The Nudix Hydrolase Ndx1 from *Thermus thermophilus* HB8 Is a Diadenosine Hexaphosphate Hydrolase with a Novel Activity*

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The *ndx1* gene, which encodes a Nudix protein, was cloned from the extremely thermophilic bacterium *Thermus thermophilus* HB8. This gene encodes a 126-amino acid protein that includes the characteristic Nudix motif conserved among Nudix proteins. Ndx1 was overexpressed in *Escherichia coli* and purified. Ndx1 was stable up to 95 °C and at extreme pH. Size exclusion chromatography indicated that Ndx1 was monomeric in solution. Ndx1 specifically hydrolyzed (di)adenosine polyphosphates but not ATP or diadenosine triphosphate, and it always generated ATP as the product. Diadenosine hexaphosphate (Ap6A), the most preferred substrate, was hydrolyzed to produce two ATP molecules, which is a novel hydrolysis mode for Ap6A, with a catalytic constant (*k*cat) of 1.4 μM and a *Km* of 4.1 s⁻¹. These results indicate that Ndx1 is a (di)adenosine polyphosphate hydrolase. Ndx1 activity required the presence of the divalent cations Mn²⁺, Mg²⁺,Zn²⁺, and Co²⁺, whereas Ca²⁺,Ni³⁺, and Cu²⁺ were not able to activate Ndx1. Fluoride ion inhibited Ndx1 activity via a non-competitive mechanism. Optimal activity for Ap₆A was observed at around pH 8.0 and about 70 °C. We found two important residues with *pK*ₐ values of 6.1 and 9.6 in the free enzyme and *pK*ₐ values of 7.9 and 10.0 in the substrate-enzyme complex. Kinetic studies of proteins with amino acid substitutions suggested that Glu-46 and Glu-50 were conserved residues in the Nudix motif and were involved in catalysis. Trp-26 was likely involved in enzyme-substrate interactions based on fluorescence measurements. Based on these results, the mechanism of substrate recognition and catalysis are discussed.

The Nudix hydrolase family comprises enzymes that catalyze a reaction where the substrate is a nucleoside diphosphate linked to another moiety, X (1). This family is characterized by the Nudix motif GXXEX₆EXELY, where X is any amino acid, and U is one of the bulky hydrophobic amino acids, Ile, Leu, or Val (1). This motif forms a loop-helix-loop structure that is involved in substrate binding and catalysis (2, 3). These enzymes are found in all kingdoms. It has been proposed that the function of Nudix proteins is housecleaning to eliminate potentially toxic nucleotide metabolites from the cell and to regulate the concentrations of nucleoside diphosphate derivatives (1).

A recent Pfam (4) search of the data bases for the Nudix signature sequence has revealed about 1100 open reading frames from more than 250 species ranging from viruses to humans, and about 70 of the gene products have been identified. These products hydrolyze nucleoside diphosphate derivatives such as (deoxy)nucleotide triphosphate (5), nucleotide sugar (6–8), dinucleotide polyphosphate (9–11), NADH (12), and coenzyme A (13). One example is the MutT protein, which degrades 8-oxo-deoxyguanine triphosphate to prevent mutations caused by oxidation of guanine nucleotides (14). Furthermore, diphosphoinositol polyphosphate (DIPP) (15) and phosphoribosyl pyrophosphate have also been reported as substrates for Nudix hydrolases (16). Although preferred substrates have been identified for some Nudix enzymes, the functions and molecular mechanisms, including substrate recognition, remain to be elucidated.

*Thermus thermophilus* HB8 is a Gram-negative bacterium that grows at temperatures above 75 °C (17). It is the most thermophilic eubacterium for which a gene manipulation system has been established (18–20). Proteins from this bacterium are stable against heat and are, thus, suitable for physicochemical studies, including x-ray crystallography. We selected *T. thermophilus* HB8 for the systematic study of the structures and functions of all proteins from a single organism in a project named the Whole Cell Project (21, 22). Interestingly, *Deinococcus radiodurans*, which is very closely related to *T. thermophilus*, possesses twenty-three Nudix genes (23). This bacterium is characterized by extraordinary resistance to ionizing radiation; it is thought that some of its Nudix proteins may be associated with novel DNA repair pathways (24). However, the diversity of substrates and functions of Nudix proteins in vivo remain unclear. Such unique features make proteins of this family good targets for structural and functional proteomics.

Therefore, we aimed to investigate the molecular mechanism and physiological functions of Nudix proteins from *T. thermophilus* HB8. In this work, we describe the overexpression and purification of *T. thermophilus* HB8 Ndx1 protein. The enzymatic activity and the biochemical properties of Ndx1 are also described.

**EXPERIMENTAL PROCEDURES**

*Materials—*DNA-modifying enzymes, including restriction enzymes, were from Takara Shuzo and New England Biolabs. Yeast extract and polypeptone were from Difco. Isopropyl-β-D-thiogalactopyranoside was

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‡ The abbreviations used are: DIPP, diphosphoinositol polyphosphate; Ap₆A, diadenosine hexaphosphate; Ap₅A, diadenosine pentaphosphate; Ap₄A, diadenosine tetraphosphate; Ap₃A, diadenosine triphosphate; Gp₆G, dguanosine pentaphosphate; Gp₅G, dguanosine tetraphosphate; Ap₃A, adenosine tetraphosphate; CD, circular dichromism; Ap₅A (n is the number of phosphate groups), diadenosine polyphosphate; h-, human.

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from Wako Pure Chemicals. *Escherichia coli* strains BL21(DE3) and DH5α and plasmids pET-11b and pETBlue were from Novagen. Toyopearl-SuperQ and Toyopearl-Phenyl 650M were from Tosoh. Superdex 75 10/30 was from Amersham Bioscience. The CAPCELL PAK C18 column was from Shiseido. The molecular weight marker kit was from Sigma.

Dianucleoside polyphosphates (d(ADP)₆, d(ATP)₆, d(CTP)₆, d(GTP)₆, d(UTP)₆) and diadenosine monophosphate (d(AMP)₆) were from Sigma.

**Overexpression of the ndx1 Gene**—Preliminary sequence data for the *T. thermophilus* HB8 ndx1 gene, which contains the Nudix motif, was provided by the *T. thermophilus* HB8 genome project (21, 22). Using this information, two primers for amplification of the target gene were synthesized, and PCR (polymerase chain reaction) was carried out using these primer and LA Taq polymerase. The primer sequences were 5′-ATATCATATGGAAGCCGGCGGGGCGTGTC-3′ and 5′-ATATGACAATTTAATAGGGGATCACTGAAAAG-3′, and the underlining indicates NdeI and BamHI sites. The amplified gene fragment was ligated into pET-11b (Novagen) and BamHI sites following TA cloning and sequencing. *E. coli* BL21(DE3) cells transformed with the resulting plasmid were grown at 37 °C to 0.5% (cell/wet cell) on 1.5 liters of LB medium containing ampicillin. The cells were then harvested by centrifugation, and stored at −20 °C.

**Purification of Ndx1**—All purification steps described below were carried out at room temperature. Frozen cells (3 g) were suspended in 30 ml of lysis buffer (50 mM Tris-HCl, 0.1 mM EDTA, 10% (w/v) glycerol, and 20% (w/v) ammonium sulfate. The proteins were eluted with a linear gradient of 50% methanol. Nucleotides were detected at 260 nm, and their identifications were based on their retention times. The activity of Ndx1 was measured by adding 100 μl of 100 mM EDTA, and the protein was removed by ultrafiltration using a membrane filter. The 100-μl aliquot of the filtrate was applied to a reversed-phase column (CAPCELL PAK C18, 4.6 × 75 mm), which was equilibrated with 20 mM sodium phosphate (pH 7.0), 5 mM tetra-n-butyl ammonium phosphate, and 10% methanol. Elution was performed by a gradient of 10–50% methanol. Nucleotides were detected at 260 nm, and their identifications were based on their retention times. Their concentrations were calculated by integrating their respective peak areas. Concentrations of substrates were varied between 0.2 and 16 μM. Initial velocity was calculated from product concentration and plotted against substrate concentration. These were fitted to the Michaelis–Menten equation and Hanes–Woelfl plot, and the kinetic constant was calculated using the following software Igor Pro 3.1 (Wave Metrics). *Fitting Plot of pH-dependent Assay*—Assuming Scheme 1 for the Ndx1 reaction, the following equations could be generated (26). In short, there are two residues that are related to catalysis by Ndx1.

\[
K_e = K_e(1 + [H^+]K_{es1} + [K^+]K_{es2}) \left(1 + [H^+]K_{es3} + [K^+]K_{es4} \right)^{-1}
\]

(1)

\[
V = k_v[E_p] \left(1 + [H^+]K_{es1} + [K^+]K_{es2} \right)
\]

(2)

\[
k_{in} = k_v[E_p] \left(1 + [H^+]K_{es1} + [K^+]K_{es2} \right)
\]

(3)

\[
VK_e^{-1} = k_v[E_p] \left(1 + [H^+]K_{es1} + [K^+]K_{es2} \right)
\]

(4)

\[
k_{out}/K_e = k_v/k_v[E_p] \left(1 + [H^+]K_{es1} + [K^+]K_{es2} \right)
\]

(5)

The ρKₐ values of one residue are ρKₐ and ρKₑ, at free enzyme and complex, respectively. Similarly, those of the other residue are ρKₑ and ρKₑ. These ρKₑ values were calculated by fitting the data to Equations 1–3 using the software Igor Pro 3.1 (Wave Metrics).

**Fluorescence Measurements**—The fluorescence emission of Ndx1 was measured with a Hitachi spectrofluorometer, model F-4500. All measurements were taken with an excitation wavelength of 295 nm in a 5 × 5-mm quartz cuvette at 25 °C. The fluorescence titration was carried out by measuring the emission intensity at 328 nm. The reaction mixture (200 μl) contained 50 mM Tris-HCl, 100 mM KCl, 5 mM MgCl₂, 0.5 μM Ndx1, and various concentrations of ATP (7.5 μM).

**Site-directed Mutagenesis**—Pairs of oligonucleotides about 30 bases in length with melting temperatures of about 67 °C and containing the desired substitutions were designed. The ndx1 gene includes NruI and PshAI sites at the 60th and 155th positions, respectively. For W26A substitution, the region between the NruI site and BglII site at the 3′ terminus of the gene was amplified by PCR. For R45K, E46Q, E49Q, and E50Q substitution, the regions between the 5′ terminal NdeI site and PshAI site were amplified. The amplified DNA fragments were cloned into the pPTBlue vector by TA cloning. *E. coli* DH5α were transformed with the constructed plasmid DNA and cultured. Substitution at the desired positions was confirmed by sequencing. Then the wild-type fragments in the expression vector were replaced by the confirmed fragments containing the mutations.

**RESULTS**

**Preparation of Ndx1**—Using information from the *T. thermophilus* HB8 genome project, we identified eight open reading frames containing Nudix motifs and named them ndx1 to ndx8. The ndx1 gene product (DDBJ/EMBL/GenBank™ accession number AB125632; project code 1331) comprises 126 amino acids, has a molecular mass of 14.2 kDa, and has a theoretical pI of 4.8. When a BLAST search was carried out using the Ndx1 sequence as a query, Ndx1 was the most similar (about 25% identity) to Ap₃A hydrolases from *Caenorhabditis elegans*, hu-
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Fig. 1. Alignment of Ndx1 and other Nudix proteins. A, an amino acid sequence alignment between Ndx1 and several diadenosine polyphosphate hydrolases. Values in parentheses are the specific substrates for each Nudix protein. Hsa, H. sapiens; Cel, C. elegans; Spo, S. pombe; Soc, S. cerevisiae. Ap₆A hydrolases are from H. sapiens (P50583) and C. elegans (CA63535). Ap₆A from S. pombe (Q09790), YOR163w from S. cerevisiae (275071), and DIPPP from H. sapiens (AF62529) are enzymes that hydrolyze Ap₆A and diphosphoinositol polyphosphate. The accession numbers are presented in parentheses to identify amino acid sequences deposited in the NCBI protein data base. Numbers at the end of respective rows represent residue numbers.

B, an amino acid sequence alignment near the Nudix motif in several Nudix proteins. The accession numbers of the proteins are as follows; C. elegans (Cel), CA63535; H. sapiens (Hsa), P50583; Bartonella bacilliformis (Bba), L25278; Hordeum vulgare (Hvu), Z99996; L. angustifolius (Lan), U98841; S. pombe (Spo), Q09790; E. coli (Eco), 8-oxo-dGTP, P08337; E. coli (Eco; ADP-ribose), P36651. The black background indicates the tryptophan residues that are conserved in diadenosine polyphosphate hydrolases. Asterisks (*) indicate residues that are highly conserved in the Nudix motif.

man, and pig (Fig. 1A). However, the sequence similarity was restricted to the surroundings of the Nudix motif and was not enough to determine whether Ndx1 is an Ap₆A hydrolase or not.

Thus, Ndx1 was overexpressed in E. coli BL21(DE3) under the control of an isopropyl-β-D-thiogalactopyranoside-induced T7 promoter. The induced band at ∼15 kDa (corresponding to the size of Ndx1) was observed in the soluble fraction (Fig. 2), and we purified the protein to homogeneity utilizing three column chromatography steps (see details under “Experimental Procedures”). The sequence of the N-terminal nine residues of the overexpressed protein agreed with the residues predicted from the ndx1 sequence, confirming that the purified protein was Ndx1. Approximately 25 mg of Ndx1 was obtained from 3 g of cells.

Physicochemical Properties—Size exclusion chromatography was performed to investigate the oligomeric state of Ndx1. The elution profile of Ndx1 showed a single peak (Fig. 2B). The apparent molecular mass corresponding to the peak was estimated to be 17 kDa, which was similar to the 14.2-kDa mass calculated from the sequence. This indicates that Ndx1 exists in a monomeric state in solution.

The far-UV CD spectrum of the purified Ndx1 showed negative double maxima at 209 and 220 nm (Fig. 3A), characteristic of an α-helical structure. The stability of Ndx1 to temperature and pH was examined based on the mean residue ellipticity at 222 nm (θₑ,222). Ndx1 was stable up to 95 °C at pH 7.5 (Fig. 3B) and stable in a wide range of pH at 25 °C (Fig. 3C).

Enzymatic Activity—Most Nudix proteins examined to date are nucleotide pyrophosphatases that hydrolyze a nucleoside diphosphate linked to another moiety (1). Therefore, enzymatic activity of Ndx1 was examined for a wide range of nucleotides known to be substrates of other Nudix proteins. Ndx1 was inactive toward the following nucleotides when assayed at 50 μM: 5′-(deoxy)nucleoside triphosphates, 5′-nucleoside diphosphates, nucleoside diphosphate sugars, NADH, NAD⁺, CoA, and acetyl CoA. Significant activity was found toward dinucleotide polyphosphates and nucleotide phosphatases. The respective substrates yielded products as follows: Ap₆A, 2ATP; Ap₅A, ATP and ADP; Ap₄A, ATP and AMP; Ap₃A, ATP and inorganic orthophosphate (Table I). In all cases ATP was generated as a product. ATP and Ap₆A were not hydrolyzed by Ndx1. These data indicate that Ndx1 protein has ATP-generating (di)nucleotide polyphosphate hydrolase activity.

Table I shows the steady-state kinetic constants for each active substrate assuming Michaelis-Menten type reactions. Whereas the Km values for these substrates were all about 1 μM, the catalytic constants (kcat) varied among the tested substrates (Table I). Based on the catalytic efficiencies (kcat/Km), the highest activity was observed for Ap₆A followed by p₄A, Ap₅A, and Ap₄A (Table I). The substrate preference was Ap₆A > p₄A >> Ap₅A > Ap₄A for polyphosphates and adenosine > guanosine for the base. Therefore, we conclude that Ndx1 is an ATP-generating Ap₆A hydrolase. Among the known enzymes that specifically hydrolyze Ap₆A, Ndx1 is the only enzyme that symmetrically hydrolyzes Ap₆A.

Divalent metal ions were essential for Ndx1 activity, which is a common property of Nudix proteins (27). The effect of several divalent metal ions (each 5 mM) on Ndx1 activity for Ap₆A was investigated. Significant activity was observed in the presence of Mn²⁺, Mg²⁺, and Zn²⁺ with the apparent rate constant of kₐp values of 8.6, 4.2, and 4.1 s⁻¹, respectively. In contrast, there was low activity in the presence of Ca²⁺ (kₐp 0.51 s⁻¹), and there was no activity in the presence of Ca²⁺, Ni²⁺, and Cu²⁺. Among monovalent ions, fluoride ion showed strong inhibition of the Ndx1 activity. Dependence of the inhibition effect on fluoride ion concentration revealed that the inhibition was in a non-competitive manner with a Ki of 424 μM toward free enzyme and a Ki of 80 μM toward complex.

Ndx1 exhibited higher activity at higher pH and little activity at lower pH. This observation is typical for the Nudix class of enzymes, which usually have optimal pH values in the alkaline range. The presence of two pKa values (7.9 and 10.0) was found in the plot of kₐp against pH (Fig. 4A). The optimal pH for Ndx1 activity was about 8, judged by the catalytic efficiency kₐp/Km (Fig. 4B). The plot of the kₐp against temperature was bell-shaped; the optimal temperature for Ndx1 activity was 70 °C. This higher activity at higher temperature reflects a common property of enzymes from T. thermophilus.

Protein-Substrate Interaction—The Km values of Ndx1 for several (d)adenosine polyphosphates were about 1 μM. Because these results suggest that the affinity for these substrates is almost the same, we hypothesized that Ndx1 recognized a common moiety of the substrates at the initial binding phase. Although several tertiary structures of Nudix proteins have been reported (2, 3, 28, 29), detailed investigations of
substrate binding mechanisms have not often been performed by biochemical methods. The adenosine phosphate moiety is commonly contained in Ndx1 substrates, and Ndx1 has no activity toward ATP. Therefore, we measured the intrinsic fluorescence spectrum of Ndx1 upon binding to ATP to investigate the interaction between the protein and the adenosine phosphate moiety. The emission spectra were measured using an excitation wavelength of 295 nm, which excited only tryptophan residues. When ATP was added to Ndx1, the fluorescence intensity at around 328 nm decreased (Fig. 5A, bold solid line). As the ATP concentration increased, the fluorescence intensity gradually decreased. These results suggest that the decrease in fluorescence intensity reflects the protein-substrate interaction. When dATP was used as a ligand, the fluorescence intensity also decreased but not equivalently to ATP. When the change in fluorescence intensity was plotted against ligand concentration, $K_a$ was determined to be 13 $\mu$M for ATP and 36 $\mu$M for dATP based on bimolecular binding reaction (Fig. 5B). The affinity of Ndx1 for these nucleotides was also investigated by examining the inhibition of ATP and dATP on Ap$_{\alpha}$A hydrolysis by Ndx1 (not data shown). These results showed that $K_i$ was 13 $\mu$M for ATP and 41 $\mu$M for dATP. When Mg$^{2+}$ was omitted from the reaction mixture, no fluorescence change was observed (Fig. 5A, dotted line). Also, when GTP was used, the fluorescence intensity did not decrease, as is predicted by the low affinity of Ndx1 toward diguanosine polyphosphates.

Among the four Trp residues of Ndx1, Trp-26 was conserved in the N-terminal half of the Nudix motif in the sequence of dinucleotide polyphosphate hydrolases (Fig. 1B). This raised the possibility that the observed fluorescence changes could be ascribed to Trp-26. This hypothesis was confirmed by the observation that the mutant W26A Ndx1, in which Trp-26 was replaced by alanine, showed no decrease in fluorescence intensity upon the addition of ATP (Fig. 5C).

**Catalytic Residues**—From the dependence of Ndx1 hydrolysis activity on pH, $pK_a$ values of 7.9 and 10.0 were obtained from the $k_{\text{cat}}$ plot (Fig. 4A), and $pK_a$ values of 6.1 and 9.6 were obtained from the plot of $k_{\text{cat}}/K_m$ (Fig. 4B). These $pK_a$ changes demonstrated that when free Ndx1 bound to the substrate, the $pK_a$ of one residue changed from 6.1 to 7.9, and the $pK_a$ of a second residue changed from 9.6 to 10.0. It is thought that two residues whose $pK_a$ values change play an important role in Ndx1 hydrolysis.

In Ndx1, the conserved Nudix motif is located between Gly-31 and Val-53. In this motif, Glu-45, Arg-46, Glu-49, and Glu-50 are highly conserved across the sequences of Nudix proteins from plants, animals, and bacteria (1). When the glutamic acid residues (Glu-46, Glu-49, and Glu-50) in the Nudix motif were replaced by glutamine, the E46Q and E50Q mutants showed a 2.2 $\times$ 10$^4$-fold reduction and a 1.3 $\times$ 10$^5$-fold reduction in $k_{\text{cat}}$, respectively (Table II). In contrast, the E49Q mutation had very little effect on activity (Table II). Moreover, mutation of the conserved Arg-46 residue to lysine only slightly reduced the activity (Table II).

**DISCUSSION**

Many Nudix genes have been found in organisms whose genomes have been sequenced. The human genome project has identified at least 18 Nudix genes, some of which are 90% alike in amino acid sequence (30). In contrast, the sequences of bacterial Nudix proteins have weaker similarity to each other within a species and among species. For example, the E. coli genome contains 11 Nudix genes, whose products do not highly resemble each other (about 20% identity or less) and have different substrate specificities. The genome of D. radiodurans contains at least 23 Nudix genes, which is the largest number of Nudix genes found in any species (23). The products of these genes show diversity in size as well as in sequence; they include larger proteins (about 40–60 kDa) than the average Nudix protein (15–25 kDa). The T. thermophiles HB8 genome project has revealed eight Nudix genes, designated as ndx1 to ndx8. These gene products are not highly similar to each other (about 20% identity) except for the region around the Nudix motif. Although T. thermophilus is very closely related to D. radiodurans, only 2 of its Nudix proteins show a relatively high sequence similarity (54 and 40%) to Nudix proteins of D. radiodurans. The low sequence similarity found in the Nudix proteins in respective bacteria may suggest that these proteins have diverse functions.

In this study we have demonstrated that Ndx1 has the ability to hydrolyze diadenosine polyphosphates and showed the highest activity when Ap$_{\alpha}$A was used as a substrate. Proteins known to hydrolyze Ap$_{\alpha}$A include Schizosaccharomyces

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**Fig. 2. Expression and purification of Ndx1.** A, expressed and purified Ndx1. SDS-PAGE was run using a 10–20% gradient polyacrylamide gel. Lane 1, molecular marker containing reference proteins; lane 2, about 10 $\mu$g of cell extract; lane 3, preparation after purification. B, size exclusion chromatography. Ndx1 was loaded onto a Superdex 75 HR 10/30 column equilibrated with 50 mM Tris-HCl and 100 mM KCl (pH 7.5) and eluted with the same solvent at 25 $^\circ$C. Ndx1 was detected by absorbance at 280 nm. Molecular weight markers are: bovine serum albumin, 66 kDa (k); bovine carbonic anhydrase, 29 kDa; horse heart cytochrome c, 12.4 kDa.
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Fig. 3. CD spectrum, thermostability, and pH stability of Ndx1. A, far-UV CD spectrum. The measurement was performed at 25 °C in buffer solution containing 5 μM Ndx1, 50 mM potassium phosphate, and 100 mM KCl (pH 7.5). B, the temperature dependence of the residue molar ellipticity [θ] at 222 nm ([θ]₂₂₂). The measurements were performed in the same solution as for the far-UV CD spectrum measurements. The heating rate was 1 °C/min. C, pH-dependent changes in [θ]₂₂₂. The measurements were performed at 25 °C for 1 h (circles) or 24 h (triangles). The buffers used were: 50 mM glycine-HCl (pH 2.0–2.4), 50 mM sodium acetate (pH 4.0–4.8), 50 mM potassium phosphate (pH 5.0–6.9), 50 mM Tris-HCl (pH 7.7–8.8), 50 mM glycine-NaOH (pH 10.0–10.9), and 50 mM sodium phosphate (pH 11.8), and 100 mM KCl.

Ap₆A hydrolases cleave their substrates at multiple sites. YOR163w and Aps1 produce ADP and ATP, and hDIPP-2 always produces ATP and ADP (31, 32). Ndx1 has two features not found in other Ap₆A hydrolases. The first is that Ndx1 cleaves Ap₆A at Trp-26, a highly conserved residue. This implies a phylogenetic relationship between Ndx1 and Ap₆A hydrolases.

The features described above may be due to highly specific substrate recognition by Ndx1. The lower value of Kₘ for Ap₆A than Gp₄G indicates that Ndx1 prefers adenine to guanine as a base moiety. In addition, Ndx1 had lower affinity for dATP than for ATP, suggesting involvement of the sugar moiety in substrate recognition. ATP was not hydrolyzed by Ndx1, but its Kₘ was 13 μM, based on the intrinsic fluorescence change. ADP did not decrease the fluorescence intensity, indicating no binding to the enzyme. In addition, Ndx1 showed almost the same kₕcat values (about 1 μM) toward diadenosine polyphosphates (Ap₆A) (n = 4–6). These data strongly suggest that the ATP moiety of Ap₆A (n = 4–6) significantly contributes to the affinity of these substrates for Ndx1. However, the kₕcat values were different with each substrate, and its value was highest for Ap₆A. We hypothesize that the ATP moiety contributes mainly at the initial binding phase.

Fluorescence measurements using ATP also provided some information about the recognition site of Ndx1 for the ATP moiety. The substitution of Trp-26, a highly conserved residue, for Ala resulted in no fluorescence change. The reaction kinetics of the W26A mutant showed a lower rate (kₕcat = 1.3 s⁻¹) and lower affinity (Kₘ = 19 μM) than the wild-type protein (Table II). In the crystal structure of Ap₆A hydrolase from Lupinus angustifolius (29), the tryptophan residue corresponding to Trp-26 of Ndx1 is located at the substrate-binding site. These observations imply that Trp-26 of Ndx1 interacts with the substrate, possibly via stacking of the adenine base moiety of the substrate.

In addition, it should be noted that at extreme pH (11.5), minor products (p₄A + ADP) were observed in addition to the major products (2 ATP) (data not shown). This supports the notion that the residue whose the pKₐ value changed from 9.6 to 10.0 when free Ndx1 bound to the substrate is related to binding the substrate. This residue is probably Lys or Arg and

| Substrate | Product | kₕcat | Kₘ | kₕcat/kₘ |
|-----------|---------|-------|----|---------|
| Ap₆A      | ATP     | 4.1   | 1.4| 2.9 × 10⁶|
| p₄A       | ATP, P_i| 1.4   | 1.0| 1.4 × 10⁶|
| Ap₆A      | ATP, ADP| 0.52  | 1.1| 4.7 × 10⁴|
| Ap₆A      | ATP, AMP| 0.27  | 1.1| 2.5 × 10⁵|
| Gp₄G      | GTP, GDP| 0.27  | 1.4| 2.0 × 10⁵|
| Gp₄G      | GTP, GMP| 0.09  | 9.3| 9.7 × 10⁴|

The second feature that distinguishes Ndx1 from others is that Ndx1 produced ATP from several substrates. For example, Ndx1 produced ATP not only from Ap₆A but also from Ap₄A, Ap₄A, and p₄A (31). Ndx1 produced ATP from Ap₄A in a strict manner, and hAps1, hAps2, and hDIPP-2 always produce ATP. In short, Ndx1 produces ATP as a product in a strict manner, which always generates ATP. Generation of ATP as a product does not decrease the fluorescence intensity, indicating no binding to the enzyme. In addition, Ndx1 showed almost the same kₕcat values (about 1 μM) toward diadenosine polyphosphates (Ap₆A) (n = 4–6). These data strongly suggest that the ATP moiety of Ap₆A (n = 4–6) significantly contributes to the affinity of these substrates for Ndx1. However, the kₕcat values were different with each substrate, and its value was highest for Ap₆A. We hypothesize that the ATP moiety contributes mainly at the initial binding phase.

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may function as strict recognition and cleavage of substrates. A candidate is Arg-45 in the Nudix motif. However, Arg-45 is unlikely to be this residue because the R45K mutant strictly cleaved Ap6A. Similar to the activity of Ndx1, other known Nudix hydrolases show high activity in alkaline pH (around 8.0) (34). This feature may be related to a substrate, which includes some dissociative groups in its structure.

Another characteristic of Ndx1 reaction is that the $k_{cat}$ value for ApnA was related to the length of the phosphate.

**Fig. 4. Nudix activity depending on pH.** The Ndx1 activity assay was performed at several pH levels, and the kinetic parameters were calculated using the Michaelis-Menten equation. The buffers used were: 50 mM HEPES (pH 6.74–7.05), 50 mM Tris-HCl (pH 7.5–8.8), and 50 mM Gly-NaOH (pH 9.06–10.66), each of which contained 100 mM KCl. $k_{cat}$, plotted relative to pH. This plot was fitted to Equation 1 (see “Experimental Procedures”), and the theoretical curve was drawn. $B$, $k_{cat}/K_m$, plotted relative to pH. This plot was fitted as in A using Equation 3. $C$, $K_m$, plotted relative to pH. This plot was fitted as in A using Equation 5.

**Fig. 5. Fluorescence measurements of Ndx1.** Emission spectra were measured at 25 °C using an excitation wavelength of 285 nm. $A$, fluorescence spectra were measured using 0.5 μM wild-type Ndx1. The symbols used are as follows: solid line, in the presence of 5 mM MgCl$_2$; bold solid line, in the presence of 50 μM ATP and 5 mM MgCl$_2$; dotted line, in the presence of 50 μM ATP; broken line, protein alone. $B$, the changes in emission intensity at 328 nm dependent upon substrate concentration. The substrates used in this measurement were ATP (circles) and dATP (triangles). These plots were fitted on bimolecular binding reaction. $C$, fluorescence measurements were performed using 0.5 μM W26A mutant Ndx1. The symbols used are as follows: solid line, in the presence of 50 μM ATP and 5 mM MgCl$_2$; dotted line, in the presence of 5 mM MgCl$_2$. The intensity of the W26A protein alone was significantly smaller than that of wild type.

**Fig. 11.** Fluorescence measurements of Ndx1. Emission spectra were measured at 25 °C using an excitation wavelength of 285 nm. $A$, fluorescence spectra were measured using 0.5 μM wild-type Ndx1. The symbols used are as follows: solid line, in the presence of 5 mM MgCl$_2$; bold solid line, in the presence of 50 μM ATP and 5 mM MgCl$_2$; dotted line, in the presence of 50 μM ATP; broken line, protein alone. $B$, the changes in emission intensity at 328 nm dependent upon substrate concentration. The substrates used in this measurement were ATP (circles) and dATP (triangles). These plots were fitted on bimolecular binding reaction. $C$, fluorescence measurements were performed using 0.5 μM W26A mutant Ndx1. The symbols used are as follows: solid line, in the presence of 50 μM ATP and 5 mM MgCl$_2$; dotted line, in the presence of 5 mM MgCl$_2$. The intensity of the W26A protein alone was significantly smaller than that of wild type.
group of the substrate. Comparison of the \( k_{\text{cat}} \) for ApA and ApA suggests that P6 is important for catalysis. In addition to the ATP moiety containing the P1-P3 region, a contact of Ndx1 at around the P6 site and/or the terminal adenine may be required for stability of the substrate in the transition state. As mentioned above, Ndx1 has a higher sequence similarity to

\[ \text{Mn}^2+ \]

hydrolases than Ap6A hydrolases and generates ATP as mentioned above, Ndx1 has a higher sequence similarity to

\[ \text{Cu}^2+ \]

Ap4A hydrolases than Ap6A hydrolases and generates ATP as mentioned above, Ndx1 has a higher sequence similarity to

\[ \text{F}^- \]

of Mg2+. It has been reported that ApA is synthesized by acyl-CoA synthetase (46). Diadenosine polyphosphates have been found in E. coli (50), erythrocytes (45), plant leaves (49), and mamma

\[ \text{K}^- \]

lions (50, 51) and are supposed to function as signaling mole-

\[ \text{Mg}^2+ \]

ules (1). At present there is no evidence that diadenosine polyphosphates function as a signaling molecule in prokaryotes including T. thermophilus. However, some kinases are known to be inhibited by diadenosine polyphosphates including ApA (52, 53). Because diadenosine polyphosphates are very stable (these hardly hydrolyze by incubation at 100 °C for 20 min (50, 51) and can accumulate in the cell, Ndx1 may function as a housecleaning enzyme to prevent accumulation of those compounds. While considering whether such a molecule exists in T. thermophilus and whether it is meaningful that Ndx1 symmetrically hydrolyzes ApA, we are studying the physiological function of Ndx1 in relationship to ApA.

As mentioned earlier, proteins from T. thermophilus are stable against heat and extreme pH. This property makes these proteins suitable for structural and functional analysis. Actually, the high stability of Ndx1 enabled us to examine the dependence of the activity on pH. Now crystallization of Ndx1 is under way to determine the tertiary structure. We have already succeeded in crystallization of another Nudix protein (Ndx4) from T. thermophilus (54). Further details concerning the mechanisms of hydrolysis and substrate specificity of Ndx1 will be uncovered by structural analysis of Ndx1.

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