Identification and Characterization of the Nudix Hydrolase from the Archaeon, *Methanococcus jannaschii*, as a Highly Specific ADP-ribose Pyrophosphatase*

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The *MJ1149* gene from the Archaeon, *Methanococcus jannaschii*, has been cloned and expressed in *Escherichia coli*. The 19-kDa protein containing the Nudix box, GX\_EX\_REUXEXGU, has been purified and identified as a highly specific enzyme catalyzing the Mg\(^{2+}\)-dependent hydrolysis of ADP-ribose according to the equation: ADP-ribose + H\(_2\)O → AMP + ribose-5-phosphate. The enzyme retains full activity when heated to 80 °C, and the rate of hydrolysis is 15-fold higher at 75 °C than at 37 °C in keeping with the thermophilicity of the organism. This is the first Nudix hydrolase identified from the Archaea, indicating that the family of enzymes containing the Nudix signature sequence is represented in all three kingdoms.

The Nudix family of proteins is widely distributed in nature and is structurally related by a highly conserved amino acid signature sequence designated the “Nudix” box: GX\_EX\_REUXEXGU, where U is one of the bulky, hydrophobic amino acids I, L, or V (1). In addition to this common structural feature, those proteins of the family characterized as enzymes, all catalyze the hydrolysis of nucleoside diphosphates, linked to some other moiety x, hence the acronym “Nudix.” BLAST searches (2) of the data banks have uncovered over 130 putative proteins containing the Nudix box, and we have been systematically cloning the genes into expression vectors and attempting to identify, purify and then characterize the enzymes related to these genes. The Nudix family is well represented in *Eukaryota* and *Prokaryota*, and we have proposed that the physiological function of these enzymes is to sanitize the cell by removing potentially toxic nucleoside diphosphate derivatives or to modulate the accumulation of these derivatives during intermediary metabolism (1).

In this paper, we report the cloning, purification, and characterization of a highly specific ADP-ribose pyrophosphatase expressed from a gene in the archaeabacterium, *Methanococcus jannaschii*. This report is the first example of a Nudix hydrolase present in the Archaea. It documents the conservation of these enzymes in all three kingdoms, and it attests to the primordial origin of the Nudix hydrolase family.

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**Experimental Procedures**

**Materials**

Common biochemicals were obtained from Sigma unless otherwise noted. Oligonucleotide primers used in the polymerase chain reaction were from Integrated DNA Technologies (Coralville, Iowa). Calf intestinal alkaline phosphatase was from Stratagene, human semen phosphatase was from Sigma, and enzymes used in standard cloning procedures were from Life Technologies, Inc., Stratagene, and United States Biochemicals.

**Methods**

Cloning—*MJ1149*, the gene for ADP-ribose hydrolase in *M. jannaschii* (GenBank™ accession number D6445) was amplified from the construct, AMJAM72, obtained from the American Type Culture Collection. Oligonucleotide primers containing restriction sites NdeI (start site) and BamHI (end site) were used to amplify the gene by the polymerase chain reaction. The amplified gene was purified and digested with NdeI and BamHI using the standard protocol as outlined by Sambrook et al. (4), and ligated into pET11b under control of a T7 lac promoter. The resultant plasmid, pMJ1149, was transformed into HB101 cells for storage and into BL21 (DE3) cells for expression.

**Purification of the Enzyme**—Cells were grown at 37 °C in LB medium to an *A*\(_{600}\) of about 0.6 and induced by the addition of isopropyl-β-D-thiogalactopyranoside to a concentration of 1 mM. The cells were allowed to grow for an additional 2 h, harvested, washed, with an isotonic saline solution, and stored at −80 °C. All subsequent steps were conducted at 0–4 °C. To extract the protein, the cells were resuspended in 3 volumes of buffer A (50 mM Tris-Cl, pH 7.5, 1 mM EDTA, 0.1 mM dithiothreitol) and sonicated in a Branson sonifier. The crude extract was centrifuged to remove cellular debris, and the supernatant was adjusted to a protein concentration of 10 mg/ml with buffer A (Fraction I).

To Fraction I (100 ml), 10% streptomycin sulfate prepared in buffer A was slowly added with continuous stirring to a final concentration of 1%. After 20 min, the precipitate containing the enzyme was collected by centrifugation and resuspended in 100 ml of buffer A containing 1 mM ammonium sulfate (Fraction II).

Fraction II was loaded on a column of DEAE-Sepharose (2.5 × 24 cm) equilibrated with buffer A. Most proteins including ADP-ribose pyrophosphatase were eluted by washing the column with buffer A, whereas nucleic acids were retained on the column. Fractions containing the enzyme were pooled and dialyzed against buffer A (Fraction III).

To Fraction III, 15 ml of 0.2 N acetic acid was slowly added with continuous stirring. The precipitate containing the enzyme activity was collected by centrifugation and resuspended in 20 ml of buffer A (Fraction IV).

Fraction IV was applied to a hydroxylapatite column (1.5 × 10 cm) equilibrated with buffer A and eluted with a 400-mM linear gradient from 0–2 mM ammonium sulfate in buffer A. The fractions containing enzyme were pooled and concentrated by pressure filtration (Fraction V).

Fraction V was again loaded onto a DEAE-Sepharose column (1.5 × 10 cm) to remove a trace amount of residual nucleic acids. The enzyme in the flow-through fractions was pooled, dialyzed, and concentrated as above (Fraction VI). All studies reported in this paper were done with this fraction.

**Enzyme Assay**—Activity was measured in a 50-μl reaction mixture containing 50 mM Tris-Cl, pH 8.0, 50 mM MgCl\(_2\), 1 mM dithiothreitol, 2
mm ADP-ribose, 10 units of calf intestinal alkaline phosphatase, and 0.2–2 milliunits of enzyme. The reactants were incubated at 37 °C for 15 min, terminated by the addition of 250 μl of 20 mM EDTA, and the inorganic orthophosphate produced was quantitated by the colorimetric assay of Ames and Dubin (5).

**Enzyme Assay 2**—The standard reaction mixture contained 50 mM Tris-Cl, pH 8.0, 50 mM MgCl2, 1 mM dithiothreitol, 2 mM ADP-ribose, and 0.5–3 milliunits of enzyme. The reactants were incubated at 37 °C for 15 min and terminated by the addition of 50 μl of a mixture of four parts of Norit (20% packed volume) and one part of 7% HClO4. After centrifugation, 50-μl aliquots were withdrawn and added to 250 μl of 100 mM sodium acetate, pH 5.2. Semen phosphatase was added to hydrolyze the ribose-5-phosphate formed, and the mixture was incubated an additional 15 min. The liberated phosphate was determined as described above. A unit of enzyme hydrolyzes 1 μmol of ADP-ribose/min in either assay.

**Product Determination**—The standard assay mixture was scaled up 20-fold. At different time intervals, 100 μl were withdrawn and added to 500 μl of 20 mM EDTA, and an aliquot was analyzed on an high performance liquid chromatography column to determine the amount of ADP-ribose depleted and AMP formed. To another aliquot, alkaline phosphatase was added and incubated for 15 min, and the amount of Pi formed was determined.

**RESULTS**

**Cloning, Expression, and Purification**

The gene, MJ1149, from *M. jannaschii* was cloned into pET11b as described under "Methods," sequenced, and found to be in agreement with that reported by Bult et al. (6). MJ1149 is the only gene in the entire genome of *M. jannaschii* containing the Nudix box signature sequence, characteristic of hydrolases specific for derivatives of nucleoside diphosphates. Expression of pMJ1149 in *Escherichia coli* BL21(DE3) resulted in the appearance of a new band on an SDS-polyacrylamide gel not found for the parent vector, as shown in Fig. 1. Purification was carried out in several steps as outlined under “Methods.” It is interesting to note that the enzyme co-precipitates with streptomycin is used to remove nucleic acids from the crude extract. This could be because of the high content of basic amino acids in the protein (calculated pI of 9.4) interacting with the polynuclear nucleic acids. This association of the protein with nucleic acids during purification appears to be merely adventitious because addition of nucleic acids has no effect on the activity of the purified enzyme.

**Determination of Catalytic Activity**

From sequence alignments, Bult et al. (6) predicted that MJ1149 codes for a homolog of the *E. coli* MutT antimutator nucleoside triphosphatase (7, 8). This comparison is shown in Fig. 2. Accordingly, we tested the purified MJ1149 protein for nucleoside triphosphatase activity but found none. However, examination of other potential Nudix hydrolase substrates (1) uncovered a highly specific ADP-ribose pyrophosphatase activity.

**Properties of the Enzyme**

**Substrate Specificity**—A list of potential substrates for the enzyme is shown in Table I. The candidates tested represent cognate substrates of other members of the Nudix hydrolase family. Only ADP-ribose or its closely related derivative, 2’-phospho-ADP-ribose, are hydrolyzed to a significant extent. Notably absent is activity on the (deoxy)nucleoside triphosphates, characteristic of the *E. coli* MutT protein (8). The kinetic parameters for both substrates show no substantive differences between the two (Table II) and the K m values are similar to those of other characterized Nudix hydrolases (9–12).

**Products of the Reaction**—A scaled-up reaction mixture was incubated under the standard conditions of assay 2 (see "Methods"), and aliquots were removed at intervals and analyzed for remaining substrate and formation of products. Another aliquot was incubated with alkaline phosphatase to release Pi from the products. The data in Table III show that for each mole of ADP-ribose disappearing, 1 mole of AMP appears. Two moles of Pi are formed for each mole of ADP-ribose hydrolyzed when the products are treated with phosphatase. No inorganic phosphate is produced during the course of the reaction in the absence of alkaline phosphatase. Thus the stoichiometry of the reaction is: ADP-ribose + H2O → AMP + ribose-5-P.

**Fig. 2.** Amino acid sequence alignment of ADP-ribose pyrophosphatase with *E. coli* MutT. The amino acid sequence of ADP-ribose pyrophosphatase and *E. coli* MutT were aligned using Clustal W (30). To maximize homology, the program introduces gaps that are shown by dashed lines. The vertical lines represent identity, and the dots represent similarity. The boldface amino acids represent absolutely conserved amino acids in the Nudix box (see Introduction).
The enzyme is highly resistant to heat inactivation. Fig. 3 shows that it can be heated to 80 °C for 5 min and retain full activity. At 85°, it loses about 30% of its activity in 5 min. It is also highly active when the incubation is carried out at elevated temperatures. As shown in Fig. 3B, the rate of hydrolysis of ADP-ribose at 75 °C is 15-fold higher than at 37 °C. Incubations at higher temperatures were impractical because of the spontaneous hydrolysis of the substrate.

**DISCUSSION**

The identification of a Nudix hydrolase in *M. jannaschii*, a member of the Archaea, is an important extension of our systematic study of the characterization and distribution of this interesting family of enzymes. As a result of this work, Nudix hydrolases are now represented in all three kingdoms, and a recent BLAST search (2) shown in Fig. 4 reveals the diversity in nature, of putative proteins (open reading frames) containing the Nudix signature sequence. This amino acid motif first attracted our attention when it was found that only a small region of homology existed between two proteins, MutT (*E. coli*) and MutX (*Streptococcus pneumoniae*), both of which are involved in preventing mutations in their respective organisms and both are enzymes having dGTP pyrophosphohydrolase activity (13). An early search of the protein data banks against MutT and MutX revealed about a dozen sequences containing the conserved motif (14, 15) one of which we had already partly characterized as a dATP pyrophosphohydrolase (15). At that time, the small group of related open reading frames was referred to as the “MutT family”, and the highly conserved amino acid motif was considered the escutcheon of a class of enzymes having nucleoside triphosphate pyrophosphohydrolase activity (13). Shortly thereafter, we discovered another enzyme having the conserved sequence that had no activity on dGTP or any other nucleoside triphosphate, had no MutT antimutator activity, but instead was highly active on NADH (9).

Since that report, several other members of the family have

**TABLE I**

Activity of ADP-ribose hydrolase on various substrates

The activity of the enzyme was measured at 37 °C using assay 1 or 2 (see “Methods”). Each substrate was present at a concentration of 2 mM, and 0.5–3 milliunits of enzyme were used.

| Substrate                  | Specific activitya (units mg⁻¹) |
|----------------------------|--------------------------------|
| ADP-ribose                 | 5.5                            |
| pADP-ribose                | 4.0                            |
| cyclic ADP-ribose          | <0.1                           |
| ADP-glucose, GDP-glucose, TDP-glucose | <0.1                  |
| ADP-mannose, UDP-mannose   | <0.1                           |
| NAD, NADH, FAD             | <0.1                           |
| ApA, ApA, ApA, ApA         | <0.1                           |
| (deoxy)nucleoside triphosphates | <0.1               |
| (deoxy)nucleoside diphosphates | <0.1                |

a pADP-ribose, (2’)monophosphoadenosine5’diphosphoribose; ApA, adenine5’diphosphate5’adenosine. Other dinucleoside polyphosphates are abbreviated in an analogous manner.

A unit of activity is defined as 1 μmol of substrate hydrolyzed/min.

**TABLE II**

Kinetic analysis of ADP-ribose pyrophosphatase

Assay 2 (see “Methods”) was used to determine the kinetic constants with substrate concentrations of 0.05–3 mM. Km and Vmax values were calculated from non-linear regression analysis (31). A unit of enzyme hydrolyzes 1 μmol of substrate/min and Kcat was calculated from Vmax assuming 1 active site/monomer.

| Substrate       | Vmax  | Kcat | Km  | Kcat/Km |
|-----------------|-------|------|-----|---------|
|                 | units mg⁻¹ | s⁻¹ | mM  | s⁻¹     |
| ADP-ribose      | 6.2 ± 0.3 | 1.98 ± 0.08 | 0.34 ± 0.05 | 5820 |
| pADP-ribose     | 4.4 ± 0.2 | 1.40 ± 0.07 | 0.46 ± 0.08 | 3040 |

**TABLE III**

Stoichiometry of the ADP-ribose pyrophosphatase reaction

Incubation constituents for assay 2 (see “Methods”) were scaled up 20-fold, and aliquots were removed at the indicated time intervals. One aliquot was analyzed by HPLC for ADP-ribose remaining and AMP formed. The other aliquot was treated with phosphomonoesterase, and the Pi released was measured.

| Time (min) | ADP-ribose (nmol) | AMP (nmol) | ΔADP-ribose (mg⁻¹) | ΔAMP (mg⁻¹) | ΔPi/ΔADP-ribose (mg⁻¹) | ΔPi/ΔAMP (mg⁻¹) |
|------------|------------------|------------|-------------------|-------------|-----------------------|-----------------|
| 0          | 100              | 0          | 0                 | 0           | 0                     | 0               |
| 5          | 56               | 15         | -14               | +15         | +27                   | 1.9             |
| 10         | 73               | 30         | -27               | +30         | +55                   | 2.0             |
| 20         | 57               | 52         | -43               | +52         | +95                   | 2.2             |
| 30         | 46               | 57         | -54               | +57         | +114                  | 2.1             |

ΔADP-ribose, ΔAMP, and ΔPi represent the quantities of the respective moieties measured at the indicated time, minus the zero time values.
been characterized, including those that catalyze the hydrolysis of GDP-mannose (10), Ap₄A (16–18), Ap₃A, NADH, and ADP-ribose (12). Some of these are shown in Fig. 4, along with their favored substrates. Where tested, none of these other enzymes play a role in the mutT mutator phenotype. Thus the antimutator nucleoside triphosphatase of the original MutT protein represents only a small subset of the family of enzymes specified by the conserved amino acid motif. There is a nexus, however, to this varied group of substrates. As mentioned in the introduction, they are all derivatives of nucleoside diphosphates linked to some other moiety, and we have proposed the term Nudix hydrolase to designate this family. The observations reported in this paper provide another example of a putative protein miscataloged as a MutT homologue. The purified enzyme has no nucleoside triphosphate pyrophosphohydrolase activity, nor does the gene, MJ1149, complement mutT when introduced into E. coli (data not shown).

It is noteworthy that M. jannaschii does not appear to contain a MutT orthologue because the ADP-ribose pyrophosphatase is the only Nudix hydrolase predicted from its genome (6). Perhaps the ecological niche inhabited by this archaebacterium obviates the need for the MutT enzyme. The organism, isolated from a hydrothermal vent, a “white smoker” chimney at an ocean depth of about 1.5 miles, is an extreme anaerobe (19). If the mutagenic substrate of the MutT enzyme is 8-oxo-dGTP, as proposed by Maki and Sekiguchi (20), the oxidized nucleotide might be absent under these extreme anaerobic conditions, precluding the need for the enzyme. Fowler et al. (21) have reported that E. coli mutT has a normal spontaneous mutation frequency when grown anaerobically, in keeping with this notion. On the other hand, it is possible that another protein, without the Nudix box, substitutes for MutT or that characteristics of DNA replication under the unusual growth conditions of a thermal vent eliminate the need for a specialized enzyme to control the specific AT → CG mutation pathway (22). Genomes of other Archaea are being sequenced, and other putative Nudix hydrolases are emerging. Perhaps studies of these new candidates will help distinguish between the alternative possibilities.

Another interesting correlation is apparent between the habitat of the organism and the heat stability of the enzyme (Fig. 3). Because the optimal growth rate for M. jannaschii is at 85 °C (19), it is not surprising that the enzyme is stable and catalytically functional at these elevated temperatures. A recent publication by Villeret et al. (23) explains the heat stability of another archaeal enzyme on the basis of its quaternary structure. From crystallographic data, they showed that the ornithine carbamoyltransferase of Pyrococcus furiosis is a dodecamer composed of four trimers, and they suggest that oligomerization of subunits may be a strategy for conferring thermal stability. This might explain a puzzling observation we noted during the purification of the ADP-ribose pyrophosphohydrolase. The enzyme was eluted in the void volume of a Seph-

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1 S. F. O’Handley, C. A. Dunn, D. N. Frick, and M. J. Bessman, unpublished observations.
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axed G-100 gel filtration column, suggesting it was much larger than the 19-kDa protein we expected. At the time, we thought that this might represent nonspecific aggregation or interaction with contaminating nucleic acid, but it is quite possible in the light of this new observation that the thermal stability of the M. jannaschii enzyme is another example of oligomerization as that cited above.

The number of Nudix signature sequences uncovered in searches varies widely for different organisms and seems to be independent of genome size. For example, E. coli has about 11, Saccharomyces cerevisiae has 5, Caenorhabditis elegans has 5, Deinococcus radiodurans has more than 18, Arabidopsis has 2, and so far about 7 have been found in the human genome. Contrast this with the single Nudix hydrolase present in M. jannaschii. It might be tempting to speculate that the single Nudix hydrolase is a feature distinguishing Archaea from prokaryotes and eukaryotes, but at least one other archaeabacterium, M. thermoautotrophicum has 3, as yet uncharacterized.

We have proposed that the cellular function of the Nudix hydrolases is to remove potentially toxic or deleterious metabolites or to modulate the accumulation of metabolic intermediates by diverting them to alternate pathways in response to changes in cellular processes (1). ADP-ribose pyrophosphatase is an excellent representative, because it can fulfill both functions. Free ADP-ribose is a by product of the ubiquitous NAD+-linked ADP-ribosylation reactions implicated in a wide variety of cellular regulatory processes (for review, see Refs. 24 and 25). The hydrolysis of mono- or poly(ADP-ribose) from modified proteins results in free ADP-ribose, and also the large scale metabolic turnover of NAD+ itself contributes in part to the intracellular ADP-ribose pool (26). This free ADP-ribose can then be recycled by hydrolyzing it to AMP and ribose-5-phosphate. A more important role of the enzyme may be to remove the potentially hazardous ADP-ribose from the cytoplasmic pool. Because of its free aldehyde group, ADP-ribose is an excellent nonenzymatic glycating agent capable of derivatizing the lysines, cysteines, histidines, and N termini of proteins (27). These nonspecific ADP-ribosylation reactions would have detrimental effects on protein (enzyme) function, and they could confuse those recognition or regulatory systems based on specific ADP-ribosylations. For example, diphertheria toxin kills cells by catalyzing the ADP-ribosylation of EF-2, thereby shutting down protein synthesis (28). If this or similar reactions were to occur nonenzymatically because of the accumulation of ADP-ribose, it would be disastrous for the cell. In this respect, it is interesting to note that the only other substrate of the purified ADP-ribose pyrophosphatase is 2’ phospho-ADP-ribose (a catabolite of NADP) which has also been shown to derivatize proteins nonenzymatically (29).

So far, the genomes of almost every cellular organism sequenced, as well as several viruses, contain at least one Nudix box signature sequence; most genomes contain several. This argues for the primordial origin of the Nudix box and for the physiological significance of the enzymes designated by it.

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