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# Characterisation of multiple substrate-specific (d)ITP/(d)XTPase and modelling of deaminated purine nucleotide metabolism

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**Accumulation of modified nucleotides is defective to various cellular processes, especially those involving DNA and RNA. To be viable, organisms possess a number of (deoxy)nucleotide phosphohydrolases, which hydrolyze these nucleotides removing them from the active NTP and dNTP pools. Deamination of purine bases can result in accumulation of such nucleotides as ITP, dITP, XTP and dXTP. *E. coli* RdgB has been characterised as a deoxyribonucleoside triphosphate pyrophosphohydrolase that can act on these nucleotides. *S. cerevisiae* homologue encoded by YJR069C was purified and its (d)NTPase activity was assayed using fifteen nucleotide substrates. ITP, dITP, and XTP were identified as major substrates and kinetic parameters measured. Inhibition by ATP, dATP and GTP were established. On the basis of experimental and published data, modelling and simulation of ITP, dITP, XTP and dXTP metabolism was performed. (d)ITP/(d)XTPase is a new example of enzyme with multiple substrate-specificity demonstrating that multispecificity is not a rare phenomenon [BMB reports 2012; 45(4): 259-264]**

## INTRODUCTION

Under physiological conditions, nucleotides can undergo chemical modification of the bases (1). Deamination of purine base is a major chemical modification that occurs to purine nucleotides in cells (2). The deamination of adenine at C-6 or guanine at C-2 generates hypoxanthine or xanthine, respectively (Fig. 1).

Deaminated nucleotides may either accumulate in nucleotide pools or become incorporated into DNA and RNA, leading to alteration of nucleic acid structure and genetic information. The alteration of nucleic acid structure is associated with mutagenesis and carcinogenesis (3), degenerative disorders (4), senescence and ageing (5), and cell death (6). In addition to defective DNA metabolism, other biological functions of canonical nucleotides may be adversely affected by deaminated nucleotides (7).

It is therefore important to understand how deamination products are eliminated from cells. Living organisms are equipped with specific enzymes that hydrolyse these non-canonical nucleoside triphosphates to their corresponding nucleoside monophosphates and pyrophosphate, to avoid their deleterious effects (8). RdgB (E.C. 3.6.1.19), has been characterised as one of such enzymes in *E. coli* (9,10). *S. cerevisiae* homologue of RdgB is encoded by YJR069C and it has been shown to possess

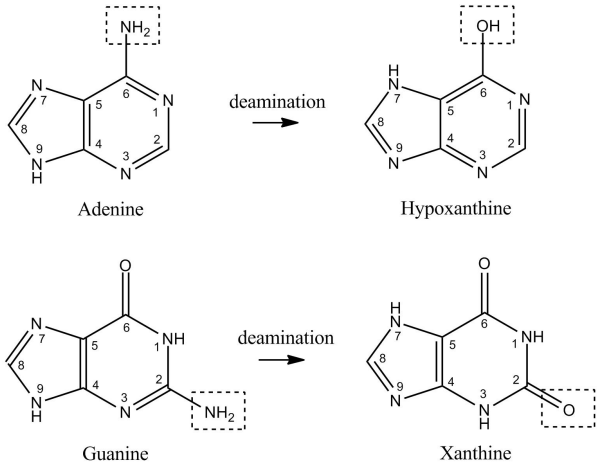


**Fig. 1.** The *deamination* of adenine and guanine generates hypoxanthine and xanthine, respectively. Chemical structures of purine bases are shown.

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deoxyribonucleoside triphosphate pyrophosphohydrolase activity (E.C. 3.6.1.19) on deoxy-*N*-6-hydroxylaminopurine triphosphate and deoxyinosine triphosphate (9). Due to ability to control 6-*N*-hydroxylaminopurine sensitivity, it has been named HAM1 (11).

In the present study, we characterise the YJR069C-encoded enzyme of *S. cerevisiae*. Fifteen different nucleotide substrates were tested for phosphatase activity. ITP, XTP and dITP were identified as major substrates and their kinetic parameters were determined. The effects of ATP, dATP and GTP on pyrophosphatase activity towards substrates were studied. Using data from this work, and cell nucleotide concentrations (12), we built a simple mathematical model for removal of ITP, dITP, XTP and dXTP in purine nucleotide metabolism.

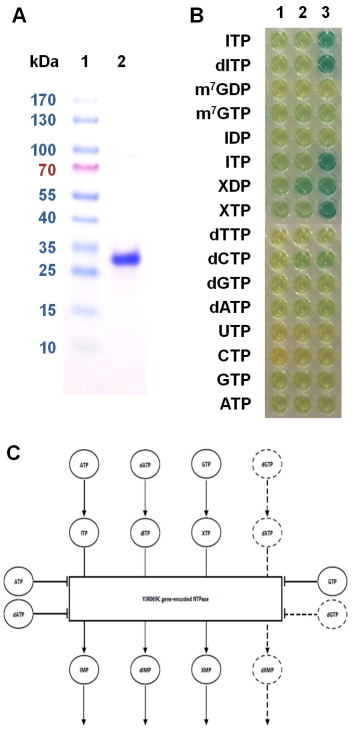


**Fig. 2.** (A) The purification of (d)ITP/(d)XTPase was confirmed by SDS-PAGE electrophoresis. Protein preparation (lane 2) was compared to pre-stained protein ladder (lane 1, RunBlue™ obtained from Expedeon Protein Solutions) and was found to well-correspond to molecular weight of 26.9 kDa. Enzyme concentration determined by BCA assay (Sigma) was $13.1 \times 10^{-3} \pm 0.024 \times 10^{-3}$ mM. (B) Assays of pyrophosphatase activity on fifteen different di- and tri-phospho-nucleotides in 96-well plate. Release of inorganic phosphate by coupled (d)ITP/(d)XTPase and inorganic pyrophosphatase activities was determined by using PiColorLock™ Gold reagent resulting in a purple colour. Abbreviations of nucleotides used in the reactions are provided on the left side of panel. Reactions contain either (d)ITP/(d)XTPase only (lane 1), inorganic pyrophosphatase only (lane 2) or both (d)ITP/(d)XTPase and inorganic pyrophosphatase (lane 3). (C) Three-step kinetic model of (d)ITP/(d)XTPase hydrolysis of ITP, dITP, XTP and dXTP.

## RESULTS

Deaminated nucleotides have several degenerative effects on cell processes involving nucleic acids, which may lead to mutagenesis resulting in carcinogenesis, degenerative disorders, senescence, cell death, defective DNA metabolism and other adverse affects on various biological functions (3-5, 7). Cell has evolved mechanisms and relevant enzymatic activities that ensure removal of deaminated nucleotides. The removal of deaminated purines has been little studied so far.

Previously, it has been suggested that the ITPA (inosine triphosphate pyrophosphatase, (E.C. 3.6.1.19) protein can hydrolyse these type of nucleosides and its homologues were at some extent characterised in *E. coli* (9, 10). In order to characterise the *S. cerevisiae* counterpart, a homologues enzyme encoded by YJR069C (HAM1) and shown to possess deoxy-*N*-6-hydroxylaminopurine triphosphate and deoxyinosine triphosphate pyrophosphohydrolases activities (9) was investigated. A corresponding protein was expressed and purified as described in Materials and Methods; its purity was checked by SDS-PAGE electrophoresis (Fig. 2A) and enzymatically by testing for relevant background activity. Fifteen substrates were assayed for nucleotide pyrophosphatase activity (Fig. 2B). The enzyme was found to hydrolyse efficiently ITP, dITP and XTP

**Table 1.** Experimentally-determined (d)ITP/(d)XTPase activities and kinetic parameters for different substrates at pH 7.5 and 30°C, with 6.55 nM enzyme, determined using an inorganic pyrophosphatase coupled assay

| Substrate | Activity (μmol/min mg protein) |
|---|---|
| dITP | 59.096 ± 5.750 |
| ITP | 39.262 ± 9.031 |
| XTP | 36.155 ± 2.352 |
| dATP | 0.488 ± 1.350 |
| dGTP | 0.235 ± 0.166 |
| dCTP | 0.073 ± 0.026 |
| dTTP | 0.055 ± 0.087 |
| GTP | 0.023 ± 0.163 |
| $m^7$GTP | <0.003 |
| IDP | <0.003 |
| XDP | <0.003 |
| $m^7$GDP | <0.003 |
| UTP | <0.003 |
| CTP | <0.003 |
| ATP | <0.003 |

| Substrate | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($M^{-1}s^{-1}$) |
|---|---|---|---|
| ITP | 2.38 ± 0.41 | 0.992 ± 0.056 | $4.17 \times 10^5$ |
| dITP | 3.06 ± 0.60 | 1.288 ± 0.016 | $4.21 \times 10^5$ |
| XTP | 3.70 ± 0.68 | 1.000 ± 0.024 | $2.70 \times 10^5$ |
| GTP | ~$3 \times 10^3$ ($K_i$) | - | - |
| dATP | ~$1 \times 10^3$ ($K_i$) | - | - |
| ATP | >$1 \times 10^4$ ($K_i$) | - | - |

(Table 1) releasing pyrophosphate and IMP, dIMP and XMP, respectively (data not shown). Besides, statistically reliable pyrophosphatase activities towards dATP, dGTP, dCTP, dTTP and GTP were also detected. There was no significant activity towards ATP, CTP, UTP, $m^7$GTP, IDP, XDP and $m^7$GDP. Due to the high pyrophosphatase activities towards ITP, dITP, XTP, we hypothesise that dXTP is also a good substrate for this enzyme. YJR069C-encoding enzyme has shown around two magnitude and higher differences in activity between "key" substrates (dITP, ITP and XTP) and dGTP or dATP, which is somewhat similar to what was reported previously in (9, 10). Since YJR069C-encoding protein was found to have pyrophosphatase activity on ITP, dITP and XTP, and same activity was proposed for dXTP, enzyme is annotated as (d)ITP/(d)XTPase.

The optimal pH for the inorganic pyrophosphatase coupled assay was found to be around 7.5. It should be noted that optimal pHs in alkaline range 7.5 to 9.5 were reported for (d)ITPases in other organisms (9, 10, 13). Assays for determination of pyrophosphatase $K_m$ and $k_{cat}$ were performed on ITP, dITP and XTP (Table 1). All three substrates showed high affinity ($K_m \sim 3$ μM) and moderate activity ($k_{cat} \sim 1$ $s^{-1}$). Michaelis-Menten constants of ITP, dITP, and XTP differ significantly less than has been observed with RdgB (10). However, the $K_m$ values follow the same order in both organisms *S. cerevisiae* and *E. coli*: ITP < dITP < XTP.

The inhibitive characteristics of ATP, dATP, GTP and IMP were tested. GTP, dATP and ATP were found to inhibit (d)NTPase activity. However their $K_i$ of 1 mM and less shows them to have three orders of magnitude less affinity for the enzyme than its natural substrates (Table 1).

These experimentally-determined parameter values were combined in a simple mathematical model as depicted in Fig. 2C. The four pathways cross-talk and interact through competition of ITP, dITP, XTP and dXTP for NTPase hydrolysis. Sensitivity analysis can quantify the level of cross-talk, through revealing how steady state concentrations and fluxes change when parameters are perturbed.

**Table 2.** Scaled sensitivity coefficients for ITP concentration

| Parameter | Sensitivity |
|---|---|
| (NTPase) | −1.03 |
| (ATP) | 1.19 |
| (dATP) | 0.0966 |
| (GTP) | 0.184 |
| (dGTP) | 0.0179 |
| $K_{m,ITP}$ | 1.00 |
| $K_{i,ATP}$ | −0.164 |
| $K_{i,dATP}$ | −0.0954 |
| $K_{i,GTP}$ | −0.177 |
| $K_{i,dGTP}$ | −0.0172 |
| $k$ (ATP deamination) | 1.02 |
| $k_{cat}$ (NTPase:ITP) | −1.02 |
| $k$ (GTP deamination) | 0.00693 |
| $k_{cat}$ (NTPase:XTP) | −0.00692 |

For example, in order to reduce the ITP concentration, a cell has a number of choices. It can naturally achieve this through increase (d)NTPase concentration; this is represented through a scaled sensitivity coefficient of −1.03 (see Table 2). As the coefficient is negative, an increase in [NTPase] leads to a decrease in [ITP]. The specific value of the sensitivity coefficient means that a 1% increase in [NTPase] leads to a 1.03% decrease in [ITP].

[ITP] can also be reduced through other expected methods: decreasing [ATP], decreasing the ATP to ITP deamination rate, or increasing the $k_{cat}$ for ITP hydrolysis by (d)NTPase. However, the cellular objective of reducing [ITP] can also be achieved through unexpected means, such as reducing cellular [GTP], or increasing the $k_{cat}$ for XTP hydrolysis by NTPase. Such nonlinear interactions can only elucidated from complex biological problems such as these, through application of mathematical modelling.

## DISCUSSION

We report characterisation of (d)ITP/(d)XTPase in *S. cerevisiae*, which in a line with ADP-ribose-specific hydrolase Ysa1 (14), deoxyuridine triphosphate diphosphatase Dut1 (15, 16), extracellular nucleotide pyrophosphatases/phosphodiesterases Npp1/Npp2 (17), scavenger mRNA decapping hydrolase Dcs1 (18) or heterodimeric enzyme form Dcs1/Dcs2 (19), phosphoribosyl-ATP pyrophosphatase His4 (20) and others (21) form more complete knowledge on nucleotide-related pyrophosphatases. Here, a first model of deaminated purine nucleotide metabolism is implemented and MCA (metabolic control analysis) performed. This model could be further strengthened through acquisition of data presenting concentrations of nucleoside triphosphates in *S. cerevisiae* under various conditions, including findings on new enzymatic activities that contribute to removal of deaminated nucleotides (e.g. Dut1 (16)) and a more detailed understanding of the mechanism underlying the process of deamination. Finally, (d)ITP/(d)XTPase is a new example of enzyme with multiple substrate-specificity demonstrating that multispecificity is not a rare phenomenon in the protein world (22).

## MATERIALS AND METHODS

### Yeast strains

Yeast ORF clone of *Saccharomyces cereviaise* strain Y258 (*MATa pep*4-3, *his*4-580, *ura*3-53, *leu*2-3,112) (23) containing the expression plasmid BG1805 with YJR069C ORF was purchased from Open Biosystems (YSC3869-9517320).

### Nucleotides

All nucleoside diphosphates and nucleoside triphosphates used in assays were obtained from Jena Biosciences.

## Chemicals

IgG sepharose, glutathione sepharose 4B and GST-3C protease (GE Heathcare), all amino acids, rafinose and galactose (Sigma), Pi ColorLock™ Gold reagent with stabilizer and 0.1 mM phosphate stock (Innova Biosciences).

## Expression and purification of (d)ITP/(d)XTPase

Proteins were expressed and purified according to (24) with some modifications. Briefly, yeast ORF strain were first grown overnight in 5 ml synthetic medium minus uracil with 2% raffinose (for recipe see (25)) at 30°C, then overnight culture was diluted into 50 ml same and grown for 24 hours at 30°C, followed by dilution into 600 ml of same medium and overnight growth to $OD_{600}$ 0.5-0.8. Expression was induced by addition of 30 ml 40% galactose, followed by growth for 4-5 hours. Cells were harvested by centrifugation, washed two times with 50 ml ice-cold water, centrifuged and stored in aliquots at −80°C.

Cells from 600 ml culture, resuspended in 1 ml LB buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$) with 2 μg leupeptin and 2 μg pepstatin, were lysed at 4°C by beating 5 times for 30 sec each, with 1 min interval on ice between each round with 0.8 mm glass beads in MiniBead-Beater-16 (Stratech Scientific). Resulting crude extract was centrifuged for 10 min at maximum speed in pre-cooled minicentrifuge.

To affinity purify proteins on IgG sepharose, crude extract was diluted to 10 ml by adding LB buffer and NP40 (up to concentration of 0.1%), and incubated with 100 μl of IgG beads (GE Healthcare) at 4°C for 2 hrs. Unbound proteins were removed by 5 subsequent washes of the IgG beads with 10 ml LB buffer each time. To elute (d)ITP/(d)XTPase, IgG beads resuspended in 100 μl of 3C protease cleavage buffer: 10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 0.1% NP40) were incubated with 0.5-2 units of GST-3C protease (GE Healthcare) with vigorous mixing on an orbital shaker at 4°C for 16 hours. Resulting protein sample was cleared from beads by passage through a SPIN-X filter centrifuge tube, 0.22 μm cellulose acetate, (Corning, Inc) and after extensive dialysis stored in 50 mM HEPES, pH 7.5, 150 mM KCl, 2 mM $MgCl_2$) at −80°C. Protein purity and concentration was determined by Commassie staining and quantitative comparison with the BSA protein standards.

## Enzymatic assay

Pyrophosphatase activity was assayed by measuring the inorganic phosphate (Pi) release in a coupled reaction with inorganic pyrophosphatase (26) at pH 7.5 and 30°C. 100 μl reactions were performed in 96-well plate and inorganic phosphate was measured by adding 25 μl PiColorLock™ Gold reagent at defined time points as described bellow. Then, after 5 min 10 μl of stabilizer was added and resulting purple colour was allowed to develop for another two hours. Absorbance was measured at 635 nm using FluoroStar Omega plate-reader (BMG Labtech). Two types of assay, one without inorganic pyrophosphatase and other without pyrophosphatase ((d)ITP/(d)XTPase) were used as negative controls. A standard calibration curve for inorganic phosphate (0-50 μM) was used to calculate release of Pi.

Enzyme specificity to different substrates was assayed in reaction mixture A (50 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 0.5 unit/ml inorganic pyrophosphatase, 52.4 nM (d)NTPase) with 50 μM substrate (ITP, dITP, $m^7$GTP, $m^7$GDP, IDP, XDP, XTP, dTTP, dCTP, dGTP, dATP, UTP, GTP, CTP, or ATP). Reactions were stopped after 0, 30 and 90 minutes of incubation. All assays were performed three times in duplicates.

Assays to determine effects of ATP, dATP and GTP on NTPase activity on substrates were performed for 80 minutes in reaction mixture A containing either ATP (0.8-3,000 μM) and 3μM ITP; dATP (0.8-9,000 μM) and 3.1 μM dITP; or GTP (0.8-4,500 μM) and 3.8 μM XTP. All assays were performed in duplicates.

Assays to determine kinetic parameters of (d)ITP/(d)XTPase activity on ITP, dITP and XTP were performed in reaction mixture A containing varying substrate concentrations of 2-50 μM. 100 μl reactions were stopped at intervals up to 80 minutes. All assays were performed in triplicates.

## Calculation of kinetic parameters

$K_m$ and $k_{cat}$ for (d)ITP/(d)XTPase with respect to ITP, dITP and XTP were determined using nonlinear regression using GraphPad. $K_i$ of GTP, dATP and ATP were determined using the Cheng and Prusoff (27) equation.

## Modelling

The model structure is presented in Fig. 2C. Deamination reactions (e.g. ATP→ITP) and use reactions (e.g. IMP→) are modelled using irreversible mass-action kinetics. (d)NTPase reactions (e.g ITP→IMP) are modelled using competitively inhibited Michaelis-Menten-type kinetics:

$$v = \frac{[NTPase]kcat\frac{[S]}{KS}}{1+\frac{[P]}{KmITP}+\frac{[dITP]}{KmdITP}+\frac{[XTP]}{KmXTP}+\frac{[dXTP]}{KmdXTP}+\frac{[GTP]}{KiGTP}+\frac{[dGTP]}{KidGTP}}$$

where S denotes substrate. Literature values were used for cell nucleotide concentrations (12) and protein copy number (3,490 molecules/cell) were taken from the literature (28), and a cell volume of $4 \times 10^{-14}$ l was assumed (29). The first-order rate constants for the deamination and use reactions were estimated as $10^{-6}$ $s^{-1}$ and $10^{-5}$ $s^{-1}$, respectively, from the known concentrations of ATP, ITP and IMP (12). While the characteristics of the dGTP branch were not determined experimentally, they were assumed to be similar to the GTP branch. The model is available in SBML (30) format; model analysis is performed using COPASI (31).

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