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Thiamin Pyrimidine Biosynthesis in *Candida albicans*: A Remarkable Reaction between Histidine and Pyridoxal Phosphate

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Supporting Information

ABSTRACT: In *Saccharomyces cerevisiae*, thiamin pyrimidine is formed from histidine and pyridoxal phosphate (PLP). The origin of all of the pyrimidine atoms has been previously determined using labeling studies and suggests that the pyrimidine is formed using remarkable chemistry that is without chemical or biochemical precedent. Here we report the overexpression of the closely related *Candida albicans* pyrimidine synthase (THI5p) and the reconstitution and preliminary characterization of the enzymatic activity. A structure of the *C. albicans* THI5p shows PLP bound at the active site via an imine with Lys62 and His66 in close proximity to the PLP. Our data suggest that His66 of the THI5 protein is the histidine source for pyrimidine formation and that the pyrimidine synthase is a single-turnover enzyme.

The thiazole (**7**) and pyrimidine (**10**) heterocycles of thiamin are biosynthesized in *Saccharomyces cerevisiae* using chemistry that is fundamentally different from the bacterial pathway[1,2] (Figure 1). In bacteria, the thiazole is formed from deoxy-D-xylulose 5-phosphate, glycine (or tyrosine), and cysteine, and the pyrimidine is formed from aminoimidazole ribotide.[3] In contrast, in *S. cerevisiae*, thiazole 7 is formed from nicotinamide adenine dinucleotide (NAD), glycine, and an active-site cysteine of the THI4 protein,[4] and the 4-amino-2-methyl-5-hydroxymethylpyrimidine phosphate (HMP-P, **10**) is formed from histidine and pyridoxal phosphate (PLP).[5−9] The origin of all of the atoms of HMP-P has been determined (Figure 1) and suggests that HMP-P is formed using remarkable chemistry that is without chemical or biochemical precedent. Genetic studies have demonstrated that HMP-P formation requires only a single gene (*THI5*).[10] Here we report the overexpression of THI5p, the reconstitution of the enzymatic activity, a preliminary characterization of the reaction, and a structure of the enzyme/PLP complex. These experiments suggest that the THI5 protein is the histidine source for HMP-P formation and that THI5p is a single-turnover enzyme.

The *THI5* gene from *Candida albicans* was overexpressed in *Escherichia coli* BL21(DE3), grown in minimal medium from a pET28b vector, and purified by Ni-NTA chromatography. The purified protein has the expected molecular mass of 40 408 Da.

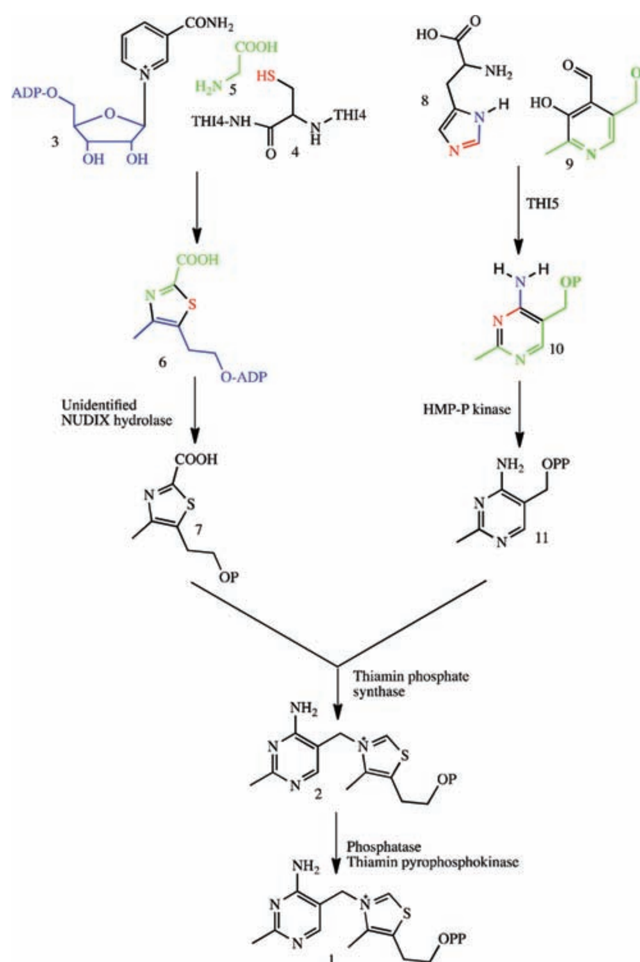

Figure 1. Thiamin pyrophosphate biosynthesis in *S. cerevisiae*.

The active form of the enzyme was prepared by treatment of this protein with Fe(II) or Fe(III) under anaerobic conditions followed by removal of excess iron. Addition of PLP to this preparation under aerobic conditions yielded HMP-P (Figure 2). This reaction product was characterized by comigration with an authentic standard, by dephosphorylation to give HMP,

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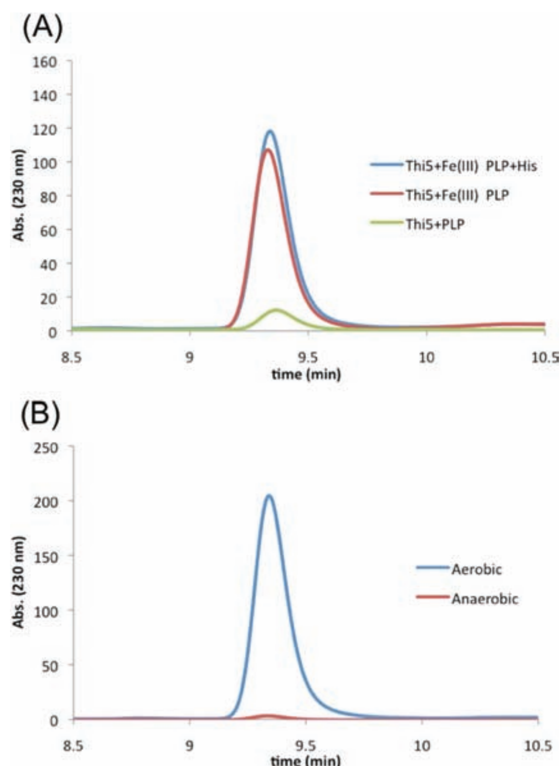

Figure 2. Reconstitution of the THI5-catalyzed reaction. (A) The reconstitution reaction requires PLP and Fe(III) but is independent of added histidine. The small amount of HMP-P shown in the green trace is due to product that copurifies with THI5 and not to synthesis. (B) The reaction to form HMP-P requires oxygen.

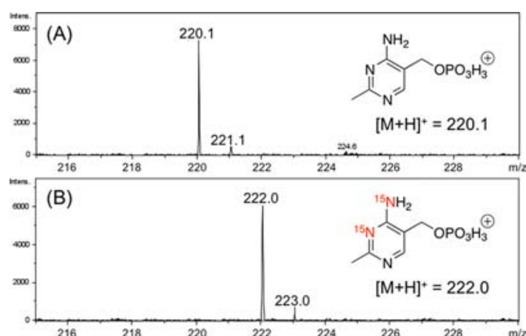

Figure 3. MS analysis of HMP-P formed using (A) ^{14}N-THI5p and (B) ^{15}N-THI5p.

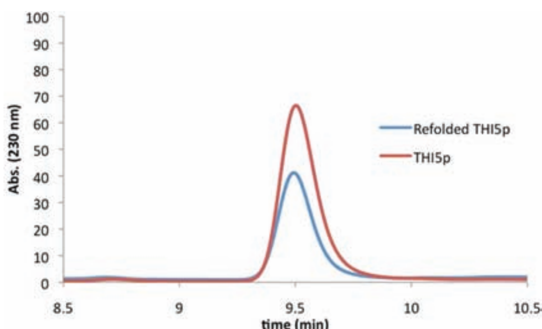

Figure 4. THI5p denatured in urea and refolded is active. Red trace: HMP produced by THI5p before urea denaturation. Blue trace: HMP produced by THI5p after denaturation in 8 M urea and renaturation by slow dialysis.

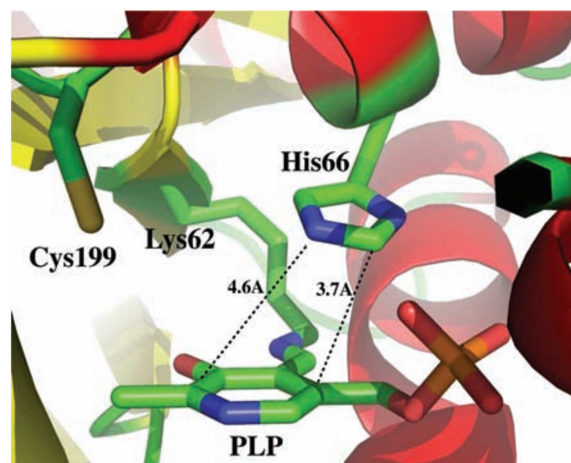

Figure 5. Active site of *C. albicans* THI5p showing PLP bound via an imine to Lys62 and His66 in close proximity to the PLP.

by enzymatic conversion to thiamin monophosphate, and by mass spectrometry (MS) analysis. After optimization and scale-up, it was also possible to characterize the product by ^{1}H NMR analysis. Two remarkable features of this reaction are the following: (1) HMP-P formation does not require the addition of histidine or a histidine-derived metabolite, and (2) the reaction is not catalytic (THI5p:HMP-P = 1:0.5).

^{15}N-THI5p, which was prepared by growing the over-expression strain in ^{15}N-ammonium chloride-containing minimal medium, produced HMP-P that upon MS analysis showed the expected [M + 2] peak corresponding to incorporation of two atoms of ^{15}N from the protein (Figure 3). This suggested the possibility that THI5p serves as the donor of the histidine-derived atoms of HMP-P.

To eliminate the possibility that HMP-P formation was dependent on the copurification of a tightly bound histidine or histidine metabolite, THI5p was treated with 8 M urea until no activity remained and then renatured by slow dialysis. After purification by Ni-NTA chromatography, the resulting protein showed 59% of the specific activity of the original preparation (Figure 4). Since denaturation destroys the active-site structure and dialysis removes any released metabolite, this experiment provides strong support for the hypothesis that the histidine-derived N=C−N fragment is extracted from the THI5 protein.

The *C. albicans* THI5p sequence has six histidine residues. Of these, His18 and His66 are absolutely conserved in all species (Figure S5). If one of these histidine residues is the N=C−N donor, we would expect THI5p after HMP-P formation (inactive THI5p) to show a mass deficit. However, MS analysis of a tryptic digest of inactive THI5p gave fragments containing only five of the six-histidine residues; we were unable to identify a peptide containing His66 or modified His66 in samples of active and inactive enzyme. We do not yet understand why the MS analysis failed to identify this peptide. Each of the six histidine residues was then mutated in an alternative attempt to identify the histidine donor for HMP-P formation. The H18A, H128N, H234N, H273N, and H323N mutants were active, while the H66N mutant was inactive. This suggests that the absolutely conserved His66 might be the source of the N=C−N fragment.

X-ray crystallography of the *C. albicans* THI5p complexed to PLP provided further insight into the N=C−N source. The active site of this enzyme (Figure 5) shows PLP bound as an

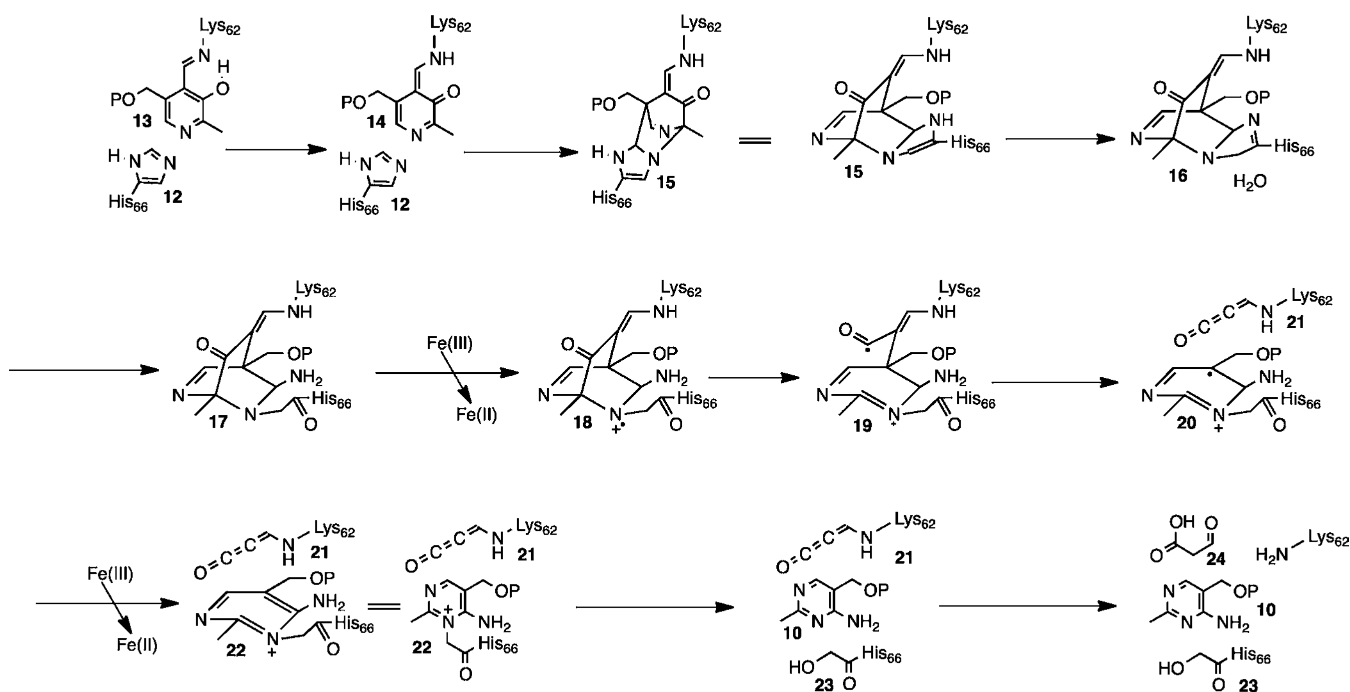

Figure 6. Mechanistic proposal for the formation of HMP-P from PLP and the active-site His66.

imine with Lys62 and His66 stacked on top of it. On the opposite face of the PLP is a cavity lined with conserved residues, presumably providing the iron binding site and the acid/base residues needed to catalyze the conversion of a His–PLP adduct to HMP-P.

A starting mechanistic proposal for the conversion of PLP and histidine to HMP-P is outlined in Figure 6. In this mechanism, the active-site PLP imine **13** undergoes a tautomerization to give **14**. The azadiene part of **14**, activated by electron-donating and electron-withdrawing substituents, undergoes a formal Diels–Alder reaction with the CN double bond of His66 to give **15**. Tautomerization to **16** followed by imine hydrolysis gives **17**. Fe(III)-mediated oxidation of the tertiary amine of **17** gives **18**, which can then undergo two radical-mediated β-scission reactions to give **20**. After oxygen-mediated regeneration of Fe(III), oxidation of **20** gives **22**. A substitution reaction releases HMP-P (**10**) from the protein. Hydrolysis of heterocumulene **21** to **24** completes the reaction.

In conclusion, active THI5p can be readily overexpressed in *E. coli*. The protein uses PLP and the active-site His66 to form HMP-P. This reaction requires oxygen and Fe(III) and generates inactive THI5p. A structure of *C. albicans* THI5p shows PLP bound at the active site via an imine with Lys62 and His66 in close proximity to the PLP. A mechanistic proposal for a reaction between His66 and PLP to generate HMP-P is described. Many features of this proposal are readily testable, and studies to elucidate the mechanism of this remarkable reaction are currently underway.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

THI5p purification, assay conditions, reaction characterization, MS analysis, crystallization, and structure determination. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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