Short Communication

Enzymatic characteristics of Nudix hydrolase 2 (Nud2), an 8-oxo-dGTP hydrolase from Myxococcus xanthus

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Myxococcus xanthus Nudix hydrolase 2 (Nud2) hydrolyzed oxidized deoxynucleotides, such as 8-oxo-dGTP, 8-oxo-dGDP, 8-OH-dTP, and 2-OH-dATP, and showed the highest specific activity toward 8-oxo-dGTP. Mn$^{2+}$ was the most effective co-factor for stimulating oxidized deoxynucleotide hydrolase activity. The $K_m$ of Nud2 with 8-oxo-dGTP for Mn$^{2+}$ was 19-fold lower than that for Mg$^{2+}$, and was 2-fold lower than that with dGTP for Mn$^{2+}$. The specificity constant ($k_{cat}/K_m$) for 8-oxo-dGTP was 6-fold higher than that for dGTP. Nud2 contains a similar Nudix motif ($^{84}AX_5^99GX_7REX_2EEXGX$). Replacement of Ala84 and/or Gly90 in the Nudix motif of Nud2 by Gly or Glu had negligible effects on 8-oxo-dGTP hydrolase activity, suggesting that a strict Nudix motif sequence is not essential for complete hydrolase activity of Nud2.

Key Words: 8-oxo-dGTP; Myxococcus xanthus; Nudix hydrolase

Nudix hydrolases are a family of pyrophosphatases containing the highly conserved 23-residue sequence segment (GX$_2$EX$_3$REUXEEXGU, where U is a bulky hydrophobic amino acid such as Ile, Leu, or Val, and X is any amino acid) called the Nudix motif (Bessman et al., 1996). They are metal-dependent enzymes found in all classes of organism, and can hydrolyze a wide range of organic pyrophosphates, including nucleoside di- and triphosphates, dinucleoside and diphosphoinositol polyphosphates, nucleotide sugars, RNA caps, NAD(P)H, coenzyme A, and guanosine-3',5'-tetraphosphate (ppGpp) with varying degrees of substrate specificity (McLennan, 2006). As these substrates are involved in energy metabolism and signal transduction, they are generally believed to be involved in metabolic regulation and stress response.

In addition, potentially mutagenic oxidized dNTPs, such as 7,8-dihydro-8'-oxo-dGTP (8-oxo-dGTP) and 2-hydroxy-2'-deoxyadenosine-5'-triphosphate (2-OH-dATP), are also hydrolyzed by Nudix hydrolases such as Escherichia coli MutT, Orf135 protein, and human MTH1 (Iida et al., 2005; Maki and Sekiguchi, 1992; Sakumi et al., 1993). In the cell, 8-oxo-dGTP and 2-OH-dATP formed by reaction with reactive oxygen species are readily incorporated into nascent DNA strands during replication, which would cause base substitution mutations (Hayakawa et al., 1995; Kamiya and Kasai, 2000; Maki and Sekiguchi, 1992). 8-oxo-dGTP is inserted by DNA polymerases opposite dATP in a template, whereas 2-OH-dATP is incorporated opposite dGTP and dCTP. Thus, the activities of these Nudix hydrolases toward oxidized purine deoxynucleoside triphosphates prevent genomic mutations and maintain the fidelity of protein synthesis under oxidative stress.

In bacteria, the number of Nudix genes varies from 0 to >30 (McLennan, 2006). We have previously reported that Myxococcus xanthus contains 12 Nudix hydrolases (Kimura et al., 2018). Among them, 11 His-tagged Nudix hydrolases (Nud1-Nud11) have been expressed in E. coli, and their substrate specificities have been studied (Kimura et al., 2018). Nud2 (MXAN_1246) showed high hydrolyase activity towards Ap4A, Ap5A, ATP, ADP, GTP, GDP, and especially 8-oxo-dGTP. In this study, we further characterized the enzymatic features of M. xanthus Nud2, which possesses the highest hydrolyase activity toward 8-oxo-dGTP and does not contain a strict Nudix motif sequence.

Most previously characterized Nudix hydrolases require divalent cation cofactors for activity. Many Nudix hydrolases require Mg$^{2+}$ for maximum activity, and Mn$^{2+}$ or Zn$^{2+}$ for partial activity. We have previously reported...
that *M. xanthus* Nudix hydrolases for Ap₄A were stimulated by Mn²⁺, Mg²⁺, and Co²⁺ (Kimura et al., 2018). When Nud2 activities for 13 substrates (four each of oxidized purine deoxynucleotides, unoxidized purine deoxynucleotides, and unoxidized purine nucleotides, and a diadenosine triphosphate (Ap₄A)) were measured in the presence of 5 mM each of Mn²⁺, Mg²⁺, or Co²⁺, Mn²⁺ was found to stimulate all the activities (Fig. 1A). Mg²⁺ (5 mM) was not effective in stimulating Nud2-mediated 8-oxo-dATP, ATP, and Ap₄A hydrolysis; however, 5 mM Mg²⁺ was the most effective in hydrolysing dADP and dGDP. Co²⁺ (5 mM) also stimulated most Nud2 hydrolase activities, with the exception of 8-oxo-dATP and Ap₄A. The Ap₄A hydrolase activity of Nud2 was stimulated only by
Mn^{2+}.

The optimal concentrations of Mn^{2+} for Nud2 hydrolyase activity toward 8-oxo-dGTP and dGTP were 0.25 mM and 0.75 mM, respectively (Fig. 1B). The Michaelis constant ($K_m$) of Nud2 with 8-oxo-dGTP and dGTP for Mn^{2+} were 0.09 mM and 0.20 mM, respectively. On the other hand, the optimal concentration of Mg^{2+} for 8-oxo-dGTP was similar to that for dGTP (Fig. 1C); however, the $K_m$ of Nud2 with 8-oxo-dGTP for Mg^{2+} (1.72 mM) was 1.7-fold lower than that with dGTP (2.97 mM). Furthermore, the $K_m$ of Nud2 with 8-oxo-dGTP for Mg^{2+} was 19-fold higher than that for Mn^{2+}. These results indicated that Nud2 can hydrolyze oxidized dGTP at lower concentrations of Mn^{2+} and Mg^{2+} than unoxidized dGTP.

Nud2 hydrolyase activity toward 2-OH-dATP and dATP did not differ significantly with respect to the optimal concentration of Mn^{2+} (2.5 mM) or Mg^{2+} (7.5 mM) (Figs. 1C and D); however, compared to the Nud2 hydrolyase activity toward dATP, activity toward 2-OH-dATP was also observed at low concentrations of Mn^{2+} (0.25–0.5 mM). The $K_m$ of Nud2 for Mn^{2+} with 2-OH-dATP and dATP as substrates were 0.47 mM and 1.19 mM, respectively, whereas that for Mn^{2+} with 2-OH-dATP was approximately 5-fold higher than that with 8-oxo-dGTP.

As the optimal Mn^{2+} concentration (0.25 mM) required for Nud2 activity with 8-oxo-dGTP was considerably low, Nud2 activities toward other substrates were also determined with 0.25 mM Mn^{2+}, 1 mM Mn^{2+}, and 1 mM Mg^{2+}. As shown in Fig. 1A, more than 25% of the relative activities were observed when Nud2 was incubated with four oxidized deoxynucleotides and dATP at 0.25 mM Mn^{2+}. In contrast, Nud2 negligibly hydrolyzed ATP and GTP in the presence of 0.25 mM Mn^{2+}, 1 mM Mn^{2+}, or 1 mM Mg^{2+}. Small et al. (2000) have reported that concentrations of Mg^{2+} and Mn^{2+} in forest and arable soil solutions were 0.5–0.8 mM and 6–23 μM, respectively. The intracellular concentration of Mg^{2+} in E. coli is identical to the extracellular concentration over a range of 1 μM to 10 mM (Hurwitz and Rosano, 1967). These results suggested that, in vivo, Nud2 in M. xanthus inhabiting the soil may preferably hydrolyze oxidized deoxynucleotides instead of unoxidized deoxynucleotides.

To investigate the kinetic parameters of Nud2 for 13 substrates, these substrates were incubated with 0.04–1.5 μg of Nud2 at 37°C for 10–15 min. $K_m$ and the catalytic constant ($k_{cat}$) were determined from the Michaelis-Menten plots (Table 1). Nud2 had low $K_m$ values (25–30 μM) toward oxidized deoxynucleotides, whereas it showed low affinity toward unoxidized nucleotides, with the exception of dGTP. The $K_m$ of Nud2 for 8-oxo-dGTP was similar to those of other Nudix hydrolases, with the exception of E. coli MutT (0.081–0.53 μM) (Supplementary Table S1). The $K_m$ of Nud2 for 8-oxo-dGTP was 1.7-fold lower than that for dGTP; however, the difference in $K_m$ of Nud2 (1.7-fold) for 8-oxo-dGTP and dGTP was significantly lower than that observed with E. coli MutT (approximately 550–13,600-fold) and human MTH1 (11–17-fold). In addition, the $K_m$ values of Nud2 for 8-oxo-dGDP, 8-oxo-dATP, and 2-OH-dATP were 5-, 10-, and 12-fold lower than that for dGDP, dATP, and dATP, respectively. The $K_m$ value of Nud2 for 2-OH-dATP was 3-4-fold higher than that of human MTH1 (Fujikawa et al., 1999). In contrast, the $K_m$ of Nud2 for dATP and dADP were approximately 3-fold lower than that of E. coli MutT; furthermore, the $K_m$ of Nud2 for dGDP was similar to that of E. coli MutT (Ito et al., 2005).

Nud2 hydrolyzed nucleoside triphosphates to corresponding monophosphates and pyrophosphates, and exhibited the highest activity toward 8-oxo-dGTP (Table 1). The $k_{cat}$ of Nud2 for 8-oxo-dGTP was 3.7-fold higher than that for dGTP. The specificity constant ($k_{cat}/K_m$) of Nud2 for 8-oxo-dGTP was 6-fold higher than that for dGTP; however, the ratio of ($k_{cat}/K_m$)_{8-oxo-dGTP}/($k_{cat}/K_m$)_{dGTP} was also significantly lower than those of E. coli MutT (approximately 1,600–6,200) and human MTH1 (14–44) (Table S1). Nud2 showed the second highest activity against 8-oxo-dGDP. 8-oxo-dGDP is hydrolyzed by E. coli MutT, but not human MTH1 (Ito et al., 2005). While, E. coli MutT hydrolyzes neither 8-OH-dATP nor 2-OH-dATP; however, human MTH1 hydrolyzes 2-OH-dATP more efficiently than 8-oxo-dGTP (Fujikawa et al., 1999). Nud2 hydrolyzed 2-OH-dATP; however, the $k_{cat}$ of Nud2 for 2-OH-dATP was 12-fold lower than that for 8-oxo-dGTP. As the M. xanthus genome has a high GC content (69%), dGTP oxidation is more risky than dATP oxidation. Therefore, the higher hydrolyase activity of Nud2 toward 8-oxo-dGTP than

### Table 1. Kinetic parameters of M. xanthus Nud2.

| Substrate       | Major Product | $K_m$ (μM) | $k_{cat}$ (min⁻¹) | $k_{cat}/K_m$ (min⁻¹ μM⁻¹) |
|-----------------|---------------|------------|-------------------|---------------------------|
| 8-oxo-dGTP      | 8-oxo-dGMP    | 27 ± 2     | 67.7 ± 2.1        | 2.5                       |
| 8-oxo-dGDP      | 8-oxo-dGMP    | 27 ± 1     | 16.4 ± 0.7        | 6.1 ± 10⁻¹                |
| 8-oxo-dATP      | 8-oxo-dAMP    | 30 ± 1     | 6.2 ± 0.3         | 2.1 ± 10⁻¹                |
| 2-OH-dATP       | 2-OH-dAMP     | 25 ± 2     | 5.8 ± 0.6         | 2.3 ± 10⁻¹                |
| ATP             | AMP           | 455 ± 31   | 9.5 ± 0.7         | 2.1 ± 10⁻²                |
| GTP             | GMP           | 598 ± 38   | 20.3 ± 1.0        | 3.4 ± 10⁻²                |
| ADP             | AMP           | 1173 ± 25  | 6.1 ± 0.2         | 5.2 ± 10⁻²                |
| GDP             | GMP           | 319 ± 26   | 11.2 ± 0.1        | 3.5 ± 10⁻²                |
| dATP            | dAMP          | 298 ± 28   | 9.0 ± 0.8         | 3.0 ± 10⁻²                |
| dGTP            | dGMP          | 46 ± 1     | 18.5 ± 1.2        | 4.0 ± 10⁻²                |
| dADP            | dAMP          | 447 ± 7    | 8.6 ± 0.6         | 1.9 ± 10⁻²                |
| dGDP            | dGMP          | 134 ± 2    | 7.7 ± 0.9         | 5.7 ± 10⁻²                |
| Ap4A            | AMP           | 385 ± 10   | 6.2 ± 0.8         | 1.6 ± 10⁻²                |

*1 0.25 mM Mn^{2+}.

*2 1 mM Mn^{2+}.

*3 5 mM Mn^{2+}.

*4 5 mM Mg^{2+} were used for enzyme assay.

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**Fig. 2.** 8-oxo-dGTP hydrolyase activities of wild-type Nud2 and its variant mutants.

The assay was performed with 0.1 mM 8-oxo-dGTP in 50 mM HEPES (pH 8.0), 0.25 mM MnCl₂, and 0.06 μg enzyme at 37°C for 15 min.
E. coli MutT negligibly hydrolyzed dATP and ATP, whereas Nud2 hydrolyzed dATP and ATP with a catalytic constant ($k_{\text{cat}}$) of 9.0 and 9.5 min$^{-1}$, respectively. $K_{\text{m}}$ of Nud2 for dATP was 7–8-fold lower than that for oxidized dATP. Nud2 also hydrolyzed GDP, ADP, dGDP, and dGDP; however, specificity constants ($5.2 \times 10^{-3}–5.7 \times 10^{-2}$) of Nud2 for these substrates were lower than those for oxidized deoxyribonucleotides (2.1 $\times$ 10$^{-2}$–2.5), suggesting that oxidized deoxyribonucleotides may be better substrates for Nud2 than unoxidized nucleotides in M. xanthus cells.

M. xanthus produces Ap$_4$A and Ap$_5$A under various stress conditions, and high concentrations of intracellular Ap$_4$A and/or Ap$_5$A inhibit M. xanthus sporulation under starvation (Kimura et al., 2017; Oka et al., 2015). Ap$_4$A is hydrolyzed by M. xanthus Nud2, a hydrolase (ApAH) with a $K_{\text{m}}$ value of 0.86 mM (Sasaki et al., 2014). Nud2 has a higher affinity toward Ap$_4$A than ApAH; however, the $k_{\text{cat}}$ of Nud2 for Ap$_4$A was 70-fold lower than that of ApAH. M. xanthus ApAH also had 8-oxo-dGTP hydrolysis activity, and it mainly hydrolyzed 8-oxo-dGTP to 8-oxo-dGDP and phosphate (data not shown). The $K_{\text{m}}$ and $k_{\text{cat}}$ of ApAH for 8-oxo-dGTP were 57 ± 9.6 mM and 4.3 ± 0.04 min$^{-1}$, respectively.

Nud2 is a small protein consisting of 193 amino acids, with a molecular weight of 21.8 kDa. The amino acid sequence of Nud2 has minimal identity with that of E. coli MutT and human MTH1 (6.5% and 12.3% identity, respectively) (Fig. S1). Although the sequence identity between E. coli MutT and human MTH1 is 23%, the overall folding of these proteins, except for that at the C terminal region, are similar, whereas the secondary structure of Nud2, with the exception of the N and C-terminal regions, was partially similar to those of E. coli MutT and human MTH1 (Supplementary Fig. S1). Nudix hydrolases contain a highly conserved Nudix motif (GX$_{5}$EX7REUXEEXGU), and, furthermore, E. coli MutT and human MTH1 possess a homologous region (GGK$_{5}$EX-RELXEEXG), which is designated as the phosphohydrolase module (Fujii et al., 1999; Nakabeppu, 2001). Nud2 does not contain the strict Nudix motif sequence; in contrast, it possesses a similar conserved motif ($8^{\text{AX}}_{5}^{\text{GX}-\text{REX}_2^{\text{EEXG}}}$). Structural analyses of E. coli MutT revealed that the phosphohydrolase module constitutes the active center of dGTPase (Lin et al., 1996, 1997). The Gly38 and Gly44 (corresponding to Ala84 and Gly90 of Nud2) in the module of E. coli MutT are essential amino acids that are involved in the coordination of enzyme-bound metal and stabilization of the active site region, respectively (Nakamura et al., 2010; Shimokawa et al., 2000). In human MTH1, Gly37 and Gly43 (corresponding to Ala84 and Gly90 in Nud2) are also essential for its catalytic function (Cai et al., 1997; Fujii et al., 1999).

Hence, Ala84 and Gly90 in Nud2 were replaced by Gly and Glu, respectively, and a double mutant was also constructed using site-directed mutagenesis. As shown in Fig. 2, the hydrolase activity of the Ala84Gly (A84G) mutant toward 8-oxo-dGTP decreased by 29%, whereas that of the Gly90Glu (G90E) mutant increased by 27%. The double mutant (A84G/G90E) showed an only slightly higher 8-oxo-dGTP hydrolase activity than the wild-type enzyme, suggesting that these two residues in the Nudix motif are not essential for complete 8-oxo-dGTP hydrolase activity of Nud2.

In summary, M. xanthus Nud2 does not share sequence identity with E. coli MutT and human MTH1, and does not contain a strict Nudix motif; however, Nud2 had the highest hydrolase activity toward 8-oxo-dGTP; it also hydrolyzed 8-oxo-dGDP, 8-oxo-dATP, and 2-OH-dATP. The hydrolase activity of Nud2 for 8-oxo-dGTP was stimulated by Mn$^{2+}$ with a $K_{\text{m}}$ of 0.09 mM. The $K_{\text{m}}$ of Nud2 with 8-oxo-dGTP for Mn$^{2+}$ was 2-fold lower than that for dGTP for Mn$^{2+}$. The specificity constant of Nud2 for 8-oxo-dGTP and 2-OH-dATP were 6- and 8-fold higher than that for dGTP and dATP, respectively. Replacement of Ala84 and Gly90 with Gly and Glu, respectively, in the Nudix motif of Nud2 did not change the Nudix hydrolase activity, suggesting that these residues (Gly and Glu) in the Nudix motif of Nud2 are not essential for complete hydrolase activity of Nud2. Further research with the nud2 mutant is needed to understand the role of Nud2 as an 8-oxo-dGTP hydrolase in M. xanthus.

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Supplementary Materials

Supplementary figure and table are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/gjam).

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