Studies on the ADP-ribose Pyrophosphatase Subfamily of the Nudix Hydrolases and Tentative Identification of \textit{trgB}, a Gene Associated with Tellurite Resistance*

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Four Nudix hydrolase genes, \textit{ysa1} from \textit{Saccharomyces cerevisiae}, \textit{orf209} from \textit{Escherichia coli}, \textit{yqkg} from \textit{Bacillus subtilis}, and \textit{hi0398} from \textit{Hemophilus influenzae} were amplified, cloned into an expression vector, and transformed into \textit{E. coli}. The expressed proteins were purified and shown to belong to a subfamily of Nudix hydrolases active on ADP-ribose. Comparison with other members of the subfamily revealed a conserved proline 16 amino acid residues downstream of the Nudix box, common to all of the ADP-ribose pyrophosphatase subfamily. In this same region, a conserved tyrosine designates another subfamily, the diadenosine polyphosphate pyrophosphatases, while an array of eight conserved amino acids is indicative of the NADH pyrophosphatases. On the basis of these classifications, the \textit{trgB} gene, a tellurite resistance factor from \textit{Rhodobacter sphaeroides}, was predicted to designate an ADP-ribose pyrophosphatase. In support of this hypothesis, a highly specific ADP-ribose pyrophosphatase gene from the archaebacterium, \textit{Methanococcus jannaschii}, introduced into \textit{E. coli}, increased the transformant’s tolerance to potassium tellurite.

The Nudix hydrolases comprise a large family of proteins characterized by the highly conserved array of amino acids \textit{GX}_{\alpha}EX, REUXEEXGU, where \textit{U} represents a bulky, hydrophobic, amino acid, usually Ile, Leu, or Val (1). A recent BLAST (2) search of the sequence data banks has revealed more than 300 putative proteins from over 80 species containing this amino acid motif, the Nudix box (Fig. 1). We have been systematically identifying and characterizing the enzymatic activities associated with these proteins, and we have found that almost all of the major substrates for these enzymes are nucleoside diphosphates linked to some other moiety, \(\chi\), hence the acronym “Nudix.” The range of substrates acted on by various members of the family includes ribo- and deoxynucleoside triphosphates, nucleotide sugars, dinucleoside polyphosphates, NADH, and ADP-ribose. These substances are potentially toxic to the cell, signaling molecules, or metabolic intermediates whose concentrations require modulation during changes in the cell cycle or during periods of stress. We have suggested that the role of the Nudix hydrolases is to sanitize or modulate the accumulation of these metabolites (1). Since the Nudix box is common to all of these enzymes, their specificity for the individual substrates must lie somewhere distal to the conserved region. In this paper, we describe the cloning and characterization of four ADP-ribose pyrophosphatases, and we identify a proline residue downstream of the conserved sequence common to members of this subfamily of Nudix hydrolases. Furthermore, we have observed that other recurring amino acids in this same region are predictive of two other subfamilies of the Nudix hydrolases, the dinucleoside polyphosphate pyrophosphatases and the NADH pyrophosphatases.

We also demonstrate that ADP-ribose pyrophosphatase activity may play a role in tellurite resistance, since overexpression of this enzyme markedly increases the survival of cultures of \textit{Escherichia coli} exposed to this toxic metalloid oxyanion.

**EXPERIMENTAL PROCEDURES**

**Materials**

Primers were obtained from Integrated DNA Technologies (Corvalive, IA). Biochemicals and enzymes were obtained from Sigma unless otherwise noted. Calf intestinal alkaline phosphatase was from Stratagene, and enzymes used in standard cloning procedures were from Life Technologies, Inc. and U.S. Biochemical Corp. \textit{E. coli} strain MG1655 was kindly provided by Dr. Frederick R. Blattner (University of Wisconsin), and strains of \textit{Saccharomyces cerevisiae}, \textit{Bacillus subtilis}, \textit{Hemophilus influenzae}, and \textit{E. coli} BL21 (DE3) were departmental stocks.

**Cloning**

Genes of interest were amplified from genomic DNA with forward primers incorporating an \textit{NdeI} site and reverse primers incorporating a \textit{BamHI} site. The insert was prepared by digestion with \textit{NdeI} and \textit{BamHI} followed by gel purification and it was ligated with the corresponding restriction sites of \textit{pET11b} under control of the \textit{T7 lac} promoter for expression. The cloned genes, with their accession numbers in parentheses are as follows: \textit{ysa1}, \textit{S. cerevisiae} (Q09176); \textit{orf209}, \textit{E. coli} (P98551); \textit{yqkg}, \textit{B. subtilis} (PS4570); \textit{hi0398}, \textit{H. influenzae} (AAC22057). The plasmid constructs are designated pYSA1, pOrf209, pYQKG, and pH0398, respectively.

**Expression and Purification of the Enzymes**

BL21 (DE3) cells containing the respective plasmid were grown at 37 °C in LB broth on a shaker at an \(A_{600}\) of about 0.6 and induced by the addition of isopropyl-\(\beta\)-thiogalactopyranoside to a concentration of 1 mM. The cells were allowed to grow for an additional 3 h, harvested, washed by suspension in isotonic saline, and centrifuged in preweighed centrifuge tubes, and the packed cells were stored at \(-80^\circ\text{C}\). A summary of the steps involved in the purification of each of the enzymes follows.

**YSAI**—Cells were suspended in 3 volumes of 50 mM Tris, pH 7.5, 1 mM EDTA (buffer A) supplemented with 0.1 mM diithiothreitol and 30% glycerol and disrupted in a French press. Glycerol was absolutely necessary for stabilization of the enzymatic activity throughout the purification procedure for YSA1. The protein was adjusted to 10 mg/ml, and nucleic acids were precipitated by adding streptomycin sulfate to a
concentration of 1%. Ammonium sulfate was added to a final concentration of 50% saturation, and the precipitate was discarded after centrifugation. The supernatant was dialyzed and chromatographed on DEAE-Sepharose. The active fractions were pooled, dialyzed, and chromatographed on a gel filtration column (Sephadex G-100), and the active fractions were pooled, concentrated by precipitation in 80% ammonium sulfate, dialyzed, and chromatographed on DEAE-Sepharose.

**YQKG and HI0398**—The purification of these two enzymes was considerably simplified, because almost all of the expressed protein leaked out of the frozen and thawed cells merely by washing them in buffer A. Endogenous proteins remained within the cells, resulting in an extract highly enriched for the expressed enzyme. The YQKG and HI0398 enzymes were recovered in an essentially pure state (>90%) by precipitating them in 70 or 30% ammonium sulfate, respectively.

**FIG. 1.** Representatives of the Nudix hydrolase family. A recent BLAST search (2) revealed more than 300 putative members of the Nudix hydrolase family from over 70 species. Shown is a sample of 70 entries from 43 species illustrating the highly conserved Nudix signature sequence.
Enzyme Assay

Enzyme velocities were quantitated by measuring the conversion of a phosphatase insensitive substrate, ADP-ribose, to the phosphatase-sensitive products, AMP and ribose 5-phosphate. The liberated inorganic orthophosphate was measured by the procedure of Ames and Dubin (3). The standard incubation mixture (50 μl) contained 50 mM Tris-Cl, pH 8.0, 2 mM MgCl₂, 2 mM ADP-ribose, 0.2–2 μl units of enzyme, and 2 units of alkaline intestinal phosphatase. After 15 min at 37 °C, the reaction was terminated by the addition of EDTA, and inorganic orthophosphate was measured. A unit of enzyme hydrolyzes 1 μmol of substrate/min under these conditions. Note that 2 mol of phosphate are liberated per mol of ADP-ribose hydrolyzed.

Product Determination

The standard assay mixture (minus alkaline intestinal phosphatase) was scaled up, and at various time intervals aliquots were analyzed by paper electrophoresis (4), and additional aliquots were used for the determination of inorganic orthophosphate in the presence and absence of added alkaline intestinal phosphatase.

RESULTS AND DISCUSSION

Expression and Purification of Proteins—Induction of BL21 (DE3) cells transformed with the cloned genes (see “Methods”) led to the appearance of new protein bands corresponding to molecular weights calculated from the respective amino acid content. Fig. 2 shows an SDS-polyacrylamide gel comparing induced cells containing the cloned genes with control cells containing the vector, pET11b, without the inserted genes. In each case, a well-defined new band is visible. When extracts of the cells prepared as described under “Methods” were centrifuged and analyzed by gel electrophoresis, the bulk of the newly expressed protein was in the soluble fraction (data not shown). It is interesting that two of the expressed proteins HI0398 (from H. influenzae) and YQKG (from B. subtilis) were extracted without mechanically disrupting the frozen cells. At present, it is not apparent why these proteins behave differently from most of the other Nudix hydrolases expressed in E. coli. Fig. 1 also shows the highly purified proteins resulting from the protocol described under “Methods.” These fractions were used for characterization of the enzymes reported below.

Enzymatic Activities—Our initial studies of this group of enzymes began with Orf209. Although we did not know its enzymatic activity, we were influenced by our earlier work (1) indicating that all of the major substrates for the Nudix hydrolase family are derivatives of nucleoside diphosphates. Accordingly, we screened a number of candidates in this structural category and found that ADP-ribose was an excellent substrate for the enzyme. This is shown in Table I along with the three other enzymes included in this study. For comparison, the two additional ADP-ribose pyrophosphatases described in earlier works (7, 8) are also reported in the table. The activities toward ADP-ribose are compared with rates with other naturally occurring nucleoside diphosphate derivatives known to be favored substrates for other members of the Nudix hydrolase family (8). In each case, ADP-ribose is the preferred substrate although there is a wide range in absolute specificities. For example, MJ1149 from the archaeobacterium, Methanococcus jannaschii, has no significant activity toward any of the other substrates, whereas Orf186 from E. coli and HI0398 from H. influenzae have substantial activities on NADH and GDP-mannose, respectively. However, more rigorous kinetic analyses would be required for each of the putative substrates if a more substantive interpretation of the relative rates is in order. Kinetic parameters for ADP-ribose are compared in Table II, and a broad distribution in some of the constants is noted. These comparative values, derived under standard assay conditions, should be interpreted with caution, because differences in the physiology and ecology of the individual strains could have large effects on the data. For example, we have shown that the V_max of MJ1149 increases 15-fold when assayed at 75 °C (8), raising the rate from 6.2 to 93 units/mg, and this temperature is still 10 °C below the normal habitat of the organism (9).

Other Properties of the Enzymes—As with most of the Nudix
The ADP-ribose pyrophosphatase subfamily of Nudix hydrolases studied so far, the ADP-ribose pyrophosphatase subfamily members have distinctly alkaline pH optima, ranging from pH 8 to 9. All absolutely require a divalent cation for activity, with \( \text{Mg}^{2+} \) at approximately 2 mM the preferred metal. \( \text{Mn}^{2+} \) at equal concentrations is 10–20% as effective. One of the enzymes from this study, Orf209, has approximately 40% of maximal activity when \( \text{Mg}^{2+} \) is replaced by \( \text{Zn}^{2+} \), and this is similar to the results seen previously for Orf186 (7).

**Products of the Reaction**—Aliquots of standard reaction mixtures (omitting alkaline phosphatase), appropriately scaled up, were analyzed as described under “Methods.” No inorganic phosphate was formed during the course of the reactions. The disappearance of substrate ADP-ribose was coincident with the appearance of AMP, and inorganic orthophosphate was released upon incubation of the products with alkaline intestinal phosphatase. The course of the reaction may be written as

\[
\text{ADP-ribose} \rightarrow \text{AMP} + \text{inorganic orthophosphate}
\]
ADP-ribose + H₂O → AMP + ribose 5-phosphate.

Identification of Nudix Hydrolase Subfamilies—The discovery of a nucleoside triphosphatase activity associated with the *E. coli* mutT1 mutator gene (10) and also with its ortholog, *mutX*, of *S. pneumoniae* (11, 12) suggested that the small region of amino acid identity in the two otherwise dissimilar proteins comprised the catalytic site of these two enzymes. A BLAST (2) search at that time revealed 13 other open reading frames present in organisms ranging from viruses to humans (11, 13), and Koonin (13) suggested that the conserved MutT signature sequence might designate nucleoside triphosphate pyrophosphohydrolase activity. However, subsequent work has revealed that the MutT enzyme is only one member of a large family of different enzymes with different substrates including sugar nucleotides, NADH, dinucleoside polyphosphates, and as shown in the present work, ADP-ribose (for a review, see Ref. 8). Despite the large body of evidence to the contrary, the MutT signature sequence (GX₃EX₂REUXEXGXU) has been erroneously linked to the MutT enzymatic activity and to antimutagenesis. For example, MJ1149 of *M. jannaschii* has been designated MutT in the TIGR sequencing project (14), whereas in reality it has neither nucleoside triphosphatase nor antimutagenic activity and is in fact a highly specific ADP-ribose pyrophosphatase (8). Also, a recent report (15) attributes the extreme radiation resistance of *Deinococcus radiodurans* to its large number of MutT genes insulating it from oxidative stress. In fact, only one of the genes has been identified so far, *gdr8*, designating a new enzyme, UDP-glucose pyrophosphatase,¹ unrelated to the MutT enzyme. This ambiguity between the “MutT motif,” an amino acid array shared by several different enzymes, and the “MutT enzyme,” connoting a specific physiological function, has caused considerable confusion. For this reason, we introduced the acronym, Nudix hydrolase, to define the family of different enzymes sharing the Nudix box signature sequence of amino acids, the MutT enzyme being only one member of this large family. Fig. 1 shows a Clustal (16) alignment of a partial list of putative enzymes containing the Nudix box (MutT motif) uncovered in a recent search of the data banks using the BLAST program (2). Fig. 1 contains 70 entries selected from a total of 300 putative proteins from 75 different species. Sequenced in this list are different families of enzymes acting on the substrates mentioned above and almost certainly some enzymes with novel, undiscovered activities. Since the Nudix box is common to all of these proteins, the determinant(s) of specificity must be supplementary to the Nudix signature sequence. One of our objectives in discovering and characterizing new members of this interesting family of enzymes is to recognize distinguishing features of the subfamilies in order to predict the enzymatic activity of undetermined entries. As the collection of characterized enzymes grows, alignments of those with similar activities become more revealing. Fig. 3A is an alignment of the ADP-ribose pyrophosphatase subfamily showing a highly conserved proline, 15 or 16 amino acids downstream of the terminal glycine of the Nudix box. The checked entries have all been identified, and the remaining entries are predicted. Actually, we predicted the activity of the *H. influenza* and *B. subtilis* proteins before their respective genes were cloned and expressed, and we have recently learned that a human EST (Fig. 3A, line 17) is also an ADP-ribose pyrophosphatase.² Similar alignments are shown in Fig. 3, B and C, for diadenosine polyphosphate hydrolases and the NADH hydrolases, respectively. In the former, there is a conserved tyrosine 16–18 amino acids downstream from the Nudix box, and in the latter, there is a consensus of eight amino acids in this region. When this 8-amino acid consensus sequence was first uncovered, there was only one known NADH pyrophosphatase with the MutT motif. We have recently cloned, expressed, purified, and determined the activities of the two additional entries XGLO67w (*S. cerevisiae*), and F13H10³ (*C. elegans*), both of which have the enzymatic activity predicted from the downstream signals. The three-dimensional solution structure for one of the Nudix hydrolases, MutT, has been solved by NMR spectroscopy (17), and it has a unique, loop 1-helix-loop 2 motif encompassing its catalytic and nucleotide binding site. The characteristic amino acids distinguishing the three families mentioned above would all be in loop 2 if this structural feature were conserved in all of the Nudix hydrolases. Preliminary studies on the crystal structure of the Orf209 ADP-ribose pyrophosphatase suggest⁴ that the loop-helix-loop motif is present in this enzyme as well. The three-dimensional structures of two additional enzymes, Orf17, the dATPase (5), and Orf1.9, the GDP-mannose hydrolase (18), are also in the process of being solved, the former by x-ray crystallography⁵ and the latter by NMR spectroscopy,⁶ so that we should soon have insight into the generality of the loop-helix-loop motif in the Nudix hydrolase family. It is interesting to note that one of the enzymes, Orf186, has both the conserved proline and the conserved tyrosine (see Fig. 3, A and B). This correlates well with the specificity of Orf186, which is almost equally active on Ap₅A and ADP-ribose (Table I). On the other hand, these amino acid predictors of activity should, at present, be viewed only as clues to narrow down the possibilities in identifying new members of the family, since not all of the enzymes fit the present patterns. For example, two Ap₅A hydrolases from the yeasts *Schizosaccharomyces pombe* (19) and *S. cerevisiae* (20) do not have the conserved tyrosine of the Ap₅A hydrolases, and Orf186, which also has substantial activity on NADH (see Table I) does not have the 8-amino acid consensus predictive of this activity.

**ADP-ribose Pyrophosphatase and Tellurite Resistance—**An opportunity to test our classification scheme and to demonstrate its potential utility was provided by the trgB gene of *R. sphaeroides*, which has been shown to be a tellurite resistance determinant (21). Fig. 3A, line 7, shows that TRGB contains the Nudix box and, in addition, the signal proline tentatively categorizing the protein as an ADP-ribose pyrophosphatase. To test whether this enzyme could confer the tellurite resistance phenotype, we transformed *E. coli* with a plasmid, pTRC, containing the ADP-ribose pyrophosphatase gene (MJ1149) from the archaebacterium *M. jannaschii*, because the enzyme from this organism is highly specific for ADP-ribose (see Table I). The graphs in Fig. 4 clearly demonstrate that the ADP-ribose pyrophosphatase gene increases resistance to tellurite. In Fig. 4A, it can be seen that the transformed culture retained almost 100% viability at a 50% survival rate for the parent culture. Likewise, there was approximately 60% survival versus 100% killing, at 0.5 µg/ml K₂TeO₃. Fig. 4B shows the differential effect of tellurite on growing cultures of the transformed and parent cells. That is this is a specific effect of the ADP-ribose pyrophosphatase gene and not a general property of the Nudix hydrolases is shown in Fig. 4C. None of the other genes tested conferred tellurite resistance. Commensurate with the increase in tellurite resistance was a 5-fold increase in ADP-ribose pyrophosphatase in crude extracts of the transformed cells compared with the parent culture.⁶

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¹ C. A. Dunn, S. Desni, and M. J. Bessman, unpublished results.
² H. Yang, M. Slupska, and J. H. Miller, personal communication.
³ W-L. Xu and M. J. Bessman, unpublished results.
⁵ P. Leggett, M. J. Bessman, and A. S. Miledi, unpublished results.
⁶ C. A. Dunn and M. J. Bessman, unpublished observations.
ADP-ribose Pyrophosphatase Subfamily of Nudix Hydrolases

Tellurite resistance has been used for many years in the differential diagnosis of pathological microorganisms and is a complex process implicating many genes found in prokaryotes and eukaryotes (for a review, see Ref. 22). Our experiments and classification scheme strongly suggest that one of these genes, trgB, designates an ADP-ribose pyrophosphatase, implicating ADP-ribose itself as a factor in tellurite sensitivity. Although the role played by ADP-ribose in this process is not known, these experiments are an example of how functional genomics, the prediction of protein function from amino acid sequence, can aid in identifying the activities of unknown proteins involved in physiological processes.

It is, perhaps, not surprising that ADP-ribose is directly involved in, or potentiates, tellurite toxicity. Cellular ADP-ribose arises from the hydrolysis of mono- and poly(ADP-ribosylated proteins during the regulation of metabolic processes (for a review, see Ref. 23) and also by the large scale turnover of NAD$^+$, amounting to approximately 30 and 90% of the total NAD$^+$ synthesis in E. coli and HeLa cells, respectively (24). Because of its free aldehyde group, ADP-ribose can derivatize terminal amino groups, lysines, and cysteines in proteins nonenzymatically (25, 26), thereby inactivating enzymes or leading to proteins targeted for apoptosis (27–29) or to non-specifically tagged proteins confusing the ADP-ribosylation recognition system. Recently, the accumulation of ADP-ribose has been implicated in the liver damage caused by high levels of acetaminophen (N-acetyl-p-aminophenol, Tylenol$^\text{®}$) when it was shown that the acetaminophen metabolite, N-acetyl-p-benzoquinonimine, inhibits rat liver ADP-ribose pyrophosphatase (30). We have also seen that this derivative inhibits YSA1, the yeast enzyme described in this paper. It has also been reported that inhibitors of poly(ADP-ribose) polymerase, one of the major sources of cellular ADP-ribose, prevent the liver damage caused by overdoses of acetaminophen (31). These recent experiments support the large body of data implicating free ADP-ribose as a cytotoxic agent.

As mentioned in the Introduction, we have suggested that the members of the Nudix hydrolase family of enzymes share common features including a conserved amino acid signature sequence and a specificity for nucleoside diphosphate derivatives and that one of their physiological functions is to sanitize the cell of potentially toxic metabolites. The ubiquitous ADP-ribose itself as a factor in tellurite sensitivity. Although ADP-ribose pyrophosphatases described in this paper meet these three criteria and qualify this subfamily as a bona fide member of the Nudix hydrolases.

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