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NUDT5 hydrolyzes oxidized deoxyribonucleoside diphosphates with broad substrate specificity

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ABSTRACT

The human NUDT5 protein catalyzes the hydrolysis of 8-hydroxy-dGDP. To examine its substrate specificity, four oxidized deoxyribonucleotides (2-hydroxy-dADP, 8-hydroxy-dADP, 5-formyl-dUDP, and 5-hydroxy-dCDP) were incubated with the NUDT5 protein. Interestingly, all of the nucleotides, except for 5-hydroxy-dCDP, were hydrolyzed with various efficiencies. The kinetic parameters indicated that 8-hydroxy-dADP was hydrolyzed as efficiently as 8-hydroxy-dGDP. The hydrolyzing activities for their triphosphate counterparts were quite weak. These results suggest that the NUDT5 protein eliminates various oxidized deoxyribonucleoside diphosphates from the nucleotide pool and prevents their toxic effects.

Keywords: NUDT5 / oxidized nucleotide / nucleotide pool sanitization / substrate recognition

1. Introduction

An increasing body of evidence suggests that endogenous oxidation of DNA and DNA precursors by reactive oxygen species (ROS) seems to induce spontaneous mutations, various diseases including cancer and neurodegeneration, and normal aging [1-4]. 8-Hydroxy-2'-deoxyguanosine 5'-triphosphate (8-OH-dGTP, also known as 7,8-dihydro-8-oxo-2'-deoxyguanosine 5'-triphosphate) is an oxidized form of dGTP, and its presence in the mitochondrial nucleotide pool was recently shown [5]. The amount of 8-OH-dGTP was ~10% of that of dGTP in rat liver mitochondria. In *Escherichia coli*, half of the 8-hydroxyguanine base formed in DNA is attributed to the incorporation of 8-OH-dGTP into DNA [6]. The *E. coli* MutT protein catalyzes the hydrolysis of 8-OH-dGTP to 8-hydroxy-2'-deoxyguanosine 5'-monophosphate (8-OH-dGMP, 7,8-dihydro-8-oxo-2'-deoxyguanosine 5'-monophosphate) [7]. The spontaneous mutation frequency in a *mutT*-deficient strain is much higher than that in the wildtype strain [6,8], indicating that 8-OH-dGTP is a major source of spontaneous mutations. In addition, oxidized DNA precursors, including 8-OH-dGTP, 2-hydroxy-2'-deoxyadenosine 5'-triphosphate (2-OH-dATP), 5-hydroxy-2'-deoxycytidine 5'-triphosphate (5-OH-dCTP), and 5-formyl-2'-deoxyuridine 5'-triphosphate (5-CHO-dUTP), are mutagens in living cells when exogenously added [9-12]. These results indicate that the oxidized DNA precursors are the subjects of genotoxicity-avoidance systems in cells.

Organisms possess mechanisms to prevent the mutations caused by oxidized DNA precursors. The specific hydrolysis of 8-OH-dGTP by the *E. coli* MutT protein is important for the suppression of endogenous and exogenous 8-OH-dGTP [6,13]. MutT contains the “phosphohydrolase module” or “MutT signature” amino acid residues [14,15]. Several *E. coli* and eukaryotic proteins with the module have been shown to catalyze the hydrolysis of oxidized DNA precursors [7,16-23]. Among these enzymes, the human NUDT5 protein is unique, since the hydrolysis of 8-hydroxy-2'-deoxyguanosine 5'-diphosphate (8-OH-dGDP, 7,8-dihydro-8-oxo-2'-deoxyguanosine 5'-diphosphate) by this protein is much more efficient than that of 8-OH-dGTP [19]. The expression of the NUDT5 protein in *mutT*-deficient *E. coli* mutant cells decreases the spontaneous mutation frequency to the normal level, suggesting its importance in nucleotide pool sanitization [19]. Since the human MTH1, yeast PCD1, and *E. coli* Orf135 and Orf17 proteins act on multiple substrates containing different damaged nucleobases [17,18,21,23], NUDT5 might also recognize other oxidized deoxyribonucleoside diphosphates besides 8-OH-dGDP.

In this study, the NUDT5 protein was incubated with various oxidized deoxyribonucleoside diphosphates [8-hydroxy-2'-deoxyadenosine 5'-diphosphate (8-OH-dADP, 7,8-dihydro-8-oxo-2'-deoxyadenosine 5'-diphosphate), 2-hydroxy-2'-deoxyadenosine 5'-diphosphate (2-OH-dADP), 5-formyl-2'-deoxyuridine 5'-diphosphate (5-CHO-dUDP), and 5-hydroxy-2'-deoxycytidine 5'-diphosphate (5-OH-dCDP), as well as 8-OH-dGDP] *in*

vitro. Unexpectedly, all of the nucleotides, except for 5-OH-dCDP, were hydrolyzed by this protein. Thus, the NUDT5 protein is a hydrolyzing enzyme with broad substrate specificity for oxidized deoxyribonucleoside diphosphates.

2. Materials and methods

2.1. Unmodified deoxyribonucleotides

dADP, dGDP, dCDP, and TDP were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and purified by HPLC using a YMC-pack ODS-AM-303 column (ϕ 4.6 X 250 mm, 5 μ m, YMC Co., Kyoto, Japan), with an isocratic system consisting of 12.5 mM citric acid, 25 mM sodium acetate, 10 mM acetic acid, and 30 mM sodium hydroxide. They were desalted by HPLC using two Ultrasphere ODS columns (ϕ 4.6 X 250 mm, Beckman Coulter, Fullerton, California, USA) connected in series.

2.2. Synthesis of oxidized deoxyribonucleotides

8-OH-dGTP, 2-OH-dATP, and 5-OH-dCTP were prepared as described previously [10,24,25]. 5-CHO-dUTP was synthesized from dTTP by oxidation with Cu (II) and peroxydisulfite [26]. 8-Hydroxy-2'-deoxyadenosine 5'-triphosphate (8-OH-dATP, 7,8-dihydro-8-oxo-2'-deoxyadenosine 5'-triphosphate) was prepared by alkali treatment of 8-bromo-dATP, which was synthesized by the established procedures from dATP [27]. The corresponding diphosphates were prepared by similar procedures. These purified deoxyribonucleotides were eluted as a single peak in both

reverse-phase and anion-exchange HPLC, and their purities were estimated to be more than 99% (data not shown). They were stable under the assay conditions in the absence of NUDT5 (data not shown).

2.3. *NUDT5* assay

The amino-terminal His-tagged NUDT5 protein was synthesized by the *E. coli* overexpression system, as described [19] with slight modifications. Namely, NUDT5 expression was induced by treatment with 1 mM isopropyl- β -D-thiogalactopyranoside at 37°C for 4 hr, and the protein was purified by Ni Sepharose 6 Fast Flow and HiLoad 16/60 Superdex 75 pg (GE Healthcare Bio-Sciences, Piscataway, New Jersey, USA). The NUDT5 activities were assayed in a reaction mixture (100 μ l) containing 20 mM Tris-HCl, pH 8.0, 8 mM MgCl₂, 40 mM NaCl, 80 μ g/ml bovine serum albumin, 5 mM dithiothreitol, 2% glycerol, and the nucleotide substrates. Following a preincubation at 30°C for 5 min, the mixtures were incubated at 30°C with the NUDT5 protein. Reactions were terminated by the addition of 100 μ l of 0.5% SDS. All samples were injected into a TSK-gel DEAE-2SW column (ϕ 4.6 mm X 250 mm, Tosoh, Tokyo, Japan), with isocratic elution by 75 mM sodium phosphate buffer, pH 7.0, at a flow rate of 1 ml/min. The amounts of the deoxyribonucleoside diphosphates and their hydrolyzed products were quantitated by measuring the area of UV absorbance. Hanes-Woolf plots were drawn from the initial velocity of the deoxyribonucleoside diphosphatase reaction for the NUDT5 protein. The K_m and k_{cat} values were derived from the intercepts of regression lines.

3. Results

3.1. Hydrolysis of oxidized deoxyribonucleotides by NUDT5

In this study, we chose 8-OH-dADP, 2-OH-dADP, 5-CHO-dUDP, and 5-OH-dCDP (in addition to 8-OH-dGDP) as substrate candidates (Fig. 1), since the corresponding deoxyribonucleosides are formed in *in vitro* oxidation reactions [28].

First, the deoxyribonucleoside diphosphates (10 μ M) were incubated with the NUDT5 protein (111 nM). As shown in Fig. 2, all of the deoxyribonucleoside diphosphates, except for 5-OH-dCDP, were hydrolyzed by this enzyme. When the enzyme reactions were conducted for 30 min, 8-OH-dADP was hydrolyzed slightly more efficiently than 8-OH-dGDP (Table 1). Meanwhile, the hydrolyses of 2-OH-dADP and 5-CHO-dUDP were less effective, with 0.4- and 0.5-fold efficiencies relative to that of 8-OH-dGDP, respectively (Table 1).

We also examined canonical deoxyribonucleoside diphosphates under the same conditions. Unmodified dGDP and dADP were hydrolyzed by the NUDT5 protein (data not shown). The hydrolysis of TDP was less efficiently than the two purine nucleotides and no hydrolysis of dCDP was observed.

Next, the corresponding deoxyribonucleoside triphosphates (10 μ M) were incubated with the NUDT5 protein under the same conditions, except that the enzyme concentration was increased to 556 nM. The hydrolyses of the triphosphate derivatives were less efficient than those of the diphosphate counterparts. Among the

triphosphate derivatives, the NUDT5 protein catalyzed the hydrolysis of 8-OH-dATP more efficiently than 8-OH-dGTP (Table 2). In contrast, the hydrolyses of 2-OH-dATP and 5-CHO-dUTP were much less effective, with only 0.1-fold efficiencies relative to that of 8-OH-dGTP.

3.2. Kinetic parameters

We then determined the kinetic parameters for the hydrolysis reactions of the oxidized deoxyribonucleoside diphosphates (Table 3). The kinetic parameters thus obtained indicated that 8-OH-dADP and 8-OH-dGDP were comparable substrates. The K_m value of 8-OH-dADP was 1.4 times higher than that of 8-OH-dGDP. The k_{cat} value of 8-OH-dADP was 1.5 times higher than that of 8-OH-dGDP. The relative k_{cat} / K_m value of 8-OH-dADP was 1.06, which means that 8-OH-dADP was hydrolyzed as efficiently as 8-OH-dGDP. In contrast, the K_m values of 2-OH-dADP and 5-CHO-dUDP were 4.2 and 1.9 times, respectively, higher than that of 8-OH-dGDP. In addition, their k_{cat} values were 1.6-1.8 times lower than that of 8-OH-dGDP. Their relative k_{cat} / K_m values were 6.8 and 3.4 times lower, which means that their hydrolyses occurred less efficiently, as compared to that of 8-OH-dGDP. The results obtained in this study suggest that the NUDT5 enzyme hydrolyzes oxidized deoxyribonucleoside diphosphates with broad substrate specificity.

We also determined the kinetic parameters for the hydrolysis reactions of unmodified dGDP and dADP (Table 3). The K_m value of dGDP was 3.6 times higher than that of 8-OH-dGDP. The k_{cat} value of dGDP was 2.5 times higher than that of

8-OH-dGDP. The relative k_{cat} / K_m value of dGDP was 0.69, which means that dGDP was hydrolyzed 1.4 times less efficiently than 8-OH-dGDP. The K_m value of dADP was 6.0 times higher than that of 8-OH-dGDP and the k_{cat} values of dADP and 8-OH-dGDP were similar. The relative k_{cat} / K_m value of dADP was 6.1 times lower, as compared to that of 8-OH-dGDP.

4. DISCUSSION

The human NUDT5 protein catalyzed the hydrolysis of 8-OH-dGDP and 8-OH-dGTP *in vitro*, although the activity for the latter was much lower ([19], and Tables 1 and 2). Previously, the expression of the NUDT5 protein in *mutT*-deficient *E. coli* mutant cells was shown to decrease the spontaneous mutation frequency to the normal level [19]. Thus, the 8-OH-dGDPase (and/or 8-OH-dGTPase) activity of the protein seemed to supplement the MutT 8-OH-dGTPase/8-OH-dGDPase activity in cells. 8-OH-dGTP is mutagenic in mammalian cells [11,12] and the knock-down of NUDT5 increased the mutations induced by 8-OH-dGTP (Hori *et al.*, unpublished results). Therefore, the NUDT5 protein appears to have the ability to prevent the mutagenesis by 8-OH-dGTP. We showed that the human NUDT5 protein catalyzed the hydrolysis of 8-OH-dADP, 2-OH-dADP, and 5-CHO-dUDP as well as 8-OH-dGDP *in vitro*, although the hydrolyzing activities for the corresponding triphosphate derivatives were much lower (Tables 1 and 2). Thus, the activities of NUDT5 for various oxidized deoxyribonucleotides could contribute to the prevention of their toxic effects in cells.

Importantly, the NUDT5 protein catalyzed the hydrolysis of the oxidized deoxyribonucleoside diphosphates more efficiently than their triphosphate counterparts. 8-OH-dGTP and 8-OH-dGDP are interconvertible within a cell [29]. Thus, the hydrolyzing activities for 8-OH-dADP, 2-OH-dADP, and 5-CHO-dUDP of NUDT5 could lead to a decrease in their corresponding triphosphates, thus preventing their incorporation into DNA. Ishibashi *et al.* proposed [19] that the hydrolysis of 8-OH-dGDP, a potent inhibitor of the MTH1 protein [17,30], could potentiate the function of MTH1. The MTH1 protein, another human MutT-type enzyme, catalyzes the hydrolysis of various deoxyribonucleoside triphosphates, including 8-OH-dGTP, 8-OH-dATP, and 2-OH-dATP *in vitro* [16,17,31,32]. Since 2-OH-dADP also inhibits the activity of MTH1 [17], and since 8-OH-dADP is probably another MTH1 inhibitor, the activities of NUDT5 for them could promote the function of MTH1.

Nakabeppu and his colleagues found that both 8-OH-dGTPase and 2-OH-dATPase activities of human MTH1 contributed to suppression of H₂O₂-induced cell dysfunction and death [33], suggesting that 8-OH-dGTP and 2-OH-dATP induce cell death. In agreement with this finding, Rai *et al.* recently reported that the suppression of MTH1 expression in cells caused senescence [34]. Thus, the 8-OH-dGDPase and 2-OH-dADPase activities of NUDT5 could also suppress the cell death induced by the corresponding deoxyribonucleoside triphosphates.

In contrast to 8-OH-dGTP and 2-OH-dATP, the functions of 8-OH-dATP and 5-CHO-dUTP in human (mammalian) cells are unclear. In *E. coli*, 5-CHO-dUTP induces chromosomal gene mutations, but the mutagenicity of 8-OH-dATP is quite weak in bacteria [10,35]. To examine effects of 8-OH-dADP and 5-CHO-dUDP on mutagenesis and cell death in human cells is important and would help us to understand the roles of the 8-OH-dADPase and 5-CHO-dUDPase activities of NUdT5. The biological significance of the hydrolyzing activities should be revealed by various experiments.

Among the *E. coli* and mammalian MutT-type enzymes, the MTH1 and *E. coli* Orf135 (NudG) proteins catalyze the hydrolysis of deoxyribonucleoside triphosphates, and their catalytic activities are inhibited by deoxyribonucleoside diphosphates [16-18]. Both deoxyribonucleoside triphosphates and diphosphates are substrates for *E. coli* MutT and Orf17 (NudB, NtpA) [21,22]. The NUdT5 protein is unique, since deoxyribonucleoside diphosphates, not deoxyribonucleoside triphosphates that could be incorporated into the growing DNA chain, are its substrates. The biological significance of this activity is still open to debate, although its possible role(s) described above have been suggested. The roles of nucleotide pool sanitization enzymes including NUdT5, and DNA repair proteins must be precisely defined to clarify the effects of oxidized DNA precursors [36,37].

The K_m value of 8-OH-dGDP obtained in this study was 2.1 μM (Table 3) and 2.7 times higher than that reported earlier (0.77 μM [19]). Meanwhile, the K_m values of dGDP and dADP were similar in the present and previous studies. The differences

in the K_m value of 8-OH-dGDP could be attributable to differences in lots of 8-OH-dGDP, enzyme preparation, and/or reaction conditions. However, 8-OH-dGDP was shown to be the best substrate among 8-OH-dGDP, dGDP, and dADP in this study, in agreement with the report by Ishibashi *et al.* [19].

The kinetic parameters of the human MTH1 protein for 8-OH-dGTP, 8-OH-dATP, and 2-OH-dATP have been reported [17]. The parameters of the NUDT5 protein for the corresponding diphosphate derivatives indicate that the enzyme catalyzed the hydrolysis of oxidized deoxyribonucleotides *in vitro* less efficiently than the MTH1 protein. For example, the K_m and k_{cat} values of MTH1 for 8-OH-dGTP are 15.2 μM and 738 min^{-1} (12.3 sec^{-1}) and the k_{cat} / K_m value is calculated to be 48.6 $\text{min}^{-1} \mu\text{M}^{-1}$. The k_{cat} / K_m value of NUDT5 for 8-OH-dGDP was 0.176 $\text{min}^{-1} \mu\text{M}^{-1}$ (Table 3), 276 times smaller than that of MTH1 for 8-OH-dGTP. These values suggest that NUDT5 may be much less important for sanitization of the nucleotide pool than MTH1. However, the knock-down of NUDT5 increased the mutations induced by 8-OH-dGTP as efficiently as that of MTH1 (Hori *et al.*, unpublished results), indicating importance of NUDT5 in living cells. Thus, it is required to evaluate enzymatic activities of NUDT5 and MTH1 in cells.

Interestingly, 8-OH-dGDP, 8-OH-dADP, 2-OH-dADP, and 5-CHO-dUDP seem to lack a common hydrogen acceptor/donor (Fig. 1). Zha *et al.* reported the crystal structure of human NUDT5 complexed with ADP-ribose [38]. The adenine base of the ADP-ribose is specifically recognized via hydrogen-bonding interactions between 1-N and 6-amino of the base and the main chain amide and carbonyl groups of

Glu-47, and between 7-N of the base and one side chain amino group of Arg-51. In addition, the Trp-28 and Trp-46 residues sandwich the adenine moiety, forming strong π - π stacking interactions. When we consider that 8-substituted purine nucleotides adopt the *syn*-conformation [39], the 8-oxo and 7-imino groups of both 8-OH-dGDP and 8-OH-dADP might be recognized by NUDT5 (possibly by Glu-47). The 3-imino-2-oxo structure of 5-CHO-dUDP and the 1-imino-2-oxo structure of 2-OH-dADP (in the 2-oxo tautomer) might also be recognized when they adopt the *anti*-conformation. The reason why the K_m values for 8-OH-dGDP, 8-OH-dADP, and 5-CHO-dUDP were lower than that for 2-OH-dADP might be due to the presence of the 2-hydroxy tautomer of 2-OH-dADP, which lacks a similar structure. TDP is a very poor substrate for the NUDT5 protein [19], although the nucleotide also contains the 3-imino-2-oxo structure. Thus, another recognition mechanism, similar to that by the Arg-51, Trp-28, and Trp-46 residues, could contribute to the preferred binding of 5-CHO-dUDP. In general, water molecules participate in substrate recognition by enzymes. Therefore, it is hard to predict the relationship between the structure and affinity from only the crystal structure of NUDT5 in complex with ADP-ribose [38]. To reveal the actual binding mode, the three-dimensional structural analysis of the complexes of a substrate analog and the protein will be very useful.

In this study, we showed that the NUDT5 protein catalyzed the hydrolysis of oxidized deoxyribonucleoside diphosphates *in vitro* with broad substrate specificity. Since a greater number of tumors were spontaneously formed in the lungs, livers, and stomachs of MTH1-deficient mice, as compared with wildtype mice [40], it would be

interesting to examine the phenotype of animals deficient in NUDT5. Experiments to reveal the actual function(s) and binding mode of the protein are in progress.

Conflict of interest

The authors declare that there are no conflicts of interest.

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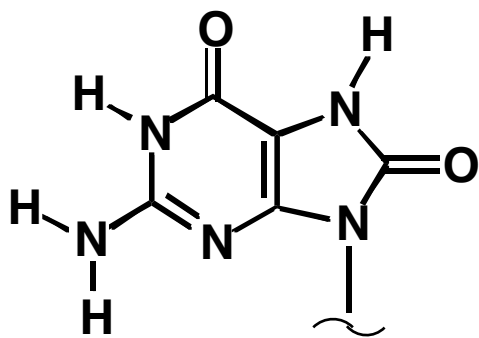
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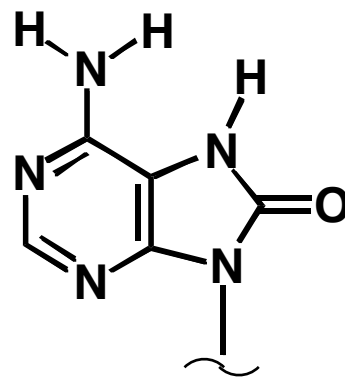
FIGURE LEGENDS

Figure 1. Structures of the nucleobase moieties of the oxidized deoxyribonucleotides used in this study.

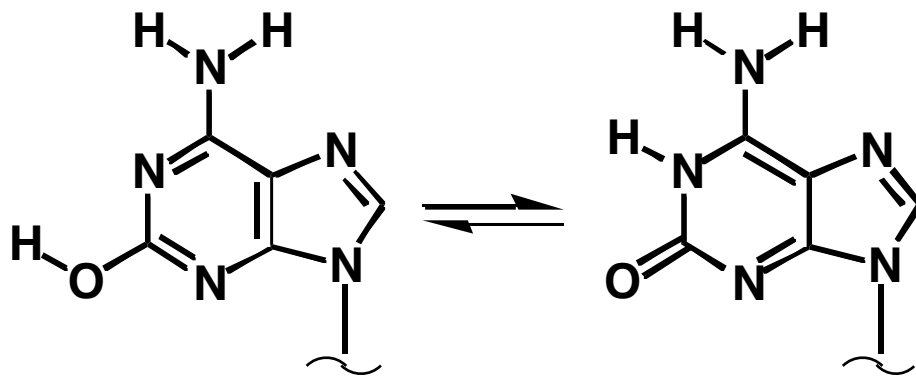
Figure 2. Hydrolysis of oxidized deoxyribonucleoside diphosphates by the human NUDT5 protein, monitored by anion exchange HPLC. Each nucleotide (10 μ M) was incubated with 111 nM of the NUDT5 protein at 30°C for 30 min. (A) 8-OH-dGDP, (B) 8-OH-dADP, (C) 2-OH-dADP, (D) 5-CHO-dUDP.



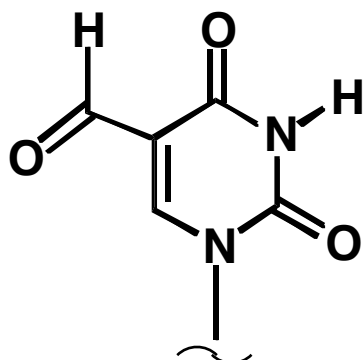
8-OH-dGDP



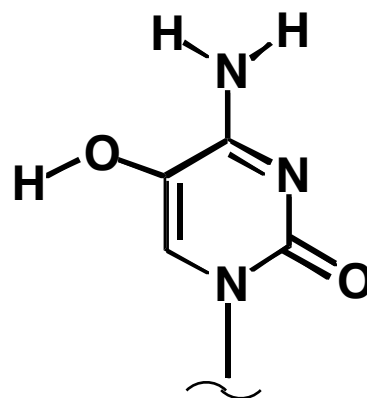
8-OH-dADP



2-OH-dADP



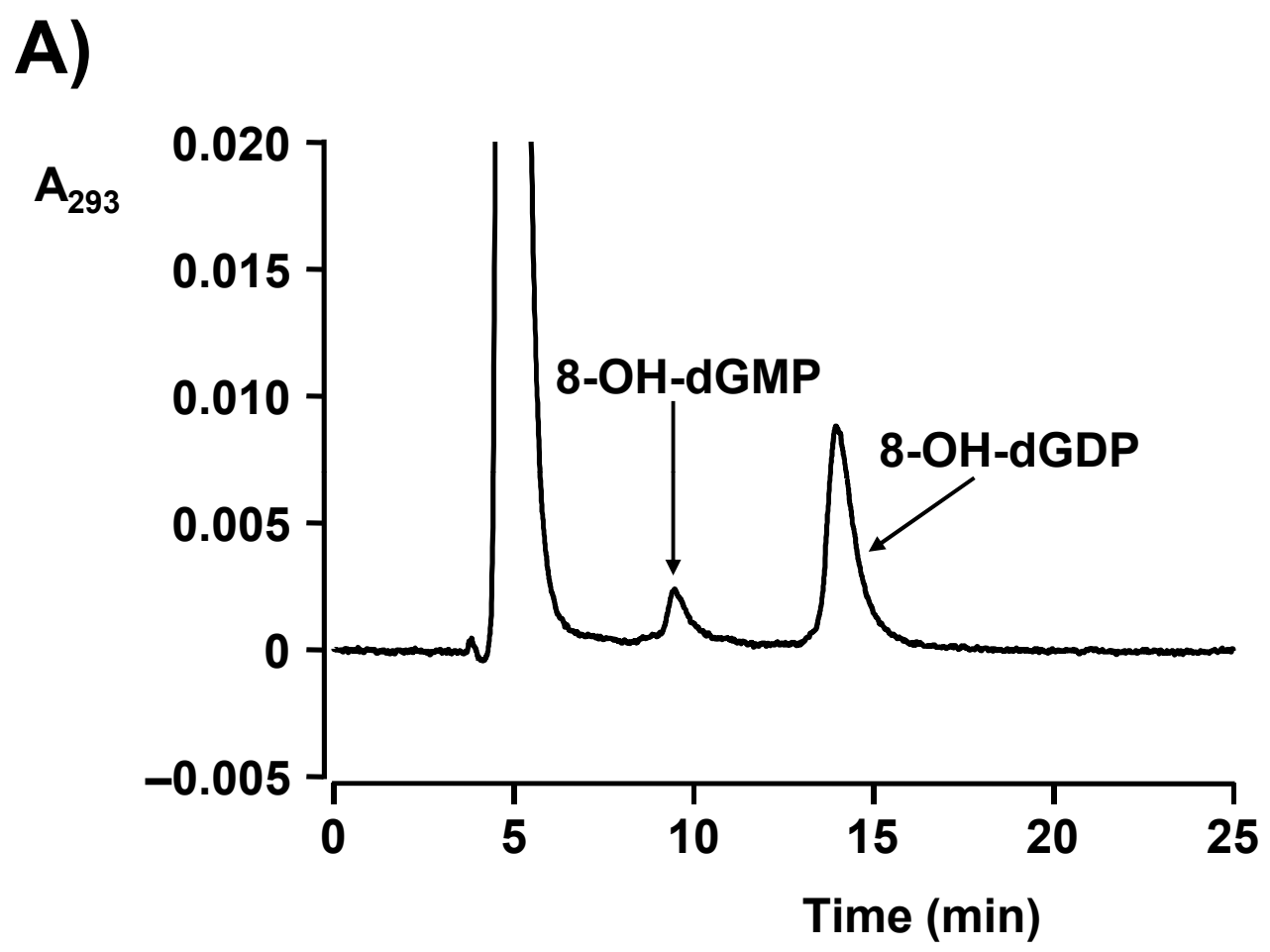
5-CHO-dUDP



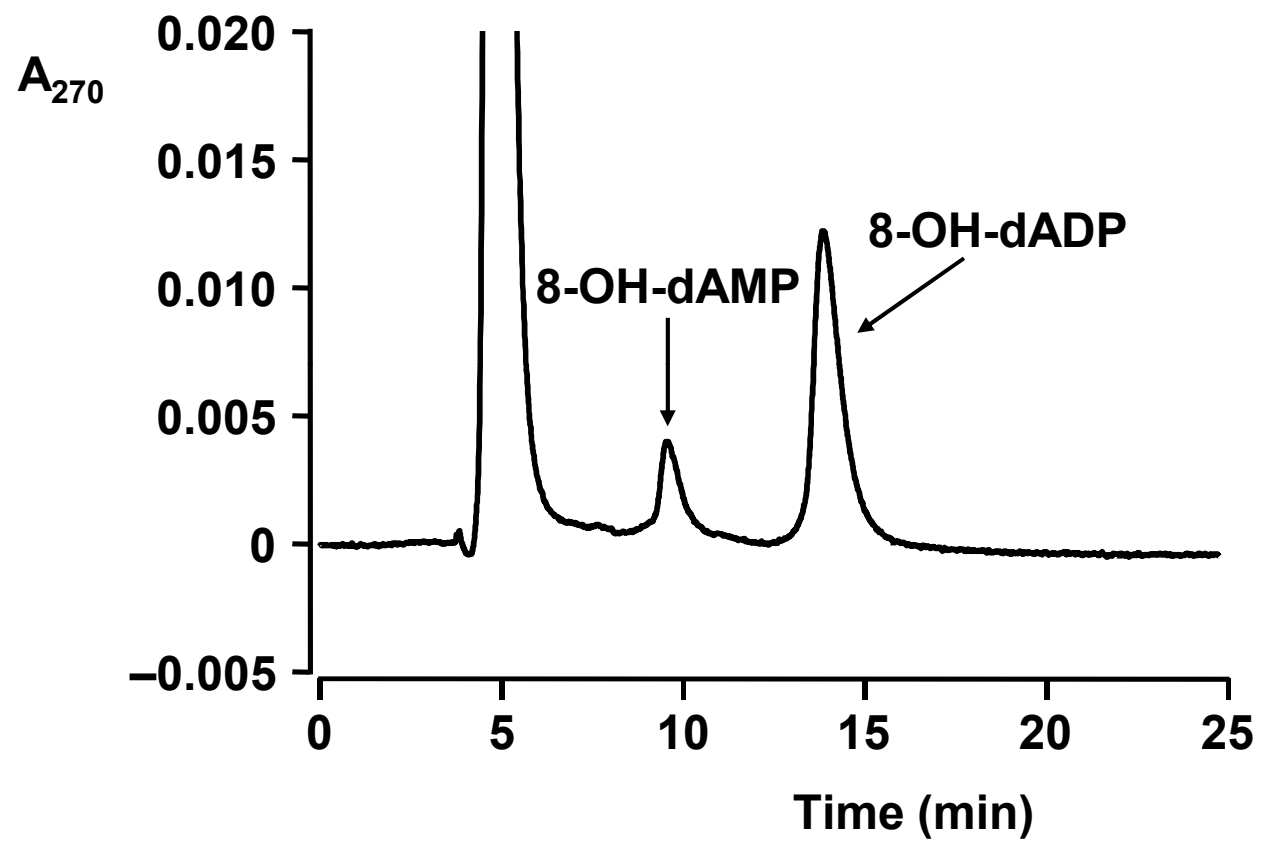
5-OH-dCDP

Fig. 1

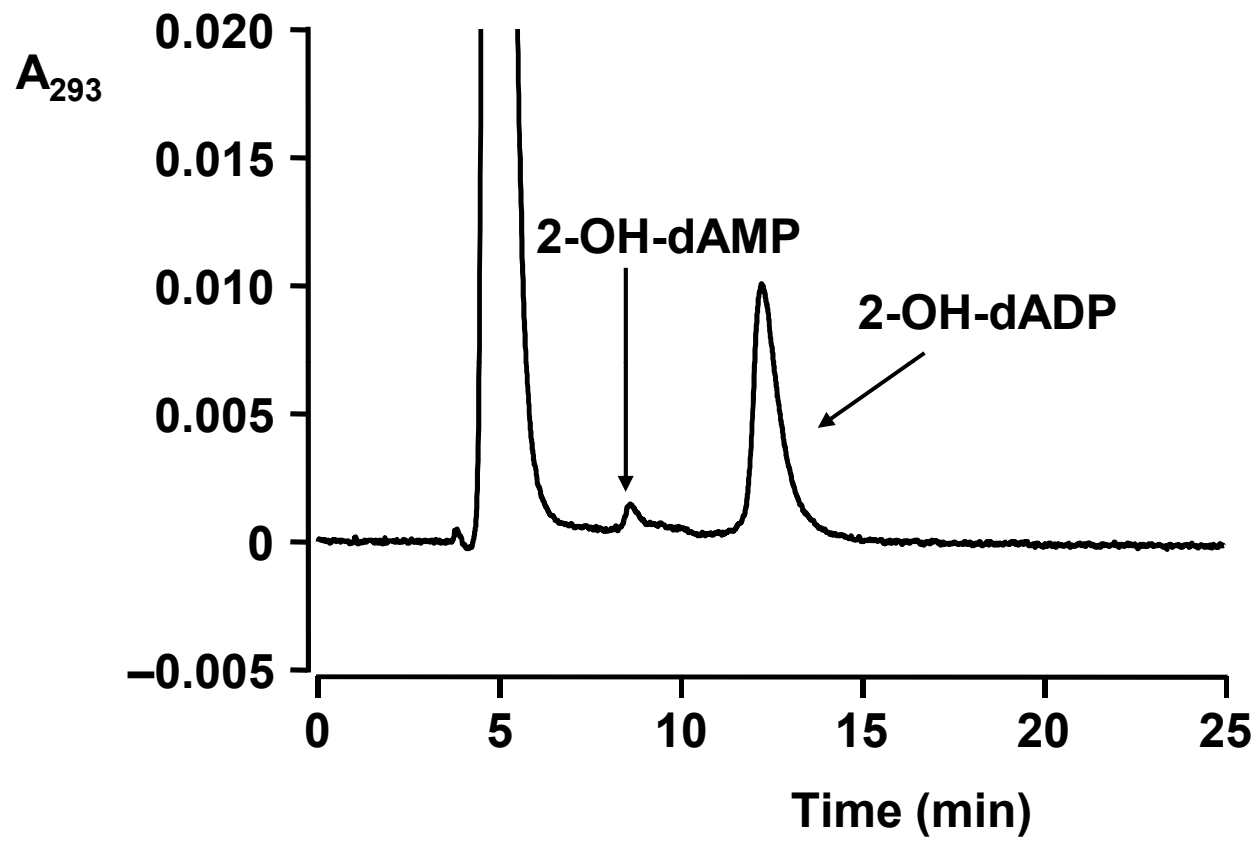
Figure 2



B)



C)



D)

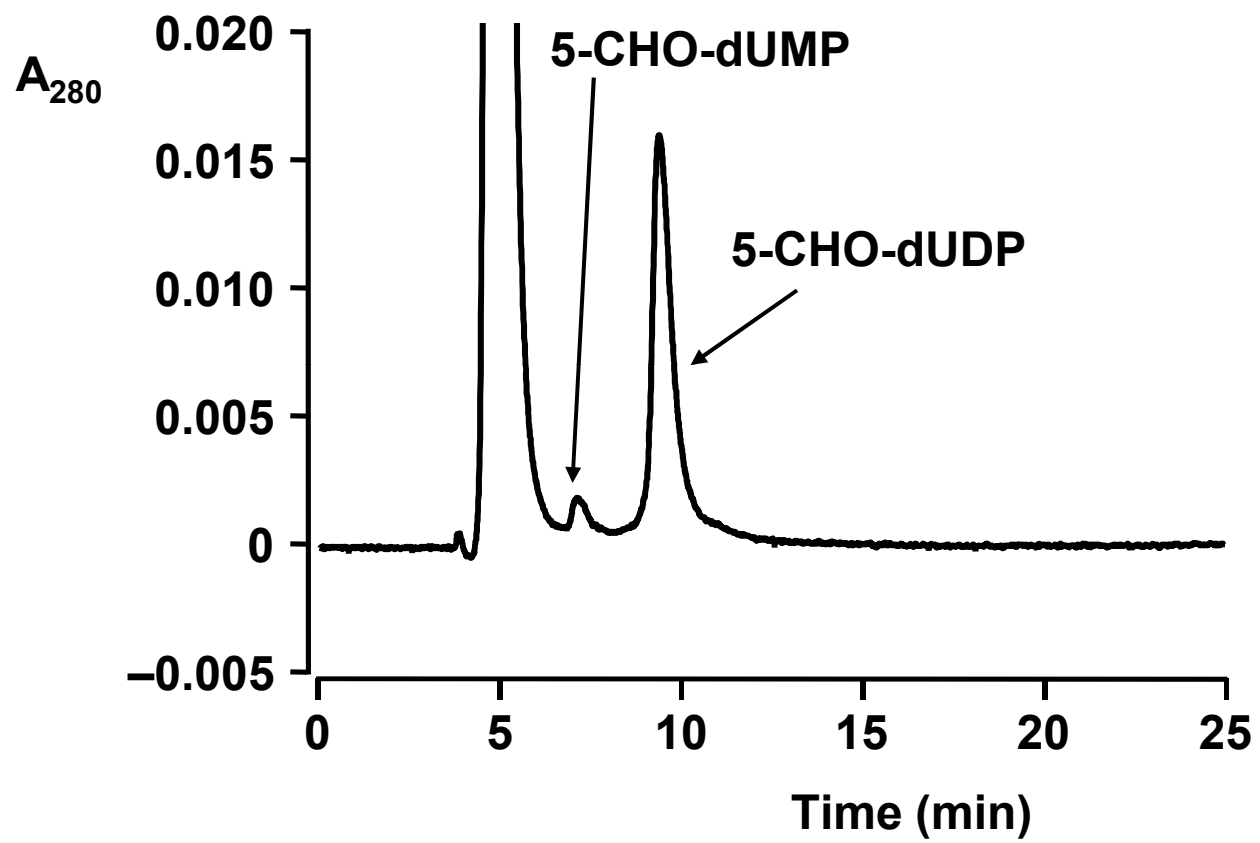


Table 1. Hydrolysis of oxidized deoxyribonucleoside diphosphates by NUDT5

substrate	hydrolysis (%) ^a
8-OH-dGDP	10.0 (1.0)
8-OH-dADP	12.7 (1.3)
2-OH-dADP	4.1 (0.4)
5-CHO-dUDP	4.8 (0.5)
5-OH-dCDP	ND

^a Each nucleotide (10 μ M) was incubated with 111 nM of the NUDT5 protein at 30°C for 30 min, and the hydrolysis percentage was measured by HPLC, as described in the “Materials and Methods”. Relative efficiencies to that of 8-OH-dGDP are shown in parentheses. Experiments were done at least in duplicate and the mean values are represented. ND, Not detected.

Table 2. Hydrolysis of oxidized deoxyribonucleoside triphosphates by NUDT5

substrate	hydrolysis (%) ^a
8-OH-dGTP	2.7 (1.0)
8-OH-dATP	3.4 (1.3)
2-OH-dATP	0.2 (0.1)
5-CHO-dUTP	0.2 (0.1)
5-OH-dCTP	ND

^a Each nucleotide (10 μ M) was incubated with 556 nM of the NUDT5 protein at 30°C for 30 min, and the hydrolysis percentage was measured by HPLC, as described in the “Materials and Methods”. Relative efficiencies to that of 8-OH-dGTP are shown in parentheses. Experiments were done at least in duplicate and the mean values are represented. ND, Not detected.

Table 3. Substrate specificity of the NUDT5 protein.

nucleotide	K_m (μM) ^a	k_{cat} (min^{-1}) ^a	k_{cat} / K_m ($\text{min}^{-1} \mu\text{M}^{-1}$) ^b
8-OH-dGDP	2.1	0.369	0.176 (1.00)
8-OH-dADP	2.9	0.538	0.186 (1.06)
2-OH-dADP	8.8	0.226	0.026 (0.15)
5-CHO-dUDP	4.0	0.209	0.052 (0.30)
dGDP	7.6	0.929	0.122 (0.69)
dADP	12.6	0.365	0.029 (0.16)

^a Experiments were done at least in duplicate and the mean values are represented.

^b Relative values to that of 8-OH-dGDP are shown in parentheses.