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Thi20, a remarkable enzyme from *Saccharomyces cerevisiae* with dual thiamin biosynthetic and degradation activities

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Abstract

Saccharomyces cerevisiae Thi20 is a fusion protein with homology to *Bacillus subtilis* ThiD and TenA. The N-terminus of Thi20 has significant sequence homology to *B. subtilis* ThiD, while the C-terminus has homology to *B. subtilis* TenA. Incubation of Thi20 with thiamin reveals that it has thiaminase II activity, in addition, incubation of Thi20 with HMP (4-amino-2-methyl-5-hydroxymethylpyrimidine) and ATP reveals that it has HMP kinase and HMP-P (4-amino-2-methyl-5-hydroxymethylpyrimidine phosphate) kinase activity. This demonstrates that Thi20 is a trifunctional protein with thiamin biosynthetic and degradative activity.

Keywords: Thiaminase II; 4-Amino-2-methyl-5-hydroxymethylpyrimidine kinase; Thiamin; Thiamin metabolism; Thiamin biosynthesis; Thiamin degradation

1. Introduction

Thi20 is the first of a three-member gene family consisting of Thi20, Thi21, and Thi22 [1]. All three are apparent gene fusions containing an N-terminus with sequence homology to *Bacillus subtilis* ThiD and a C-terminus with sequence

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homology to *B. subtilis* TenA (Fig. 1). Thi20 and Thi21 are functionally redundant and have been assigned HMP-P kinase activity [1]. No function has yet been assigned to Thi22.

ThiD from *B. subtilis* has both the HMP and HMP-P kinase activities [2] shown in Fig. 2 [3]. Since the N-terminal 303 amino acids of Thi20 have 24% sequence identity to ThiD, we hypothesized that Thi20 may also have HMP kinase activity, as well as the previously identified HMP-P kinase activity.

Bacillus subtilis TenA is a part of the thiamin biosynthetic operon [4] containing TenA-TenI-ThiO-ThiS-ThiG-THiF-ThiD. The C-terminal 206 amino acids of Thi20 have 22% sequence identity with *B. subtilis* TenA, recently discovered as thiaminase II [5].

Thiaminases are classified into two distinct groups, thiaminase I or thiaminase II. Thiaminase I has been structurally and biochemically characterized. Thiaminase I accepts a variety of nucleophiles to cleave the thiazole from the pyrimidine, resulting in several different pyrimidine adducts [6–9]. In contrast, thiaminase II is specific for the use of water as the nucleophile, resulting only in the formation of HMP (4-amino-2-methyl-5-hydroxymethylpyrimidine) and Thz (4-methyl-5-thiazole ethanol) (Fig. 2).

Thiaminase II activity has been detected in several organisms over the last 5 decades, including bacteria, plants, and fungi [10–14]. Notably, thiaminase II activity has been detected in *Saccharomyces cerevisiae* cell-free extracts [15], but the protein and the gene responsible for this activity have never been identified. Here, we report the identification of thiaminase II and hydroxymethyl pyrimidine kinase in *S. cerevisiae* as Thi20.

2. Materials and methods

2.1. Growth and purification of Thi20

Thi20 was cloned into a pDESTF1 plasmid (a Gateway-adapted vector based on the pET system from Novagen containing a 6× His tag) and transformed into the BL21Star (DE3)pRare2 expression strain of *Escherichia coli*. Thi20 was obtained by inoculating a 1 L culture of Luria–Bertani broth, containing 100 mg/L ampicillin and 20 mg/L chloramphenicol, with a 10 ml starter culture. The cells were grown to an OD_{600} of 0.6 at 37 °C, at which point expression was induced with 1 mM isopropyl β-D-thiogalactoside (IPTG). After induction, the temperature was reduced to 15 °C, the cells were grown overnight, pelleted, resuspended in lysis buffer (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, and 10 mM imidazole), lysed by sonication, and then clarified by centrifugation at 39,000*g*. The resulting cell-free extract was then bound to Ni–NTA resin (Qiagen) and washed with five column volumes of lysis buffer. The bound protein was then washed with 300 ml of wash buffer (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, and 20 mM imidazole). The pure protein was eluted from the Ni–NTA resin with elution buffer (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, and 250 mM imidazole) and desalted by dialysis against 50 mM Tris buffer, pH 8.

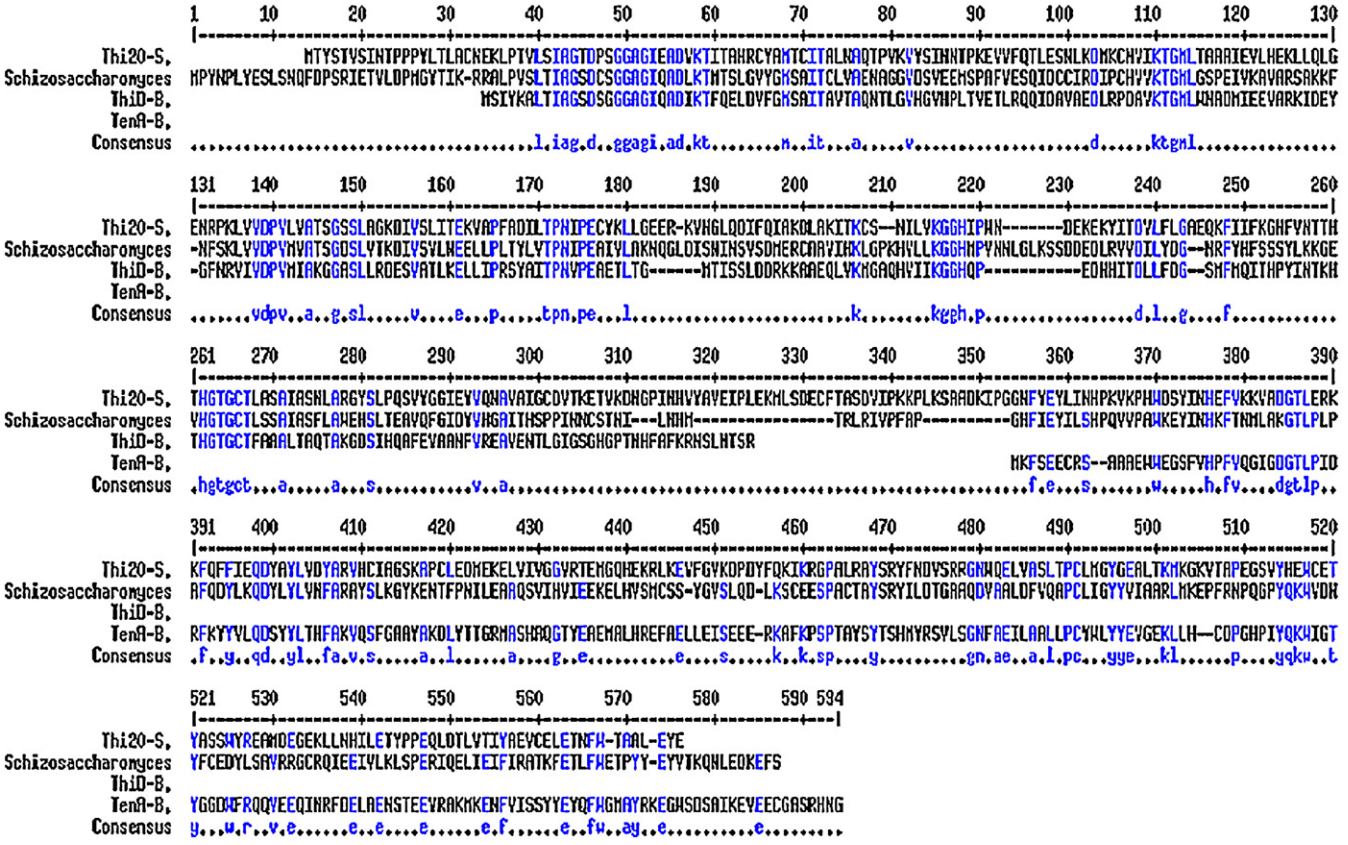

Fig. 1. Sequence alignment of Thi20, a Thi20 homolog in *Schizosaccharomyces pombe* and ThiD and TenA from *B. subtilis*.

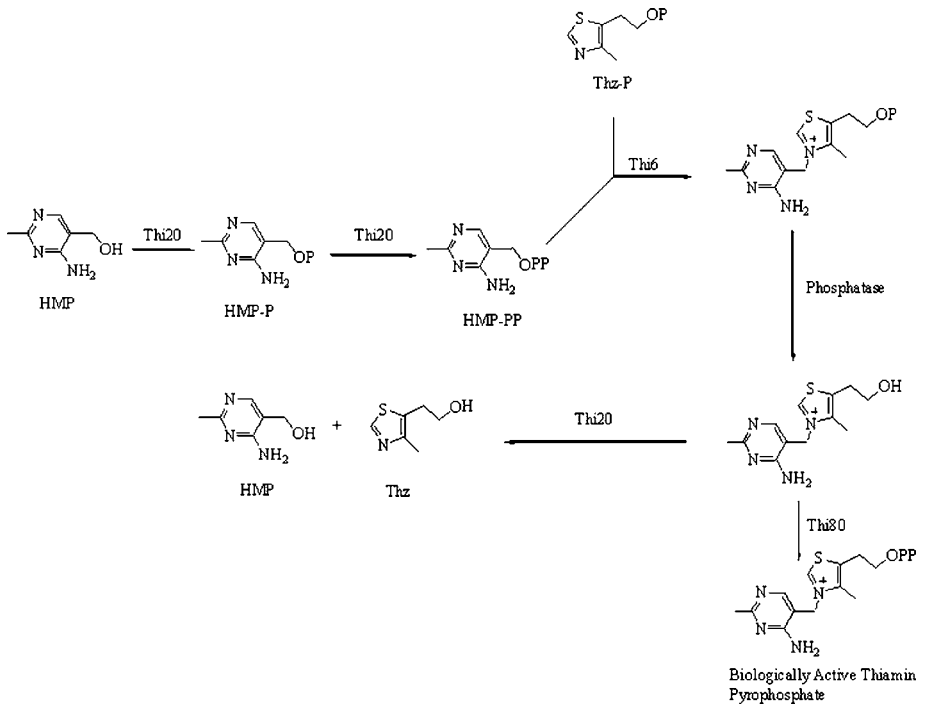

Fig. 2. Outline of thiamin metabolism in *S. cerevisiae*.

2.2. Assay for the HMP and HMP-P kinase activities

The HMP kinase activity of Thi20 was assayed by incubating 7 μM Thi20 with 200 μM HMP, 500 μM ATP, and 5 mM $MgCl_2$ in 50 mM Tris (pH 8) for 15 min at 32 °C. The reaction was terminated by filtration through a membrane with an exclusion limit of 10 kDa to remove the protein. The filtrate was mixed with an equal volume of 100 mM phosphate buffer (pH 8.2) to ensure an injection pH of 8–8.2. The HMP-P kinase activity was demonstrated in the same manner as above, extending incubation time to 30 min. Samples (200 μl) were then analyzed by HPLC on a C_{18} reverse phase column (Supelco, Supelcosil LC-18-T 15 cm × 4.6 cm, 3 μm) using the method described in Table 1 at a flow rate of 1 ml/min. The products were detected by absorbance at 242 nm and eluted in the following order: HMP (3.6 min), HMP-P (5.8 min), HMP-PP (10.2 min), ADP (11.5 min), and ATP (12.8 min). Retention times were confirmed by NMR verified standards.

2.3. Assay for the thiaminase II activity of Thi20

The thiaminase activity of Thi20 was assayed by incubating 25 μM Thi20, and 2.5 mM thiamin at room temperature in 100 mM phosphate buffer (pH 6.6). The reac-

Table 1
HPLC method for the elution of thiamin and the small molecules associated with thiamin biosynthesis and degradation

Minutes	% 4 mM TBAHS	% Buffer	% Methanol
0	75	15	10
14	25	25	50
16	50	40	10
18	75	15	10
30	75	15	10

Tetrabutylammonium hydrogen sulfate (TBAHS), buffer = 100 mM phosphate buffer at pH 6.6, containing 4 mM TBAHS.

tion was terminated by filtration through a membrane with an exclusion limit of 3 kDa to remove the protein, then analyzed by HPLC as described for the HMP kinase assay (Table 1).

2.4. Generation of the sequence alignment

The sequence alignment depicted in Fig. 1 was generated using the Multialin database, at http://prodes.toulouse.inra.fr/multalin/multalin.html.

3. Results and discussion

3.1. HMP kinase activity

The N-terminus of Thi20 shows sequence similarity to ThiD from *B. subtilis*, a protein recently shown to have both HMP and HMP-P kinase activities [2]. This suggested that Thi20 may also exhibit these activities. Figs. 3A and B confirm these assignments, showing HMP being converted to HMP-P, and then to HMP-PP. The release of HMP-P through multiple turnovers of the enzyme without producing a significant amount of HMP-PP or AMP indicates that the production of HMP-PP from HMP requires two separate binding events, rather than two tandem phosphorylations without HMP-P release, or a pyrophosphorylation. Since there is no other known use of HMP-P within the cell, this suggests that the HMP kinase activity is part of the HMP salvage pathway rather than obligatory to the in vivo synthesis of HMP-PP.

3.2. Thiaminase II activity

The C-terminus of Thi20 shows sequence similarity to *B. subtilis* TenA, a recently discovered thiaminase II [5]. This sequence similarity suggested that Thi20 may also have thiaminase II activity. Fig. 3C confirms the thiaminase II activity, showing the degradation of thiamin to HMP and Thz. Thi20 shows substrate and product inhibition and we have not yet determined its catalytic parameters.

Thi20 represents an unusual fusion protein in *S. cerevisiae* with three catalytic activities. The C-terminal domain has thiaminase II activity, while the N-terminal

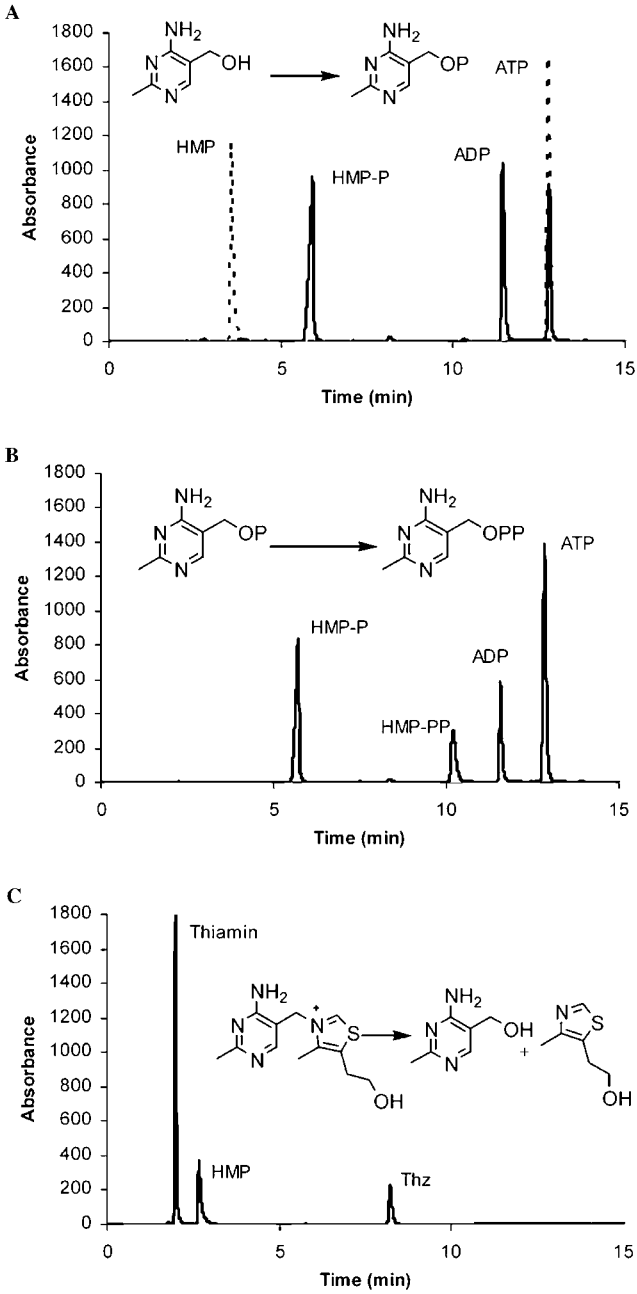

Fig. 3. HPLC chromatograms depicting Thi20 catalytic activity. (A) Chromatogram depicting the phosphorylation of HMP. (B) Chromatogram depicting the phosphorylation of HMP-P. (C) Chromatogram depicting the degradation of thiamin to HMP and Thz by Thi20.

domain has HMP and HMP-P kinase activity. A phylogenetic analysis of ThiD/TenA fusions indicates that Thi20 is narrowly distributed among Actinobacteria and Eukaryota. The physiological role of this unusual trifunctional protein with both thiamin biosynthetic and degradative activities is currently unclear.

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