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Enzymatic and structural characterization of an archaeal thiamin phosphate synthase

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ABSTRACT

Studies on thiamin biosynthesis have so far been achieved in eubacteria, yeast and plants, in which the thiamin structure is formed as thiamin phosphate from a thiazole and a pyrimidine moiety. This condensation reaction is catalyzed by thiamin phosphate synthase, which is encoded by the *thiE* gene or its orthologs. On the other hand, most archaea do not seem to have the *thiE* gene, but instead their *thiD* gene, coding for a 2-methyl-4-amino-5-hydroxymethylpyrimidine (HMP) kinase/HMP phosphate kinase, possesses an additional C-terminal domain designated *thiN*. These two proteins, ThiE and ThiN, do not share sequence similarity. In this study, using recombinant protein from the hyperthermophile archaea *Pyrobaculum calidifontis*, we demonstrated that the ThiN protein is an analog of the ThiE protein, catalyzing the formation of thiamin phosphate with the release of inorganic pyrophosphate from HMP pyrophosphate and 4-methyl-5-β-hydroxyethylthiazole phosphate (HET-P). In addition, we found that the ThiN protein can liberate an inorganic pyrophosphate from HMP pyrophosphate in the absence of HET-P. A structure model of the enzyme–product complex of *P. calidifontis* ThiN domain was proposed on the basis of the known three-dimensional structure of the ortholog of *Pyrococcus furiosus*. The significance of Arg320 and His341 residues for *thiN*-coded thiamin phosphate synthase activity was confirmed by site-directed mutagenesis. This is the first report of the experimental analysis of an archaeal thiamin synthesis enzyme.

1. Introduction

Thiamin (vitamin B_1) contains two ring structures, 2-methyl-4-amino-5-hydroxymethylpyrimidine (hydroxymethylpyrimidine, HMP) and 4-methyl-5-β-hydroxyethylthiazole (hydroxyethylthiazole, HET), and occurs in cells as free thiamin and its phosphoesters [1]. Thiamin pyrophosphate functions as a cofactor for enzymes indispensable for the metabolism of carbohydrates and amino acids, including transketolase, 2-oxoacid decarboxylase, 2-oxoacid dehydrogenase and acetolactate synthase. In addition to its coenzymatic function, thiamin may have a specific role in vertebrate neuronal activity as the triphosphate form. Most eubacteria, yeast and plants synthesize thiamin *de novo*. The thiamin biosynthetic pathway involves the independent formation of HMP pyrophosphate (HMP-PP) and HET phosphate (HET-P), as well as their subsequent condensation to form thiamin phosphate by thiamin phosphate synthase (TPS, EC 2.5.1.3), previously known as thiamin pyrophosphorylase (Fig. 1) [2]. Almost all eubacterial cells directly synthesize thiamin pyrophosphate by the phosphorylation of thiamin phosphate by thiamin phosphate kinase (EC 2.7.4.16), whereas in eukaryotic cells and some eubacterial cells, this final step occurs in two stages; hydrolysis to free thiamin and its pyrophosphorylation by thiamin pyrophosphokinase (EC 2.7.6.2). In addition, most microorganisms have kinases involved in the salvage synthesis of HMP-PP and HET-P from HMP and HET, respectively. Genes encoding adjacent enzymatic steps of the thiamin biosynthetic pathway (e.g. *thiD*, *thiE*, *thiM*) often form clusters in the bacterial genome [3,4], whereas their fused genes (e.g. *thiDE*, *thiEM*) are found in the genomes of yeast and plants [5,6].

Over the last two decades, biochemical, genetic and structural studies have clarified the thiamin biosynthetic pathway especially in eubacteria and eukaryotes [2]. In contrast, thiamin metabolism including its biosynthesis has never been experimentally investigated in archaea. Genome analyses of archaea reveal the presence of genes encoding proteins with amino acid sequence similarities to already-known thiamin biosynthetic enzymes [3,4]: HET-P synthase (yeast *THI4*), HMP-P synthase (*thiC*), HMP kinase/HMP-P kinase (*thiD*) and

Abbreviations: TPS, thiamin phosphate synthase; HMP, 2-methyl-4-amino-5-hydroxymethylpyrimidine; HMP-P, HMP phosphate; HMP-PP, HMP pyrophosphate; HET, 4-methyl-5-β-hydroxyethylthiazole; HET-P, HET phosphate

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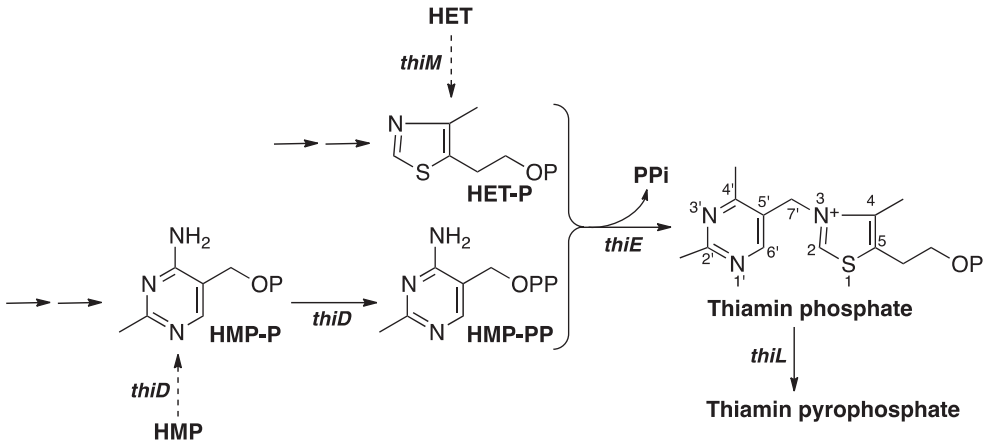

Fig. 1. The later steps in the biosynthesis of thiamin in eubacteria. Bacterial genes are indicated in italics. Dashed arrows indicate salvage reaction. Abbreviations are as defined in the text.

thiamin phosphate kinase (*thiL*). Curiously, most archaea do not seem to have a TPS gene (*thiE*), but instead their *thiD* genes code an HMP kinase/HMP-P kinase with an additional C-terminal domain of about 180 amino acids (aa). This additional domain, designated *thiN*, is not similar to *thiE*. Although *thiN* is predominantly distributed in archaea, it is also found in thermophilic eubacteria such as *Thermotoga maritima* (Supplementary Table S1). In all cases when *thiE* is absent, *thiD* exists as a *thiDN* fusion gene, whereas in some cases, *thiE* and *thiD* co-exist in the same species. A multiple sequence alignment of several putative ThiN proteins (one from each species) is shown in Fig. 2. These sequences emerged as an aldolase-2 superfamily in BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The proteins in Fig. 2 share 35–55% sequence identity with each other, and there are significant amino acid identities among all 6 sequences.

Rodinov et al. [3] predicted that the ThiN protein is involved in thiamin synthesis instead of ThiE in some archaea, and Morett et al. [4] practically demonstrated that thiamin phosphate is produced from HMP-PP and HET-P by incubation with the recombinant ThiN protein of *T. maritima*, although the enzymatic properties were not dissected. In this study, we isolated and characterized the *thiDN* gene from the hyperthermophile archaea *Pyrobaculum calidifontis*. We demonstrated that the recombinant ThiN protein is TPS catalyzing the formation of thiamin phosphate with the release of inorganic pyrophosphate from HMP-PP and HET-P. In addition, the ThiDN protein was proven to be a multifunctional enzyme capable of thiamin phosphate synthesis from HMP and HET-P in the presence of ATP and Mg^{2+}. Molecular modeling of the enzyme–product complex structure is also presented.

2. Materials and methods

2.1. Strains and growth conditions

P. calidifontis JCM 11548 was used to prepare the cell-free extract and the genomic DNA. *P. calidifontis* was cultured in 2-l Erlenmeyer flasks in medium (600 ml, pH 7.0, adjusted with NaOH) containing 10 g/l tryptone, 1 g/l yeast extract, and 3 g/l $Na_2S_2O_3 \cdot 5H_2O$ at 90 °C under aerobic conditions. *Escherichia coli* strain DH5α was used to amplify plasmids and BL21(DE3) codon plus RIL (Stratagene, CA) was used to express recombinant proteins.

2.2. Plasmids and chemicals

The intact coding sequences of *P. calidifontis thiDN* and its C-terminal 255–449 aa region (*thiN*) were PCR-amplified from the genomic DNA

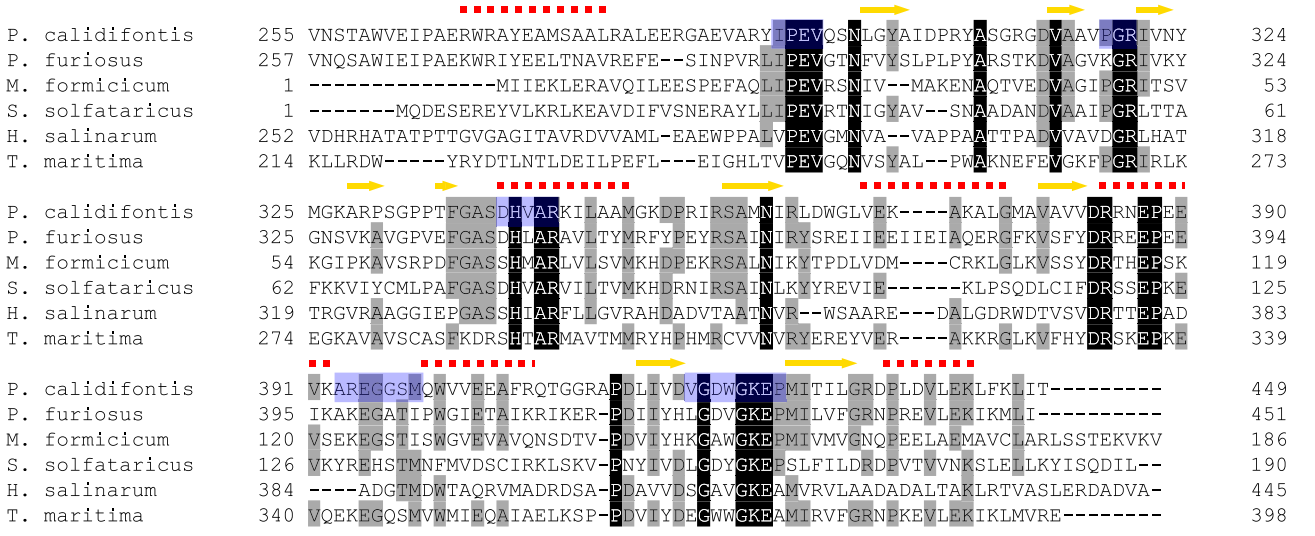

Fig. 2. Alignment of the amino acid sequences of the ThiN protein. Amino acid sequences of ThiN proteins from *Pyrobaculum calidifontis* (WP_011848934), *Pyrococcus furiosus* (WP_011012479), *Methanobacterium formicicum* (WP_004031884), *Sulfolobus solfataricus* (WP_009989596), *Halobacterium salinarum* (WP_010903947) and *Thermotoga maritima* (WP_004080887) were aligned using the ClustalW program (http://clustalw.ddbj.nig.ac.jp/). Residues identical in all sequences are highlighted in black and similar residues are highlighted in gray. For the secondary structure of the simulated *P. calidifontis* ThiN protein, red dotted lines represent α-helices and yellow arrows represent β-strands. Residues involved in the formation of the predicted active site are highlighted in purple transparently.