Structural Insights into the *Thermus thermophilus* ADP-ribose Pyrophosphatase Mechanism via Crystal Structures with the Bound Substrate and Metal*

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ADP-ribose pyrophosphatase (ADPRase) catalyzes the divalent metal ion-dependent hydrolysis of ADP-ribose to ribose 5'-phosphate and AMP. This enzyme plays a key role in regulating the intracellular ADP-ribose levels, and prevents nonenzymatic ADP-ribosylation. To elucidate the pyrophosphatase hydrolysis mechanism employed by this enzyme, structural changes occurring on binding of substrate, metal, and product were investigated using crystal structures of ADPRase from an extreme thermophile, *Thermus thermophilus* HB8. Seven structures were determined, including that of the free enzyme, the Zn$^{2+}$-bound enzyme, the binary complex with ADP-ribose, the ternary complexes with ADP-ribose and Zn$^{2+}$ or Gd$^{3+}$, and the product complexes with AMP and Mg$^{2+}$ or with ribose 5'-phosphate and Zn$^{2+}$. The structural and functional studies suggested that the ADP-ribose hydrolysis pathway consists of four reaction states: bound with metal (I), metal and substrate (II), metal and substrate in the transition state (III), and products (IV). In reaction state II, Glu-82 and Glu-70 abstract a proton from a water molecule. This water molecule is situated at an ideal position to carry out nucleophilic attack on the adenosyl phosphate, as it is 3.6 Å away from the target phosphorus and almost in line with the scissile bond.

Nudix pyrophosphatases are widely distributed in nature and share a highly conserved amino acid sequence motif called the "Nudix motif" (GX$_2$EX,-REUXEESGU, where U is one of the bulky hydrophobic amino acids I, L, or V), which adopts a unique loop-helix-loop structure (1). Enzymes in this family catalyze the hydrolysis of nucleoside diphosphates, linked to another moiety X. Their postulated role is to control the cellular concentration of toxic nucleoside diphosphate derivatives or physiological metabolites, accumulation of which could be harmful (1). ADP-ribose (ADPR)$^1$ is one such diphosphate derivative, which is produced enzymatically as part of the turnover of NAD$^+$, cyclic ADPR, ADP-ribosylated proteins, and poly-ADP-ribosylated proteins. Although certain proteins are posttranslationally modified by ADPR, high intracellular levels of ADPR could result in nonenzymatic ADP-ribosylation. This is a deleterious process that inactivates enzymes and could interfere with the recognition of enzymatic ADP-ribosylation (2). ADPR pyrophosphatases (ADPRases) catalyze the hydrolysis of ADPR to AMP and ribose 5'-phosphate to prevent ADPR accumulation.

ADPRase activity has been detected in all three kingdoms (3–7), but the specificity for ADPR over other substrates and the selectivity of metal ions required for activity vary between species. The mechanism underlying the different substrate specificity and the metal dependence is unknown at both the structural and functional levels. Elucidation of these properties requires the study of ADPRases from numerous sources.

In this article, we investigated the catalytic mechanism of ADPRase from an extreme thermophile, *Thermus thermophilus* HB8 (TtADPRase). In general, proteins isolated from *T. thermophilus* are heat-stable and suitable for physicochemical studies, including x-ray crystallography (8, 9). TtADPRase catalyzes the hydrolysis of ADPR to AMP and ribose 5'-phosphate in the presence of Mg$^{2+}$ and Zn$^{2+}$ ions. The enzyme is also heat-stable and retains ADPRase activity at 75 °C. The structures of the free enzyme, the Zn$^{2+}$-bound enzyme, the binary complex with ADPR, the ternary complexes with ADPR and metal, and the product complexes containing AMP or ribose 5'-phosphate were determined. Based on structural and functional analyses, we propose a novel reaction mechanism for ADPR pyrophosphatase hydrolysis that is different from those proposed based on the ternary complex structures of ADPRases from *Escherichia coli* (EcADPRase) and from *Mycobacterium tuberculosis* (MtADPRase) (10, 11).

**EXPERIMENTAL PROCEDURES**

Preparation of Mutant TtADPRases—pT7Blue-ndx4, which contains the ndx4 gene, was used as a template of mutagenesis (12). pT7Blue-ndx4 was digested with BamHI and HindIII, and the fragment was inserted into pKP19K (Takara Shuzo Co., Kyoto, Japan) digested with the same enzymes. Site-directed mutagenesis of E82Q, E86Q, and

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$ The abbreviations used are: ADPR, ADP-ribose; ADPRase, ADP-ribose pyrophosphatase; EcADPRase, *E. coli* ADP-ribose pyrophosphatase; MAD, multiwavelength anomalous diffraction; MtADPRase, *M. tuberculosis* ADP-ribose pyrophosphatase; OAADPR, O-acetyl-ADP-ribose; Se-Met, selenomethionyl; TtADPRase, *T. thermophilus* ADP-ribose pyrophosphatase.
ADP-ribose Pyrophosphatase from an Extreme Thermophile

Table I

| Data set name | Peak | Se-Met | Free enzyme |
|---------------|------|--------|------------|
| Data collection |      |        |            |
| Space group | P3,21 |        | P3,21      |
| Cell constant (Å) | a = b = 49.8, c = 118.5 |        | a = b = 49.6, c = 117.9 |
| Wavelength (Å) | 0.9804 |        | 0.9809      |
| Resolution (Å) | 50–1.70 (1.76–1.70) | 50–1.70 (1.76–1.70) | 50–1.70 (1.76–1.70) |
| No. of observations | 195,717 | 195,671 | 195,385 |
| Unique reflections | 19,436 | 19,430 | 19,457 |
| Data completeness (%) | 99.8 (100) | 99.7 (100) | 99.8 (100) |
| Mean I/σ | 38.3 (6.1) | 38.6 (6.1) | 38.3 (6.2) |
| Rmerge | 5.4 (21.0) | 4.4 (20.2) | 4.7 (19.9) |
| Phasing power acertic | 3.56 |        | 3.56 |
| Cullis R acertic | 0.58 |        | 0.58 |
| Model refinement statistics | (resolution for refinement) |        | (resolution for refinement) |
| Rcryst | 20.9/23.1 |        | Rcryst |
| Average B factor | 0.005 |        | 17.23 |
| Deviations from ideality | 1.25 |        | 1.25 |
| Bond lengths (Å) | 88.9 |        | 88.9 |
| Ramachandran plot | Additional allowed |        | Additional allowed |

Note: Values in parentheses refer to the highest resolution shell. The crystals of the ternary complex were obtained by mixing 1:1 the reservoir solution and the solution containing 2 mM protein and 2 mM ZnCl2. Data were collected at the wavelengths of peak (1.278 Å) and refined from 15- to 2.5-Å resolution using the phase information from the diffraction data with the CNS program (18). The three selenium atom sites deduced from the difference Fourier map coincided with the three sulfur sites of the methionine residues in the model. The N-terminal region was disordered, and the first Met was not detected. Progress in the structural refinement was evaluated at each stage by the free R factor and by inspection of stereochemical parameters calculated by the PROCHECK program (19). Final phasing and refinement statistics are shown in Table I.

Data Collection and Refinement of the Wild-type Complexes—The crystals of the Zn2+–bound enzyme were obtained by mixing 1:1 the reservoir solution and the solution containing 2 mM protein and 2 mM ZnCl2. Data were collected at the wavelengths of peak (1.278 Å) and refined from 15- to 2.5-Å resolution using the phase information from the diffraction data with the CNS program (18). The three selenium atom sites deduced from the difference Fourier map coincided with the three sulfur sites of the methionine residues in the model. The N-terminal region was disordered, and the first Met was not detected. Progress in the structural refinement was evaluated at each stage by the free R factor and by inspection of stereochemical parameters calculated by the PROCHECK program (19). Final phasing and refinement statistics are shown in Table II.

The crystals of the ternary complex were obtained by mixing 1:1 the reservoir solution and the solution containing 2 mM protein and 2 mM ZnCl2. Data were collected at the wavelengths of peak (1.278 Å) and refined from 15- to 2.5-Å resolution using the phase information from the diffraction data with the CNS program (18). The three selenium atom sites deduced from the difference Fourier map coincided with the three sulfur sites of the methionine residues in the model. The N-terminal region was disordered, and the first Met was not detected. Progress in the structural refinement was evaluated at each stage by the free R factor and by inspection of stereochemical parameters calculated by the PROCHECK program (19). Final phasing and refinement statistics are shown in Table III.

The crystals of the ternary complex were obtained by mixing 1:1 the reservoir solution and the solution containing 2 mM protein and 2 mM ZnCl2. Data were collected at the wavelengths of peak (1.278 Å) and refined from 15- to 2.5-Å resolution using the phase information from the diffraction data with the CNS program (18). The three selenium atom sites deduced from the difference Fourier map coincided with the three sulfur sites of the methionine residues in the model. The N-terminal region was disordered, and the first Met was not detected. Progress in the structural refinement was evaluated at each stage by the free R factor and by inspection of stereochemical parameters calculated by the PROCHECK program (19). Final phasing and refinement statistics are shown in Table IV.

The crystals of the ternary complex were obtained by mixing 1:1 the reservoir solution and the solution containing 2 mM protein and 2 mM ZnCl2. Data were collected at the wavelengths of peak (1.278 Å) and refined from 15- to 2.5-Å resolution using the phase information from the diffraction data with the CNS program (18). The three selenium atom sites deduced from the difference Fourier map coincided with the three sulfur sites of the methionine residues in the model. The N-terminal region was disordered, and the first Met was not detected. Progress in the structural refinement was evaluated at each stage by the free R factor and by inspection of stereochemical parameters calculated by the PROCHECK program (19). Final phasing and refinement statistics are shown in Table V.

The crystals of the ternary complex were obtained by mixing 1:1 the reservoir solution and the solution containing 2 mM protein and 2 mM ZnCl2. Data were collected at the wavelengths of peak (1.278 Å) and refined from 15- to 2.5-Å resolution using the phase information from the diffraction data with the CNS program (18). The three selenium atom sites deduced from the difference Fourier map coincided with the three sulfur sites of the methionine residues in the model. The N-terminal region was disordered, and the first Met was not detected. Progress in the structural refinement was evaluated at each stage by the free R factor and by inspection of stereochemical parameters calculated by the PROCHECK program (19). Final phasing and refinement statistics are shown in Table VI.

The crystals of the ternary complex were obtained by mixing 1:1 the reservoir solution and the solution containing 2 mM protein and 2 mM ZnCl2. Data were collected at the wavelengths of peak (1.278 Å) and refined from 15- to 2.5-Å resolution using the phase information from the diffraction data with the CNS program (18). The three selenium atom sites deduced from the difference Fourier map coincided with the three sulfur sites of the methionine residues in the model. The N-terminal region was disordered, and the first Met was not detected. Progress in the structural refinement was evaluated at each stage by the free R factor and by inspection of stereochemical parameters calculated by the PROCHECK program (19). Final phasing and refinement statistics are shown in Table VII.

The crystals of the ternary complex were obtained by mixing 1:1 the reservoir solution and the solution containing 2 mM protein and 2 mM ZnCl2. Data were collected at the wavelengths of peak (1.278 Å) and refined from 15- to 2.5-Å resolution using the phase information from the diffraction data with the CNS program (18). The three selenium atom sites deduced from the difference Fourier map coincided with the three sulfur sites of the methionine residues in the model. The N-terminal region was disordered, and the first Met was not detected. Progress in the structural refinement was evaluated at each stage by the free R factor and by inspection of stereochemical parameters calculated by the PROCHECK program (19). Final phasing and refinement statistics are shown in Table VIII.

The crystals of the ternary complex were obtained by mixing 1:1 the reservoir solution and the solution containing 2 mM protein and 2 mM ZnCl2. Data were collected at the wavelengths of peak (1.278 Å) and refined from 15- to 2.5-Å resolution using the phase information from the diffraction data with the CNS program (18). The three selenium atom sites deduced from the difference Fourier map coincided with the three sulfur sites of the methionine residues in the model. The N-terminal region was disordered, and the first Met was not detected. Progress in the structural refinement was evaluated at each stage by the free R factor and by inspection of stereochemical parameters calculated by the PROCHECK program (19). Final phasing and refinement statistics are shown in Table IX.

The crystals of the ternary complex were obtained by mixing 1:1 the reservoir solution and the solution containing 2 mM protein and 2 mM ZnCl2. Data were collected at the wavelengths of peak (1.278 Å) and refined from 15- to 2.5-Å resolution using the phase information from the diffraction data with the CNS program (18). The three selenium atom sites deduced from the difference Fourier map coincided with the three sulfur sites of the methionine residues in the model. The N-terminal region was disordered, and the first Met was not detected. Progress in the structural refinement was evaluated at each stage by the free R factor and by inspection of stereochemical parameters calculated by the PROCHECK program (19). Final phasing and refinement statistics are shown in Table X.
### Statistics for data and refinement of wild-type complexes

| Data collection | 
|-----------------|
| **Zn\textsuperscript{2+}-bound enzyme** (ligand Zn\textsuperscript{2+}) | **Binary complex** (ligand ADPR) | **Zn\textsuperscript{2+} ternary complex** (ligands Zn\textsuperscript{2+} and ADPR) |
| Peak | Low remote | Peak | Low remote | Peak | Low remote |
| Space group | P3\textsubscript{2}1 | P3\textsubscript{2}1 | P3\textsubscript{2}1 |
| Cell constant (Å) | a = b = 49.9, c = 118.31 | a = b = 49.9, c = 119.3 | a = b = 49.7, c = 118.9 |
| Wavelength (Å) | 1.278 | 1.292 | 1.54 | 1.282 | 1.287 |
| Resolution (Å) | 50–160 (1.66–1.60) | 50–160 (1.66–1.60) | 19–201 (2.12–2.01) | 20–1.8 (1.86–1.80) | 20–1.8 |
| No. of observations | 333,276 | 323,773 | 164,512 | 192,144 | 190,720 |
| Unique reflections | 99.0 (92.4) | 98.7 (91.5) | 99.9 (99.9) | 99.2 (99.2) | 99.1 (98.1) |
| Mean | I | 57.2 (3.9) | 60.7 (4.6) | 11.2 (10.3) | 35.26 (12.8) | 35.36 (12.5) |
| R\textsubscript{cryst} (%) | 2.7 (22.4) | 2.4 (20.0) | 4.0 (5.7) | 4.9 (8.9) | 4.8 (8.6) |

**Model refinement statistics**

| Resolution for refinement | 43–1.74 | 19–2.0 | 20–1.8 |
| R\textsubscript{cryst} / R\textsubscript{free} | 22.1/25.6 | 21.3/23.4 | 20.6/23.5 |
| Average B factor | 22.58 | 17.59 | 21.45 |
| Deviations from ideality | Bond lengths (Å) | 0.064 | 0.013 | 0.004 |
| Bond angles (°) | 1.28 | 2.02 | 1.28 |
| Ramachandran plot | Favored | 91.3 | 91.0 | 91.5 |
| Additional allowed | 8.7 | 9.0 | 8.5 |

**Reaction state in Fig. 8**

| I | II | II |

### Data set name

| Data collection | **Gd\textsuperscript{3+} ternary complex** (ligands Gd\textsuperscript{3+} and ADPR) | **Product complex I** (ligands AMP and Mg\textsuperscript{2+}) | **Product complex II** (ligands ribose 5'-phosphate and Zn\textsuperscript{2+}) |
|-----------------|---------------------------------|---------------------------------|---------------------------------|
| Peak | Low remote | Peak | Low remote | Peak | Low remote |
| Space group | P3\textsubscript{2}1 | P3\textsubscript{2}1 | P3\textsubscript{2}1 |
| Cell constant (Å) | a = b = 49.9, c = 118.1 | a = b = 49.7, c = 118.0 |
| Wavelength (Å) | 1.711 | 1.716 | 1.00 |
| Resolution (Å) | 50–2.3 (2.38–2.30) | 50–2.3 | 50–1.8 (1.86–1.80) |
| No. of observations | 104,941 | 105,462 | 204,961 |
| Unique reflections | 8,054 | 8,063 | 16,475 |
| Data completeness (%) | 99.9 (98.1) | 99.5 (98.1) | 99.8 (100) |
| Mean | I | 23.7 (18.0) | 40.6 (11.4) | 24.6 (3.8) |
| R\textsubscript{cryst} (%) | 9.7 (19.2) | 5.0 (13.7) | 6.2 (28.3) |

**Model refinement statistics**

| Resolution for refinement | 50–1.8 |
| R\textsubscript{cryst} / R\textsubscript{free} | 22.3/24.6 |
| Average B factor | 22.70 |
| Deviations from ideality | Bond lengths (Å) | 0.005 |
| Bond angles (°) | 1.29 |
| Ramachandran plot | Favored | 90.4 |
| Additional allowed | 9.6 |

**Reaction state in Fig. 8**

| I | II |

### Table II

**ADP-ribose Pyrophosphatase from an Extreme Thermophile**

**Values in parentheses refer to the highest resolution shell.**

\[ R_{cryst} = \frac{\sum |I_{obs} - I_{calc}|}{\sum I_{calc}} \]

\[ R_{free} = \frac{\sum |I_{obs} - I_{calc}|}{\sum I_{calc}} \]

\[ R_{cryst} = \frac{\sum |F_{obs} - F_{calc}|}{\sum F_{calc}} \]

\[ R_{free} = \frac{\sum |F_{obs} - F_{calc}|}{\sum F_{calc}} \]

\[ \Delta = I_{obs} - I_{calc} \]

Dr. Brown, J. Smith, and L. Johnson, *Journal of Biochemistry*, vol. 123, pp. 37165-37170, 2000.
ADP-ribose Pyrophosphatase from an Extreme Thermophile

**RESULTS**

The dimer structure of the Gd³⁺ ternary complex of TtADP-Pase is shown in Fig. 1A. The two identical monomers (one subunit is colored red and blue, and another subunit is colored pink and light blue) were related by a crystallographic 2-fold axis, which was consistent with the result of gel filtration chromatography (12). The structure of TtADP-Pase did not show large conformational change, regardless of whether it exists as the free enzyme, Zn²⁺-bound enzyme, binary complex with ADPR, ternary complexes with ADPR and Gd³⁺, or product complexes with AMP or ribose 5'-phosphate. When the Cα atoms of these six structures were superimposed on the Gd³⁺ ternary complex structure, the average root mean square deviation values between the Gd³⁺ ternary complex structure and the other structures ranged from 0.20 to 0.26 Å.

The subunit was divided into two distinctive structural domains (Fig. 1, A and B); an N-terminal domain (residues 1–34, colored red) and a C-terminal domain, which is also referred to as the Nudix domain (residues 35–170, colored blue). The N-terminal domain contains the ribose 5'-phosphate of ADPR are represented by letters A and B, not by α and β.

**Overall Structure**

**Table III**

| Data collection | Data set name |
|-----------------|---------------|
| **ES2Q (ligands Mg²⁺ and SO₄²⁻)** | **ES6Q (ligands Mg²⁺ and ADPR)** |
| Space group | P₃₁₂₁ | P₃₁₂₁ |
| Cell constant (Å) | a = b = 49.9, c = 118.6 | a = b = 49.7, c = 118.5 |
| Wavelength (Å) | 1.282 | 1.278 |
| Resolution (Å) | 1.65 (1.66) | 1.65 (1.71) |
| No. of observations | 1,058,696 | 1,058,696 |
| Unique reflections | 475,174 | 479,474 |
| Data completeness (%) | 96.2 (95.8) | 98.0 (98.0) |
| Mean I/σ(I) | 19.71 | 19.71 |
| Bond lengths (Å) | 0.005 | 0.005 |
| Bond angles (°) | 1.36 | 1.28 |
| Ramachandran plot | Favored 93.1 | Favored 93.8 |
| Additional allowed | 6.9 | 6.2 |

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| Data collection | Data set name |
|-----------------|---------------|
| **ES2Q (ligands Zn²⁺ and SO₄²⁻)** | **ES6Q (ligands Zn²⁺ and ADPR)** |
| Space group | P₃₁₂₁ | P₃₁₂₁ |
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The subunit was divided into two distinctive structural domains (Fig. 1, A and B); an N-terminal domain (residues 1–34, colored red) and a C-terminal domain, which is also referred to as the Nudix domain (residues 35–170, colored blue). The N-terminal domain contains the ribose 5'-phosphate of ADPR are represented by letters A and B, not by α and β.

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The subunit was divided into two distinctive structural domains (Fig. 1, A and B); an N-terminal domain (residues 1–34, colored red) and a C-terminal domain, which is also referred to as the Nudix domain (residues 35–170, colored blue). The N-
and the Nudix domain was comprised of anti-parallel $\beta$-sheet from $\beta$1 to $\beta$3, and the Nudix domain was $\alpha$ + $\beta$ fold, with mixed sheet of $\beta$4 to $\beta$9 surrounded by three $\alpha$-helices of a1 to a3 (Fig. 1B). N-terminal residues (1–10) and residues (124–130) in loop L4 were disordered, but the presence of ADPR and metal resulted in order in loop L4. The Nudix motif (residues 67–89) folded into a loop-helix structure, not a loop-helix-loop structure, stabilized by an electrostatic network formed by salt links between Arg-18 and Arg-27* in the active site. In the presence of ADPR and metal ions, the Nudix domain was disordered, but the presence of ADPR and metal resulted in order in loop L4. The Nudix motif (residues 67–89) folded into a loop-helix structure, not a loop-helix-loop structure, stabilized by an electrostatic network formed by salt links between Arg-18 and Arg-27* in the active site.

Terminal domain comprised anti-parallel $\beta$-sheet from $\beta$1 to $\beta$3, and the Nudix domain was $\alpha$ + $\beta$ fold, with mixed sheet of $\beta$4 to $\beta$9 surrounded by three $\alpha$-helices of a1 to a3 (Fig. 1B). N-terminal residues (1–10) and residues (124–130) in loop L4 were disordered, but the presence of ADPR and metal resulted in order in loop L4. The Nudix motif (residues 67–89) folded into a loop-helix structure, not a loop-helix-loop structure, stabilized by an electrostatic network formed by salt links between Arg-18 and Arg-27* in the active site. In the presence of ADPR and metal ions, the Nudix domain was disordered, but the presence of ADPR and metal resulted in order in loop L4. The Nudix motif (residues 67–89) folded into a loop-helix structure, not a loop-helix-loop structure, stabilized by an electrostatic network formed by salt links between Arg-18 and Arg-27* in the active site.

In general, thermophile proteins tend to have a short loop length, because flexibility of the loop reduces stability. Smaller loops, such as the loop-helix structure described above, are advantageous to TtADPRase. The TtADPRase structure of Gd$^{3+}$ ternary complex was quite similar to those of the EcADPRase and M. tuberculosis ADPRase (10, 11), although the detailed structures differed. N-terminal domain $\beta$-sheet was shorter, loop L4 was moved outward, and loop L5 tip was situated upward by 4 Å. TtADPRase has other properties that might contribute to stability, including a lower content of chemically unstable amino acids such as Asn, Cys, and Met (25), and a smaller surface area in the N-terminal domain than for EcADPRase and M. tuberculosis ADPRase.

Dimer Interactions

The TtADPRase homodimer was stabilized by interactions in three regions. The first region contained an interface created between the N-terminal domains of both subunits, where the side chain of Thr-15 and the amide nitrogens of Arg-18 and Ile-19 made hydrogen bonds with the side chain of Glu-29 of another subunit. The second region contained an interface created between the N-terminal domain of one subunit and the Nudix domain of another subunit, where His-33 made a stacking interaction with Phe-105 of another subunit. The third region contained an interface created between the Nudix domains of both subunits, where Phe-97 made a stacking interaction with His-145 of another subunit, Ser-102 made a hydrogen bond with Glu-108 of another subunit, and Lys-109 made a salt bridge with Glu-151 of another subunit.

ADPR Binding (Corresponds to Reaction State II in Fig. 8)

The stereo structure and the scheme of hydrogen-bonding and stacking interaction between TtADPRase and ADPR are shown in Fig. 2 (A and B). The activity of TtADPRase was dependent on the presence of metal ions and was inhibited by acidic pH. ADPR was not hydrolyzed when crystals were prepared in solutions without metal ions for the binary complex with ADPR or prepared in an acidic pH for the ternary complex with ADPR and Zn$^{2+}$ or Gd$^{3+}$ (Table II). The binary complex without metal ion gave a better Fourier map (Fig. 2A) than the ternary complex. ADPR bound to the dimeric interface, composed of a turn between $\beta$1 and $\beta$2, L1, L3, $\beta$6 and a3 of one subunit, L3*, $\beta$2*, $\beta$3* of another subunit (secondary structures and residue numbers of another subunit are indicated by asterisks). Movement of amino acid residues was not observed between the free enzyme and the substrate complexes (the binary complex and the ternary complexes), except for the side chain Arg-18 and Arg-27* in the active site. In the pres-
ence of ADPR, Arg-18 moved toward ADPR to stack on the adenine of ADPR. Arg-27* moved away to avoid the positive charge repulsion of Arg-18 and made a hydrogen bond with O2H of the adenosyl ribose (Fig. 3, compare A and B). Such rearrangement of two residues on binding of ADPR resulted in the formation of a fence with Tyr-28*.

Bound ADPR resembled a horseshoe, in which two ends come together such that the adenine N7 and β-phosphate oxygen made hydrogen bonds with the same water molecule W1 (Fig. 2). The amide nitrogen and oxygen of Glu-29* made hydrogen bonds with adenine N1 and N6, respectively. In the binary complex structure, the terminal ribose was in α-configuration, and this terminal ribose O1H made hydrogen bonds with two water molecules W2 and W3. These water molecules also made hydrogen bonds with amide nitrogen atoms of Ala-154 and Thr-155 and oxygen atoms of the side chain of Glu-63 and Thr-155. The adenine was stacked in the hydrophilic pocket composed of Arg-18, Ile-19, and Tyr-28*. Two oxygen atoms of the β-phosphate made hydrogen bonds with Arg-54. On the other hand, the α-phosphate group made a hydrogen bond with the Leu-68 amide nitrogen. Pro-103* stacked on the terminal ribose. In the binary complex, Ser-102* made hydrogen bonds with O2H and O3H of the terminal ribose. The amide oxygen and nitrogen of Gly-104* made hydrogen bonds with adenine N6 and the terminal ribose O2H, respectively. Glu-108 made a hydrogen bond with the terminal ribose O3H. These many interactions for recognition with the terminal ribose might explain the high substrate specificity of ADPR over ADP-sugar.

**Metal Binding (Corresponds to Reaction States I and II in Fig. 8)**

To identify the metal-binding sites, the structures of the Zn2+-bound enzyme and the Gd3+ and Zn2+-ternary complexes were determined. In the presence of Zn2+ ions, $k_{cat}$ was 5 times larger than that of Mg2+, as shown in Table IV. Gd3+ ion is an analog of Mg2+ ion with 30% activity for Mg2+ (data not shown). From the Zn2+-bound enzyme, two Zn2+ ions were detected based on anomalous diffraction signals in the active site, as shown in Fig. 4A and Table V. ZnI was coordinated with tetrahedral geometry by Glu-82 and three water molecules. The signal of ZnII was very weak in 2Fo − Fc map, and ZnII was therefore not included in the model.

From the ternary complexes, two Zn2+ ions or two Gd3+ ions were identified in the active site, as shown in Fig. 4 (B and C). ZnI was coordinated with octahedral geometry by the bidentate carboxylate of Glu-82, three water molecules, and α-phosphate. ZnII was coordinated by the bidentate carboxylate of Glu-70. On the other hand, two Gd3+ ions were located in the active site, although we detected four Gd3+ ions in a subunit. GdI was coordinated by the bidentate carboxylate of Glu-82, two water molecules, and α-phosphate. GdII was coordinated with octahedral geometry by the carboxyl oxygen of Ala-66, two water molecules, the bridging oxygen (O3α), and β-phosphate. The other two Gd3+ ions were located close to the positions of Glu-122 and Glu-140, respectively. Partial protonation of Glu residues may cause the longer distance to metal ions, because both ternary complex crystals were produced under pH 5.2. When the two ternary complex structures were compared with that of the binary
complex, in the binary complex the two carboxylates of Glu-82 and Glu-86 were located away from the substrate, and the distances from O1 to the carboxylate oxygen of Glu-82 and from O3 to the carboxylate oxygen of Glu-86 were 3.8 Å and 5.9 Å, respectively. On the other hand, in the ternary structures, these Glu residues were closer to the substrate, and the corresponding distances were 2.9 and 4.8 Å for Zn$^{2+}$, and 2.6 and 4.9 Å for Gd$^{3+}$, respectively, because metal ions neutralized negative charge of the two carboxylates and phosphates.

**Product Binding (Corresponds to Reaction State IV in Fig. 8)**

Two product complexes were obtained (Fig. 5, A and B): one with AMP and Mg$^{2+}$, and another with ribose 5′-phosphate and Zn$^{2+}$. In the case of product complex with AMP and Mg$^{2+}$ (Fig. 5A), the adenine was recognized by Ile-19 and Tyr-28* through stacking interactions and adenine N1 made a hydrogen bond with Glu-29*, and adenine N6 made hydrogen bonds with Glu-29* and Gly-104*, although positions of adenine and -phosphate of AMP were located away from residues in the Nudix motif.

In the case of another product complex with ribose 5′-phosphate and Zn$^{2+}$ (Fig. 5B), the ring-opened ribose O3H made hydrogen bond with N of Ser-102*. ZnI and ZnII were coordinated by Glu-82 and Glu-70, respectively.
Fig. 5. Product binding site. Residue numbers followed by asterisks indicate residues of another subunit. WB represents a water molecule. Hydrogen bonds are shown with dashed blue lines. A, the active site in the product complex with AMP and Mg\textsuperscript{2+}. AMP is shown with a violet line. Gd\textsuperscript{3+} ions (small light green balls) and ADPR (a black line) in the Gd\textsuperscript{3+} ternary complex structure are superimposed on the model. B, the active site in the product complex with ribose 5'-phosphate and Gd\textsuperscript{3+}. Ribose 5'-phosphate is shown with a violet line. Zn\textsuperscript{2+} ions (small green balls) and ADPR (a black line) in the Zn\textsuperscript{2+} ternary complex structure are superimposed on the model.

In both product complexes, Arg-18 and Arg-27\textsuperscript{*} were located away from the active site. Consequently interactions between “fence” residues, consisting of Arg-18, Arg-27\textsuperscript{*}, and Tyr-28\textsuperscript{*}, and purine ring were absent (Fig. 3, compareisks indicate residues of another subunit. Black line) and ADPR (a black line) and the product-releasing mechanism of ADPRase. The conformational changes observed in product complexes suggest the product-releasing mechanism of ADPRase.

Activity of Wild-type and Mutant TtADPRases

Based on the ternary structures, the mutants of Glu-82 or Glu-86 from T. thermophilus were selected because the replacements of Glu-82 and Glu-86 with Gln resulted in the formation of new hydrogen bonds. As a result of the loss of metal ions or the change of metal location, and the loss of hydrogen bonds, ADPR and Mg\textsuperscript{2+} were lost. Instead of ADPR, one sulfate molecule was located at the position corresponding to α-phosphate of ADPR in the Gd\textsuperscript{3+} ternary complex of wild-type enzyme. The water molecule (WB) lost interaction with Mg\textsuperscript{2+} ions, and its pK\textsubscript{a} value could be higher than that of wild-type enzyme.

In the E82Q mutant structure that was determined using crystals produced in the solution with ADPR and Mg\textsuperscript{2+}, Gln-82 moved 0.7 Å toward the amide oxygen of Leu-68 to form a hydrogen bond and lost its hydrogen bond with Arg-81 (Fig. 6A). ADPR, Mg\textsuperscript{2+} ions, and water molecule (Wn) were not observed. Instead of ADPR, one sulfate molecule was located at the position corresponding to β-phosphate of ADPR in the Gd\textsuperscript{3+} ternary complex of wild-type enzyme. The water molecule (WB) lost interaction with Mg\textsuperscript{2+} ions, and its pK\textsubscript{a} value could be higher than that of wild-type enzyme.

In the E86Q mutant structure that was determined using crystals produced in the solution with ADPR and Zn\textsuperscript{2+}, ADPR and ZnI were not observed, but one sulfate molecule was located at the position corresponding to β-phosphate of ADPR in the Zn\textsuperscript{2+} ternary complex of wild-type enzyme (Fig. 6B). Zn II and two water molecules (WB, Wn) were present. Glu-82 moved 0.8 Å toward the backbone carbonyl oxygen of Leu-68 to form a hydrogen bond, and donated a proton to the water molecule (Wn) through a hydrogen bond. Thus, replacement of Glu-82 with Gln reduced the affinity for metal ion, and formed a new hydrogen bond between Glu-82 and Leu-68.

In the E82Q mutant ternary complex with ADPR and Mg\textsuperscript{2+} (Fig. 6C), the conformation of residues in the active site was almost the same as in the Gd\textsuperscript{3+} ternary complex of wild-type enzyme, although Glu-86 moved to form hydrogen bonds with Glu-82 and Ala-66. Mg II and water molecule (Wn) were not observed. Mg I and the water molecule (WB) were present; however, Mg I was located away from the water molecule (WB).

In the E86Q mutant ternary complex with ADPR and Zn\textsuperscript{2+} (Fig. 6D), Glu-86 also moved to form hydrogen bonds with Glu-82 and Ala-66. The adenosine moiety of ADPR was observed; however, the other moiety of ADPR was disordered. The water molecule (WB) was not observed. ZnI was coordinated by Glu-82 and the water molecule (Wn). ZnI was located at different position from that of the Zn\textsuperscript{2+} ternary complex of wild-type enzyme (compare a large green ball with a small green ball around Glu-82). Thus by replacement of Glu-86 with Gln, the location of metal I (MgI and ZnI) altered, and new hydrogen bonds between Glu-86 and Glu-82 and between Glu-86 and Ala-66 were formed.

The replacements of Glu-82 and Glu-86 with Gln resulted in the loss of metal ions or the change of metal location, and resulted in the formation of new hydrogen bonds. As a result of such alteration of charge distribution in the active site, two water molecules (Wn, WB) could disappear or have pK\textsubscript{a} values different from those of the wild-type enzyme. Such situation was reported in mutagenesis studies of RNase H1 from E. coli (26). These results indicate that the configuration composed of two water molecules (Wn and WB) and metal ions, orientations and positions of which are determined by Glu-82 and Glu-86, is important for activity.
**TtADPRase Catalytic Mechanism**

Based on the eight structures (Fig. 7, A–D) and functional analyses of TtADPRase, the following mechanism for the ADPR hydrolysis reaction is proposed and its reaction scheme is represented in Fig. 8.

**Metal Binding of TtADPRase (Reaction State I in Fig. 8)**– Even in the absence of ADPR, metal I (MI) can bind to Glu-82, as shown in the Zn$^{2+}$/H11001-bound structure (Fig. 7A).

**Ternary Complex with Substrate and Metal (Reaction State II in Fig. 8)**– In the presence of ADPR, metal II (MII) can bind the ligands, as shown in the ternary structures with ADPR and Gd$^{3+}$ (C and D). Glu-82 and Glu-70 could abstract a proton from the water molecule (Wn), and could poise Wn for nucleophilic attack on the adenosyl phosphate. The distance between the nucleophile Wn and P$^5$/H9251 is 3.6 Å. The angle formed between this P$^5$/H9251-O and P$^5$/H9251-O3 is 145°, suggesting an in-line mechanism for the cleavage reaction. Arg-54 interacts with $\beta$-phosphate and the terminal ribose O5, which may stabilize the negative charge of the substrate. In the presence of a metal, the nucleophile Wn is located at an ideal position to be activated by Glu-82, and this water molecule can attack on the adenosyl phosphate. In the absence of metal ions, Glu-82 and Glu-86 are distant from the substrate as a result of ion-ion repulsion, and the distance between Glu-82 and the nucleophile Wn is increased to 4.5 Å (Fig. 7B); therefore, Glu-82 cannot abstract a proton from the nucleophile Wn.

**Transition State (Reaction State III in Fig. 8)**– In the transition state, the adenosyl phosphorus is expected to be bound to five oxygen atoms with trigonal bipyramidal geometry (Fig. 8, III). To determine the site of nucleophilic substitution, the enzymatic reaction was conducted in the presence of H$_2^{18}$O and the products were analyzed by electrospray mass spectrometry. The addition of $^{18}$OH to AMP occurred, indicating that the

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**Fig. 6.** The active site structures of the E82Q and E86Q mutants. E82Q mutant structures were superimposed onto the corresponding metal ternary complex structures of wild-type enzyme with ADPR and Gd$^{3+}$ (A) or Zn$^{2+}$ (B). E86Q mutant structures were also superimposed onto the corresponding metal ternary complex structures of wild-type enzyme with ADPR and Gd$^{3+}$ (C) or Zn$^{2+}$ (D). The mutant structures are shown as ball-and-stick models. The structures of wild-type enzymes are not shown except for ADPR molecules (orange lines or brown lines), because they are similar to those of the mutants. Large balls represent water molecules (red balls) and metal ions (green balls) in the mutant structures, and small balls represent those in the wild-type structure. The atoms that were not observed in the mutant structures are written in gray letters. Hydrogen bonds and metal coordination in the mutant structures are shown by dashed blue lines.
nucleophilic water (Wn) attacks the adenosyl phosphorus (data not shown). The pKa value of a water molecule that coordinates metal is lower than that of a normal water molecule (27). In this structure, the water molecule (WB) bound to metal ions (MI) serves as a general acid and donates a proton to the bridging oxygen O3a.

**DISCUSSION**

Recent structural studies of EcADPRase and MtADPRase proposed that a water molecule, which bridges two metal ions and is deprotonated by EcGlu-162 or MtGlu-142 in loop L9, is at an ideal position for carrying out the nucleophilic attack on the adenosyl phosphate. Although TtGlu-127 in loop L4 corresponds to EcGlu-162 and MtGlu-142 (Fig. 1B), the residues in loop L4 are distant from the substrate. Additionally, the mutagenesis studies showed that D126N, E127Q, D128N, and E129Q mutants retained activity (Table IV), suggesting that residues in loop L4 are not essential for the reaction. In the TtADPRase structures of the ternary complex with ADPR and Gd3+ or Zn2+, a water molecule is present close to Glu-82 and Glu-70, and is in a favorable position for nucleophilic attack on the adenosyl phosphate. The structural and kinetic analyses of E82Q and E86Q suggested that Glu-82 and Glu-86 in the Nudix motif contribute to the binding of metal ions and water molecules, and thus are essential for the reaction.

Degradation of pyrophosphate derivatives by Nudix family proteins depends on metal ions. TtADPRase requires the presence of Mg2+ or Zn2+ ions for its activity. Although metal coordination and position are different between the Zn2+ and Gd3+ ternary complexes, as shown in Fig. 4 (B and C), the positions and orientations of the nucleophilic water (Wn), catalytic water molecule (WB), and catalytically essential residues are similar. TtADPRase did not show activity in the presence of other metal ions, such as Ca2+, Ni2+, and Mn2+. Positions and orientations of the nucleophilic water (Wn), catalytic water molecule (WB), and catalytically essential residues in the presence of the other metal ions would be different from those in the presence of Mg2+ or Zn2+. 

**FIG. 7. The architecture of the TtADPRase active site.** Coordinations with metal ions and hydrogen bonds are shown by dashed blue lines. The nucleophile water and catalytic acid water are represented by Wn and WB, respectively. Substrates Pα, Pβ, and O3α are represented by PA, PB, and O3A, respectively. The water molecule that forms a hydrogen bond with Glu-85 is represented by WA. A, the Zn2+-bound enzyme structure. B, the binary complex structure. C, the ternary complex with ADPR and Gd3+. D, the ternary complex with ADPR and Zn2+. The Gd3+ ternary complex structure is assumed to be analogous to a Mg2+ ternary structure. The nucleophilic attack and proton abstraction events are shown by pink arrows in C and D.
Genome analysis of *T. thermophilus* HB8 suggested the absence of an ADPR-transferase like protein and the presence of an ADPR-glycohydrolase like protein, involved in ADPR transfer in mono-ADP-ribosylation (8, 9). It implies that the possibility that ADPR molecules are constantly produced by mono-ADP-ribosylation is low. Recently, Sauve et al. (28) reported that the novel molecule O-acetyl-ADPR (OAADPR) is produced during NAD-dependent protein deacetylation by Sir2. Sir2 enzymes are broadly conserved from bacteria to humans and function in transcriptional silencing, DNA repair, and life span extension (29–31). Rafty et al. (32) found that ADPRases such as yeast YSA1 and mouse NudT5 hydrolyzed OAADPR to AMP and acetylated ribose 5'-phosphate when studied in vitro. In TtADPRase binary complex with ADPR, hydroxyl groups of the terminal ribose interact with the residues through the water molecules. Acetyl group of OAADPR can be placed in the space occupied by the water molecules. A Sir2 homolog has been identified in the *T. thermophilus* HB8 genome providing a source of OAADPR, which is expected to be a natural substrate for TtADPRase.

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Structural Insights into the Thermus thermophilus ADP-ribose Pyrophosphatase Mechanism via Crystal Structures with the Bound Substrate and Metal

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