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Polyphosphatase PPN1 of *Saccharomyces cerevisiae* Is a Deoxyadenosine Triphosphate Phosphohydrolase

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Abstract

The *Saccharomyces cerevisiae* polyphosphatase PPN1 (uniprot/Q04119) degrades inorganic polyphosphates both by cleaving Pi from the chain end and by fragmenting long-chain polymers into shorter ones. In this study, we have found a new activity of this protein: it releases phosphate from dATP. The dATP phosphohydrolase activity of pure PPN1 was ~7-fold lower compared to the exopolyphosphatase activity. This activity was strongly stimulated by Co^{2+} ions, as well as by ammonium ions, and inhibited by heparin and pyrophosphate similar to the exopolyphosphatase activity of PPN1. The Km value for dATP was 0.88 ± 0.14 mM. The dATP phosphohydrolase activity in the cells of PPN1-overexpressing yeast strain was several-fold higher than that in the parent strain. The other exopolyphosphatase of *S. cerevisiae*, PPX1, did not split Pi from dATP.

Keywords

Polyphosphate, Yeast, PPN1, Polyphosphatase, Deoxyadenosine Triphosphate Phosphohydrolase, dATP

1. Introduction

Inorganic polyphosphate (PolyP) is multifunctional biopolymer performing many cellular functions in all living cells, from prokaryotic to human [1]-[8]. The ability of PolyP metabolizing enzymes to catalyze the conversion of other substrates is one of the causes of the involvement of these proteins in regulatory pathways. For example, the gppA exopolyphosphatase splits Pi from guanosine 5'-triphosphate, 3'-diphosphate and guanosine 5'-diphosphate, 3'-diphosphate, bacterial second messengers [9]. Some bac-

terial exopolyphosphatases display NTPase activities [10]. The enzymes belonging to the polyphosphate kinase 2 subfamily catalyze nucleoside monophosphate phosphorylation [11]. The exopolyphosphatase of *Pseudomonas aeruginosa* is a polyphosphate: ADP phosphotransferase [12]. The *Saccharomyces cerevisiae* protein DDP1 (a diadenosine and diphosphoinositol polyphosphate phosphohydrolase) possess an endopolyphosphatase activity [13]. The exopolyphosphatase PPX1 of *S. cerevisiae* hydrolyzes adenosine-tetraphosphate phosphohydrolase and guanosine-tetraphosphate phosphorhydrolase activities [14]. The polyphosphatase PPN1 (http://www.uniprot.org/uniprot/Q04119) of *S. cerevisiae* degrades PolyP both by cleaving Pi from the chain end and by fragmenting long-chain polymers into shorter ones [15] [16]. Pi release was predominant in the presence of Co^{2+}, while the fragmentation of high-molecular PolyP was predominant in the presence of Mg^{2+} [16]. No other substrates of this enzyme are known; the pyrophosphatase activity of PPN1 was extremely low [17]. We have attempted to use the pure recombinant PPN1 [18] to reveal PolyP in the PCR reaction products with the high level of pyrophosphate [19]. In these experiments, the treatment of dNTP mixture with PPN1 resulted in Pi release; *i.e.*, PPN1 catalyzed the reaction:

$$dNTP \rightarrow dNDP + Pi$$
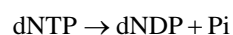
This study was aimed at characterizing the dNTP phosphohydrolase activity of PPN1 with dATP as a substrate.

2. Materials and Methods

2.1. Strain Growth

The ΔPPN1 mutant strain CRN of *S. cerevisiae* was obtained from N. Rao and A. Kornberg [15]. The strain CRN/pMB1_PPN1 Sc of *S. cerevisiae* overexpressing the polyphosphatase PPN1 was designed by Eldarov and co-authors earlier [20]. Yeast strains were grown in a synthetic minimal YNB medium containing (per 1 l) 1.7 g of bacto yeast nitrogen bases (Difco, Detroit, USA), 20 g of glucose, by 20 mg of L-tryptophane, L-histidine, L-methionine and adenine, and 60 mg of L-leucine. Uracyl (20 mg/l) was added for cultivation of the strain CRN. The cells were cultivated up to the stationary growth stage under shaking (145 rpm/h) at 29°C.

2.2. PPN1 Purification

The cellular extracts were obtained as described [18]. The cellular extract of the strain CRN/pMB1_PPN1 Sc was used for purification of polyphosphatase PPN1 by a combination of the methods designed earlier for obtaining pure PPN1 [18]. The cellular extract was supplemented by ammonium sulfate up to 50% saturation and incubated for 1 h, followed by centrifugation at 12,000 g for 20 min. The supernatant was absorbed on the Butyl-Toyopearl 650 M equilibrated with 50 mM Tris-HCl, pH 7.2, with 50% ammonium sulfate. In 45 min, the resin was precipitated at 4000 g for 3 min and washed three times with 50 mM Tris-HCl, pH 7.2, containing 50% ammonium sulfate. For polyphosphatase elution, the resin was washed four times with 50 mM Tris-HCl, pH 7.2,

containing 25% ammonium sulfate. Triton X-100 (0.05%) was added to the preparation. After ultrafiltration through an YM-10 membrane, the solution was applied to a DEAE-Toyopearl 650 M column (1.5 × 7.5 cm) equilibrated with 25 mM Tris-HCl, pH 7.2, with 0.1% Triton X-100. The column was washed with 50 ml of the same buffer and then with 50 ml of 0.1 M KCl in the same buffer. The polyphosphatase was eluted at a flow rate of 24 ml/h with an increasing KCl concentration (0.1 - 0.8 M) in the same buffer. The gradient volume was 200 ml. The fractions with the polyphosphatase activity were pooled, concentrated by ultrafiltration, and incubated with heparin-agarose for 2 h. The resin was pre-equilibrated with 25 mM Tris-HCl, pH 7.2, with 0.1% Triton X-100. The heparin-agarose washed with 10 ml of the buffer and then 5-fold with 5 ml of 0.5 M KCl in the same buffer, using centrifugation at 1500 g for 5 min. Then the resin was washed twice with 5 ml of 0.7 M KCl in the buffer; the exopolyphosphatase was desorbed with 5 ml (4-fold) of 1 M KCl in the same buffer. The preparation had a specific activity with polyP 208 - 2000 E/mg of protein. The protein was assayed with Pierce Bradford reagent after precipitation with trichloroacetic acid. The preparation was stored at −20˚C.

2.3. Enzyme Activities Assay

The enzyme activities were assayed at 30˚ in 0.1 ml of 50 mM Tris-HCl, pH 7.2, containing 0.1 mM $CoSO_4$ and 200 mM NH_4Cl. The concentrations of $MgSO_4$, $MnSO_4$, $ZnSO_4$, and other additives are indicated in the legends to the tables and figures. The amount of the enzyme releasing 1 nmole of phosphate (Pi) per 1 min was taken as a unit of enzyme activity (mU). Inorganic polyphosphate with an average chain length of 208 phosphate residues ($polyP_{208}$) (Monsanto, USA) was used as a substrate of exopolyphosphatase at the concentration of 2.5 mM (the concentration was estimated by phosphorus). $PolyP_{208}$ was purified from pyrophosphate and orthophosphate as described [21]. The dATP (GE Healthcare Bio-Sciences Corp, Lithuania), ATP, ADP and pyrophosphate (Sigma) were used at the concentration of 2 mM. Heparin was from Spofa, Czech Republic. The released Pi was assayed with malachite green [22] with an immunoplates spectrometer (Sapfir, Russia). The Pi content in the samples without the enzyme was assayed as a control.

2.4. Statistics

The assays were performed in triplicate; the mean values and standard deviations were calculated by Excel. The correlation coefficient was calculated using http://www.alcula.com/calculators/statistics/correlation-coefficient/.

3. Results

The purified PPN1 catalyzed the release of Pi from dATP (Table 1). The other exopolyphosphatase of *S. cerevisiae*, PPX1 (kindly provided by L. Lichko) [23], did not split Pi from dATP (Table 1). It is surprising, because PPX1 is more active with $polyP_3$ [23] and with adenosine-tetraphosphate and guanosine-tetraphosphate [14]. The PPN1 ac-

tivity was maximal with long-chain PolyP and weak with $PolyP_3$ [17] [18]. The dATP phosphohydrolase activity of pure PPN1 was ~7-fold lower compared to the exopolyphosphatase activity (Table 1). The activity of PPN1 with ATP was nearly twofold lower than with dATP; the activity with ADP and PPi was still lower (Table 2). The concentration dependence of dATPase activity of this enzyme corresponded to the Michaelis-Menten equation, and Km was 0.88 ± 0.14 mM.

The exopolyphosphatase activity of PPN1 displays the non-Michaelis kinetics, with the apparent Km of 0.0035 and 1.1 mM with $PolyP_{208}$ and $PolyP_3$, respectively [17]. The substrate affinity and hydrolysis rate of $PolyP_3$ [17] and dATP were similar. Both exopolyphosphatase and dATPase activities of PPN1 were stimulated by NH_4Cl (200 mM) nearly twofold (not shown).

Figure 1 shows the dependence of dATPase activity on the concentration of divalent cations. Co^{2+} was more effective than Mn^{2+}; Mg^{2+} and Zn^{2+} had no stimulatory effects. Heparin and PPi inhibited this activity and the inhibitory effect of ADP was low (Figure 2). The effects of divalent cations and the above inhibitors on the dATPase (Figure 1, Figure 2) and exopolyphosphatase [18] activities of PPN1 were similar.
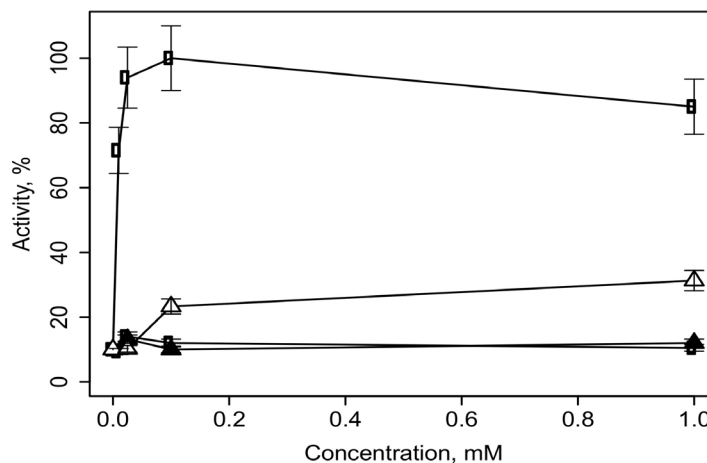
The dATP phosphohydrolase (dATPase) activity of cellular extract of the PPN1-overexpressing yeast strain was several-fold higher than that in the parent strain (Table 3). This increase was comparable with the increase in exopolyphosphatase activity. In addition, the dATPase and exopolyphosphatase activities of the cellular extract of transformant cells were similarly inhibited by heparin, the known suppressor of polyphosphatases [1], while the dATPase activity of the cellular extract of ΔPPN1 mutant was little affected by heparin. Probably, some other enzymes perform the dATPase activity in this strain.

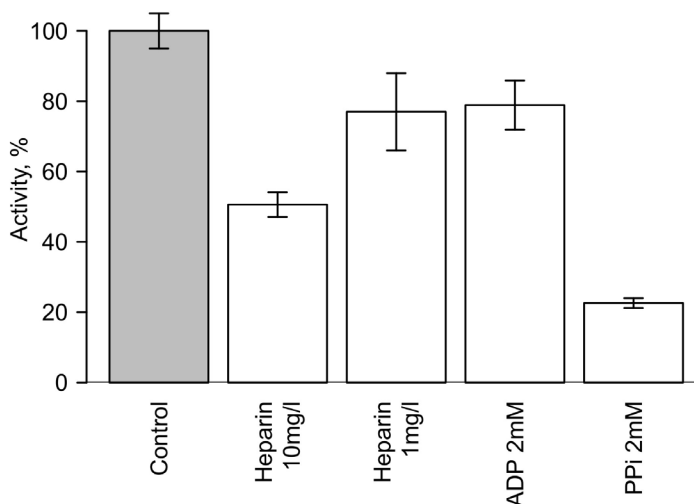**Table 1.** The exopolyphosphatase and dATP phosphohydrolase activities of pure PPN1 and PPX1 of *S. cerevisiae.*

Enzyme	Activity with different substrates	
	Substrate	Activity, mU/ml
PPN1	$PolyP_{208}$	244.0 ± 28.5
PPN1	dATP	34.9 ± 3.9
PPX1	$PolyP_{208}$	311 ± 32.0
PPX1	dATP	0

Table 2. Pi release by PPN1 from some substrates (2 mM).

Substrate	P_i release, % of control
dATP	100 ± 5.0
ATP	57 ± 5.0
ADP	20.0 ± 2.1
PPi	11.0 ± 1.0

Figure 1. The effects of Co^{2+} (white squares), Mg^{2+} (open circles), Zn^{2+} (black triangles), and Mn^{2+} (open triangles) on the dATP phosphohydrolase activity of polyphosphatase.

Figure 2. The effects of inhibitors on the dATP phosphohydrolase activity of polyphosphatase PPN1. The activity was assayed in 50 mM Tris-HCl, pH 7.2, containing 0.1 mM CoSO$_4$ and 200 mM NH$_4$Cl.

Table 3. The hydrolysis of PolyP208 and dATP by the cellular extracts of ΔPPN1 and PPN1-overexpressing strains of S. cerevisiae.

Enzyme activity, mU/mg protein	Yeast strain	
	ΔPPN1	PPN1-overexpressing strain
Polyphosphatase	88 ± 9.0	3640 ± 10
Polyphosphatase, 10 mg/l heparin	88 ± 7.0	420 ± 30
dATP phosphohydrolase	30 ± 2.8	370 ± 62
dATP phosphohydrolase, 10 mg/l heparin	26 ± 1.0	55 ± 4.0

4. Discussion

In this study, we have found the dATP phosphohydrolase activity of the *S. cerevisiae* protein PPN1. The activity is similar to the exopolyphosphatase activity of this protein in the dependence on divalent cations, stimulation by NH_4 ions, and inhibition by heparin and PPi. The activity decreased in the following order: dATP > ATP > ADP. The preliminary data on the hydrolysis of dNTP mixture suggest the ability of hydrolysis of other dNTP. It should be noted that the PPX1 exopolyphosphatase of *S. cerevisiae* hydrolyzes neither dATP (this study) nor dNTP mixture [19].

The catabolism of dNTPs is performed by many enzymes. First, the alkaline and acid phosphatases are able to hydrolyze these substrates (http://www.brenda-enzymes.org).

Second, the DEAH-box splicing factor Prp22 of yeasts can hydrolyze all common NTPs and dNTPs with a comparable efficiency [24], catalyzing the reaction:

(d) nucleoside triphosphate + H_2O → (d) nucleoside diphosphate + phosphate (EC 3.6.1.15). Finally, deoxynucleotide triphosphate triphosphohydrolases (dNTPases) hydrolyze deoxynucleotide triphosphates (dNTPs) into nucleosides and tripolyphosphate (3.1.5.B1, http://www.brenda-enzymes.org). In mammalian cells, the sterile alpha motif and HD domain-containing protein 1 (SAMHD1) is a dNTPase and a major regulator of cellular dNTP levels [25]. This protein prevents the infection of nondividing cells by retroviruses, including HIV, by depleting the cellular dNTP pool [25]. The importance of dNTP metabolism and SAMHD1 in cancer development was discussed [26]. It seems that the dNTP catabolism becomes particularly important under the conditions when a decrease in proliferative activity is necessary for the survival of an organism or a cell population. For further investigation of the role of PPN1 as a putative regulator of cellular dNTP level in yeast cells, it is necessary to take into account the following two facts. First, PPN1 gene is responsible for the exopolyphosphatase activity in yeast nuclei [27]. Second, ΔPPN1 mutants displayed the impairment of the cell cycle when the cells were grown under Pi limitation [4].

5. Conclusion

Yeast polyphosphatase PPN1 is deoxyadenosine triphosphate phosphohydrolase while the exopolyphosphatase PPX1 did not split Pi from dATP. The exopolyphosphatase and deoxyadenosine triphosphate phosphohydrolase activities of this protein are similar in ion and inhibitor effects.

Acknowledgements

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List of Abbreviations

PolyP—inorganic polyphosphates, PolyP208—inorganic polyphosphates with an average chain length of 208 phosphate residues, PolyP3—tripolyphosphate, Pi—orthophosphate, PPi—pyrophosphate; dATPase—deoxyadenosine triphosphate phosphohydrolase.

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