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Cloning, Purification, and Properties of a Novel NADH Pyrophosphatase

EVIDENCE FOR A NUCLEOTIDE PYROPHOSPHATASE CATALYTIC DOMAIN IN MutT-LIKE ENZYMES*

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David N. Frick and Maurice J. Bessman‡

From the Department of Biology and the McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland 21218

An *Escherichia coli* open reading frame containing significant homology to the active site of the MutT enzyme codes for a novel dinucleotide pyrophosphatase. The motif shared by these two proteins and several others is conserved throughout nature and may designate a nucleotide-binding or pyrophosphatase domain. The *E. coli* NADH pyrophosphatase has been cloned, overexpressed, and purified to near homogeneity. The protein contains 257 amino acids (M_r = 29,774) and migrates on gel filtration columns as an apparent dimer. The enzyme catalyzes the hydrolysis of a broad range of dinucleotide pyrophosphates, but uniquely prefers the reduced form of NADH. The V_{max}/K_m for NADH (69 μmol min^{-1} mg^{-1} mM^{-1}) is an order of magnitude higher than for any other dinucleotide pyrophosphate tested. In addition, the K_m for NADH (0.1 mM) is 50-fold lower than the K_m for NAD$^+$. The hydrolysis of dinucleotide pyrophosphates requires divalent metal ions and yields two mononucleoside 5′-phosphates. The metals that most efficiently stimulate activity are Mg^{2+} and Mn^{2+}. Although these metals support similar V_{max} values at optimal metal concentration, the apparent K_m for Mg^{2+} is 3.7 mM (at 1 mM NADH), whereas the apparent K_m for Mn^{2+} at the same NADH concentration is 30 μM.

This work was initiated during a study of a highly conserved motif that was first identified in the *Escherichia coli* MutT and *Streptococcus pneumoniae* MutX antimutator proteins by Mejean *et al.* (1). The motif is shared by enzymes from *Proteus vulgaris* (2), human cells (3), and another partially purified *E. coli* protein (4). Because these proteins all hydrolyze nucleoside triphosphates to form nucleoside monophosphates and inorganic pyrophosphate, we proposed that this small region of conserved amino acids, common to these proteins, designates a nucleoside-triphosphate pyrophosphohydrolase activity (4). Several other open reading frames in widely divergent organisms that specify this small sequence of amino acids but that otherwise have little homology were identified by a computer search of the data bases (1, 5). We have initiated a systematic study to isolate and examine whether these other genes code for proteins that share similar enzymatic activities. One of these proteins, coded for by open reading frame *orf257* (or *yjad*) between the *thi*C and *heme*E genes in the 90-min region of the *E. coli* genetic map (6) (GenBank™ accession numbers U00006 and D12624), is the subject of this study. It will be demonstrated that this protein does not hydrolyze nucleoside triphosphates, but that it has a novel NADH pyrophosphatase activity, which provides further insight into the function of the conserved amino acid motif.

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‡ To whom correspondence should be addressed. Tel.: 410-516-7316; Fax: 410-516-5213; E-mail: ZOOT@JHUVMS.HCF.JHU.EDU.

EXPERIMENTAL PROCEDURES

Materials

Primers were obtained from Integrated DNA Technologies (Coralville, Iowa). Unless otherwise noted, biochemicals and enzymes were purchased from Sigma.

Methods

Cloning of E. coli orf257

E. coli strain MG1655, kindly provided by Dr. Frederick R. Blattner, was used to prepare chromosomal DNA by the method of Silhavy *et al.* (7). Oligonucleotide primers were used in the polymerase chain reaction to attach *Nco*I and *Bam*HI restriction sites and to amplify the *orf257* gene. The 790-base pair product was purified from an agarose gel, digested with *Nco*I and *Bam*HI (Stratagene, La Jolla, CA), repurified, and ligated into the *Nco*I and *Bam*HI sites of plasmid pET11d (Novagen, Madison, WI). In the resulting plasmid, the *orf257* gene was under control of a T7(lac) promoter. The plasmid, pETorf257, was transformed into strain HB101 for storage and into strain HMS174(DE3) for protein expression using techniques from Sambrook *et al.* (8).

Purification of Orf257 Protein

Growth and Expression—Strain HMS174(DE3), harboring plasmid pETorf257, was unstable when grown to high cell density, presumably due to a selective disadvantage upon depletion of ampicillin. Consequently, cultures were not grown directly to stationary phase, and the following procedure was designed to maximize the yield of the Orf257 protein. Single colonies of strain HMS174(DE3) containing pETorf257 were inoculated into 5 ml of LB medium containing 100 μg/ml ampicillin. After the cells grew to an A_{600} of 0.5, they were collected by centrifugation, washed with 0.9% NaCl, and transferred to 100 ml of fresh medium containing ampicillin. This wash was repeated after the cells again reached an A_{600} of 0.5, and the cells were then transferred to 2 liters of fresh medium. When the cells again reached an A_{600} of 0.5, they were induced with 1 mM isopropyl-β-D-thiogalactopyranoside and grown for an additional 2 h. Under these conditions, ~100% of the cells still contained the plasmid, as determined by plating aliquots of the culture on selective media.

Crude Extract—The induced cells were harvested by centrifugation and washed with an isotonic saline solution, and the cell paste was suspended in buffer A (50 mM Tris-Cl, pH 7.5, 1 mM EDTA, and 1 mM dithiothreitol) and sonicated in a Branson sonifier for 20 min. The crude extract was clarified by centrifugation at 15,000 × *g* for 30 min (Fraction I).

Streptomycin Sulfate—Fraction I was diluted to 10 mg/ml, and a 10% solution of streptomycin sulfate was added slowly to a final concentration of 1% while the extract was stirred on ice. The precipitate, which contained the Orf257 protein, was collected by centrifugation and dissolved in 20 ml of buffer A (Fraction II).

DEAE-Sepharose—Fraction II (18 ml) was loaded onto a column (2.5 × 24 cm) of DEAE-Sepharose (Pharmacia Biotech Inc.) and washed

with 400 ml of 50 mM Tris-Cl, pH 7.5, 0.1 mM dithiothreitol (buffer B). The Orf257 protein was eluted with buffer B containing 200 mM NaCl, whereas nucleic acids remained bound to this column. The fractions containing the Orf257 protein were pooled (173 ml), and EDTA was added to a final concentration of 1 mM (Fraction III).

Ammonium Sulfate Fractionation—Solid ammonium sulfate (50 g) was added to 172 ml of Fraction III, and the precipitate was collected by centrifugation and discarded. Ammonium sulfate (53.6 g) was added to the supernatant, and the precipitate containing the Orf257 protein was dissolved in 7.5 ml of buffer A (Fraction IV).

Sephadex G-100—Fraction IV (7 ml) was loaded onto a gel filtration column (Sephadex G-100, 2.5 × 60 cm) and eluted with 50 mM Tris-Cl, 1 mM EDTA, 0.1 mM dithiothreitol, and 50 mM NaCl. The fractions containing the Orf257 protein were combined, concentrated by ultrafiltration, dialyzed against buffer A, and stored at −80 °C in 30% glycerol (Fraction V).

Assays

The purification of the enzyme was originally followed by polyacrylamide gel electrophoresis (9) because we had not yet discovered an activity associated with the Orf257 protein. Once the major substrate of the enzyme was discovered, the activity was quantitated by three assays, all based on the measurement of the AMP formed in Reaction 1.

$$\text{NADH} \xrightarrow[\text{Orf257}]{Mg^{2+}} \text{NMNH} + \text{AMP}$$

REACTION 1

The reaction mixtures (100 μl for Assays 1 and 2 and 50 μl for Assay 3) contained 50 mM Tris-Cl, pH 8.5, 20 mM $MgCl_2$, and 0.33–3.3 milliunits of enzyme. One unit of activity is 1 μmol of NADH hydrolyzed per min. After incubation for 10–30 min at 37 °C, the reactions were terminated by boiling. Stage I was then treated as follows.

Assay 1—The AMP formed was measured by converting it to ADP with adenylate kinase and ATP and coupling this to the lactate dehydrogenase indicator system, which follows the oxidation of NADH spectrally at 340 nm (10). The reaction contained 0.1 ml of Stage I, 20 mM KCl, 6 mM ATP, 10 mM $MgCl_2$, 4 mM phosphoenolpyruvate, 0.4 mM NADH, 10 units of lactate dehydrogenase, 10 units of pyruvate kinase, and 5 units of adenylate kinase in a volume of 1 ml. Calculation was based on a value of 6.22×10^3 M^{-1} cm^{-1} for ϵ_{340} NADH.

Assay 2—The AMP was measured by its spectral shift at 265 nm when hydrolyzed to IMP with AMP deaminase (11) based on the method developed by Kalckar (12). In addition to Stage I, the reaction contained 160 mM sodium succinate, pH 6.0, 10 mM $MgCl_2$, and 0.25 unit of AMP deaminase in a volume of 1 ml.

Assay 3—The AMP and NMNH[1] formed in Stage I were hydrolyzed to the respective nucleosides and inorganic orthophosphate, and the latter was determined colorimetrically. To Stage I, 1–2 units of calf intestinal alkaline phosphatase (EC 3.1.3.1) or 5′-nucleotidase (EC 3.1.3.5) were added and incubated for an additional 30 min at 37 °C. Phosphate was determined by the method of Ames and Dubin (13) as modified by Bhatnagar *et al.* (14).

RESULTS

Gene Cloning—The *orf257* gene was cloned directly from chromosomal DNA isolated from strain MG1655 as described under "Methods." The DNA was inserted into the vector pET11d, and the region containing the insert was sequenced using standard procedures (Sequenase Version 2.0). The nucleotide sequence agreed with that submitted to GenBank™ (accession number U00006) and designated a protein containing 257 amino acids as shown in Fig. 1. The region of homology to the MutT protein and other MutT-like proteins spans amino acids 159–181, and all of the amino acids in the signature sequence identified by Koonin (5) are also present in this region of the Orf257 protein. These amino acids were used as the basis for the alignment with the MutT protein.

Protein Expression and Purification—Cultures of HMS174-

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MutT    ..... ..... ..... ..... .....
Orf257  MDRIIEKLDH GWWVVSHEQK LWLPKGELPY GERANFDLVG QRALQIGEWQ
        1          11         21         31         41

MutT    ..... ..... ..... ..... .....
Orf257  GEPVWLVQQQ RRHDMGSVRQ VIDLDVGLFQ LAGRGVQLAE FYRSHKYCGY
        51         61         71         81         91

MutT    ..... ..... .MKKLQIAVG IIRNENNEIF ITRRAADAHM
                       :|||:  ||         |        |
Orf257  CGHEMYPSKT EWAMLCSHCR ERYYPQIAPC IIVAIRRDDS ILLAQHTRHR
        101        111        121        131        141

MutT    ANKLEFPGGK IEMGETPEQA VVRELQEEVG ITPQHFSLFE KLEYEFPDRH
          ::  |    :|:|||:||| |:||: || |  | :  :  :      ||
Orf257  NGVHTVLAGF VEVGETLEQA VAREVMEESG IKVKNLRYVT SQPWPFPQSL
        151        161        171        181        191

MutT    ITLWFWLVER WEGEPWGKEG QPGEWMSLVG LNADDFPPAN EPVIAKLKRL
        :|:::: :      :  ||     |        | :  :|      ::    :
Orf257  MTAFMAEYDS GDIVIDPKEL LEANWYRYDD LPLLPPPGTV ARRLIEDTVA
        201        211        221        231        241

MutT    .......

Orf257  MCRAEYE
        251
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FIG. 1. **Alignment of *E. coli* MutT and *E. coli* dinucleotide pyrophosphatase amino acid sequences.** The amino acid sequences of the MutT and Orf257 proteins are aligned based on the conserved region shown in *boldface*. Identical amino acids are indicated with *connecting lines*, and similar amino acids (*i.e.* hydrophobic, polar, or charged) are noted with *colons*.

(DE3) cells containing pETorf257 were grown, harvested, extracted, and fractionated as described under "Methods." The overexpression of the Orf257 protein in this system is shown in Fig. 2*A* by the appearance of a new band at ~29 kDa after induction of the cells with isopropyl-β-D-thiogalactopyranoside. Also shown is an aliquot of the final stage in the purification, which appears >98% pure. The protein appears virtually free of contaminants on a native gel as well (Fig. 2*B*), and assays of slices from this gel indicated that the enzymatic activity coincided with the major protein band (data not shown). It was noted that the enzyme precipitates with the nucleic acids during the streptomycin fractionation. This may indicate that the enzyme binds to DNA or RNA, although no subsequent evidence of nucleic acid involvement such as stimulation or inhibition of activity was observed in the presence of single- or double-stranded DNA or RNA. The protein eluted from the gel filtration column in Fraction V earlier than would be expected for a 29-kDa protein. A second gel filtration column was calibrated with molecular weight standards, and the Orf257 protein appeared at a region of ~60 kDa. This could mean that Orf257 exists as a homodimer in solution, although further physicochemical techniques would be necessary to substantiate this possibility.

Identification of Enzymatic Activity—We initially thought that the Orf257 protein would have a nucleoside-triphosphate pyrophosphohydrolase activity similar to that of all other members of the MutT family of proteins that have been characterized (3, 4, 15). No such activity was detected for the Orf257 protein using the eight canonical (deoxy)nucleoside triphosphates as substrates. To test the possibility that the protein was missing a pyrophosphate acceptor or a cofactor, we set up a coupled lactate dehydrogenase/pyruvate kinase/adenylate kinase assay to test for a substrate-dependent formation of ADP or AMP by monitoring NADH oxidation at 340 nm. All fractions containing the Orf257 protein were found to contain an adenylate kinase-dependent (AMP-producing) activity, and furthermore, this activity was later observed even when ATP was absent from the assay mixture. The only other direct source of AMP in the system was NADH. Accordingly, a reaction mixture containing only NADH, $MgCl_2$, and the Orf257 protein was analyzed by paper electrophoresis in 25 mM citrate at pH 4.9 as

[1] The abbreviations used are: NMNH, reduced nicotinamide mononucleotide; AppA, di(adenosine 5′)-P^1,P^2-pyrophosphate; ApppA, di(adenosine 5′)-P^1,P^3-triphosphate; AppppA, di(adenosine 5′)-P^1,P^4-tetraphosphate.

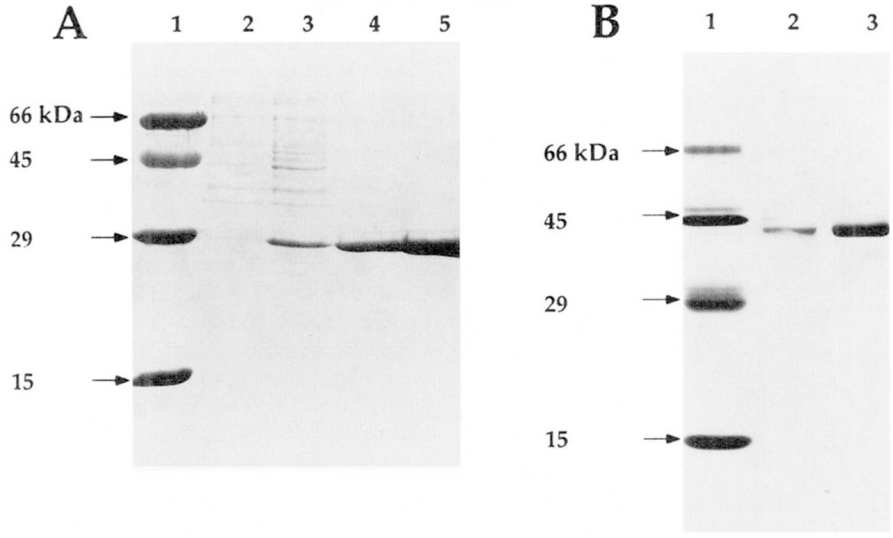


FIG. 2. **Expression and purification of the Orf257 protein.** *A*, a 15% SDS-polyacrylamide gel stained with Coomassie Blue. *Lane 1* contains 4 μg each of bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and MutT (15 kDa). *Lanes 2* and *3* show the expression of the Orf257 protein. HMS174(DE3) cells containing the plasmid pETorf257 were grown in LB/ampicillin media as described under "Methods." Cells (1.5 ml) were harvested before (*lane 2*) and 2 h after (*lane 3*) isopropyl-β-D-thiogalactopyranoside addition. The A_{600}/ml was determined for each aliquot, and the cells were boiled in 400 μl of SDS loading buffer/A_{600} unit. *Lane 2* contains 20 μl of the preinduced cell extract. *Lane 3* contains 20 μl of the induced cell extract. *Lanes 4* and *5* contain 2 and 6 μg of the purified protein (Fraction V), respectively. *B*, a 15% native polyacrylamide gel stained with Coomassie Blue. *Lane 1* contains the same standards as *lane 1* in *A*. *Lanes 2* and *3* contains 2 and 6 μg of Fraction V, respectively.

described by Markham and Smith (16). The products, visualized with UV light, comigrated with authentic AMP and NMNH, respectively. The AMP product was confirmed by its reactivity with adenylate deaminase, which is highly specific for this nucleotide (11). The adenylate deaminase assay and the phosphate assay described under "Methods" were used to measure the stoichiometry of the reaction as shown in Fig. 3. No phosphate was released upon digestion of NADH with the Orf257 protein alone. Upon subsequent addition of alkaline phosphatase, 2 mol of phosphate were detected for each mole of AMP formed (with the second mole of P_i coming from NMNH).

The pH optimum for the reaction was 8.5 (data not shown). Both MutT (4) and the MutT-like enzyme Orf17[2] also have alkaline pH optima. Although phosphate buffer inhibited the reaction slightly (20%), dithiothreitol, DNA, monovalent cations, pyrophosphate, and EDTA (at concentrations significantly lower than metal concentration) had no effect on activity. The lack of inhibition or stimulation by pyrophosphate clearly distinguished the enzyme from the reversible NAD^+ pyrophosphorylase (EC 2.7.7.1) (17). Only weak product inhibition was observed for NMNH, which indicates that the reaction is not freely reversible. A double reciprocal analysis of four titrations with NADH (0.2–1.0 mM) in the presence of various concentrations of NMNH (0–2.1 mM) suggested that NMNH is a linear competitive inhibitor, with a K_i of 4.2 mM. No inhibition was observed for the oxidized NMN^+ even at 10-fold higher inhibitor concentrations (data not shown).

Substrate Specificity—Several nucleotide pyrophosphates were tested as substrates, and their relative activities are shown in Table I. All dinucleotides were β-isomers because the α-isomer of NAD^+ was not a substrate for the enzyme. Reactions were done at 37 °C in 50 mM Tris-Cl, 20 mM $MgCl_2$, 2 mM each substrate, and 0.2–20 μg (1.5–150 milliunits) of the Orf257 protein. Because initial velocity is approximately equal to the V_{max} for NADH under these conditions, the values reported represent the specific activities of each substrate relative to that of NADH. The data in Table I were obtained using

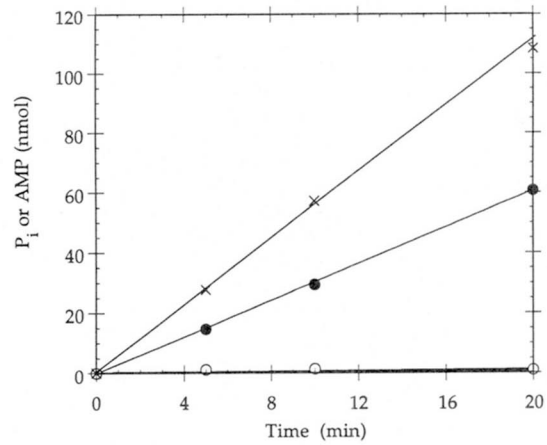


FIG. 3. **Products of the *E. coli* dinucleotide pyrophosphatase.** Fraction V (3.0 milliunits) was incubated with 1 mM NADH, 20 mM $MgCl_2$, and 50 mM Tris-Cl, pH 8.5, at 37 °C. The reaction was terminated by boiling, and aliquots were used for the determination of AMP (●), inorganic orthophosphate (○), and phosphatase-labile phosphate (×) as described under "Methods."

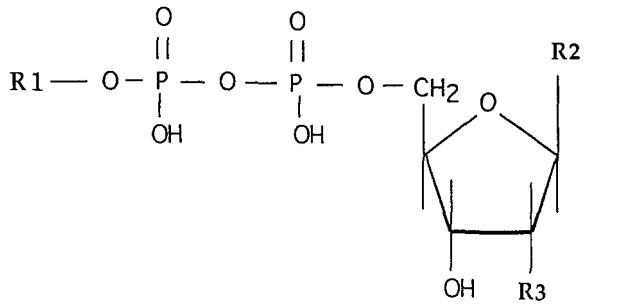
Assay 3 (phosphatase-labile P_i). In each case where AMP was a potential product, Assay 2 was used also, as confirmatory evidence, with essentially identical results (data not shown).

The enzyme was more active on NADH than on any other substrate tested, and in each case involving the pyridine nucleotides, the reduced substrate was hydrolyzed more rapidly than its respective oxidized form. Several other nucleotides with related structural features showed varying degrees of reactivity. However, it is especially noteworthy that none of the 16 canonical (deoxy)nucleoside di- and triphosphates were hydrolyzed. This is in striking contrast to the other enzymes of the family (MutT, MutX, Orf17, and human 8-oxo-dGTPase), which all show a marked preference for deoxynucleoside triphosphates. Certain patterns emerge in comparing the reactivity of several of the other substrates. For example, the rate of hydrolysis decreases as the length of the polyphosphate bridge increases in the Ap_nA series. Furthermore, a second

[2] D. N. Frick and M. J. Bessman, unpublished results.

TABLE I
Specificity of E. coli orf257 NADH pyrophosphatase

The specific activity of the enzyme on each of the nucleotides listed below was measured using Assay 3. Reactions were done at 37 °C in 50 mM Tris-Cl, pH 8.5, 20 mM $MgCl_2$, 2 mM each substrate, and 1.5–150 milliunits of the Orf257 protein (Fraction V). The specific activity with each substrate is expressed relative to the specific activity with NADH under the same conditions.



Substrate	R1	R2	R3	Relative specific activity
NADH	Nam-ribose-red[a]	Ade	OH	100
NAD^+	Nam-ribose-ox	Ade	OH	17
Deamino-NADH	Nam-ribose-red	Hyp	OH	97
Deamino-NAD^+	Nam-ribose-ox	Hyp	OH	28
NADPH	Nam-ribose-red	Ade	PO_4H_2	39
$NADP^+$	Nam-ribose-ox	Ade	PO_4H_2	5
FAD	Riboflavin	Ade	OH	22
AppA	Ado	Ade	OH	52
ApppA	AMP	Ade	OH	25
AppppA	ADP	Ade	OH	13
ADP-ribose	Ribose	Ade	OH	33
ADP-glucose	Glc	Ade	OH	4.5
UDP-glucose	Glc	Ura	OH	<1.0
(d)NTP	PO_3H_2	A,T,G, or C	H or OH	<0.1
(d)NDP	H	A,T,G, or C	H or OH	<0.1

[a] Nam-ribose-red, nicotinamide riboside, reduced form; Nam-ribose-ox, nicotinamide riboside, oxidized form.

nucleotide is essential for optimal activity. The elimination of the nicotinamide group (ADP-ribose) causes a 3-fold reduction in rate, and the replacement of the ribose with a glucose (ADP-glucose) causes a further 7-fold decrease. The enzyme has no detectable activity under these conditions toward UDP-glucose in which neither the adenine nor the nicotinamide groups are retained. These observations focus attention on structural features of the substrates that correlate with their reactivity. However, caution should be exercised in overinterpreting the observations made on this small group of compounds examined under one set of reaction conditions.

Several of the substrates showing high relative rates (in Table I) were subjected to a more detailed kinetic analysis. Double reciprocal (Lineweaver-Burk) analysis (18) and nonlinear least-squares fitting weighted to substrate concentration (19) were used to determine the K_m and V_{max} values of the substrates tested. Table II lists the K_m, V_{max}, and catalytic efficiency (V_{max}/K_m) of various substrates, which again demonstrate that NADH is the most favored substrate for the Orf257 protein identified so far. The replacement of the adenine moiety with hypoxanthine affects the K_m or V_{max} slightly (<3-fold). However, changes on the nicotinamide nucleotide have much larger effects on both the K_m and V_{max}. The substitution of the nicotinamide group with adenine (AppA) or the elimination of the base entirely (ADP-ribose) causes an order of magnitude decrease in V_{max}/K_m, and the introduction of a positive charge on the nicotinamide is associated with a decrease in V_{max}/K_m by over 2 orders of magnitude.

The striking preference for NADH over NAD^+, which may be related to the physiological function of the Orf257 protein, is clearly demonstrated in Fig. 4. For NADH, the V_{max} (7.6 units mg^{-1}) corresponds to a turnover rate of 3.8/s based on two active sites/dimer. This is similar to the k_{cat} for the MutT enzyme (14, 20).

Metal Requirement—The AMP deaminase assay (Assay 2) was used to explore the metal ion requirement of the enzyme. The chlorides of Mg^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Fe^{2+}, Cu^{2+}, and Ca^{2+} at concentrations from 0.01 to 10 mM were compared, and at optimal concentrations of each metal, Zn^{2+}, Co^{2+}, and Fe^{2+} gave 10, 8, and 8% of the activity of Mg^{2+}, respectively, whereas Cu^{2+} and Ca^{2+} supported no detectable hydrolysis. On the other hand, Mn^{2+}, as shown below, supported more than twice the rate of hydrolysis compared with Mg^{2+} at optimal concentrations. Unlike other dinucleotide pyrophosphatases (21), Co^{2+} was not as effective as Mg^{2+}, and it did not stimulate the enzyme when included with either Mn^{2+} or Mg^{2+}.

Fig. 5 shows a titration of 1 mM NADH with $MnCl_2$ or $MgCl_2$. Double reciprocal analysis (Fig. 5*B*) yields an apparent V_{max} for the Mn^{2+}-activated reaction of 22 units/mg with an apparent $K_m(Mn^{2+})$ of 30 μM and an apparent V_{max} for the Mg^{2+}-activated reaction of 8.8 units/mg with an apparent $K_m(Mg^{2+})$ of 3.7 mM. This indicates that at low metal concentrations, Mn^{2+} has a 310-fold higher V_{max}/K_m (metal) than Mg^{2+}. However, due to the inhibitory effects of Mn^{2+}, the two metals support roughly equal velocities at high metal concentrations.

DISCUSSION

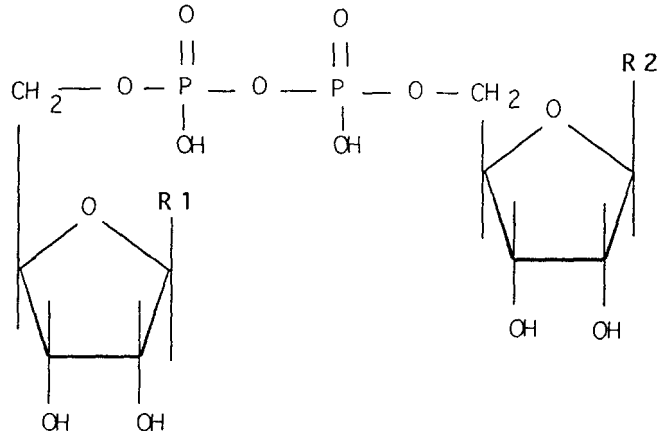
Since the purification and characterization of NAD^+ pyrophosphatase (EC 3.6.1.22) from potato extracts were described by Kornberg and Pricer (22), enzymes cleaving NAD^+ into NMN^+ and AMP have been reported in a large variety of plants, animals, and bacteria. The relatedness of these enzymes has been difficult to ascertain because few have been purified, and most have a broad spectrum of activities not only to NAD^+ and $NADP^+$, but also to FAD and ADP-ribose and, in some cases, to ATP, ADP, and the family of bis(5′-nucleosidyl) oligophosphates as represented by, for example, diadenosine tetraphosphate (AppppA). However, only one other enzymatic activity has been reported that cleaves NADH at a higher rate than NAD^+, as is the case with the enzyme described in this paper. Jacobson and Kaplan (23) partially purified a dinucleotide pyrophosphatase from pigeon liver extracts that hydrolyzed NADH at a higher rate than NAD^+, but their enzyme is clearly distinguished from the *E. coli* enzyme by several criteria including its activity on α-NAD^+ and its preference for ADP-ribose, which it hydrolyzes at 200% of the rate of NADH. The *E. coli* enzyme is not active on α-NAD^+, and its V_{max}/K_m for NADH is 20-fold higher than for ADP-ribose. Further comparisons of the *E. coli* enzyme described in this paper with previously reported related activities is complicated by the fact that the earlier work was done prior to the development of techniques for gene analysis.

Of what use to the cell is an enzyme that hydrolyzes and essentially inactivates an important cofactor in cellular metabolism? One possibility is that NMNH plays some undiscovered role in the cell. The only way known, so far, of generating NMNH is through the hydrolysis of NADH by this enzyme. A second, more general role of the enzyme could be the regulation of the intracellular NADH/NAD^+ ratio, which is known to be an important factor in maintaining a balance between anabolic and catabolic pathways in higher organisms. Oxidoreduction reactions in which NAD^+ and NADH participate as cofactors are, in most cases, freely reversible, and the selective removal of NADH would favor the oxidative pathway especially under anaerobic conditions, where the reoxidation of NADH would be

TABLE II

Kinetic analysis of the specificity of the E. coli NADH pyrophosphatase

For each substrate, initial velocities of dinucleotide hydrolysis were measured in the presence of 20 mM $MgCl_2$ and 50 mM Tris-Cl, pH 8.5, using Assay 3 described under "Methods." Initial velocities were measured at seven NADH concentrations ranging from 0.04 to 2.0 mM, at seven NAD^+ concentrations ranging from 0.2 to 10 mM, at six deamino-NADH concentrations ranging from 0.081 to 1.21 mM, at six deamino-NAD^+ concentrations ranging from 0.2 to 5.0 mM, at five AppA concentrations ranging from 0.1 to 5.8 mM, and at seven ADP-ribose concentrations ranging from 0.1 to 5 mM. K_m and V_{max} values were obtained by nonlinear regression weighted to substrate concentrations (19) using initial estimates obtained from Lineweaver-Burk analyses (18).



Substrate	R1	R2	V_{max}	K_m	V_{max}/K_m
			units mg^{-1}	*mM*	*units* mg^{-1} *mM* $^{-1}$
NADH	Nam-red[a]	Ade	7.6 ± 0.4	0.11 ± 0.02	69 ± 12
NAD^+	Nam-ox	Ade	2.9 ± 0.1	5.1 ± 0.5	0.57 ± 0.5
Deamino-NADH	Nam-red	Hyp	8.9 ± 0.5	0.29 ± 0.05	31 ± 5
Deamino-NAD^+	Nam-ox	Hyp	3.2 ± 0.1	2.6 ± 0.2	1.2 ± 0.1
AppA	Ade	Ade	4.7 ± 0.1	0.67 ± 0.07	7.0 ± 0.7
ADP-ribose	H	Ade	4.8 ± 0.2	1.8 ± 0.14	2.7 ± 0.2

[a] Nam-red, nicotinamide, reduced form; Nam-ox, nicotinamide, oxidized form.

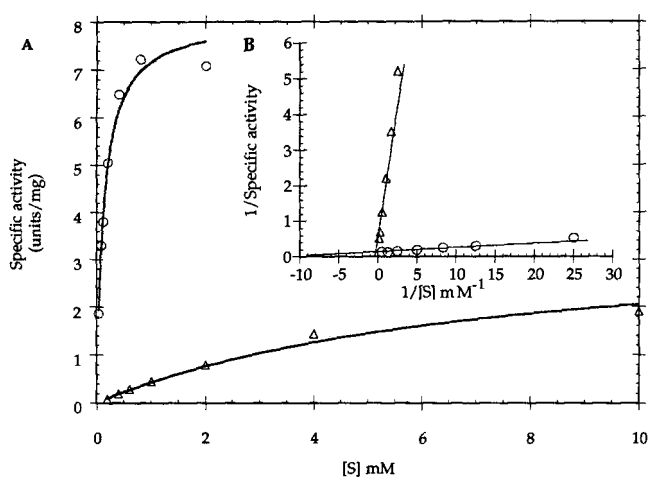


FIG. 4. **Preference of the *E. coli* dinucleotide pyrophosphatase for the reduced form of NAD.** *A*, initial velocity *versus* substrate concentration for NADH (○) and NAD^+ (△). Reactions (50 μl) contained 20 mM $MgCl_2$, 50 mM Tris-Cl, pH 8.5, and 0.5 milliunit of enzyme (Fraction V) for NADH or 3.7 milliunits of enzyme (Fraction V) for NAD^+. Reactions were terminated by boiling and analyzed by Assay 3 (see "Methods"). Curves are fit using nonlinear least-squares analyses (19). *B*, double reciprocal plot of the data in *A*.

diminished. It is interesting that Jacobson and Kaplan (23), in their early work, reconstructed a system in which the addition of partially purified pigeon liver NADH pyrophosphatase increased the rate and the extent of formation of acetaldehyde from ethanol by purified alcohol dehydrogenase. Their interpretation was that the selective hydrolysis of NADH shifted the equilibrium in favor of the oxidized substrate. Thus, the NADH pyrophosphatase could substitute for an electron acceptor and influence the equilibrium of a metabolic pathway under a specific set of circumstances. We are constructing a null mutant devoid of the enzyme in order to uncover its functional significance. Clinical interest has been generated by the report (24, 25) that Lowe's syndrome, a genetic disease with pleiotropic sequelae, is tied to an overproduction of a dinucleotide pyrophosphatase.

Our major interest in the enzyme is that it shares a signature sequence, $GXU(X)_3ET(X)_6REUXEE$ (where U represents the bulky aliphatic residue L, I, or V), with four other proteins that we have partially characterized and that all have nucleoside-triphosphate hydrolase activities producing nucleoside monophosphates and inorganic pyrophosphate (1, 4, 15). Sakumi *et al.* (3) have described an 8-oxo-dGTPase activity from human cells that also has the same amino acid motif. Besides this small region of identity, the six proteins (including the enzyme described in this paper) share very little similarity. Our analyses of site-directed mutations in MutT implicate this small region, common to all the proteins, as being important in the enzymatic activity of the enzymes (1, 26), and NMR structural analysis of MutT has revealed that this region of the protein is involved in nucleotide binding (27). Four of the six proteins from *E. coli*, *S. pneumoniae*, *P. vulgaris*, and human cells (1–3, 15) prevent mutations caused by AT → CG transversions. A fifth protein, coded for by *E. coli orf17*, has not, as yet, been shown to prevent mutations, but it is a nucleoside triphosphatase with a preference for dATP (4). These five proteins, which only have this small signature sequence in common, catalyze the hydrolysis of nucleoside triphosphates according to the following scheme: (deoxy)nucleoside triphosphate → (deoxy)nucleoside monophosphate + PP_i. Until we discovered the NADH pyrophosphatase described in this report, we believed that the signature sequence designated a

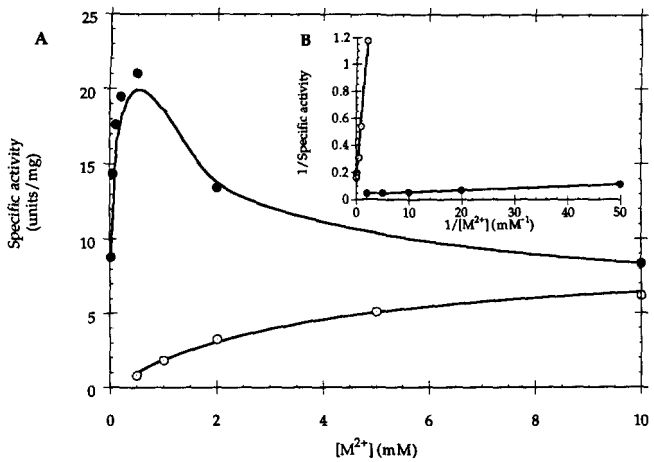


FIG. 5. **Activation of the *E. coli* dinucleotide pyrophosphatase by divalent metal cations.** *A*, initial velocity *versus* concentration of $MgCl_2$ (○) and $MnCl_2$ (●). Reactions (100 μl) were run at 37 °C with 1 mM NADH, 50 mM Tris-Cl, pH 7.5, with 0.73 milliunit of enzyme (Fraction V). Reactions were terminated by boiling, and AMP was measured in each using Assay 2. *B*, double reciprocal plot of the data in *A*, fit by least-squares analysis weighted to substrate concentration (19), excluding points above 1 mM $MnCl_2$.

catalytic site specific for the attack on the *beta*-phosphate of a nucleoside triphosphate with the elimination of pyrophosphate (28). Because NADH pyrophosphatase catalyzes the cleavage of NADH → NMNH + AMP, we believe that this signature sequence, which in the MutT protein forms a loop-helix-loop motif (27) not seen in other nucleotide-binding sites (29), has been conserved during evolution and adapted to participate in different metabolic reactions involving the cleavage of a nucleotide pyrophosphate bond. Computer searches of the sequence data banks (1, 5) have revealed several other genes of unknown function specifying the same amino acid motif in a variety of organisms ranging from viruses to eucaryotes. We are in the process of cloning, expressing, and identifying enzyme activities associated with these genes. We are also determining the three-dimensional solution structure (27) and crystal structure[3] of some of these enzymes. These approaches should lead to an understanding of the basic biochemistry associated with the conserved amino acid motif and perhaps identify functions for the other genes containing sequences that specify this arrangement of amino acids.

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