J. Biochem. 2011;149(6):731–738 doi:10.1093/jb/mvr028

JB THE JOURNAL OF BIOCHEMISTRY

Cleavage of oxidized guanine nucleotide and ADP sugar by human NUDT5 protein

Received December 16, 2010; accepted February 22, 2011; published online March 9, 2011

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MutT-related proteins, including *Escherichia coli* MutT and the human MTH1 (NUDT1), degrade 8-oxo-7, 8-dihydrodeoxyguanosine triphosphate (8-oxo-dGTP) to 8-oxo-dGMP and thereby prevent mutations caused by the misincorporation of 8-oxoguanine into DNA. The human NUDT5, which has an intrinsic activity to cleave ADP sugars to AMP and sugar phosphate, possesses the ability to degrade 8-oxo-dGDP to the monophosphate. Since 8-oxo-dGDP and 8-oxo-dGTP are interconvertible by cellular enzymes, NUDT5 has the potential to prevent errors during DNA replication. The two activities associated with NUDT5 exhibit different pH dependencies; the optimum for the cleavage of ADP ribose is pH 7–9, while that for 8-oxo-dGDPase is around pH 10. The kinetic parameters for the two types of reactions indicated that ADP ribose is a better substrate for NUDT5 compared with oxidized guanine nucleotides. The 8-oxo-dGDP cleavage was competitively inhibited by ADP ribose and its reaction product, AMP, and in reverse, the cleavage of ADP ribose was inhibited by 8-oxo-dGDP. These results imply that the two types of substrates may share the same binding site for catalysis.

Keywords: ADP ribose/Nudix hydrolase/NUDT5/8-oxo-guanine/substrate recognition.

Abbreviations: 8-oxoguanine, 8-oxo-7,8-dihydroguanine; 8-oxo-dGTP, 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-triphosphate; 8-oxo-dGDP, 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-diphosphate; 8-oxo-dGMP, 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-monophosphate; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; ROS, reactive oxygen species; IPTG, isopropyl-β-D-thiogalactopyranoside; TEAB, triethylammonium hydrogencarbonate; BSA, bovine serum albumin.

The Nudix hydrolase superfamily is widespread among eukaryotes and prokaryotes, and catalyses the hydrolysis of various phosphate-containing substances, including nucleoside triphosphates and diphosphates, nucleotide sugars and alcohols, dinucleoside polyphosphates, dinucleotide coenzymes and capped RNAs (*1*, *2*). Members of this family are characterized by the highly conserved 23-residue sequence motif, $GX_5EX_7REUXEEXGU$, where U is a bulky hydrophobic residue and X is any residue. This motif was first recognized by comparisons of the sequences of the *Escherichia coli* MutT and its human homologue, MTH1 (mutT homologue 1) (*3*). The motif was subsequently named the MutT signature, or the Nudix box (*4*). In mammals, there are presently 22 known Nudix enzymes, which are named NUDT1 (MTH1) through NUDT22, according to their order of discovery. Each of the enzymes exhibits a characteristic substrate specificity, which may reflect its biological functions.

Among these Nudix enzymes, NUDT5 has attracted special attention. This protein was first characterized as adenosine 5′-diphospho-sugar pyrophosphatase (*5*–*7*). The enzyme is most active against ADP ribose and ADP mannose, and also acts on ADP glucose and diadenosine diphosphate. Lower, but significant, activity was observed with other nucleoside diphosphate sugars and -alcohols. Among these compounds, ADP ribose is the most notable in respect to metabolism in mammalian cells. This compound is generated by the turnover of NAD^+ and protein-bound poly- and mono-(ADP ribose) and can be hazardous to the cell since it can modify histidine, lysine and cysteine residues of proteins by non-enzymatic glycation (*8*, *9*) and leads to inhibition of ATP-activated K^+ channels (*10*). Moreover, it was found that NUDT5 possesses the ability to cleave *O*-acetyl-ADP ribose (*7*). Therefore, NUDT5 is regarded as a 'sanitization enzyme' that eliminates endogenous toxic materials from the cell.

Another notable feature of NUDT5 is its capacity to degrade oxidized forms of guanine nucleotides (*11*, *12*). Reactive oxygen species (ROS), such as superoxide and hydroxyl radicals, are produced through normal metabolism, and oxidize cellular constituents. Among various oxidized purine and pyrimidine bases thus produced, 8-oxoguanine (8-oxo-7,8-dihydroguanine) is particularly important since it causes base mispairing. The 8-oxoguanine can pair with adenine, as well as cytosine, during nucleic acid syntheses, and this mispairing can cause mutations and alterations in gene expression. *Escherichia coli* MutT and human MTH1 proteins both possess the ability to degrade 8-oxoguanine-containing nucleoside triphosphate to the monophosphate, thereby preventing misincorporation of 8-oxoguanine into nucleic acids (*13*, *14*).

When the cDNA for human NUDT5 was expressed in *E. coli mutT* $^{-}$ cells, the elevated level of mutation frequency was reduced to the wild-type level, implying that the NUDT5 protein has the potential to eliminate the mutagenic oxidized substrates from the DNA precursor pool (*11*).

These studies seem to show that the NUDT5 has the ability to cleave two distantly related groups of compounds, ADP sugars and oxidized nucleotides. Since the actions of NUDT5 on ADP sugars and oxidized guanine nucleotides have been studied separately by using enzyme preparations purified in different manners, it was not clear which groups of compounds are the preferred substrates for the enzyme, and how one type of substrate affects the cleavage reactions for the other type of substrate. To better understand the roles of NUDT5 in various biological processes, it is necessary to study enzyme reactions for the two types of substrates by using the same enzyme preparation under defined conditions. The present study was undertaken to obtain and elucidate this basic information.

Materials and Methods

Purification of NUDT5, MTH1 and MutT proteins

MutT protein carrying the His tag at the N-terminal end was produced in *E. coli* strain M15 using the pQE-80L vector (QIAGEN), as described previously (*15*). NUDT5 with a His tag at its N terminus was produced in the *E. coli* over-expression system using a pQE-80L vector and *E. coli* strain M15 (QIAGEN). The *E. coli* strain M15 was transformed with pQE-80L carrying cDNA for NUDT5, and the cells were cultured in 200 ml of LB broth containing 100 μg/ml of ampicillin with shaking at 37°C. When A_{600} reached 0.6–0.7, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to give a final concentration of 1 mM, and cells were harvested 2 h after the addition of IPTG. The cells were collected by centrifugation and lysed in 10 ml of tris(hydroxymethyl)aminomethane (Tris) buffer (50 mM Tris–HCl, pH 8.0 and 300 mM NaCl) containing 10 mM imidazole by sonication. The lysate was added to TALON resin (Clontech) following clarification by centrifugation, and then the protein was eluted in Tris buffer containing 250 mM imidazole. The protein was concentrated by a Microcon YM3 (Amicon) in 10 mM Tris–HCl (pH 8.0)—0.1 mM EDTA—5 mM dithiothreitol (DTT)—10% glycerol. N-terminal His-tagged human MTH1 was produced via a cold shock expression system (TaKaRa) using pCold I DNA and *E. coli* strain BL21 at 15°C, and purified by using TALON resin as described above.

Preparation of oxidized nucleotides

8-oxo-dGTP (8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-triphosphate), 8-oxo-dGDP (8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-diphosphate) and 8-oxo-dGMP (8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-monophosphate) were prepared as described by Mo *et al.* (*16*), with slight modifications. A reaction mixture containing 100 mM sodium phosphate (pH 6.8), 6 mM nucleotide (either one of dGTP, dGDP and dGMP), 30 mM ascorbic acid and 100 mM H_2O_2 was incubated at 37°C for 4 h in the dark. Fifty microlitres of the reaction mixture were loaded onto an ion-exchange column (MonoQ HR 5/5, 5 × 50 mm, Pharmacia) equilibrated with 5% triethylammonium hydrogencarbonate (TEAB) and eluted with a 5–80% gradient of TEAB for 40 min. Fractions containing oxidized forms of nucleotides were lyophilized, dissolved in deionized–distilled water and stored at −20°C.

Enzyme assays

For the analysis of the 2 nt, enzymatic reactions and detection of the products were carried out as described previously (*11*) with slight modifications. The reaction mixture (10 μl) contained an enzyme, one of the substrates, 80 μg/ml of bovine serum albumin (BSA), 8 mM $MgCl_2$, 40 mM NaCl, 5 mM DTT and 2% glycerol in 20 mM Tris–HCl (for pH 7–9) or 20 mM glycine–NaOH (for pH 9–12). The reaction was carried out at 30°C for various times and terminated by adding sodium dodecyl sulphate (SDS) (final concentration, 1%). To determine the kinetic parameters, K_m and V_{max}, the reactions were performed with different concentrations of the substrates, 1.25–20 μM 8-oxo-dGDP using 10 ng of the NUDT5 protein, and 10–100 μM 8-oxo-dGTP using 50 ng of the protein, with glycine–NaOH buffer at pH 10 or Tris–HCl buffer at pH 8.0. After hydrolytic reactions, nucleotides were separated by high-performance liquid chromatography (HPLC) using a TSKgel DEAE-2SW column (4.6 × 250 mm, TOSOH) in an isocratic solution of 0.12 M sodium phosphate (pH 6.0) containing 40% acetonitrile. For the hydrolysis of ADP ribose, the reaction (in a 50 μl volume) was carried out with 1.25–40 μM ADP ribose and 1 ng of NUDT5 protein, as mentioned in the study by Yang *et al.* (*17*). Briefly, ADP ribose was incubated with appropriate amounts of enzyme in 50 mM of the same buffer system as for nucleotides containing 5 mM $MgCl_2$ and 1 mM DTT. The reactions were quenched by adding trifluoroacetic acid to 1% (v/v) and the reaction products were analysed according to the method of Tong *et al.* (*18*) using C18 analytical reverse phase column. The samples were loaded onto HPLC and separated in TSKgel ODS 100v (4.6 × 250 mm, TOSOH) equilibrated with 0.05% (v/v) trifluoroacetic acid in water (Buffer I). Elution was carried out in a 20-min gradient from 0% to 8% of Buffer II (0.02% trifluoroacetic acid in acetonitorile), followed by a 20-min gradient to 40% of Buffer II. The amounts of nucleoside monophosphate and AMP, produced by each hydrolysis reaction, were quantified by measuring the area of peak of UV absorbance. K_m and V_{max} values were determined from the intercepts of both axes, x (1/S) and y (1/v), of Lineweaver–Burk plots, respectively.

Results

Hydrolysis of 8-oxoguanine-containing nucleotides and ADP ribose

An N-terminal His-tagged form of human NUDT5 was produced in *E. coli* and purified by affinity chromatography. As references, we prepared human MTH1 and *E. coli* MutT proteins, each of which was His-tagged at the N-terminal end. On SDS–polyacrylamide gel electrophoresis (PAGE), all three enzyme preparations exhibited a single band at positions corresponding to the molecular weights of the fusion proteins (Fig. 1).

By using these preparations, we performed reactions against three types of compounds, 8-oxo-dGDP, 8-oxo-dGTP and ADP ribose, and the results obtained are shown in Fig. 2. It was evident that human NUDT5 can hydrolyse 8-oxo-dGDP to 8-oxo-dGMP but hardly acts on 8-oxo-dGTP. The same NUDT5 preparation was able to hydrolyse ADP ribose (Fig. 2B). Thus, NUDT5 is indeed capable of cleaving the two different types of compounds, 8-oxo-dGDP and ADP ribose. It should be noted, however, that a 20-fold higher concentration of the enzyme was used for achieving the former reaction compared with that for the latter, indicating that the ADP ribose cleavage activity of NUDT5 is considerably more potent than is its 8-oxo-dGDPase activity.

Human MTH1 and *E. coli* MutT showed no activity towards ADP ribose, even through considerably higher levels of the enzymes were used for these assays (Fig. 2C and D). These human and *E. coli* proteins were active on oxidized forms of guanine nucleotides, as described previously (*3*, *12*, *13*, *15*). MTH1 preferentially degraded 8-oxo-dGTP to 8-oxo-dGMP whereas the MutT hydrolysed both 8-oxo-dGTP and 8-oxo-dGDP to the monophosphate. Thus, NUDT5 is unique in possessing two distinct activities towards

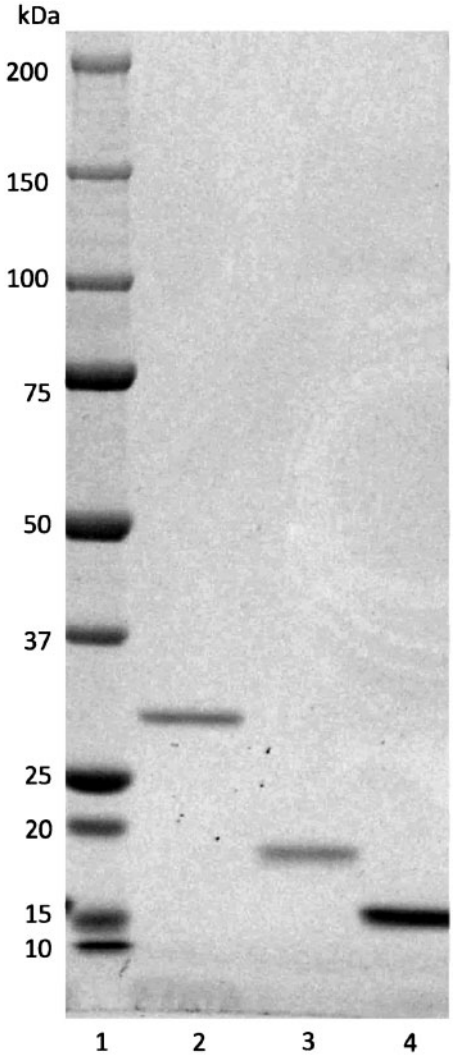

Fig. 1 SDS–polyacrylamide gel electrophoresis of purified N-terminal His-tagged enzyme preparations. Five hundred nanogram of each purified protein were subjected to 5–20% SDS–PAGE. Lane 1, molecular weight markers; lane 2, human NUDT5; lane 3, human MTH1; lane 4, *E. coli* MutT.

different types of compounds, namely oxidized guanine nucleotide and ADP sugar.

Comparison of the two types of hydrolytic reactions

The kinetic parameters of the NUDT5 enzyme were measured for the hydrolysis of various compounds (Table I). We first determined the enzymatic parameters for the hydrolysis of the two types of substrates at physiological pH (pH 8). The apparent K_m values for the hydrolysis of both substrates were nearly the same, whereas the V_{max} value for the ADP ribose cleavage was far greater than that for the 8-oxo-dGDP hydrolysis. As a result, the V_{max}/K_m value for ADP ribose was ~400 times higher than that for 8-oxo-dGDP, indicating that ADP ribose is a better substrate for the enzyme compared with 8-oxo-dGDP.

At this stage of investigation, we noticed that NUDT5 exhibited different pH optima for the two types of substrates. As shown in Fig. 3, the pH optimum for the hydrolysis of ADP ribose is in the range of pH 7–9, whereas the maximum activity for 8-oxo-dGDP is attained around pH 10. When the kinetic parameters of NUDT5 were determined at different pH levels, we obtained the results shown in Table I. The K_m value for the hydrolysis of 8-oxo-dGDP at pH 10 was nearly the same as that determined at pH 8, but the V_{max} at pH 10 was four times higher than that obtained at pH 8. Thus, the 8-oxo-dGDP hydrolytic reaction proceeds more efficiently at pH 10 than at pH 8, which further reflects to the differences in V_{max}/K_m values obtained at the two pH levels. Even under the alkaline conditions, the preference for 8-oxo-dGDP over 8-oxo-dGTP was evident; the V_{max}/K_m value for 8-oxo-dGTP was <0.4% of that for 8-oxo-dGDP.

Interactions of the two types of substrates

The finding that NUDT5 is capable of cleaving both 8-oxo-dGDP and ADP ribose raises the question of how one type of compound affects the cleavage reaction for the other. We first examined the effects of ADP ribose and its cleavage products on the 8-oxo-dGDP cleavage reaction (Experiment I in Table II). Relatively low levels of ADP ribose significantly inhibited the cleavage of 8-oxo-dGDP; the rate of cleavage in the presence of 5 μM ADP ribose, which corresponds to the concentration of the substrate, 8-oxo-dGDP, was less than half of the control (without addition). A similar degree of inhibition was achieved with AMP, which is the product of the ADP ribose cleavage reaction. On the other hand, no effect was observed with the addition of ribose 5′-phosphate, another product of the cleavage.

We then determined the effects of ADP ribose and AMP on the cleavage of 8-oxo-dGDP by NUDT5 in a more quantitative manner. Figure 4 shows Lineweaver–Burk plots for the hydrolysis of 8-oxo-dGDP in the presence of different amounts of ADP ribose and AMP. From the figures, it is evident that the other type of substrate, ADP ribose and its reaction product, AMP, competitively inhibit the cleavage of 8-oxo-dGDP.

We also examined the reverse; the effect of 8-oxo-dGDP on the ADP ribose cleavage reaction and the results are also presented in Table II. A considerable degree of inhibition was observed when 10 μM 8-oxo-dGDP was present in the reaction mixture. These results also show that there is competition between the two types of substrates. No inhibitory effect was observed with 8-oxo-dGMP, the cleavage product.

Discussion

The NUDT5 protein belonging to the Nudix hydrolase family has been characterized as an adenosine 5′-phosphosugar pyrophosphatase possessing a 'MutT' motif. Hydrolases that can cleave ADP ribose to AMP and ribose phosphate were first found in *E. coli* and *Saccharomyces cerevisie* (*4*), and thereafter several related enzymes were found in eukaryotes (*19–21*). In human, NUDT5 and NUDT9 possess such a hydrolase (*21*). The NUDT9 gene codes for two types of

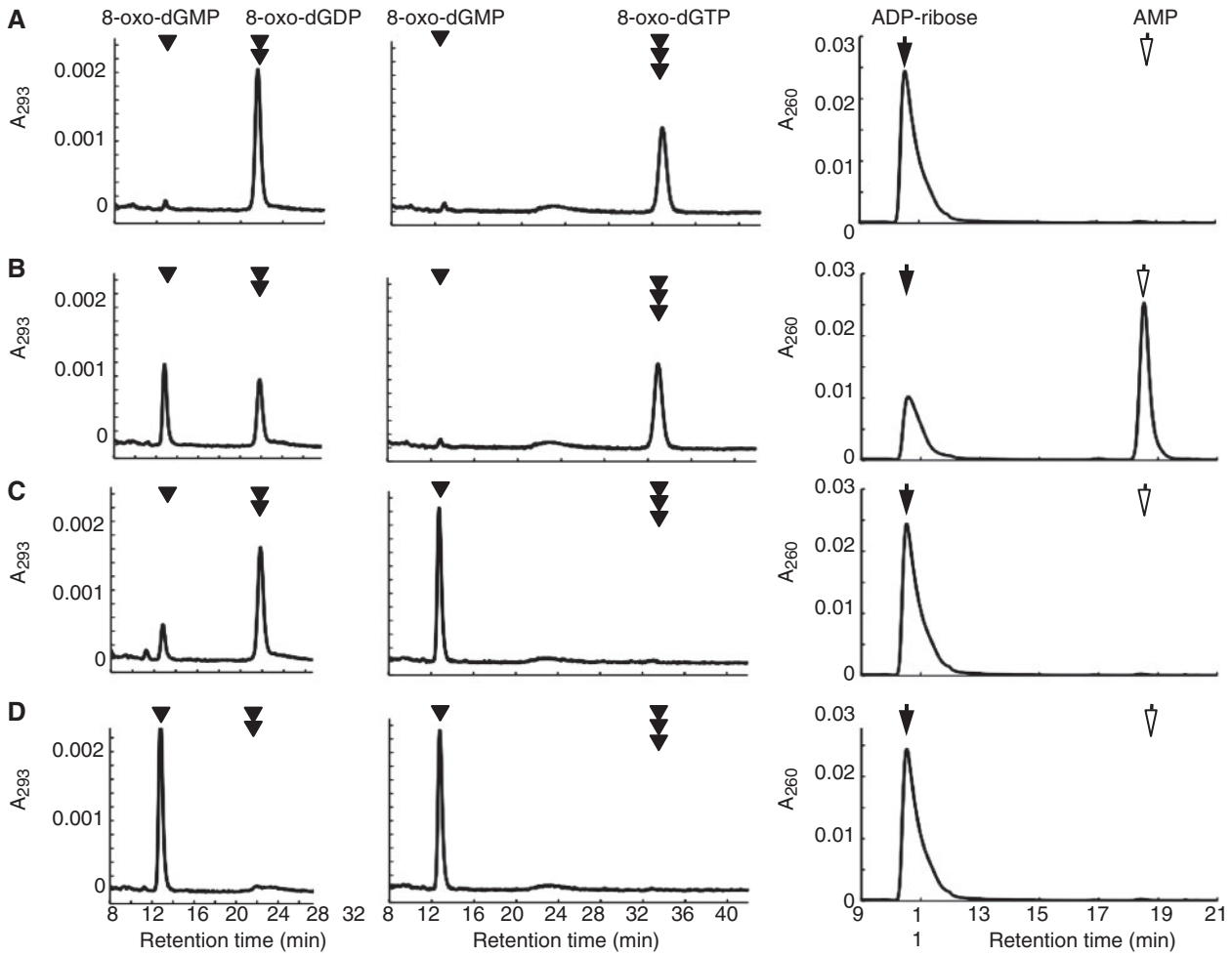

Fig. 2 HPLC elution profiles of the reaction mixtures. Left column: conversion of 8-oxo-dGDP (double inverted filled triangle) to 8-oxo-dGMP (inverted filled triangle). Central column: conversion of 8-oxo-dGTP (triple inverted filled triangle) to 8-oxo- dGMP (inverted filled triangle). Right column: conversion of ADP ribose (filled arrow head) to AMP (open arrow head). (A) No enzyme (control). (B) For the left and the central columns, 10 μM each of 8-oxo-dGDP and 8-oxo-dGTP were incubated with 100 ng of NUDT5 in 10 μl of reaction mixture I (20 mM glycine–NaOH, pH 10, containing 80 μg/ml of BSA, 8 mM $MgCl_2$, 40 mM NaCl, 5 mM DTT and 2% glycerol) at 30°C for 30 min. For the right column, 10 μM ADP ribose were incubated with 5 ng of NUDT5 in 50 μl of reaction mixture II (50 mM Tris–HCl, pH 7, containing 5 mM $MgCl_2$ and 1 mM DTT) at 37°C for 30 min. (C) For the left and the central columns, 10 μM each 8-oxo-dGDP and 8-oxo-dGTP were incubated with 5 ng of MTH1 in 10 μl of reaction mixture III (20 mM Tris–HCl, pH 8, containing 80 μg/ml of BSA, 8 mM $MgCl_2$, 40 mM NaCl, 5 mM DTT and 2% glycerol) at 30°C for 30 min. For the right column, 10 μM ADP ribose were incubated with 50 ng of MTH1 in 50 μl of reaction mixture II at 37°C for 30 min. (D) For the left and central columns, 10 μM each of 8-oxo-dGDP and 8-oxo-dGTP were incubated with 5 ng of MutT in 10 μl of reaction mixture III at 30°C for 30 min. For the right column, 10 μM ADP ribose were incubated with 50 ng of MutT in 50 μl of reaction mixture II at 37°C for 30 min.

Table I. Kinetic parameters of NUDT5.

Exp. No.	pH	Substrate	K_m (μM)	V_{max} (pmol/min/μg)	V_{max}/K_m
I	8.0	8-oxo-dGDP	3.8	11	2.9
		ADP ribose	1.9	2400	1300
II	10.0	8-oxo-dGDP	3.5	46	13
		8-oxo-dGTP	36	1.7	0.048

protein, NUDT9α and NUDT9β, and the former is the full-length product which possesses a mitochondrial signal (*22*). The degradation of ADP ribose may be important for various organisms, since ADP ribose contains a reactive aldehyde group and is able to glycate non-enzymatically various proteins (*8*, *9*). The glycation by ADP ribose forms ketoamine adducts, which are unstable and often yield protein glycoxidation products (*9*, *23*). Under physiological conditions, long-lived proteins become markedly modified and extensively cross-linked by the glycoxidation products, and such modification has been implicated in certain diseases, including diabetes and Alzheimer's disease (*23*, *24*). Moreover, it has been suggested that ADP ribose might act as a regulator of the ATP-sensitive K^+ channel (*10*) and also induce cation channel gating (*25*). Though the precise function of ADP ribose is still unclear, it may be necessary to remove any excess levels of ADP ribose from living cells to minimize its potential detrimental effect.

Beside the activity to cleave sugar-phosphates, NUDT5 protein possesses an enzymatic activity to

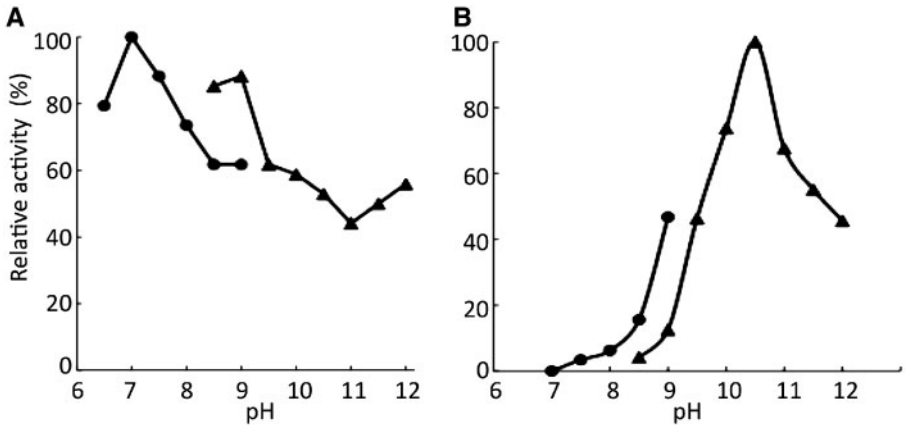

Fig. 3 The effects of pH on the hydrolysis of ADP ribose and 8-oxo-dGDP by NUDT5. (A) Hydrolysis of ADP ribose. The reaction mixtures (50 µl) contained 14 ng of NUDT5 protein and 500 µM ADP ribose and the mixture was incubated at 37°C for 30 min in 50 mM Tris buffer (pH 7–9), filled circle, or in 50 mM glycine buffer (pH 8.5 to 12), filled triangle. (B) Hydrolysis of 8-oxo-dGDP. The enzyme reactions were carried out at 30°C for 30 min with 40 ng of NUDT5 protein and 15 µM 8-oxo-dGDP in the sane buffer systems as in (A).

Table II. The effects of related compounds on the two types of cleavage reaction catalysed by NUDT5.

Exp. No.	Reaction	Related compounds (µM)	Extent of cleavage (%)	Relative efficiencies
I	8-oxo-dGDP to 8-oxo-dGMP and Pi	None		
		0	23.8	1.00
		ADP ribose		
		1	11.6	0.50
		5	5.1	0.22
		AMP		
		1	10.5	0.46
		5	6.8	0.30
		Ribose phosphate		
		10	21.7	0.95
		100	22.5	0.99
II	ADP ribose to AMP and ribose phosphate	None		
		0	12.7	1.00
		8-oxo-dGDP		
		2	11.5	0.91
		10	8.6	0.68
		8-oxo-dGMP		
		2	13.1	1.03
		10	11.3	0.89

In Experiment I, 50 ng of purified NUDT5 protein and 50 pmol of 8-oxo-dGDP were incubated with ADP ribose, AMP or ribose phosphate in 10 µl of a reaction mixture containing 20 mM glycine–NaOH buffer, pH 10, 80 µg/ml BSA, 8 mM $MgCl_2$, 40 mM NaCl, 5 mM DTT and 2% glycerol at 30°C for 30 min. In Experiment II, 2.5 ng of NUDT5 and 500 pmol of ADP ribose were incubated with 8-oxoguanine-containing nucleotides in 50 µl of a reaction mixture containing 50 mM Tris–HCl buffer, pH 7.0, 5 mM $MgCl_2$ and 1 mM DTT at 37°C for 30 min.

degrade oxidized forms of nucleotides. Ishibashi *et al.* (*11*) first found that human NUDT5 can hydrolyse 8-oxo-dGDP into 8-oxo-dGMP. More recently, a study was extended to various other forms of oxidized deoxyribonucleotides with mutagenic potentials for DNA synthesis (*26*). Several enzymes that catalyse the degradation of oxidized guanine nucleotides have been described, among which the *E. coli* MutT has been most extensively characterized. MutT can hydrolyse both 8-oxo-dGTP and 8-oxo-dGDP to nucleoside monophosphate with almost equal efficiencies (*15*). In mammalian cells, three distinct species of enzymes, MTH1 (NUDT1), NUDT5 and MTH2 (NUDT15), function to fulfill the role that is carried out by a single enzyme, MutT, in *E. coli*. MTH1 preferentially cleaves 8-oxo-dGTP, and has only a minimal effect on 8-oxo-dGDP. On the other hand, NUDT5 has a distinct preference for degrading 8-oxo-dGDP, while MTH2 possesses a relative low activity towards 8-oxo-dGTP (*27*). It has been shown that MTH2 makes a complex with PCNA, and that this complex undergoes degradation in response to UV irradiation (*28*). In addition to the intrinsic activity to degrade 8-oxo-dGTP, MTH2 might play other role(s) in the regulation of cell reproduction.

Thus far, the two activities associated with NUDT5, one to degrade oxidized nucleotides and the other to cleave ADP sugars, have been studied with different enzyme preparations. In some of the early studies, in which the NUDT5 was characterized as an enzyme that hydrolyses ADP ribose, attempts were made to detect any activity towards nucleotides. However, no such activity was detected at that time. In retrospect, this failure might have been due to the use of a relatively low level of the enzyme for such assays. As found in the present study, at least 10 times more NUDT5 protein is needed to detect an 8-oxo-dGDPase activity compared with the activity against ADP ribose. With the data shown in the present study, it is now evident that the human NUDT5 possesses two distinct enzyme activities against oxidized guanine nucleotides and ADP ribose. The fact that the NUDT5 possesses two independent enzyme activities raises the question about which of the activities plays the more important role in cellular functions. In this regard, it is noteworthy that the two activities associated with NUDT5 exhibit different pH preferences. The optimum pH for the ADP ribose cleavage reaction is pH 7–9 while that for 8-oxo-dGDPase is around pH 10. This phenomenon itself deserves special attention considering the

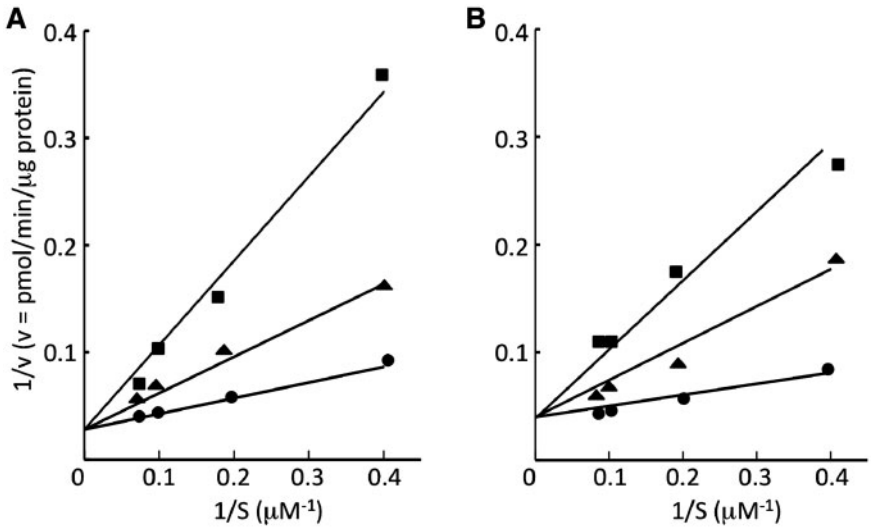

Fig. 4 The effects of ADP ribose or AMP on the hydrolysis of 8-oxo-dGDP by NUDT5 protein. These figuires show the Lineweaver–Burk plots of the reaction with the coexistence of 8-oxo-dGDP and ADP ribose or AMP. The reactions were performed with 10 ng of NUDT5 protein in the presence of 2.5–12.5 μM 8-oxo-dGDP at pH 10 and 30°C. (A) The reactions were carried out with 1 μM (filled triangle) or 5 μM (filled square) ADP ribose. (B) The reaction mixtures contained 1 μM (filled triangle) or 5 μM (filled square) AMP. The control reactions (filled circle) were carried out without ADP ribose and AMP.

molecular actions of the enzyme that acts on the different types of substrates.

Based on the kinetic parameters determined under physiological conditions, ADP ribose is more efficiently cleaved by NUDT5 compared with 8-oxo-dGDP. These situations may be related to the cellular levels of the two compounds, both of which are unfavorable for cells, particularly when they accumulate. It is also necessary to emphasize that the NUDT5 enzyme has the potential to eliminate mutagenic oxidized nucleotides from the DNA precursor pool. When the enzyme is overpexpressed in *E. coli mutT*$^-$ bacteria, the elevated level of spontaneous mutation is decreased to the normal level (*11*). To obtain a better understanding of the molecular mechanism(s) responsible for the activities of NUDT5, we have examined how one type of substrate, ADP ribose, affects the cleavage of the other, 8-oxo-dGDP. The quantitative analyses have revealed that the hydrolysis of 8-oxo-dGDP is competitively inhibited by ADP ribose. This implies that the two types of substrates, 8-oxo-dGDP and ADP ribose, may occupy the same substrate binding site of the NUDT5 enzyme. Since AMP, the cleavage product of ADP ribose, exerted a similar degree of inhibition on the hydrolysis of 8-oxo-dGDP as did ADP ribose itself, it may be inferred that AMP can also occupy this site. There is, of course, a possibility that AMP binds to the other region of the enzyme to exert its effect.

The crystal structures of the human NUDT5 in apo form and in a ternary complex with ADP ribose and Mg^{2+} have been resolved, which indicated that the enzyme exists as a homodimer and that the active site is surrounded by several amino acid residues, including Try28, Try46, Aerg51, Glu112 and Glu116 (*29*, *30*). More recently, the crystal structure of NUDT5 complexed with 8-oxo-dGDP has been presented, thus indicating that the binding of 8-oxo-dGDP occurs in the opposite direction of the pyrophosphate group, compared with that of the ADP ribose complex, although both substrates occupy the same site (T. Arimori *et al.*, unpublished data). In support of this notion, an isotope labelling experiment indicated that 8-oxo-dGDP is attacked by nucleophilic water at Pβ, whereas ADP ribose is attacked at Pα. These results showing that the two types of substrates occupy the same binding site are in accord with our present finding that cleavage of 8-oxo-dGDP is competitively inhibited by ADP ribose.

The elimination of oxidized nucleotides from the DNA precursor pool is important for preventing mutations, as well as cancer. In *E. coli*, the MutT protein, which potently degrades 8-oxo-dGTP and 8-oxo-dGDP, is almost solely responsible for reducing the mutagenic nucleotide level, as *mutT*$^-$ strains show an ~1000-times higher frequency of spontaneous mutations compared with wild-type cells (*31–33*). In human cells, however, more than two types of enzymes seem to be involved in the process to fulfill such functions. MTH1 and MTH2 both specifically hydrolyse 8-oxo-dGTP, and NUDT5 possesses the ability to cleave 8-oxo-dGDP. Taking into account the parameters for these enzyme reactions, MTH2 appears to play a smaller role than do MTH1 and NUDT5. In MTH1 gene-knockout mouse lines, a greater number of tumours were formed in the lungs, livers, and stomachs, compared with wild-type mice (*34*). It will be of interest to see if mice deficient in NUDT5 and those lacking both thte MTH1 and NUDT5 genes are more susceptible to spontaneous and induced tumourigenesis than are wild-type mice. The mouse NUDT5 gene is located on the A1 segment of chromosome 2 and is composed of 10 exons. We recently succeeded in constructing NUDT5-deficient mice (Y. Nakatsu *et al.*,

unpublished data), and various studies are now in progress to answer these questions.

Acknowledgements

We thank Dr Yasumitsu Takagi for his gift of the MTH1 preparation, and Drs Teruhisa Tsuzuki and Masumi Hidaka for helpful discussions.

Funding

Ministry of Education, Culture, Sports, Sciences and Technoloby of Japan, grants-in-aid for scientific research (partial).

Conflict of interest

None declared.

References

1. Mildvan, A.S., Xia, Z., Azurmendi, H.F., Saraswat, V., Legler, P.M., Massiah, M.A., Gabelli, S.B., Bianchet, M.A., Kang, L.-W., and Amzer, L.M. (2005) Structures and mechanisms of Nudix hydrolases. *Arch. Biochem. Biophys.* **433**, 129–143
2. McLennan, A.G. (2006) The Nudix hydrolase superfamily. *Cell. Mol. Life Sci.* **63**, 123–143
3. Sakumi, K., Furuichi, M., Tsuzuki, T., Kakuma, T., Kawabata, S., Maki, H., and Sekiguchi, M. (1993) Cloning and expression of cDNA for a human enzyme that hydrolyzes 8-oxo-dGTP, a mutagenic substrate for DNA synthesis. *J. Biol. Chem.* **268**, 23524–23530
4. Bessman, M.J., Frick, D.N., and O'Handley, S.F. (1996) The MutT proteins or 'nudix' hydrolases, a family of versatile, widely distributed, 'housecleaning' enzymes. *J. Biol. Chem.* **271**, 25059–25062
5. Gasumi, L., Cartwright, J.L., and McLennan, A.G. (1999) Cloning, expression and characterization of YSAIH, a human adenosine 5'-phosphosugar pyrophosphatase possessing a MutT motif. *Biochem. J.* **344**, 331–337
6. Yang, H., Slupska, M.M., Wei, Y.-F., Tai, J.H., Luther, W.M., Xia, Y.-R., Shih, D.M., Chiang, J.-H., Baikalov, C., Fitz-Gibbon, S., Phan, I.T., Conrad, A., and Miller, J.H. (2000) Cloning and characterization of a new member of the Nudix hydrolases from human and mouse. *J. Biol. Chem.* **275**, 8844–8853
7. Rafty, L.A., Schmidt, M.T., Perraud, A.-L., Scharenberg, A.M., and Denu, J.M. (2002) Analysis of O-acetyl-ADP-ribose as a target for Nudix ADP-ribose hydrolases. *J. Biol. Chem.* **277**, 47114–47122
8. Jacobson, E.L., Cervantes-Laurean, D., and Jacobson, M.K. (1994) Glycation of proteins by ADP-ribose. *Mol. Cell. Biochem.* **138**, 207–212
9. Cervantes-Laureant, D., Jacobson, E.L., and Jacobson, M.K. (1996) Glycation and glycoxidation of histones by ADP-ribose. *J. Biol. Chem.* **271**, 10461–10469
10. Kwak, Y.-G., Park, S.-K., Kim, U.-H., Han, M.-K., Eun, J.-S., Cho, K-.P., and Chae, S.-W. (1996) Intracellular ADP-ribose inhibits ATP-sensitive K^+ channels in rat ventricular myocytes. *Am. J. Physiol.* **271**, C464–C468
11. Ishibashi, T., Hayakawa, H., and Sekiguchi, M. (2003) A novel mechanism for preventing mutations caused by oxidation of guanine nucleotides. *EMBO Rep.* **4**, 479–483
12. Ishibashi, T., Hayakawa, H., Ito, R., Miyazawa, M., Yamagata, Y., and Sekiguchi, M. (2005) Mammalian enzymes for preventing transcriptional errors caused by oxidative damage. *Nucleic Acids Res.* **33**, 3779–3784
13. Maki, H. and Sekiguchi, M. (1992) MutT protein specifically hydrolyzes a potent mutagenic substrate for DNA synthesis. *Nature* **355**, 273–275
14. Taddei, F., Hayakawa, H., Bouton, M.-F., Cirinesi, A.-M., Matic, I., Sekiguchi, M., and Radman, M. (1997) Counteraction by MutT protein of transcriptional errors caused by oxidative damage. *Science* **278**, 128–130
15. Ito, R., Hayakawa, H., Sekiguchi, M., and Ishibashi, T. (2005) Multiple enzyme activities of *Escherichia coli* MutT protein for sanitization of DNA and RNA precursor pools. *Biochemistry* **44**, 6670–6674
16. Mo, J.-Y., Maki, H., and Sekiguchi, M. (1992) Hydrolytic elimination of a mutagenic nucleotide, 8-oxodGTP, by human 18-kilodalton protein: sanitization of nucleotide pool. *Proc. Natl. Acad. Sci. USA* **89**, 11021–11025
17. Yang, H., Slupska, M.M., Wei, Y.-F., Tai, J.H., Luther, W.M., Xia, Y.-R., Shih, D.M., Chiang, J.-H., Baikalov, C., Fitz-Gibbon, S., Phan, I.T., Conrad, A., and Miller, J.H. (2000) Cloning and characterization of a new member of the nudix hydrolases from human and mouse. *J. Biol. Chem.* **275**, 8844–8853
18. Tong, L., Lee, S., and Denu, J.M. (2009) Hydrolase regulates NAD^+ metabolites and modulates cellular redox. *J. Biol. Chem.* **284**, 11256–11266
19. Dunn, C.A., O'Handley, S.F., Frick, D.N., and Bessman, M.J. (1999) Studies on the ADP-ribose pyrophosphatase subfamily of the Nudix hydrolases and tentative identification of *trgB*, a gene associated with tellurite resistance. *J. Biol. Chem.* **274**, 32318–32324
20. Tong, L., Lee, S., and Denu, J.M. (2009) Hydrolase regulates NAD^+ metabolites and modulates cellular redox. *J. Biol. Chem.* **2874**, 11256–11266
21. Lin, S., Gasmi, L., Xie, Y., Ying, K., Gu, S., Wang, Z., Jin, H., Chao, Y., Wu, C., Zhou, Z., Tang, R., Mao, Y., and McLennan, A.G. (2002) Cloning, expression and characterisation of a human Nudix hydrolase specific for adenosine 5'-diphosphoribose (ADP-ribose). *Biochim Biophys Acta.* **1594**, 127–135
22. Perraud, A.-L., Shen, B., Dunn, C.A., Rippe, K., Smith, M.K., Bessman, M.J., Stoddard, B.L., and Scharenberg, A.M. (2003) NUDT9, a member of the Nudix hydrolase family, is an evolutionarily conserved mitochondrial ADP-ribose pyrophosphatase. *J. Biol. Chem.* **278**, 1794–1801
23. Hogan, M., Cerami, A., and Bucala, R. (1992) Advanced glycosylation endproducts block the antiproliferative effect of nitric oxide - Role in the vascular and renal complications of diabetes mellitus. *J. Clin. Invest.* **90**, 1110–1115
24. Vitek, M.P., Bhattacharya, K., Glendening, J.M., Stopa, E., Vlassara, H., Bucala, R., Manogue, K., and Cerami, A. (1994) Advanced glycation end products contribute to amyloidosis in Alzheimer disease. *Proc. Natl. Acad. Sci. USA* **91**, 4766–4770
25. Perraud, A.-L., Takanishi, C.L., Shen, B., Kang, S., Megan Smith, K., Schmitz, C., Knowles, H.M., Ferraris, D., Li, W., Zhang, J., Stoddard, B.L., and Scharenberg, A.M. (2005) Accumulation of free ADP-ribose from mitochondria mediates oxidative stress-induced gating of TRPM2 cation channels. *J. Biol. Chem.* **280**, 6138–6148
26. Kamiya, H., Hori, M., Arimori, T., Sekiguchi, M., Yamagata, Y., and Harashima, H. (2009) NUDT5 hydrolyzed oxidized deoxyribonucleoside diphosphates with broad substrate specificity. *DNA Repair* **8**, 1250–1254
27. Cai, J.-P., Ishibashi, T., Takagi, Y., Hayakawa, H., and Sekiguchi, M. (2003) Mouse MTH2 protein which prevents mutations caused by 8-oxoguanine nucleotides. *Biochem. Biophys. Res. Commun.* **305**, 1073–1077

28. Yu, Y., Cai, J.-P., Tu, B., Wu, L., Zhao, Y., Liu, X., Li, L., McNutt, M.A., Feng, J., He, Q., Yang, Y., Wang, H., Sekiguchi, M., and Zhu, W.-G. (2009) Proliferating cell nuclear antigen is protected from degradation by forming a complex with MutT homolog2. *J. Biol. Chem.* **284**, 19310–19320
29. Zha, M., Zhong, C., Peng, Y., Hu, H., and Ding, J. (2006) Crystal structures of humen NUDT5 reveal insights into the structural basis of the substrate specificity. *J. Mol. Biol.* **364**, 1021–1033
30. Zha, M., Guo, Q., Zhang, Y., Yu, B., Ou, Y., Zhong, C., and Ding, J. (2008) Molecular mechanism of ADP-ribose hydrolysis by human NUDT5 from structural and kinetic studies. *J. Mol. Biol.* **379**, 568–578
31. Yanofsky, C., Cox, E.C., and Horn, V. (1966) The unusual mutagenic specificity of an *Escherichia coli* mutator gene. *Proc. Natl. Acad. Sci. USA* **55**, 274–281
32. Akiyama, M., Maki, H., Sekiguchi, M., and Horiuchi, T. (1989) A specific role of MutT protein: to prevent dG dA mispairing in DNA replication. *Proc. Natl. Acad. Sci. USA* **86**, 3949–3952
33. Tajiri, T., Maki, H., and Sekiguchi, M. (1995) Functional cooperation of MutT, MutM and MutY proteins in preventing mutations caused by spontaneous oxidation of guanine nucleotide in *Escherichia coli*. *Mutat. Res.* **336**, 257–267
34. Tsuzuki, T., Egashira, A., Igarashi, H., Iwakuma, T., Nakatsuru, Y., Tominaga, Y., Kawate, H., Nakao, K., Nakamura, K., Ide, F., Kura, S., Nakabeppu, Y., Katsuki, M., Ishikawa, T., and Sekiguchi, M. (2001) Spontaneous tumorigenesis in mice defective in the MTH1 gene encoding 8-oxo-dGTPase. *Proc. Natl. Acad. Sci. USA* **98**, 11456–11461