multigel 2/15 (Daiichi Pure Chemicals, Tokyo, Japan) as recommended by manufacturer. Proteins in the gel were visualized by Coomassie Brilliant Blue R-250. Molecular mass of the enzyme was estimated by gel filtration chromatography on Sephacryl S-200 HR column (2.7 × 54 cm) with Gel Filtration Calibration Kit (Amersham Pharmacia Biotech, Tokyo, Japan) as recommended by manufacturer. For activity staining of NAD kinase, purified enzyme was electrophoresed on polyacrylamide gel (17) without SDS or native-gradient PAGE gel (Multigel 2/15), and the gel was immersed in a reaction mixture for assay of NAD kinase activity as described above. The protein with NAD kinase activity was stained with phenazine ethosulfate/3-(4,5-dimethyl-2-thiazoyl)-2, 5-diphenyltetrazolium bromide/glucose-6-phosphate/glucose-6-phosphate dehydrogenase system (18). Thin-layer chromatography (TLC) for the analysis of phosphorylated nucleosides was performed on silica gel with the following solvent systems: I, isobutyrate-0.5 M $NH_4OH$ (5:3 v/v); II, 95% ethanol-1.0 M $CH_3COONa$ (7:3 v/v); III, saturated $(NH_4)_2SO_4$-0.1 M $CH_3COONa$-isopropanol (40:10:1 v/v). Phosphorylated compounds were detected by exposing the silica gel to UV light. Fasta (19) on worldwide web site of DNA Databank of Japan (http:// www.ddbj.nig.ac.jp/Welcome-j.html) were used for homology search and alignment. Molecular mass of polypeptide sequence was calculated with GENETYX program (Software Development, Tokyo, Japan).

*Purification of poly(P)/ATP-NAD kinase from M. flavus.* Centrifugation was carried out at 20,000*g*, 4°C, for 20 min and dialysis was at 4°C overnight against KND (10 mM potassium phosphate (pH 7.0), 0.1 mM NAD, and 0.5 mM dithiothreitol). The cells of *M. flavus* were pre-grown aerobically at 30°C for 24 h in 650 ml of LB medium. The whole culture was used to inoculate a 65 liter liquid medium (pH 7.2) consisting of 0.1% $(NH_4)_2SO_4$, 0.05% $MgSO_4 7H_2O$, 0.1% $KH_2PO_4$, 0.4% $Na_2HPO_4$, 0.5% yeast extract, and 0.5% glucose, and the cells were grown aerobically at 30°C for 24 h. The cells (440 g wet weight) were collected, suspended in 400 ml of KND, and treated with 0.2 mg/ml lysozyme at 37°C for 20 min. After addition of 0.4 ml of 1 M phenylmethylsulfonyl fluoride, the cells were disrupted by Sonifire (Branson, Danbury, CT) and the resultant cell extract was fractionated with ammonium sulfate (20–40%). The precipitate with both poly(P)- and ATP-dependent NAD kinase activities was dissolved in 250 ml of KND, dialyzed, and then the solution was applied onto a DEAE-Toyopearl 650 M column (4.2 × 48 cm) (Tosoh) equilibrated with KND. The kinases were eluted with a linear gradient of NaCl in KND (0–700 mM, 2,000 ml). The fractions with the two activities, which were eluted with 450–500 mM NaCl, were combined, saturated with ammonium sulfate (15%), and then directly applied onto a Butyl-Toyopearl 650 M column (2.6 × 9.2 cm) (Tosoh) equilibrated with KND containing ammonium sulfate (15%). The kinases were eluted with a linear gradient of ammonium sulfate in KND (15–0%, 600 ml). The fractions with the two activities, which were eluted with 9–6% ammonium sulfate, were combined, dialyzed, and after supplementation with 10 mM $MgCl_2$, the dialysate was loaded onto an AF-Blue Toyopearl 650 ML column (0.8 × 20 cm) (Tosoh) equilibrated with KNDMg (KND containing 10 mM $MgCl_2$). The kinases were eluted stepwise with 30 ml of KNDMg containing 1.0 M NaCl, 30 ml of KNDMg containing 2.0 M NaCl, and then with 30 ml of KNDMg containing 3.0 M NaCl. The fractions with the two activities, which were eluted with 1.0–3.0 M NaCl, were combined, concentrated to about 3 ml by ultrafiltration with an Amicon model 8200 (Amicon, Beverly, Mass), and then loaded onto a Sephacryl S-200 HR column (2.7 × 54 cm) (Amersham Pharmacia Biotech) equilibrated with KND containing 0.15 M NaCl. The enzymes were eluted with KND containing 0.15 M NaCl into 3.0 ml fractions every 4.4 min, and the fractions containing two NAD kinase activities (fraction nos. 64–66) were combined, dialyzed against 10 mM Tris–HCl buffer (pH 7.0) and used as the purified enzyme in this study.

*Cloning of Rv1695 from M. tuberculosis H37Rv genomic DNA.* Genomic DNA of *M. tuberculosis* H37Rv was isolated as previously described (12). Rv1695 was amplified from the DNA with PCR (Takara Biomedicals, Kyoto, Japan). PCR was performed in a reaction mixture (100 $\mu$l) containing 2.5 U KOD polymerase (Toyobo), 0.25 $\mu$g *M. tuberculosis* H37Rv genomic DNA, 40 pmol *Nde*I primer 5′CCCATATGACCGCTCATCGCAGTGTTCTG3′, 40 pmol *Bam*HI primer 5′CGGATCCCTACTTTCCGCGCCAACCGGTC3′, 20 nmol dNTPs, 100 nmol $MgCl_2$, and the reaction buffer supplied with KOD polymerase. *Nde*I and *Bam*HI primers had *Nde*I and *Bam*HI sites, respectively. Cycle condition of PCR was as follows: 98°C 15 s, 67°C 2 s, 74°C, 30 s, 25 cycles. PCR products were separated on a 0.8% agarose gel electrophoresis and 0.93 kb PCR product was isolated with gene clean kit (Bio 101, Vista, CA). The nucleotide sequence of 0.93 kb PCR product was determined and identified to be Rv1695. The 0.93 kb PCR product was digested with *Nde*I and *Bam*HI and ligated into pET-3a (Novagen) digested with *Nde*I and *Bam*HI, yielding pSK27. Derivative strains, SK27 and SK35 (control), were obtained by transforming *E. coli* BL21(DE3)pLysS with pSK27 and pET-3a, respectively.

*Expression of Rv1695 in E. coli.* SK27 was inoculated into 400 ml LB medium (100 $\mu$g/ml ampicilin, 34 $\mu$g/ml chroramphenicol) and cultured overnight at 37°C aerobically until $A_{600}$ was 1.5. This culture was transferred into 15 liter of the same medium with same antibiotics and cultivation was continued at 37°C aerobically for 2 h until the $A_{600}$ reached 0.7; then isopropyl-$\beta$-D-thiogalactopyranoside was added to a final concentration of 0.4 mM, and the cultivation was continued again at 18°C aerobically for 3 days. SK37 was treated

**TABLE 1**

Purification of Poly(P)/ATP-NAD Kinase of *M. flavus*

| | | Poly(P)-dependent activity | | | | ATP-dependent activity | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Step | Total protein (mg) | Total activity (unit) | Yield (%) | Specific activity (a) (unit/mg) | Purification (fold) | Total activity (unit) | Yield (%) | Specific activity (b) (unit/mg) | Purification (fold) | Ratio (b/a) |
| Cell extract | 23,562 | 1,389 | 100 | 0.059 | 1.0 | 1,574 | 100 | 0.067 | 1.0 | 1.1 |
| AS[a] (20–40%) | 17,000 | 1,360 | 98 | 0.080 | 1.4 | 1,360 | 86 | 0.080 | 1.2 | 1.0 |
| DEAE-Toyopearl | 270 | 960 | 69 | 3.56 | 60.3 | 990 | 63 | 3.67 | 54.8 | 1.0 |
| Butyl-Toyopearl | 20.3 | 577 | 42 | 28.4 | 481 | 616 | 39 | 30.3 | 452 | 1.1 |
| AF-Blue Toyopearl | 3.00 | 296 | 21 | 98.7 | 1,673 | 314 | 20 | 104.7 | 1,563 | 1.1 |
| Sephacryl S-200 | 0.35 | 111 | 8.0 | 317.1 | 5,375 | 104 | 6.6 | 297.1 | 4,434 | 0.9 |

[a] Ammonium sulfate.

with the same manner in 10 ml medium. Cell extracts were prepared as above and used for the expression study of Rv1695.

*Purification of recombinant-poly(P)/ATP-NAD kinase of M. tuberculosis H37Rv expressed in E. coli.* Cell extract of SK27 obtained as above was fractionated with ammonium sulfate (20–40%). The precipitate was dissolved in 50 ml of KND, dialyzed, and then the solution was applied onto a DEAE-Toyopearl 650M column (5 × 27 cm) equilibrated with KND. The enzyme was eluted with a linear gradient of NaCl in KND (0–0.7 M, 1,600 ml). The fractions showing poly(P)/ATP-NAD kinase activities, which were eluted with 0.35–0.45 M NaCl, were combined, saturated with ammonium sulfate (30%), and then directly applied onto a Butyl-Toyopearl 650M column (2.7 × 10 cm). The enzyme was eluted with a linear gradient of ammonium sulfate in KND (30–0%, 150 ml). The fractions with the enzyme activities, which were eluted with 20–15% ammonium sulfate, were combined, dialyzed and, after supplementation with 10 mM $MgCl_2$, the dialysate was loaded onto an AF-Blue Toyopearl 650 ML column (0.8 × 20 cm). The enzyme was eluted with a linear gradient of NaCl in KND containing 10 mM $MgCl_2$ (0–3.0 M, 400 ml). The fractions with the activities, which were eluted with 0.7–1.0 M NaCl, were combined, dialyzed against 10 mM Tris–HCl (pH 7.0) and used as the purified recombinant enzyme (Ppnk) in this study.

## RESULTS

*Purification of poly(P)/ATP-NAD kinase from M. flavus.* Both poly(P)- and ATP-dependent NAD kinases were purified approximately 4,000–5,000 fold from cell extract of *M. flavus* with 6–8% of activity yield (Table 1). The purified enzyme migrated as a single protein band on SDS–PAGE (34 kDa) (Fig. 1A) and native–PAGE (Fig. 1B, lane 1). By activity staining for NAD kinase, only one position, corresponding to that of the purified enzyme, was stained in the presence of poly(P) (Fig. 1B, lane 2) or ATP (Fig. 1B, lane 3), but no positions were stained in the absence of both poly(P) and ATP (Fig. 1B, lane 4). The protein corresponding to the stained band was extracted from the gel, analyzed with SDS–PAGE (12.5%), and was revealed to be 34 kDa (data not shown). Furthermore, poly(P)- and ATP-dependent NAD kinase activities were co-eluted in a single peak in every chromatography (data not shown) and always recovered with an approximately constant activity ratio (Table 1). On the gel filtration chromatography on Sephacryl S-200 HR column, the two activities were eluted in a single peak as a 68 kDa protein. On the basis of these results described above, we concluded that the single enzyme, a 68 kDa protein consisting of two identical 34 kDa subunits, was responsible for both poly(P)- and ATP-dependent NAD kinase activities and we designated the 68 kDa protein poly(P)/ATP-NAD kinase. N-terminal amino acid sequence of the purified enzyme was determined to be $^{1}$PYTPGRRILVLTHTGREDAIS AALQATR$^{28}$. Internal amino acid sequences of the enzyme were also determined to be $^{1}$FRLPTDGWRGPVTAQE$^{16}$, $^{1}$ALFTRPLVVGPR$^{12}$, $^{1}$GYNVPLLAVNLGHVGFLAES ER$^{22}$, $^{1}$MALDVVVH VE$^{10}$, $^{1}$TVQAIASESYVVIER$^{15}$, and $^{1}$TWALNEASVEK$^{11}$.

*Identification of poly(P)/ATP-NAD kinase gene.* As a result of homology search with fasta, N-terminal and internal amino acid sequences of poly(P)/ATP-NAD kinase of *M. flavus* showed homologies with some amino acid sequences of function-unknown proteins released on GenBank. Among these proteins, we proposed that hypothetical Rv1695 protein of *M. tuberculosis* H37Rv (Accession No. Z98268-16) (Fig. 2) is poly(P)-dependent NAD kinase on the two bases: (i) *M. tuberculosis* H37Rv possessed poly(P)/ATP-glucokinase (20), (ii) our previous studies suggested that microorganisms exhibiting poly(P)-dependent glucokinase activities also show poly(P)-dependent NAD kinase ones (6). The Rv1695 protein is a polypeptide of 307 amino acids with a calculated molecular mass of 32,873 Da and is coded on a gene named Rv1695 which was sequenced at the Sanger Center, Cambridge, United Kingdom (21). Rv1695 consists of 924 nucleotides, has start codon which is preceded by probable ribosome binding site (GAGG), and stop codon followed hairpin loop structure, implying Rv1695 is expressed in *M. tuberculosis* H37Rv as a functional gene. To confirm our

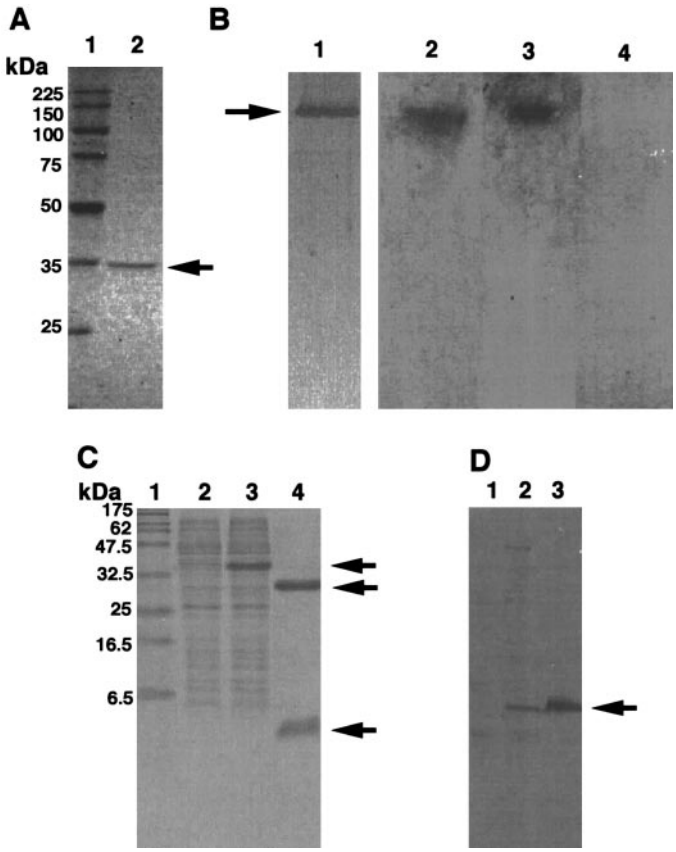


**FIG. 1.** PAGE of poly(P)/ATP-NAD kinase and Ppnk. (A) SDS–PAGE (12.5%) of purified poly(P)/ATP-NAD kinase of *M. flavus*. Lane 1: Protein markers (Novagen). Lane 2: Purified enzyme (3.0 $\mu$g). (B) Activity staining for purified poly(P)/ATP-NAD kinase (5.0 $\mu$g) of *M. flavus* in the presence of poly(P) (lane 2), ATP (lane 3), and in the absence of both poly(P) and ATP (lane 4). Before activity staining, purified enzyme was electrophoresed on native–PAGE gel (lane 1). (C) SDS–PAGE (15.0%) of cell extract of SK35, SK27, and purified Ppnk of *M. tuberculosis* H37Rv. Lane 1: Protein markers (Bio-Rad, Hercules, CA). Lane 2: Cell extract of SK35 (control strain, 5.0 $\mu$g). Lane 3: Cell extract of SK27 (5.0 $\mu$g). Lane 4: Purified enzyme (5.0 $\mu$g). (D) Native-gradient PAGE of cell extract of SK35, SK27, and purified Ppnk of *M. tuberculosis* H37Rv. Lane 1: Cell extract of SK35 (control strain, 5.0 $\mu$g). Lane 2: Cell extract of SK27 (5.0 $\mu$g). Lane 3: Purified enzyme (5.0 $\mu$g). Arrows indicate positions of poly(P)/ATP-NAD kinase (A, B) and Ppnk (C, D).

proposition that Rv1695 encodes poly(P)-dependent NAD kinase, Rv1695 was cloned from *M. tuberculosis* H37Rv genomic DNA with PCR method and was expressed in *E. coli* as described under Materials and Methods. Cell extract of SK27 showed 31.0 unit/mg and 42.0 unit/mg of poly(P)- and ATP-dependent NAD kinase activities, respectively (Table 2), while that of control strain, SK35 exhibited 0.25 unit/mg ATP-dependent NAD kinase activity and no detectable poly(P)-dependent one. Furthermore, in cell extract of SK27, a major band of 35 kDa was found by SDS–PAGE analysis (Fig. 1C, lane 3). Likewise, a major band was also recognized by native-gradient PAGE analysis (Fig. 1D, lane 2). The major bands were thought to correspond to the expressed proteins, since no such bands were observed in cell extract of control strain, SK35 (Fig. 1C, lane 2 and Fig. 1D, lane 1). The results of SDS–PAGE indicate that the subunit molecular mass of the expressed protein was 35 kDa which was comparable with calculated molecular mass of hypothetical Rv1695 protein (32,873 Da). From the expression studies described above, we concluded that Rv1695 encodes poly(P)/ATP-NAD kinase and named the gene *ppnk*.

*Purification of recombinant-poly(P)/ATP-NAD kinase (Ppnk) of M. tuberculosis H37Rv expressed in E. coli.* To characterize *ppnk* product, recombinant-poly(P)/ATP-NAD kinase (Ppnk) of *M. tuberculosis* H37Rv, we purified the Ppnk from cell extract of SK27 (Table 2). Contrary to the case of SDS–PAGE analysis of cell extract of SK27 (Fig. 1C, lane 3), the purified Ppnk migrated as 28 kDa and 4.6 kDa proteins on SDS–PAGE (Fig. 1C, lane 4). However, on native-gradient PAGE, the purified Ppnk migrated as a single protein band (Fig. 1D, lane 3) with the same mobility as that of the Ppnk in cell extract of SK27 (Fig. 1D, lanes 2). The positions corresponding to the Ppnk on native-gradient PAGE gel were stained by activity staining only in the presence of phosphoryl donors [ATP or poly(P)] (data not shown). On the gel filtration chromatography on Sephacryl S-200 HR column, the purified Ppnk was eluted as a 158 kDa protein, indicating that the Ppnk forms a tetramer consisting of 35 kDa subunits when expressed in *E. coli*, although the 35 kDa subunit was cleaved to 28 kDa and 4.6 kDa subunits during purification procedures.

N-terminal amino acid sequences of the purified Ppnk and the Ppnk in native-gradient PAGE gel showed that the purified Ppnk consists of two kinds of polypeptides, of which N-terminal amino acid sequences are $^{1}$TAHRSV-LLVV$^{10}$ and $^{1}$GVEIEVVDAD$^{10}$ (Fig. 2). Furthermore, 28 kDa protein in SDS–PAGE gel exhibited N-terminal amino acid sequence of $^{1}$GVEIEVVDAD$^{10}$ (Fig. 2). These PAGE and N-terminal amino acid sequence analyses revealed that, during purification procedures, the 35 kDa subunit of Ppnk was excised at a peptide bond between $^{59}$Met and $^{60}$Gly residues (Fig. 2), yielding 28 kDa and 4.6 kDa subunits.

*Properties of poly(P)/ATP-NAD kinase of M. flavus and Ppnk of M. tuberculosis H37Rv.* Poly(P)/ATP-NAD kinases of *M. flavus* and Ppnk of *M. tuberculosis* H37Rv specifically and completely phosphorylated NAD to NADP utilizing poly(P) or ATP (data not shown). Other compounds (adenosine, AMP, ADP, ATP, NADH, and ADP-ribose) were not phosphorylated in the presence of poly(P) or ATP by both enzymes, although the TLC results for the recognition of phosphorylated compounds are not presented. Nucleoside triphosphates were utilized by both enzymes (Table 3). ATP and dATP were utilized more effectively

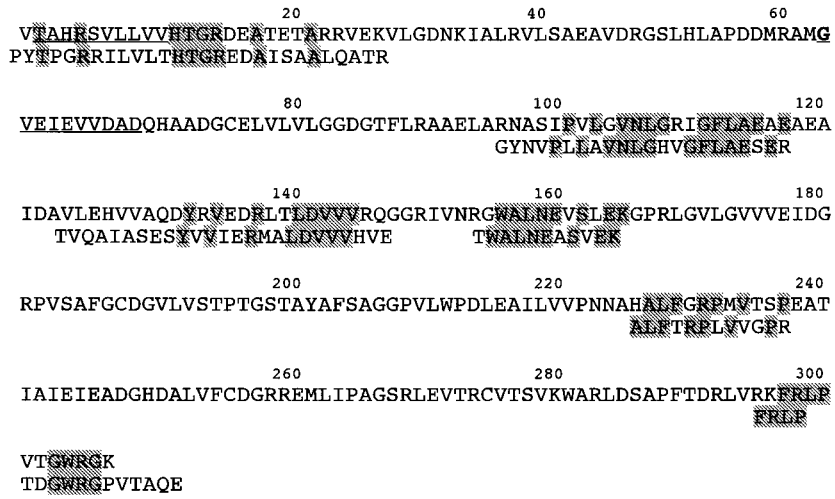


**FIG. 2.** Polypeptide sequence of hypothetical Rv1695 protein of *M. tuberculosis* H37Rv. Amino acids of polypeptide sequence of hypothetical Rv1695 protein are numbered from deduced N-terminal amino acid (V). N-terminal amino acid sequences of purified *ppnk* product, Ppnk, determined in this study are underlined. $^{60}$Gly is shown in boldface. N-terminal and internal amino acid sequences of poly(P)/ATP-NAD kinase of *M. flavus* are aligned under Rv1695 protein sequence. Identical amino acids obtained by the alignment are shaded.

than other nucleoside triphosphates (Tables 3 and 4). Commercially available poly(P)s were utilized by both enzymes, except for trimetaphosphate [cyclic form of tripolyphosphate] (Table 3). Furthermore, the Ppnk of *M. tuberculosis* H37Rv utilized ATP and poly(P) [tetrapolyphosphate, poly(P)$_4$] with similar efficiencies, while the enzyme of *M. flavus* preferred ATP to poly(P) [poly(P)$_4$] (Table 4). *p*-Nitrophenylphosphate was not utilized by the enzymes, indicating that NAD phosphorylation was not due to the reverse (phosphatase) reaction of the enzyme (14) (Table 3). The enzymes did not show glucose-6-phosphate-dependent NAD kinase activity, although such activity has been found in some coryneform bacteria isolated from sewage sludge (22) (Table 3). In the absence of NAD, both poly(P)/ATP-NAD kinase of *M. flavus* and Ppnk of *M. tuberculosis* H37Rv showed no phosphatase activities for ATP and poly(P)$_4$.

Both enzymes required bivalent metal ions such as $Mg^{2+}$ and $Mn^{2+}$ for their poly(P)/ATP-NAD kinase activities. $Mn^{2+}$ was more effective activator for both enzymes. Relative activities of poly(P)/ATP-NAD kinase of *M. flavus* in the presence of 1.0 mM metal ions were: [poly(P)-dependent activity/ATP-dependent activity] $Mg^{2+}$, 100/100; $Mn^{2+}$, 143/136; $Ca^{2+}$, 65/61; $Co^{2+}$, 51/28; $Cu^{2+}$, 33/48; $Zn^{2+}$, 30/51; and $Al^{3+}$, 0/0; and those of Ppnk of *M. tuberculosis* H37Rv were: $Mg^{2+}$, 100/100; $Mn^{2+}$, 268/246; $Ca^{2+}$, 34/39; $Co^{2+}$, 55/29; $Cu^{2+}$, 8/25; $Zn^{2+}$, 30/51, and $Al^{3+}$, 0/0; respectively. 1.0 mM *p*-chloromercuribenzoate completely inhibited poly(P)/ATP-NAD kinase activities of both enzymes. Optimum pH and temperature of poly(P)/ATP-NAD kinase of *M. flavus* were pH 7.0 in Tris–HCl and 55°C, respectively, irrespective of phosphoryl donors. Those of Ppnk of *M. tuberculosis* H37Rv were pH 6.5 in sodium-acetate and 50°C [for poly(P)-dependent activity], pH 8.0 in Tris–

**TABLE 2**

Purification of Ppnk of *M. tuberculosis* H37Rv

| | | Poly(P)-dependent activity | | | | ATP-dependent activity | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Step | Total protein (mg) | Total activity (unit) | Yield (%) | Specific activity (a) (unit/mg) | Purification (fold) | Total activity (unit) | Yield (%) | Specific activity (b) (unit/mg) | Purification (fold) | Ratio (b/a) |
| Cell extract | 8,100 | 251,100 | 100 | 31 | 1.0 | 340,200 | 100 | 42 | 1.0 | 1.4 |
| AS[a] (20–40%) | 2,300 | 177,100 | 70.5 | 77 | 2.5 | 133,400 | 39.2 | 58 | 1.4 | 0.8 |
| DEAE-Toyopearl | 1,260 | 112,140 | 44.7 | 89 | 2.9 | 107,100 | 31.5 | 85 | 2.0 | 1.0 |
| Butyl-Toyopearl | 100 | 18,500 | 7.4 | 185 | 6.0 | 18,100 | 5.3 | 181 | 4.3 | 1.0 |
| AF-Blue Toyopearl | 33 | 3,470 | 1.4 | 94 | 3.6 | 3,710 | 1.1 | 116 | 2.8 | 1.2 |

[a] Ammonium sulfate.

**TABLE 3**

Phosphoryl Donor Specificities of Poly(P)/ATP-NAD Kinase of *M. flavus* and Ppnk of *M. tuberculosis* H37Rv

| Phosphoryl donor | Relative activity (%) | Phosphoryl donor | Relative activity (%) |
|---|---|---|---|
| ATP | 100 (100) | Orthophosphate | ND (ND) |
| dATP | 91 (96) | Pyrophosphate | ND (ND) |
| AMP | ND[a] (ND) | Trimetaphosphate | ND (ND) |
| ADP | ND (ND) | Tripolyphosphate | 40 (38) |
| GTP | 88 (47) | $Poly(P)_4$ | 151 (163) |
| CTP | 73 (16) | Phosphate glass (type 35) | 49 (13) |
| dTTP | 74 (33) | Polyphosphate | 138 (151) |
| UTP | 87 (88) | Hexametaphosphate | 88 (118) |
| Glucose-6-phosphate | ND (ND) | Metaphosphate | 88 (178) |
| *p*-Nitrophenylphosphate | ND (ND) | | |

*Note.* Activities of poly(P)/ATP-NAD kinase and Ppnk for 5.0 mM ATP were relatively taken as 100%. Relative activities of Ppnk are in parentheses. Metaphosphate, hexametaphosphate, and polyphosphate (Wako Pure Chemical Industries) were used at 1.0 mg/ml. Others were from Sigma-Aldrich Japan (Tokyo, Japan) and used at 5.0 mM. A number of phosphoryl residues and molecular weight of the phosphate glass (type 35) are estimated to be 32 and 3,292, respectively.

[a] ND, not detected.

HCl and 50°C [for ATP-dependent activity], respectively. 0.05 mM NADPH decreased poly(P)- and ATP-dependent NAD kinase activities of *M. flavus* enzyme to 61 and 62%, and those of Ppnk to 71 and 92%, respectively (activities in the absence of NADPH were taken as 100%).

## DISCUSSION

Due to the lack in detailed properties of NAD kinase and a gene for the enzyme, it was currently impossible to investigate the regulation of NADP biosynthetic pathway and the influence of NADP synthesis on the activity of other metabolic pathways that require participation of NADP or NADPH as a co-factor. To begin gaining insight into the regulation of NAD phosphorylation in microorganisms, we first purified NAD kinase of *M. flavus* and found that the enzyme was a poly(P)/ATP-NAD kinase utilizing ATP or poly(P) as a phosphoryl donor. To make the finding more definitive one, the gene for poly(P)/ATP-NAD kinase was sought in *M. tuberculosis* H37Rv. As a result, the Rv1695 in genomic DNA sequences of *M. tuberculosis* H37Rv was also confirmed to be a gene for poly(P)/ATP-NAD kinase and was named *ppnk* after *ppgk* for poly(P)/ATP-glucokinase (20).

**TABLE 4**

Kinetic Parameters of Poly(P)/ATP-NAD Kinase of *M. flavus* and Ppnk of *M. tuberculosis* H37Rv

| | $K_m$ (mM) | $V_{max}$ ($\mu$mol/unit/h) | Efficiency ($V_{max}/K_m$) | Relative efficiency (%) |
|---|---|---|---|---|
| ATP | 0.13 (1.80) | 1.09 (1.40) | 8.39 (0.78) | 100 (100) |
| dATP | 0.16 (3.80) | 1.27 (1.70) | 7.94 (0.45) | 95 (58) |
| $Poly(P)_4$ | 1.04 (1.60) | 1.58 (1.60) | 1.52 (0.94) | 18 (121) |
| NAD (+ATP)[a] | 0.83 (0.90) | 1.76 (1.20) | 1.76 (1.33) | 20 (171) |
| NAD (+poly(P)4)[b] | 0.58 (2.90) | 2.55 (1.80) | 2.55 (0.62) | 30 (79) |

*Note.* Kinetic parameters were calculated with Lineweaver-Burk plot. 1.0 unit (determined in the presence of 5.0 mM ATP) of the enzyme was used. Kinetic parameters of Ppnk are in parentheses.

[a] Kinetic parameters for NAD in the presence of 5.0 mM ATP.

[b] Kinetic parameters for NAD in the presence of 5.0 mM $poly(P)_4$.

Among the characteristics of the poly(P)/ATP-NAD kinase of *M. flavus* and Ppnk of *M. tuberculosis* H37Rv, comparison of molecular structures (subunit molecular mass) of NAD kinase is of great interest. NAD kinases purified so far from pigeon liver (2), pigeon heart (3), *S. cerevisiae* (4), and *C. utilis* (5) have been reported to be octamer (34 kDa), monomer (45 kDa), tetramer (31 kDa), and octamer (32 kDa), respectively. The NAD kinases of *M. flavus* and *M. tuberculosis* H37Rv were also revealed to be a dimer and a tetramer consisting of 34 kDa and 35 kDa subunits, respectively (although the 35 kDa subunit of *M. tuberculosis* H37Rv Ppnk was split into two nonidentical polypeptides during purification procedures). These observations indicate that NAD kinases in any organisms are built as multimers of the subunit with a molecular mass of 31–40 kDa.

Other than poly(P)/ATP-NAD kinase, we have already purified poly(P)/ATP-glucokinase from *M. flavus* (23). The *ppgk* (poly(P)/ATP-glucokinase gene) has been cloned from *M. tuberculosis* H37Rv (20). Therefore, the isolations of poly(P)/ATP-NAD kinase from *M. flavus* and *ppnk* from *M. tuberculosis* H37Rv give an additional evidence to our previous observation that microorganisms having poly(P)-dependent NAD kinase activity also exhibit poly(P)-dependent glucokinase activity (6). This fact may imply some physiological consequence concerning metabolic activity of microorganisms. Briefly, for example, phosphogluconate pathway beginning with an intermediate glucose-6-phosphate is

known as a main route for generation of reductant NADPH required for the biosynthesis of long-chain fatty acids and steroids (24). Therefore, it is likely that the abilities to utilize poly(P) for phosphorylation of NAD and glucose give much energetical advantages to produce NADP and glucose-6-phosphate, and thus, to supply NADPH physiologically via phosphogluconate pathway.

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