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A continuous fluorescence assay for the characterization of Nudix hydrolases

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The common substrate structure for the functionally diverse Nudix protein superfamily is nucleotide-diphosphate-X, where X is a large variety of leaving groups. The substrate specificity is known for less than 1% of the 29,400 known members. Most activities result in the release of an inorganic phosphate ion or of a product bearing a terminal phosphate moiety. Reactions have typically been monitored by a modification of the discontinuous Fiske–SubbaRow assay, which is relatively insensitive and slow. We report here the development of a continuous fluorescence assay that enables the rapid and accurate determination of substrate specificities in a 96-well format. We used this novel assay to confirm the reported substrate characterizations of MutT and NudD of Escherichia coli and to characterize DR_1025 of Deinococcus radiodurans and MM_0920 of Methanosarcina mazei. Novel findings enabled by the new assay include the following. First, in addition to the well-characterized hydrolysis of 8-oxo-dGTP at the α–β position, MutT cleaves at the β–γ phosphate bond at a rate of 3% of that recorded for hydrolysis at the α–β position. Second, MutT also catalyzes the hydrolysis of 5-methyl-dCTP. Third, 8-oxo-dGTP was observed to be the best substrate for DR_1025 of the 41 compounds screened.

Although several of the Nudix hydrolases catalyze the hydrolysis of aberrant deoxynucleoside triphosphates efficiently, thereby reducing the extent of incorporation of the corresponding undesired base into DNA. The function of these enzymes is to “sanitize the nucleotide pool”, thereby serving to prevent incorporation of non-canonical bases into DNA [1]. The best-characterized member of this group is MutT from Escherichia coli [13]. This gene product exhibits a nearly diffusion controlled $k_{cat}/K_m$ value of approximately $10^4$ M$^{-1}$ s$^{-1}$ for the hydrolysis of mutagenic 8-oxo-dGTP; however, it also catalyzes the hydrolysis of the canonical dGTP with a $k_{cat}/K_m$ value of approximately $10^3$ M$^{-1}$ s$^{-1}$ [14]. This last figure likely represents unavoidable collateral damage due to the structural similarity of the substrates.

The standard assay for those Nudix hydrolases that expose a product phosphate ester or P$_i$ is a variant of the classical Fiske–SubbaRow method [15] following treatment of the product, if necessary, with alkaline phosphatase (APase) [12]. This discontinuous assay necessitates removing aliquots during the time course from the reaction mixture. Thus, it is slow, labor intensive, and subject to pipetting error, and it is not optimal for rapid screening of candidate substrates.
We describe here a continuous assay that employs the fluorescently tagged *E. coli* phosphate binding protein (PBP) [16]. It is free of the described limitations of the Fiske–SubbaRow assay and is suitable for substrate and inhibitor screening in a moderate throughput format using 96-well plates.

The enzymes characterized here via this assay are MutT (*E. coli*), NudD (*E. coli*), DR_1025 (*Deinococcus radiodurans*), and MM_0920 (*Methanosarcina mazei*). X-ray or nuclear magnetic resonance (NMR) structures are available for all four enzymes. The first two were well characterized by the Fiske–SubbaRow assay [4] and were selected to validate the new assay. DR_1025 was chosen because *D. radiodurans*, which is highly resistant to radiation damage, has 26 putative Nudix enzymes that likely are responsible for some of the protection [17]. Some substrate screening of the DR_1025 activity has been reported [17]. MM_0920 is a putative Nudix enzyme. A phylogenetic analysis of MM_0920 with 142 biochemically or structurally characterized Nudix proteins has been performed (J.R. Srouji, unpublished work). It was determined that MM_0920 is located in a clade where protein function appears to change frequently. The biochemical characterization of MM_0920 would allow us to infer the function of many other uncharacterized Nudix hydrolases, and it would greatly aid in more accurate function predictions for the entire Nudix family.

**Materials and methods**

**Materials**

The *E. coli* enzymes MutT (UniProt AC: P08337) [18] and NudD (UniProt AC: P32056) [19] were gifts from Albert Mildvan (Johns Hopkins University School of Medicine), and DR_1025 of *D. radiodurans* (UniProt AC: Q9RVK2) [17] was a gift from Maurice Bessman (Johns Hopkins University). MM_0920 (UniProt AC: Q8PYE2) of *M. mazei* is encoded in the plasmid constructed by the New York SGX Research Center for Structural Genomics and was purchased from the PSI:Biology-Materials Repository (clone ID: MmCD00291907) [20]. The PBP A197C [21] *E. coli* expression strain was a gift from Martin Webb (National Institute for Medical Research, UK). The BL21(DE3) *E. coli* expression strain was purchased from Invitrogen (Carlsbad, CA, USA).

The Malachite Green Phosphate Detection Kit was purchased from R&D Systems (Minneapolis, MN, USA). The Novagen BugBuster protein extraction reagents were obtained from EMD4Bio-sciences (Gibbstown, NJ, USA). The Gene Jet Plasmid Miniprep Kit was obtained from Fermentas (Glen Burnie, MA, USA). Amicon Ultra-15 and Ultra-4 NMWL 10,000 and 0.22-μm PES syringe membranes were purchased from Millipore (Bedford, MA, USA). Modified nucleotide triphosphates (e.g., 8-oxo-dGTP, 5-methyl-dCTP) were purchased from TriLink Biotechnologies (San Diego, CA, USA). N-(2-[(1-Maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide (MDCC), common biochemicals, and enzymes were obtained from Sigma–Aldrich (St. Louis, MO, USA). Fast-performance liquid chromatography (FPLC) for protein purification was performed on a BioLogic DuosFlow 10 workstation (Bio-Rad, Hercules, CA, USA). All liquid chromatography columns were supplied by GE Healthcare (Piscataway, NJ, USA): HiPrep Q FF 16/10 anion exchange, Mono-Q 4.6/100 PE anion exchange, Superdex 75 10/300 GL size exclusion, and PD-10 (Sephadex G-25M) desalting columns. P$_1$-sensor assays were performed on either a FluoroMax-4 spectrofluorometer (HORIBA Jobin Yvon, Edison, NJ, USA) or a GENios microplate reader (Tecan, Männedorf, Switzerland). The Fiske–SubbaRow assay was monitored on a NanoDrop 2000c spectrophotometer (Thermo Scientific, Rockford, IL, USA).

**Nudix enzyme purification**

MutT was expressed and purified as described by Harris and coworkers [18] with the following changes. The BL21(DE3) cells bearing the pET-MutT plasmid were grown overnight in LB medium at 37 °C and then diluted (1:50) to 2 L and grown at 37 °C for approximately 2 h until $A_{600}$ was between 0.5 and 1.0. Isopropyl-$\beta$-D-thiogalactoside (IPTG) was added to a final concentration of 0.5 mM to induce protein production for another 2 h. The cells were harvested by centrifugation at 4500g and 4 °C for 20 min. The cell pellets were stored at –80 °C. Frozen cell pellets corresponding to 0.5 L of cell culture were lysed with BugBuster reagents as described in the kit protocol. Cell debris was removed by centrifugation at 14,000g and 4 °C for 20 min.

MutT precipitates between 30 and 60% (w/v) ammonium sulfate [22]. The pellet was dissolved in 4 ml of 10 mM Tris–HCl (pH 7.6), desalted through two PD-10 columns, and filtered through a 0.22-μm PES syringe membrane. The filtrate was applied to a HiPrep Q FF 16/10 column, equilibrated with 10 mM Tris–HCl (pH 7.6), and eluted with a 0 to 100% gradient of 1 M NaCl. Fractions that contained MutT were collected, combined, concentrated to less than 500 μl using Amicon Ultra-15 Centrifugal Filter Units (Millipore), and loaded on a Superdex 75 10/300 GL column. Eluted fractions that contained MutT were shown to be more than 95% pure by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). These fractions were combined, concentrated to approximately 100 μM with Amicon Ultra-4 Centrifugal Filter Units, divided into 10-μl aliquots, and stored at –80 °C.

NudD was similarly purified. It precipitated between 45 and 80% of ammonium sulfate [23]. DR_1025 precipitated at 2.4 M ammonium sulfate. In addition, 1 mM dithiothreitol (DTT) was included throughout the whole purification procedure of DR_1025 to protect its cysteine residue.

The vector harboring MM_0920 fused with the C-terminal 6-His tag was extracted from the storage strain of the PSI:Biology-Materials Repository (kanamycin resistant, grown in LB medium) using the standard protocol of the Gene Jet Plasmid Miniprep Kit. The plasmid was transformed into BL21(DE3) cells and was induced, harvested, and lysed as described above for MutT. The cell lysate was filtered through a 0.22-μm PES syringe membrane, and the filtrate was applied to a HisTrap HP column equilibrated with 50 mM Tris–HCl buffer (pH 7.6) containing 30 mM imidazole. MM_0920 was eluted with a 0–100% gradient of buffer containing 500 mM NaCl and 500 mM imidazole. MM_0920 fractions were combined and concentrated to less than 500 μl by Amicon filtration. The final preparation of MM_0920 was more than 95% pure as judged from SDS–PAGE. These fractions were combined, concentrated to approximately 100 μM, divided into 200-μl aliquots, and stored at –80 °C.

**Preparation of P$_1$ sensor**

PBP was expressed and purified as described by Brune and coworkers [21] with the following changes. The supernatant from the sucrose osmotic shock was adjusted to 10 mM Tris–HCl (pH 8.2) and loaded onto a HiPrep Q FF 16/10 anion exchange column equilibrated with the same buffer. The column was washed with 100 ml of 10 mM Tris–HCl (pH 8.2) and eluted with a 100 ml linear gradient from 0 to 250 mM NaCl in 10 mM Tris–HCl (pH 8.2). PBP eluted at approximately 100 mM NaCl. PBP-containing fractions were concentrated to approximately 2 mM by Amicon filtration. The final protein solution was stored in 0.5-ml aliquots at –80 °C. Protein concentration was determined from $A_{280}$, assuming a calculated extinction coefficient of 61,880 M$^{-1}$ cm$^{-1}$ as calculated from amino acid composition [24].
The P$_i$ sensor was prepared by labeling PBP with a fluorescent tag, MDCC, as described by Webb [25] with the following changes. The 20-mM MDCC solution in N,N-dimethylformamide (DMF) was prepared immediately prior to the labeling step to avoid precipitation that was observed when the solution had been stored for a long period at −20°C [25]. The PBP elution was diluted to 100 μM in 10 mM Tris–HCl (pH 8.2) and reacted with 150 μM MDCC (20 mM stock solution in DMF) in a scale of 5 to 50 ml. We found that the extent of labeling was incomplete when the reaction solution was less than 5 ml. The labeling step was performed at room temperature for 45 min in a foil-wrapped tube. The reactant was filtered through a 0.22-μm PES syringe membrane and desalted with a PD-10 desalting column. The filtered desalted sample was loaded on a Mono-Q anion exchange column, which was washed with 10 ml of 10 mM Tris–HCl (pH 8.2), followed by a 20-ml gradient of 0 to 10 mM NaCl. The bright yellow eluate contained the active P$_i$ sensor, whereas the inactive P$_i$ sensor remained in the column, as reported previously [21]. The protein was concentrated to approximately 1 mM by Amicon filtration and stored in 50-μl aliquots at −80°C. The characterization of the P$_i$ sensor was performed as described by Webb [25]. Typically, 5 μM P$_i$ sensor had a linear responsive range to phosphate up to 60% of the sensor concentration.

**Enzyme assays**

The P$_i$-sensor kinetic assay was performed on either a spectrofluorometer (reaction volume = 500 μl) or a microplate reader (reaction volume = 100 μl). The standard reaction mixture contained 10 mM Tris–HCl (pH 7.6), 1 mM MgCl$_2$, 5–10 μM PBP–MDCC (depending on the concentration of background phosphate introduced by the substrate impurities), 0.005 U/ml inorganic pyrophosphatase (PPase) where pyrophosphate was one of the products, or 1–4 U/ml APase where a nucleoside monophosphate was a Nudix enzyme product. Experiments were done in all cases to verify that sufficient coupling enzyme was present to ensure that the rates of reaction were linearly dependent on the concentration of the Nudix hydrolase. The concentrations of the Nudix enzymes and substrates were as follows: 1 nM MutT with 0.2–20 μM 8-oxo-dGTP, 5 nM MutT with 1–20 μM dCTP or 5-methyl-dCTP, 30 nM NudD with 1–6 μM GDP-mannose, and 100 nM DR$_{1025}$ with 5–50 μM 8-oxo-dGTP. The mixtures were incubated at 37°C and monitored continuously for 10 min (GENios: $\lambda_{ex} = 425$ nm, $\lambda_{em} = 465$ nm, gain 50, 100 cycles, 37°C; FluoroMax-4: $\lambda_{ex} = 430$ nm, slit width $= 1–2.5$ nm, $\lambda_{em} = 465$ nm, slit width $= 1–5$ nm, 37°C). Data for the linear portions of the initial velocities were fitted by regression (OriginPro 8.1; see below).

The Fiske–SubbaRow assay was performed in 400-μl reaction volumes that contained the same components as listed above minus the P$_i$ sensor. The reaction mixtures were incubated at 37°C for periods of 3–10 min, quenched with 5 mM ethylenediaminetetraacetic acid (EDTA), and stored on ice. The liberated inorganic phosphate was quantitated with the Malachite Green Phosphate Detection Kit (R&D Systems), which essentially employs a classical Fiske–SubbaRow assay with those yielded by the continuous assay, thereby enabling broader screening of potential Nudix substrates and more facile determination of kinetic parameters.

**Data processing**

The regression calculations described below were performed with OriginPro 8.1 (OriginLab). P$_i$-sensor standard curves were obtained by titrating inorganic phosphate under the same conditions as were used in the experimental reactions. Fluorescence readings were plotted against reaction time, and the linear regions of the plots (relative fluorescence units [RFU]/s) yielded initial velocities, $v_i$ (μM P$_i$/s). Plots of $v_i/[E]$ were fit to the Michaelis–Menten equation or its transformation by nonlinear regression to yield values of $k_{cat}/K_m$. For some reactions, nonlinear regression failed to converge or the standard errors for $k_{cat}$ and $K_m$ were comparable to the fitted values themselves. In these cases, only the $k_{cat}/K_m$ ratio was obtained by linear regression with a fixed intercept of zero. Two equivalents of inorganic phosphate are ultimately produced for those reactions that initially yield pyrophosphate in the presence of PPase. The values of $v_i$ were corrected for this factor. Weighted average values of the kinetic parameters are reported from multiple trials.

**Results**

**Description and validation of P$_i$-sensor assay**

Fig. 1 summarizes the range of Nudix-catalyzed hydrolytic reactions investigated here and diagrams the assay strategy. Nudix enzymes typically catalyze hydrolysis at a diphosphate bond yielding two products, at least one of which has a terminal phosphate group. A coupling enzyme, either PPase or APase, releases at least one equivalent of free phosphate, which is captured by the P$_i$ sensor, generating an increase in fluorescence (Fig. 1A). This is recorded continuously to measure initial velocities. No coupling enzyme is required where free phosphate is a direct product of the Nudix hydrolysis (Fig. 1B, part 1, lower branch).

Fig. 2 compares kinetic data recorded by the discontinuous classical Fiske–SubbaRow assay with those yielded by the continuous P$_i$-sensor assay for three Nudix hydrolases. The results from the two methods are in excellent agreement when the measurements were carried out with the same batch of enzyme. Our preparation of NudD, which catalyzes the hydrolysis of GDP-mannose, was approximately 50% less active than that reported in the literature [26]. The kinetic constants obtained here for the MutT-catalyzed hydrolysis of 8-oxo-dGTP are in agreement with reported values [14], although the experimental conditions differed slightly. These results establish that the continuous P$_i$-sensor assay can replace the discontinuous assay, thereby enabling broader screening of potential Nudix substrates and more facile determination of kinetic parameters.

**Substrate screening**

A screening library of 41 commercially available putative substrates was assembled from compounds that had been shown to be active with one or more Nudix hydrolases (J.R. Srouji, J.F. Kirsch, and S.E. Brenner, unpublished work). Fig. 3 shows results of the substrate screening of four Nudix hydrolases: MutT, DR$_{1025}$, NudD, and MM$_{0920}$. Each substrate was assayed twice: in the presence and absence of PPase or APase as appropriate.

8-Oxo-dGTP is the most reactive MutT substrate (Fig. 3), a result that is consistent with the literature [13]. It is followed by dGTP, which is approximately 10% as reactive as 8-oxo-dGTP, a result that is also in accord with the reported value [27].

Two novel results were uncovered by the screening process: (i) the rate of 5-methyl-dCTP hydrolysis is also approximately 10% of that exhibited by 8-oxo-dGTP, and (ii) the rate of phosphate
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liberation from 5 μM 8-oxo-dGTP in the absence of added PPase was approximately 10% of that observed in its presence, indicating that MutT catalyzes hydrolysis of 8-oxo-dGTP at both the α–β and β–γ positions.

The order of substrate reactivity of DR_1025 is 8-oxo-dGTP > p4G ≈ GTP > Gp4G > dGTP (Fig. 3). This confirms the previously reported DR_1025 activity against dGTP [17]. The reported ApA hydrolysis [17] was not detected in the screening but was confirmed in a detailed kinetics study (Table 1). The apparent preference for GTP over dGTP noted in the screening experiment was not confirmed in the subsequent analysis (Table 1). The experimentally determined kinetic parameters for the DR_1025-catalyzed hydrolysis of dGTP are essentially the same in the presence and absence of PPase, indicating exclusive cleavage at the β–γ bond (Table 1). NudD shows highest activity toward GDP-mannose and GDP-glucose (Fig. 3), a result that is consistent with the literature [11,23]. Substrate screening of MM_0920 failed to identify a candidate molecule. A structure similarity search using the DALI server [28] showed that MM_0920 has overall structural similarity with MutT (PDB: 3GRN and 3A6U, with a Z score of 16.2), so we tried carefully to measure activity toward 8-oxo-dGTP. The kcat/Km value is very low (Table 1) and is increased approximately 2-fold when the assay pH is raised to 9.0 (data not shown).

Novel substrate specificities and cleavage pattern of MutT

The substrate screening results revealed that MutT has significant activity toward 5-methyl-dCTP. An expanded investigation of this reaction showed that the v_i/E_i0 values in the presence of PPase are substantially greater than those recorded in its absence, indicating that MutT cleaves mainly the α–β pyrophosphate bond of 5-methyl-dCTP (Fig. 4A). The kcat/Km value for this reaction is approximately 0.01% for that of 8-oxo-dGTP hydrolysis (Table 1), and no rate saturation was observed up to 20 μM substrate (data not shown). MutT reacted only with 5-methyl-dCTP in an activity screening with dCTP, 5-Me-dCTP, 5-OH-dCTP, 5-MeOH-dCTP, 2'-O-Me-dCTP, and N4-Me-dCTP (data not shown). The rate of phosphate release from a 5-μM solution of 5-methyl-dCTP is linear for at least 10% of the reaction course (data not shown), a result that argues that the observed activity is not due to an impurity. The purity of the compound was judged to be approximately 95% by electrospray ionization mass spectrometry (ESI MS; data not shown).

MutT predominantly catalyzes the hydrolysis of 8-oxo-dGTP at the α–β pyrophosphate bond (see Ref. [18] and Table 1); however, the substrate screening experiments showed modest hydrolytic activity for dGTP and 8-oxo-dGTP in the absence of PPase (Table 1). The kcat/Km value for the β–γ hydrolysis is approximately 3% of the α–β cleavage reaction rate of 8-oxo-dGTP (Table 1).

Discussion

Comparison of P_i-sensor and Fiske–SubbaRow assays

The major advantage of the P_i-sensor assay introduced here for Nudix hydrolases is that it continuously monitors reaction progress and greatly reduces the time and materials required to determine initial velocities (v_i) relative to the Fiske–SubbaRow assay. It can be performed in 96-well plates and, thus, is suitable for simultaneous analysis of several enzymes and substrates and for inhibitor discovery. The accurate determination of kcat/Km values requires high-quality data for v_i at [S] < K_m. Such data are more readily obtained in a continuous versus discontinuous assay.

The P_i-sensor assay can distinguish 0.05 μM P_i (Fig. 4A) when monitored continuously. In comparison, the lower detection limit of the Fiske–SubbaRow assay is 1 μM [15,29]. However, the high
sensitivity of the \(P_i\)-sensor assay makes it susceptible to traces of inorganic phosphate present in many of the test molecules. A typical assay solution might contain 5 \(\mu\)M \(P_i\) sensor and 100 \(\mu\)M substrate. Thus, even a small percentage of contamination with phosphate would deplete the \(P_i\) sensor. This issue might be remedied by adding, for example, unlabeled PBP to the substrate, followed by separation of the PBP–\(P_i\) complex from the substrate prior to assay. Alternatively, the concentration of the \(P_i\) sensor can be increased and the fluorescence change may be monitored at a wavelength that is off the emission maximum or by reducing the slit widths. These measures would also alleviate inner filter issues should they be problematic. Fortunately the objective of most screening assays is the determination of \(k_{\text{cat}}/K_m\) values, which is readily accomplished in low substrate concentrations where phosphate contamination is minimal.

**Range of applicability of the assay**

Although the \(P_i\) sensor assay can be employed to monitor the vast majority of known Nudix substrates, it is not universally applicable. For example, the mouse protein RP2p has been shown to be a Nudix hydrolase with acyl-CoA (coenzyme A) diphosphatase activity [30]. APase would, however, likely cleave the unblocked 3' phosphate of CoA (and its derivatives), releasing free phosphate and, thus, compromising the \(P_i\)-sensor assay. Some cleavage patterns of nucleotide tetra- or pentaphosphate might also escape detection. The protein IalA from *Bartonella bacilliformis* has been shown to hydrolyze adenosine pentaphosphate (\(p\text{A}\)) to ATP and pyrophosphate [31]. This reaction falls into B.1 of Fig. 1, which uses PPase as the coupling enzyme. However the \(P_i\)-sensor assay would fail to detect cleavage at the \(\beta\)-\(\gamma\) pyrophosphate bond, giving ADP and inorganic triphosphate as products. These limitations, of course, also pertain for the discontinuous assay.

Fig. 2. Comparison of \(v_i/[E_0] \text{ (s}^{-1}\text{)}\) exhibited by Nudix hydrolases determined by discontinuous assays as measured here and as reported in the literature versus the continuous \(P_i\)-sensor assay. Experimental data are shown as dots or triangles, and fitted data are shown as lines. The values of the kinetic parameters are collected in Table 1. (A) Comparison of Fiske–SubbaRow assay (F–S assay) \(pH 8.5, 37^\circ C, \text{blue triangles and solid blue line}\) and calculated data from [26] \(pH 8.5, 37^\circ C, \text{green solid line}\) for NudD-catalyzed hydrolysis of GDP-mannose. (B) Comparison of \(P_i\)-sensor assay \(pH 8.5, 37^\circ C, \text{red dots and solid red line}\), calculated data from Ref. [26] \(pH 8.5, 37^\circ C, \text{green solid line}\), and extrapolated data fitting from the Fiske–SubbaRow assay of panel A to low substrate concentration (blue dashed lines) for NudD-catalyzed hydrolysis of GDP-mannose. (C) Comparison of \(P_i\)-sensor assay \(pH 7.6, 37^\circ C, \text{red dots and solid red line}\), Fiske–SubbaRow assay \(pH 7.6, 37^\circ C, \text{blue triangles and solid blue line}\), and calculated data from Ref. [14] \(pH 8.0, 30^\circ C, \text{solid green line}\) for MutT-catalyzed hydrolysis of 8-oxo-dCTP. (D) Comparison of \(P_i\)-sensor assay \(pH 7.6, 37^\circ C, \text{red dots and solid red line}\) and Fiske–SubbaRow assay \(pH 7.6, 37^\circ C, \text{blue triangles and solid blue line}\) for DR_1025-catalyzed hydrolysis of 8-oxo-dCTP. Coupling enzymes, where used, are listed in Table 1.

**5-Methyl-dCTP hydrolase activity of MutT and its possible physiological role**

5-Methylcytosine in DNA is a source of mutation [32]. Interestingly, MutT has low but distinguishable hydrolytic activity toward 5-methyl-dCTP in the presence of PPase. The hydrolysis is undetectable when the substrate is dCTP or when PPase is omitted. These observations suggest that MutT cleaves at the \(\beta\)-\(\gamma\) phosphate bond of 5-methyl-dCTP. Another *E. coli* enzyme, NudG, when assayed at \(pH 9.1, 37^\circ C\), has a reported \(k_{\text{cat}}/K_m\) value of \(3 \times 10^7 \text{M}^{-1} \text{s}^{-1}\) for 5-methyl-dCTP [33]. This figure is approximately 100-fold higher than that found here for the MutT-catalyzed hydrolysis rate constant. The relative contribution to the in vivo hydrolysis rate of this aberrant NTP is, thus, a function of enzyme abundance. The possibility that our MutT sample is contaminated by approximately 0.5% NudG, which would generate an apparent \(k_{\text{cat}}/K_m\) value similar to that reported in Table 1 for 5-methyl-dCTP, was excluded, because our mass spectroscopy result of MutT indicates that NudG, if any, was presented at less than 0.1% (data not shown).

**\(\beta\)-\(\gamma\) Cleavage in MutT-catalyzed hydrolysis of 8-oxo-dGTP**

The predominant cleavage site in the MutT-catalyzed hydrolysis of 8-oxo-dGTP is at the reported \(\alpha\)-\(\beta\) position [18]. Such activity requires PPase for detection by the \(P_i\)-sensor assay. A small rate of cleavage over the MutT minus control was recorded in the absence of PPase, indicating that \(\beta\)-\(\gamma\) hydrolysis occurs at approximately 3%
Table 1

Kinetic parameters for Nudix hydrolase-catalyzed reactions

| Nudix hydrolase | Substrate | Assay       | pH | Phosphatase | \(k_{cat} \) (s\(^{-1}\)) | \(K_m \) (\(\mu\)M) | \(k_{cat}/K_m \) (M\(^{-1}\) s\(^{-1}\)) |
|-----------------|-----------|-------------|----|-------------|-----------------|-----------------|-----------------|
| MutT            | 8-Oxo-dGTP| F–S assay   | 7.6| PPase       | 6.9 ± 0.3       | 0.53 ± 0.09     | (9.3 ± 1.1) x 10^6 |
|                 |           | Pi sensor   | 7.6| PPase       | 5.5 ± 0.3       | 0.26^6          | 2.1 x 10^7       |
|                 |           | Pi sensor   | 7.6| None        | 0.89 ± 0.04     | 3.0 ± 0.4       | 3.0 ± 0.2        |
|                 |           | 5-Methyl-dCTP| PPase | 7.6     | 11.2 ± 0.9     | 0.9 ± 0.2       | (1.3 ± 0.2) x 10^6 |
|                 |           | Pi sensor   | 7.6| PPase       | 2.8 ± 0.3       | ND              | (2.8 ± 0.3) x 10^6 |
|                 |           | ND          |    | ND          | 240 ± 200       |                |                 |
| NudD            | GDP-mannose| P\(_i\) sensor | 8.5 | ND          | ND              | 1.5 ± 0.1       |                |
|                 |           | F–S assay   | 8.5| APase       | 0.35 ± 0.02     | 220 ± 60        | (1.6 ± 0.3) x 10^6 |
|                 |           | P\(_i\) sensor | 8.5 | APase | 0.63^b        | 250^b           | 2.5 ± 0^b       |
|                 |           | ND          |    | ND          | 1.5 ± 0.1       |                |                 |
|                 |           | ND          |    | ND          | 280 ± 50        |                |                 |
|                 |           | ND          |    | ND          | 57 ± 6          |                |                 |
|                 |           | ND          |    | ND          | 110 ± 6         |                |                 |
| DR_1025         | 8-Oxo-dGTP| P\(_i\) sensor | 7.6 | None | 0.18 ± 0.01   | 26 ± 1          | (4.4 ± 0.1) x 10^6 |
|                 |           | F–S assay   | 7.6| None        | 0.29 ± 0.04     | 28 ± 7          | (6.8 ± 0.8) x 10^6 |
|                 |           | Pi sensor   | 7.6| None        | 0.13 ± 0.01     | 22 ± 5          | (5.9 ± 0.8) x 10^6 |
|                 |           | dGTP        |    | ND          | ND              | 860 ± 20        |                |
|                 |           | Pi sensor   | 7.6| ND          | ND              | 950 ± 40        |                |
|                 |           | Pi sensor   | 7.6| ND          | ND              | 820 ± 50        |                |
|                 |           | Pi sensor   | 7.6| ND          | ND              | 57 ± 6          |                |
|                 |           | Pi sensor   | 7.6| ND          | ND              | 110 ± 6         |                |
| MM_0920         | 8-Oxo-dATP| Pi sensor   | 7.6| P\(_i\) sensor | 9.6 ± (0.8) x 10^-4 | 12 ± 3          | 78 ± 12         |
|                 |           | Pi sensor   | 7.6| P\(_i\) sensor | (1.9 ± 0.2) x 10^-3 | 13 ± 3          | 149 ± 25        |

Note. T = 37° C except for data from Ref. [14], where T = 30° C. F–S assay, Fiske–SubbaRow assay. ND, not determined because \(k_{cat}/K_m\) was obtained from linear regression fitting.

\(^a\) Calculated from data in Table 2 of Ref. [14].

\(^b\) Calculated from data in Fig. 2A of Ref. [26].

Fig. 4. Novel MutT catalytic activities. The reactions were monitored by P\(_i\) sensor at pH 7.6 and 37° C. (A) Hydrolysis of 5 mM 5-methyl-dCTP (circles) and dCTP (triangles). Initial velocities, \(v_0\) (nM P\(_i\)/s), are plotted against MutT concentration (nM) in the presence (solid circles and triangles) or absence (empty circles and triangles) of PPase. The background (no MutT) activities of 0.23 nM P\(_i\)/s (solid circles and triangles) and 0.36 nM P\(_i\)/s (empty circles) were subtracted from each data point. (B) Hydrolysis of 8-oxo-dCTP either in the presence (solid circles and solid line) or in the absence (empty circles and dashed line) of PPase.

of the \(\alpha-\beta\) site cleavage. The significance of this observation is not known. Minor alternate cleavage patterns would likely escape detection in most assays of nucleoside triphosphate hydrolase activity, so it could be a fairly common phenomenon. Alternatively, the evolutionarily acquired promiscuity of the substrate specificity of the Nudix enzyme family may restrict, to some extent, the precision of placement of the catalytic residues near the scissile bond.

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