Cloning, Purification, and Properties of a Novel NADH Pyrophosphatase

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

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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 (Mᵋ = 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 NADPH. The $V_{\text{max}}/K_m$ for NADPH (69 μmol min⁻¹ mg⁻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 Mg²⁺ and Mn²⁺. Although these metals support similar $V_{\text{max}}$ values at optimal metal concentration, the apparent $K_m$ for Mg²⁺ is 8.7 mM (at 1 mM NADH), whereas the apparent $K_m$ for Mn²⁺ 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 thiC and hemeE 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.

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 Ncol and BamHI restriction sites and to amplify the orf257 gene. The 790-base pair product was purified from agarose gel, digested with Ncol and BamHI (Stratagene, La Jolla, CA), repurified, and ligated into the Ncol and BamHI 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 A₅₉₀ of 0.5, they were collected by centrifugation, washed with 0.9% NaCl, and transferred to 100 ml of fresh medium containing ampicillin. This was repeated after the cells again reached an A₅₉₀ of 0.5, and the cells were then transferred to 2 liters of fresh medium. When the cells again reached an A₅₉₀ 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). Streptomyces 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 (25 × 24 cm) of DEAE-Sepharose (Pharmacia Biotech Inc.) and washed

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with 400 ml of 50 mM Tris-Cl, pH 7.5, 0.1 mM diithiothreitol (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 12 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. Anne (Sephadex G-100, 2.5 × 60 cm) and eluted with 50 mM Tris-Cl, 1 mM EDTA, 0.1 mM diithiothreitol, 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 substrates of the enzyme were discovered, the activity was quantitated by three assays, all based on the measurement of the AMP formed in Reaction 1.

\[
\text{Mg}^{2+} \quad \text{NADH} \quad \text{NMNH} + \text{AMP}
\]

**Orf257**

**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 MgCl₂, and 0.33–3.33 milliliters 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 MgCl₂, 4 mM phosphoenolpyruvate, 0.4 mM NADH, 19 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 × 10²⁻¹⁶ cm⁻¹ for ε₉₅ 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 Kallakur (12). In addition to Stage I, the reaction contained 160 mM sodium succinate, pH 6.0, 10 mM MgCl₂, and 0.25 unit of AMP deaminase in a volume of 1 ml.

**Assay 3—**The AMP and NMNH 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 Bhattachary (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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1 The abbreviations used are: NMNH, reduced nicotinamide mononucleotide; AppA, dideoxinosine 5'-P,5'-pyrophosphate; ApppA, dideoxynosine 5'-P,5'-triphosphate; AppppA, dideoxynosine 5'-P,5',5'-tetraphosphate.

**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. 24 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. 2B), 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 pyrophosphatase 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 mix. The only other direct source of AMP in the system was NADH. Accordingly, a reaction mixture containing only NADH, MgCl₂, and the Orf257 protein was analyzed by paper electrophoresis in 25 mM citrate at pH 4.9 as
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 phosphatase 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, coming from NMNH).

The pH optimum for the reaction was 8.5 (data not shown). Both MutT (4) and the MutT-like enzyme Orf17 also have alkaline pH optima. Although phosphate buffer inhibited the reaction slightly (20%), diithiothreitol, DNA, monovalent cations, pyrophosphatase, and EDTA (at concentrations significantly lower than metal concentration) had no effect on activity. The lack of inhibition or stimulation by pyrophosphatase 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 pyrophosphatases were tested as substrates, and their relative activities are shown in Table I. All dinucleotides were \(\beta\)-isomers because the \(\alpha\)-isomer of NAD\(^+\) was not a substrate for the enzyme. Reactions were done at 37 \(^\circ\)C in 50 mM Tris-Cl, 20 mM MgCl\(_2\), 2 mM each substrate, and 0.2–20 \(\mu\)g (1.5–150 milliunits) of the Orf257 protein. Because initial velocity was approximately equal to \(V_{\max}\) 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

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2 D. N. Frick and M. J. Bessman, unpublished results.
TABLE I

<table>
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<tr>
<th>Substrate</th>
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<td>Ura</td>
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<td>(d)NDP</td>
<td>H</td>
<td>A.T.G, or C.H or OH</td>
<td>&lt;0.1</td>
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</table>

* Nam-ribose-red, nicotinamide riboside, reduced form; Nam-ribose-ox, nicotinamide riboside, oxidized form.

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 MgCl₂, 2 mM each substrate, and 1.5-150 millimicrons 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.

**Metal Requirement**—The AMP deaminase assay (Assay 2) was used to explore the metal ion requirement of the enzyme. The chlorides of Mg²⁺, Mn²⁺, Zn²⁺, Cu²⁺, Fe²⁺, Cu²⁺, and Ca²⁺ at concentrations from 0.01 to 10 mM were compared, and at optimal concentrations of each metal, Mn²⁺, Zn²⁺, and Fe²⁺ gave 10, 8, and 8% of the activity of Mg²⁺, respectively, whereas Cu²⁺ and Ca²⁺ supported no detectable hydrolysis. On the other hand, Mn²⁺, as shown below, supported more than twice the rate of hydrolysis compared to Mg²⁺ at optimal concentrations. Unlike other dinucleotide pyrophosphatases (21), Cu²⁺ was not as effective as Mg²⁺, and it did not stimulate the enzyme when included with either Mn²⁺ or Mg²⁺.

Fig. 5 shows a titration of 1 mM NADH with MnCl₂ or MgCl₂. Double reciprocal analysis (Fig. 5B) yields an apparent $V_{\text{max}}$ for the Mn²⁺-activated reaction of 22 units/mg with an apparent $K_m$(Mn²⁺) of 30 μM and an apparent $V_{\text{max}}$ for the Mg²⁺-activated reaction of 8.8 units/mg with an apparent $K_m$(Mg²⁺) of 3.7 mM. This indicates that at low metal concentrations, Mn²⁺ has a 310-fold higher $V_{\text{max}}/K_m$ (metal) than Mg²⁺. However, due to the inhibitory effects of Mn²⁺, the two metals support roughly equal velocities at high metal concentrations.

**DISCUSSION**

Since the purification and characterization of NAD⁺ pyrophosphatase (EC 3.6.1.122) from potato extracts were described by Kornberg and Price (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 $V_{\text{max}}/K_m$ 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 MgCl₂ 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 5.0 mM, and at seven ADP-ribose concentrations ranging from 0.1 to 5.0 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).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>R1</th>
<th>R2</th>
<th>( V_{max} ) units mg⁻¹</th>
<th>( K_m ) max</th>
<th>( V_{max}/K_m ) units mg⁻¹ mM⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td>Nam-red*</td>
<td>Ade</td>
<td>7.6 ± 0.4</td>
<td>0.11 ± 0.02</td>
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<td>NAD⁺</td>
<td>Nam-ox</td>
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<td>0.57 ± 0.05</td>
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<tr>
<td>Deamino-NADH</td>
<td>Nam-red</td>
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<td>Deamino-NAD⁺</td>
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<td>Hyp</td>
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<tr>
<td>AppA</td>
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<td>ADP-ribose</td>
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<td>4.8 ± 0.2</td>
<td>1.8 ± 0.14</td>
<td>2.7 ± 0.2</td>
</tr>
</tbody>
</table>

* Nam-red, nicotinamide, reduced form; Nam-ox, nicotinamide, oxidized form.

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**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 MgCl₂, 50 mM Tris-Cl, pH 8.5, and 0.6 millilunit of enzyme (Fraction V) for NADH or 3.7 millilunits 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.**

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...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(X₃)₃ET(X₃)₃REUXEE (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 implicated 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 + PP₂. Until we discovered the NADH pyrophosphatase described in this report, we believed that the signature sequence designated a...
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 MuT 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 nucleoside 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 structure3 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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3 S. Quirk and M. J. Bessman, unpublished results.