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Cloning and Characterization of the First Member of the Nudix Family from *Arabidopsis thaliana**

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Marta Dobrzanska‡§, Blanka Szurmak‡, Aleksandra Wyslouch-Cieszynska¶, and Elzbieta Kraszewska‡

From the ‡Departament of Plant Biochemistry, ¶Mass Spectrometry Laboratory, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawinskiego 5A, 02-106 Warsaw, Poland

The sequence motif commonly called a Nudix box, represented by (GX_5EX_7REVXEEXGU) is the marker of a widely distributed family of enzymes that catalyze the hydrolysis of a variety of nucleoside diphosphate derivatives. Here we describe the cloning and characterization of an *Arabidopsis thaliana* cDNA encoding a Nudix hydrolase that degrades NADH. The deduced amino acid sequence of AtNUDT1 contains 147 amino acids. The recombinant AtNUDT1 was expressed in *Escherichia coli* and purified. In the presence of Mn^{2+} and the optimal pH of 7. 0, the recombinant AtNUDT1 catalyzed the hydrolysis of NADH with a K_m value of 0. 36 mM. A V_{max} of 12. 7 units mg $^{-1}$ for NADH was determined. The recombinant AtNUDT1 migrated as a dimer on a gel filtration column. Biochemical analysis of recombinant AtNUDT1 indicated that the first characterized member of the Nudix family from *A. thaliana* is a NADH pyrophosphatase.

Nudix hydrolases are a family of proteins defined by a conserved array of amino acids: GX_5EX_7REVXEEXGU, where U is usually Ile, Leu, or Val (1). This motif was first described as a MutT motif in a MutT protein from *Escherichia coli* (2). MutT is a nucleoside triphosphatase that hydrolyzes all canonical nucleoside triphosphates with a preference for deoxyguanosine triphosphate (dGTP) and its oxidized form 7,8-dihydro-8-oxodeoxyguanosine (8-oxo-dGTP). Inactivation of the *mutT* gene increases the occurence of A:T → C:G transversion mutations in the *E. coli* genome due to the incorporation of 8-oxo-dGTP paired with adenine during DNA replication (3). Apart from dGTP and 8-oxo-dGTP (the major substrates of MutT protein), there is a wide spectrum of substrates hydrolyzed by other Nudix enzymes, predominantly **nu**cleoside **di**phosphates linked to some other moiety **x** (1). It has been proposed that Nudix hydrolases may be divided into subfamilies based on their major substrates: dinucleoside polyphosphates, ADP-ribose, NADH, nucleotide sugars, or ribo- and deoxyribonucleoside triphosphates (4). As all these substrates are either potentially toxic, cell signaling molecules, or metabolic intermediates whose concentrations require modulation during the cell cycle, it has been postulated that the role of Nudix hydrolases is to sanitize or regulate the accumulation of these metabolites (4). To date, a number of Nudix hydrolases from different organisms have been characterized with respect to their major substrates such as nucleotide sugars (5), Ap_nA (n = 3, 4, 5, and 6) (6–12), deoxynucleoside triphosphates (13–17), ADP-ribose (18–23), GDP-mannose (5, 25), NADH (26–29), coenzyme A (30–32), and diphosphoinositol polyphosphates (33–35).

Recently, almost 800 open reading frames containing the Nudix motif from over 200 species, with representatives in procaryota, archea, and eucaryota, have been identified (36). The number of Nudix hydrolases varies from 0 in *Mycoplasma genitalium* (37) to 24 in *Deinococcus radiodurans* (38).

A BLAST search (39) revealed eight open reading frames with the Nudix (MutT) signature in the genome of *Arabidopsis thaliana.* In this work, we report the cloning and characterization of AtNUDT1, the first characterized member of the Nudix family from *A. thaliana*.

EXPERIMENTAL PROCEDURES

Materials

Seeds of *A. thaliana* ecotype Columbia were obtained from the Arabidopsis Biological Resource Center, Columbus, OH. Oligonucleotide primers used in polymerase chain reaction were provided by the DNA Sequencing and Oligonucleotide Synthesis Service from the Institute of Biochemistry and Biophysics, Polish Academy of Science. Enhanced reverse transcriptase-PCR kit, biochemicals, and calf intestinal alkaline phosphatase were purchased from Sigma, and *E. coli* inorganic pyrophosphatase was obtained from Merck. pGEM-T Easy vector system I was supplied by Promega, and *E. coli* expression vector pQE31 and QIA-express kit type IV were purchased from Qiagen. Enzymes used in standard cloning procedures were obtained from Fermentas. Plants were grown in a greenhouse (standard conditions), and total RNA was isolated from 14-day-old seedlings using Tri Reagent from the Molecular Research Center, Inc., Cincinnati, OH, according to the procedure suggested by the supplier.

Methods

Cloning of the AtNUDT1 cDNA—The cDNA corresponding to the gene, assigned in the National Center for Biotechnology Information data base as F14K14.13 from *A. thaliana*, coding 147 amino acids, was amplified by reverse transcriptase-PCR from the total RNA isolated from 14-day-old seedlings, using forward d(GGTACCGTCGACAGGAGAAGCGATA) and reversed d(CTGCAGTTAGTCTCCACCACCATGAGT) primers. The synthesized primers provided *Kpn*I and *Pst*I restriction sites at the start and at the end of the amplified AtNUDT1 cDNA, respectively. After amplification, the DNA was gel-purified and ligated into the pGEM-T Easy vector. The resulting pGEMAtNUDT1 construct was digested with *Kpn*I and *Pst*I, gel-purified, and inserted between the *Kpn*I and *Pst*I sites of the pQE31 vector. The resulting plasmid, pQE31AtNUDT1 (with the His-tag fusion upstream of the coding sequence), was used to transform *E. coli* M15 cells carrying the pREP4 repressor plasmid.

Expresssion of AtNUDT1 in E. coli and Purification of Recombinant AtNUDT1 Protein—The expression and purification of AtNUDT1 was performed according to the Qiagen procedure. A single colony was used to inoculate 10 ml of LB medium containing 100 μg/ml ampicilin and 25 μg/ml kanamycin. After overnight growth at 37 °C, 2. 5 ml of the culture was transferred to 50 ml of LB medium (with antibiotics) and grown to

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§ To whom correspondence should be addressed. Tel.: 48-22-6583848; Fax: 48-22-6584636; E-mail: martad@ibb.waw.pl.

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[1]MSTGEAIPRVAVVVFILNGNSILLGRRRSSIGNSTFALPG
[41]GHLEFGESFEECAAREVMEETGLKIEKMKLLTVTNNVFKE
   GXXXXXEXXXXXXXREVXEEXGU
[81]APTPSHYVSVSIRAVLVDPSQEPKNMEPEKCEGWDWYDWE
[121]NLPKPLFWPLEKLFGSGFNPFTHGGGD[147]
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FIG. 1. **Primary sequence of the AtNUDT1 protein.** The Nudix amino acid motif is given in *bold type* below the homologous amino acids of AtNUDT1 protein.

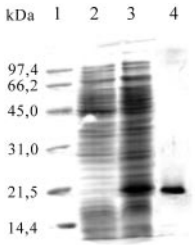

FIG. 2. **Expression and purification of recombinant AtNUDT1 hydrolase.** 12% SDS-polyacrylamide gel stained with Coomasie Blue contains: molecular mass standards (*lane 1*) (Bio-Rad), as indicated on the *left*; *E. coli* cell lysate with pQE31AtNUDT1 plasmid with no induction (*lane 2*); *E. coli* cell lysate with pQE31AtNUDT1 after 3-h induction by 1 mM isopropyl-1-thio-β-D-galactopyranoside (*lane 3*); and AtNUDT1 protein fraction purified from an nickel-nitrilotriacetic acid column (*lane 4*).

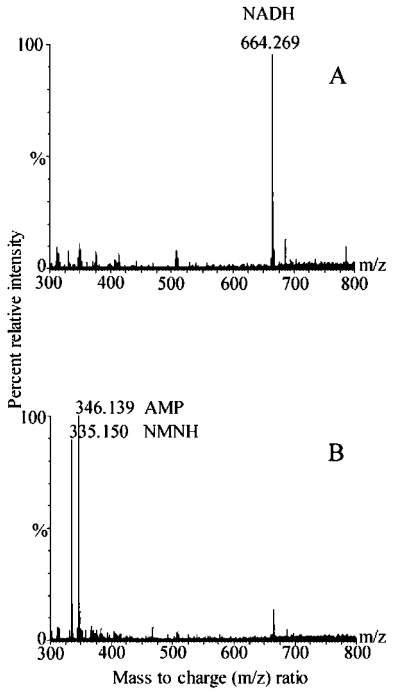

FIG. 3. **Negative ion mass spectra of the NADH hydrolysis.** MS analyses were conducted in the absence (*A*) or presence (*B*) of AtNUDT1 protein with 2 mM NADH under standard reaction conditions (without calf alkaline phosphatase) as described under "Methods." The positions of the standards (NADH, NMNH, AMP) are indicated.

an A_{600} of 0.7. Isopropyl-1-thio-β-D-galactopyranoside was added to 1 mM and the cells were incubated for another 3 h, harvested, washed with an isotonic saline buffer, and resuspended in 1 ml of lysis buffer. The cells were subsequently lysed by five consecutive freeze/thaw cycles with freezing in liquid nitrogen and thawing at room temperature, cleared by centrifugation at 14,000 × *g* for 10 min at 4 °C. The lysate (1 ml) was mixed with 0. 25 ml of 50% nickel-nitrilotriacetic acid agarose (Qiagen) and incubated at 4 °C for 1 h, with gentle shaking. The lysate-nickel-nitrilotriacetic acid mixture was loaded onto a column and washed three times with 1 ml of washing buffer, then the protein was eluted four times with 0.5 ml of elution buffer. All the buffers were prepared according to the Qiagen handbook.

The fractions were analyzed by SDS-PAGE, and those containing pure protein were collected and concentrated by ultrafiltration on a Microcon membrane YM-10 (Millipore). The amount of protein was estimated by the method of Bradford (40).

TABLE I
Substrate specificity of At NUDT1

All substrates were at a concentration of 2 mM and were assayed as described under "Methods." A unit of enzyme catalyzes the hydrolysis of 1 μmol of substrate/min under optimal conditions.

Substrate	Relative activity
	%
NADH	100
Ap_4A	24
Ap_3A	11
NAD^+	5
ADP-ribose	4

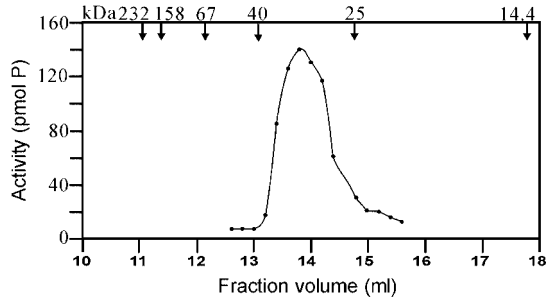

FIG. 4. **FPLC analysis of the recombinant AtNUDT1.** FPLC chromatography was performed as described under "Methods." Enzymatic activity was measured colorimetrically in each fraction. The positions of the molecular mass standards are indicated.

Enzyme Assay—Hydrolysis of substrates was assayed colorimetrically. 50 μl of the standard reaction mixture contained: 50 mM Tris, pH 7. 0, 5 mM $MnCl_2$, 1 mM dithiothreitol, 2 mM substrate, 1–2 units of alkaline phosphatase or inorganic pyrophosphatase, and 0. 2–2 milliunits of AtNUDT1 protein. After incubation at 37 °C for 15 min, the reaction was terminated by the addition of 250 μl of 20 mM EDTA. The resulting inorganic ortophosphate was determined using the method of Ames and Dubin (41) as modified by Bhatnagar *et al.* (13). A unit of enzyme catalyzed the hydrolysis of 1 μmol of substrate/min under optimal conditions.

Complementation of the E. coli mutT Mutation—Plasmids pQE31 and pQE31AtNUDT1 were transformed into *E. coli mutT* strain EC5976 (*pro, thi* Δ *(lac, ara) F' lacZ101, pro*) *mutT*, and tested for complementation of an *E. coli mutT* mutator phenotype using protocols described elsewhere (42).

FPLC[1] *Analysis*—Fast performance liquid chromatography was performed using a Superose 12 column. The proteins were eluted with 100 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 10% glycerol. 200-μl fractions were collected, and their enzymatic activities were measured as described above.

Electrospray Ionization Mass Spectrometry (ESI-MS)—Prior to ESI-MS analysis, a purified protein sample was loaded onto a Vydac C18 HPLC column and eluted with an acetonitrile gradient to remove excess salt. Samples were then injected directly into a Q-Tof1 spectrometer (Micromass) at 4 μl/min using a Hamilton syringe pump. Bovine pancreatic trypsin inhibitor was used as an internal mass standard. Positive ion spectra were obtained for the eluted protein, and the MaxEnt 1 program (Micromass) was used to deconvolute the spectra. To confirm the identity of the protein, the AtNUDT1 protein band was excised from a polyacrylamide gel, reduced, alkylated, digested overnight with trypsin, and tandem mass spectrometry of the tryptic digest was performed using the Q-Tof1 mass spectrometer (Micromass) combined with a nano-HPLC system (LC Packings). The MaxEnt3 program (Micromass) was used to identify the obtained peptide sequences that were then compared with the amino acid sequences predicted for the His-tagged AtNUDT1 protein.

The enzymatic reaction products were analyzed using negative ion spectra. Calf intestine phosphatase was omitted for these analyses. The

[1] The abbreviations used are: FPLC, fast performance liquid chromatography; ESI-MS, electrospray ionization mass spectrometry; HPLC, high performance liquid chromatography; NMNH, nicotinamide mononucleotide (reduced form).

standards were dissolved in NH_4OH/water/acetonitrile (20:40:40) to 1 mM concentration and immediately injected into the spectrometer. The enzyme reaction mixtures (2 μl) were diluted 100 times in NH_4OH/water/acetonitrile (20:40:40) and used for injection. Masses were determined as for M-H ions.

RESULTS

Identification and Cloning of the AtNUDT1 Gene—A search for the Arabidopsis homologue of the *mutT* gene in the National Center for Biotechnology Information data base revealed eight open reading frames with the Nudix (MutT) domain. For further analysis, the F14K14.3 gene from chromosome 1 was selected. The nucleotide sequence of the full-length DNA, including one intron, for the F14K14.13 gene comprises 518 base pairs. It contains an open reading frame of 444 nucleotides coding for a 147-amino acid protein (Fig. 1) with a predicted molecular mass of 16,356.61 Da and pI 5.12 (43). A cDNA clone of the F14K14.13 gene (designated AtNUDT1) was obtained from *A. thaliana* total RNA by reverse transcriptase-PCR as described under "Methods."

TABLE II
Kinetic parameters for AtNUDT1

Substrates at a concentration from 0.1 to 3 mM were used for colorimetric measurements. The K_m and V_{max} were determined from nonlinear regression analysis. The K_{cat} was calculated from the V_{max} assuming one active site per monomer. A unit of enzyme hydrolyzes 1 μmol of substrate/min.

Substrate	K_m	V_{max}	K_{cat}	K_{cat}/K_m
	mM	*units/mg*	s^{-1}	$s^{-1}\ M^{-1}$
NADH	0.36 ± 0.05	12.7 ± 0.5	3.92	1.0×10^4
NAD^+	0.48 ± 0.06	0.62 ± 0.06	0.19	0.4×10^3

Expression and Purification of the Recombinant AtNUDT1 Protein—The hexahistidine-tagged recombinant protein, AtNUDT1, was expressed in the *E. coli* M15 cells and purified to near homogeneity (Fig. 2). The approximate molecular mass on the SDS-PAGE was 19 kDa. Mass spectrometry was used as a more accurate method to determine the molecular mass of the recombinant protein.

Mass Spectrometry—Electrospray mass spectrometry was used to measure the precise mass of the purified hexahistidine-tagged AtNUDT1 protein. The obtained mass spectrum revealed microheterogeneity within the sample. The main peak of molecular mass 18,517.00 ± 1.8 Da was different from the

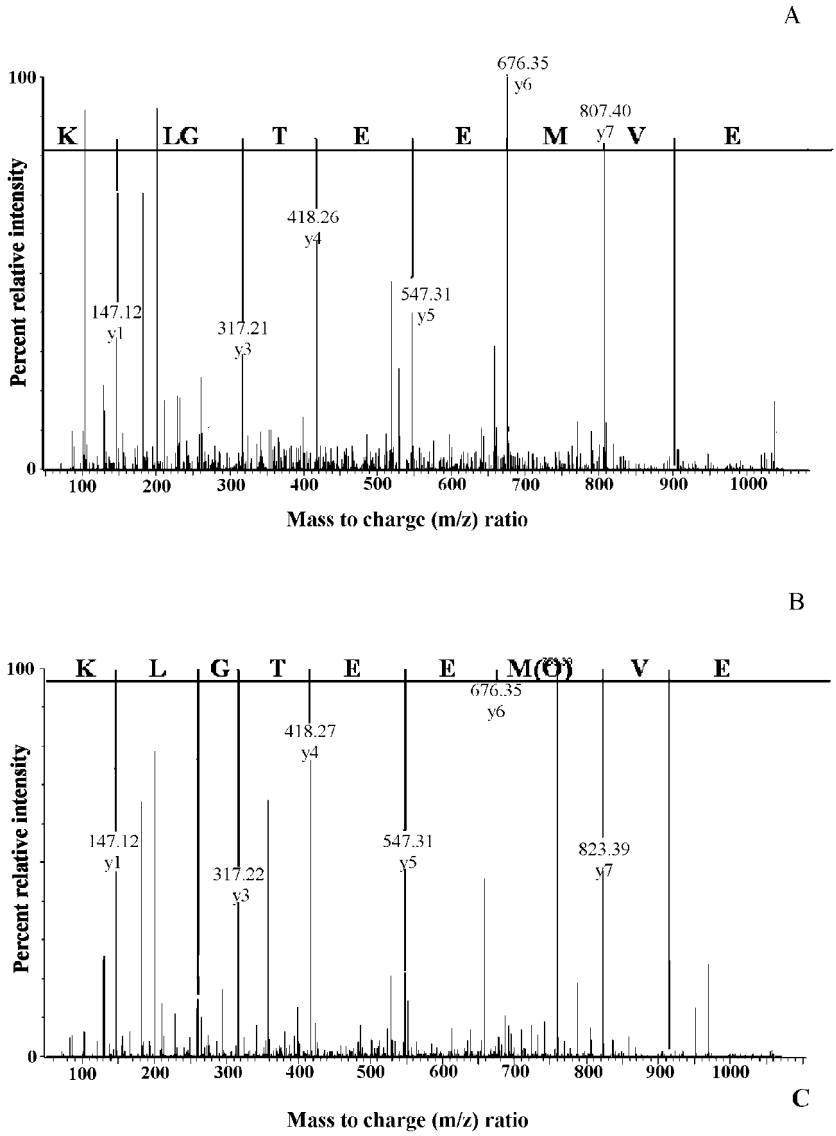

FIG. 5. **Tandem mass spectrometry of trypsin digest of AtNUDT1.** Tandem MS analysis of AtNUDT1 protein digested with trypsin was performed. An example of peptide sequencing data (tryptic peptide EVMEETGLK having a reduced (*A*) or oxidized (*B*) methionine) is presented. All the observed tryptic peptides are *underlined*, and oxidized methionines are in *bold type* (*C*).

calculated mass (18,502.9 Da). Two other low intensity components of 18,532.59 Da and 18,552.69 Da were also observed. To address this discrepancy, tandem mass spectrometry analysis for a tryptic digest of the AtNUDT1 protein was performed. The MS sequencing experiment confirmed the identity of the hexahistidine-tagged AtNUDT1 with 65% sequence coverage. The only modification in the observed tryptic peptides was methionine oxidation (Fig. 3). Thus, the +15 and +30 Da mass differences most probably result from the presence of one or two oxidized methionines of the five methionines present in the recombinant protein. This oxidation might be due to sample preparation for ESI-MS.

Substrate Specificity of the Recombinant AtNUDT1—The first attempt to characterize AtNUDT1 was to check whether this protein displayed MutT activity. Complementation of the *E. coli mutT* mutator phenotype was tested using an *E. coli mutT* strain devoid of its own 8-oxo-dGTPase activity and expressing the AtNUDT1 gene cloned into the pQE31 plasmid. The pQE31 vector with no insert was used as a control. Since the *A. thaliana* AtNUDT1 protein did not decrease the rate of the spontaneous mutations caused by the accumulation of 8-oxo-dGTP in the nucleotide pool, it was concluded that the AtNUDT1 was not functionally related to the MutT enzyme. Therefore, the purified protein was used for further characterization of specific substrates found for other Nudix hydrolases. A variety of nucleoside diphosphate derivatives were used as potential substrates (Table I). At the optimal pH of 7.0 and in the presence of 5 mM Mn^{2+}, AtNUDT1 favored NADH while less efficiently hydrolyzing Ap_4A. The rate of hydrolysis decreased 20-fold when NADH was substituted by NAD^+. No significant activity was observed for Ap_3A and ADP-ribose as substrates. No activity was observed for deoxyribonucleoside triphosphates (data not shown), which was consistent with the inability to complement the *E. coli mutT* mutation by AtNUDT1.

The reaction products of NADH hydrolysis were subjected to mass spectrometry analysis, and the results are shown in Fig. 4. The reaction products contain two new molecules with molecular mass corresponding to NMNH (335.150 Da) and AMP (346.139 Da), indicating that AtNUDT1 acts as a NADH pyrophosphatase. AtNUDT1 has an optimal activity with NADH as a substrate, at pH 7.0 and in the presence of 5 mM Mn^{2+}. When the Mn^{2+} ions were substituted by the same concentration of Mg^{2+}, an 85% decrease was observed in the catalytic activity.

ACCESSION	PARTIAL SEQUENCE
AAD25835	KVPT**G**TIKEG**E**SIWAGAV**REV**K**EE**T**D**IDAEFVEVLSFMESHQAVWQ
AAD25833	KLPT**G**VVKEG**E**NIWEGAL**REV**E**EE**T**G**IKTKFVEVLAFRESHQAFLE
AAF78493	VFPK**G**GWEDD**E**TVLEAAS**REA**I**EE**A**G**VKGILRELPLGVWEFRSKSS
AAG52038*	ALPG**G**HLEFG**E**SFEECAA**REV**M**EE**T**G**LKIEKMKLLTVTNNVFKEAP
AAL47357	KFPT**G**VVNEG**E**DIHDGSV**REV**K**EE**T**G**VDTEFDQILAFRQTHKAFFG
BAA97158	KLPT**G**FINES**E**EIFSGAV**REV**K**EE**T**G**VDTEFSEVIAFRHAHNVAFE
CAB78314	KLPT**G**VINEG**E**DIWTGVA**REV**E**EE**T**G**IIADFVEVLAFRQSHKAILK
NP189303	VFPK**G**GWEDD**E**TVLEAAS**REA**M**EE**A**G**VKGILREDPLGVWEFRSKSS

FIG. 6. **Partial sequence alignments of all members of the Nudix family from *A. thaliana*.** The conserved Nudix box is in *bold type*. AtNUDT1 protein is indicated by the *asterisk*.

The kinetic parameters for the most and least favored substrates are presented in Table II. Although the K_m for NADH was nearly the same as for NAD^+ (0. 36 and 0. 48 mM, respectively), the V_{max} values differed considerably (12.7 units/mg and 0.62 units/mg, respectively).

FPLC analysis of AtNUDT1 protein was performed to investigate the structure of this enzyme. The active protein was found at around 36 kDa, suggesting that AtNUDT1 might form a dimer under native conditions (Fig. 5).

DISCUSSION

In this report, we describe the isolation and functional characterization of AtNUDT1 protein, the first characterized member of Nudix hydrolases from *A. thaliana*, which catalyzes the hydrolysis of NADH to NMNH and AMP.

The biological role of NADH pyrophosphatases is unclear; however, it has been postulated (26) that NADH pyrophosphatase are involved in the regulation of the cellular NADH/NAD^+ ratio, an important factor in maintaining a balance between anabolic and catabolic pathways in the cell. Jacobson and Kaplan (44) demonstrated that the addition of a partially purified pigeon liver NADH pyrophosphatase to the assay increased the rate of ethanol oxidation catalyzed by alcohol dehydrogenase. The authors (44) suggested that the NADH pyrophosphatase might play an intracellular regulatory role by changing the equilibrium of a particular metabolic pathway under specific conditions. Moreover, NADH pyrophosphatase is the only known source of NMNH in the cell. Thus, it may have a specialized physiological function, although the role of NMNH in the cell remains to be discovered.

NADH pyrophosphatases were described for *E. coli* (26), *Saccharomyces cerevisiae*, and *Caenorhabditis elegans* (27, 29) and purified from *Lens esculenta* (28). Twenty-five putative members of the NADH pyrophosphatase subfamily of Nudix hydrolases have been detected by a BLAST search in a variety of species, including *Homo sapiens*. They are characterized by the presence of an array of eight conserved amino acids (SQPWPFP) around 12 amino acids downstream of the Nudix box (27). However, the analysis of amino acid sequences revealed that none of the eight members of the Nudix family from *A. thaliana* possesses the SQPWPFP consensus (Fig. 6). In this regard, the AtNUDT1 described here as a NADH pyrophosphatase resembles an open reading frame 186 hydrolase from *E. coli*, which has a substantial activity for NADH but also does not have the eight-amino acid consensus that predicts such an activity (7).

The catalytic properties of NADH pyrophosphatase from *A. thaliana* resembles those described for enzymes from *E. coli*, *S. cerevisiae*, and *C. elegans* (Table III). The striking aspect of the specificity of these enzymes is the marked preference for NADH over NAD^+. Similarly, the AtNUDT1 hydrolase favors NADH as a substrate with a 20-fold difference in the rate of hydrolysis of NADH over NAD^+, with a V_{max} similar to that of the *S. cerevisiae* protein and K_m similar to the value described

TABLE III
Comparison of the catalytic properties of NADH pyrophosphatases
All proteins expressed and isolated from transformed *E. coli* cells.

	E. coli	*S. cerevisiae*[a]	*C. elegans*[a]	*A. thaliana*
Optimal pH	8.5	7.7	8.5	7.0
Metal requirment	Mg^{2+}, Mn^{2+}	Mg^{2+}, Mn^{2+}	Mg^{2+}, Mn^{2+}	Mn^{2+}
Substrate preference	NADH	NADH	NADH	NADH
V_{max} (units/mg)	7.6	12.3	6.6	12.7
K_m (mM)	0.1	1.6	1.4	0.36
Relative specificities (%):				
NADH	100	100	100	100
NAD^+	17	18	8	5

[a] From Frick and Bessman (26) and Xu *et al.* (27).

for the *E. coli* hydrolase. The *A. thaliana* AtNUDT1 hydrolase differs from the enzyme purified from another plant, *L. esculentum*. The latter has a lower rate (1.5-fold) of NADH over NAD^+ hydrolysis, a broader substrate specificity, a distinctly alkaline pH, and is monomeric. It is possible that the lentil hydrolase belongs to the Nudix family; however, the protein sequence is not yet known.

The important common feature of NADH pyrophosphatases from *E. coli*, *S. cerevisiae*, *C. elegans*, and *A. thaliana* is the formation of homodimers. The other Nudix subfamily known to be dimeric is a family of the ADP-ribose pyrophposphatases. Recently, the crystal structure of the ADP-ribose pyrophosphatase from *E. coli* was described (24). The structure revealed a symmetric homodimer, requiring dimerization through domain swapping for substrate recognition and catalytic activity. It has been suggested that the Nudix motif is the binding and catalytic site for hydrolysis of the pyrophosphate bonds, but the specificity for individual substrates lies outside this region. The segregation of the catalytic activity and substrate specificity provided the basis for the versatility of the Nudix hydrolases.

The properties of the AtNUDT1 enzyme resemble those of the NADH hydrolases from *E. coli*, *S. cerevisiae*, and *C. elegans*; therefore, we propose the name NADH pyrophosphatase for the AtNUDT1 protein. The results presented extend the growing knowledge about the family of the Nudix hydrolases. Currently, we are investigating two other members of the Nudix family from *A. thaliana*.

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