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Thiamin Biosynthesis in Eukaryotes:

Characterization of the Enzyme Bound Product of Thiazole Synthase from *Saccharomyces cerevisiae* and Its Implications in Thiazole Biosynthesis

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Abstract

The biosynthesis of thiamin pyrophosphate in eukaryotes is different from the prokaryotic biosynthesis and is poorly understood to date. Only one thiazole biosynthetic gene has been identified (Thi4 in *Saccharomyces cerevisiae*). Here we report the identification and characterization of a Thi4 bound metabolite that consists of the ADP adduct of 5-(2-hydroxyethyl)-4-methylthiazole-2-carboxylic acid. The unexpected structure of this compound yields the first insights into the mechanism of thiamin thiazole biosynthesis in eukaryotes.

Thiamin pyrophosphate is an essential cofactor in all living systems where it plays a key role in amino acid and carbohydrate metabolism.[1, 2] The biosynthesis of thiamin involves separate syntheses of the thiazole and the pyrimidine moieties, which are then linked to form the cofactor.[3, 4] The biosynthesis of the thiazole moiety in bacteria has been elucidated and involves a complex oxidative condensation where five different enzymes interact with three different substrates (1-deoxy-D-xylulose-5-phosphate, cysteine and glycine or tyrosine).[4] Labeling studies in *Saccharomyces cerevisiae* have demonstrated that the thiamin thiazole is biosynthesized from an unidentified five carbon carbohydrate, glycine and cysteine.[5-7] Only one thiazole biosynthetic enzyme has been identified (Thi4).[8] This biosynthesis has not yet been reconstituted *in vitro*. In this communication we report the identification and characterization of the tightly bound reaction product at the active site of the *S.cerevisiae* thiazole synthase. The unanticipated structure of this metabolite provides the first insights into the mechanism of thiazole biosynthesis in eukaryotes.

The *S.cerevisiae* thiazole synthase (Thi4) was overexpressed and purified**.** The overexpressed protein failed to catalyze the formation of the thiazole moiety from cysteine (or sulfide), glycine and a variety of C5 carbohydrates. Thi4 also failed to complement an *E.coli* thiazole biosynthetic mutant (ThiF$^-$). This suggested that additional enzymes may be required for thiazole biosynthesis in *S.cerevisiae*, or that the correct substrates have not yet been identified.

In the process of searching for the activity of Thi4, we found that denaturing the protein by heat releases four major enzyme bound metabolites (Figure 1a). Each of these four species had an absorption maximum at 260nm. This absorption, combined with sequence analysis that predicted an adenine dinucleotide binding motif, suggested that the Thi4 metabolites might be adenylated. Treating the released metabolites with nucleotide pyrophosphatase and demonstrating the production of AMP by HPLC analysis confirmed this.

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The component with retention time of 14 min (compound A) was sufficiently stable for purification by HPLC; the components eluting after 11 (compound D) and 12 min (compound C) were not and therefore could not be characterized further. Intensity of compound B (eluting at 12.5min) was low and varied between experiments and has not yet been characterized. 1H NMR (Figure 1c) and COSY analysis of compound A confirmed the presence of the adenosyl moiety. Additionally, two strongly coupled methylene protons (2.7 and 3.8 ppm) and an isolated methyl (2.05 ppm) were observed. HMBC analysis demonstrated that the methyl group was connected to one of the methylene groups by two aromatic/olefinic carbons. This suggested partial structure **1**.

Negative mode ESI-MS analysis of compound A identified the molecular mass to be 596Da (monoanionic m/z=595; dianionic m/z=297). This demonstrated that the missing atoms from Structure **1** (Figure 1d) have a combined mass of 103Da. Since the thiamin thiazole is biosynthesized from glycine and contains a sulfur atom, this extra mass is consistent with Structure **2**. Extensive fragmentation studies were carried out on Compound A and the results were consistent with this structural assignment.

To confirm the presence of the thiazole moiety in **2**, Compound A was again treated with nucleotide pyrophosphatase to generate AMP and the putative thiazole **3** (Figure 2a). Treatment of this reaction mixture with hydroxymethyl pyrimidine pyrophosphate and thiamin phosphate synthase (ThiE)[4, 9] should yield **4**, provided ThiE can process **3** as a substrate. Compound **4** would then undergo rapid decarboxylation to give thiamin phosphate **5**, which can be detected by HPLC analysis with high sensitivity after its oxidation to the intensely fluorescent thiochrome phosphate **6**. The results of this experiment are shown in Figure 2c and demonstrate the production of thiochrome phosphate confirming the assigned thiazole structure. It also shows that ThiE can process the carboxylated thiazole phosphate **3**.

The recently solved structure of Thi4 supports this analysis. This structure shows that **2** is present at the active site of Thi4 and that the carboxylic acid of the thiazole forms hydrogen bonding and electrostatic interactions with Arg301 (Figure 3).

A crystal structure of Thi1 (the Arabidopsis ortholog of Thi4) has recently been deposited in the Protein Data Bank (1RP0). This structure also shows a bound adenylated intermediate but the electron density does not allow for an unequivocal assignment of the thiazole structure. Thi1 was overexpressed as a soluble protein in *E.coli* and HPLC analysis revealed the existence of compound 2 as well as the metabolites B and D. This mixture of enzyme bound metabolites might explain the ambiguous electron density corresponding to the thiazole moiety in the structure.

The identification of **2** at the active site of Thi4 suggests that the ADP-pentose moiety of **2** is probably derived from NAD and that the chemistry involved in the early steps of thiazole formation may be similar to the chemistry involved in ADP-ribosylation.[10, 11] One possible mechanism is outlined in Figure 4. Cleavage of the N-glycosyl bond of NAD **7** followed by ring opening, tautomerization and loss of water gives **11**. Imine formation, tautomerization, sulfide addition and cyclization gives **15**. Elimination of two water molecules followed by a tautomerization completes the formation of the thiazole moiety.

All attempts to release the product without denaturing the enzyme have failed and we do not yet understand the high stability of this enzyme product complex. The observation that the *N.crassa* Thi4 forms a stable complex with a cyclophilin[12, 13] suggests that a proline isomerization mediated conformational change may be required for product release.

Thiamin phosphate synthase, another enzyme on the thiamin biosynthetic pathway in prokaryotes, also contains tightly bound reaction product.[9] These observations suggest that

the analysis of a protein of unknown function for tightly bounds metabolites can be a productive strategy for functional assignment. The characterization of the reaction product bound at the active site of the yeast thiazole synthase yields the first insights into the biosynthesis of the thiamin thiazole in eukaryotes and represents yet another example of a non-redox function for NAD.[11, 14]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

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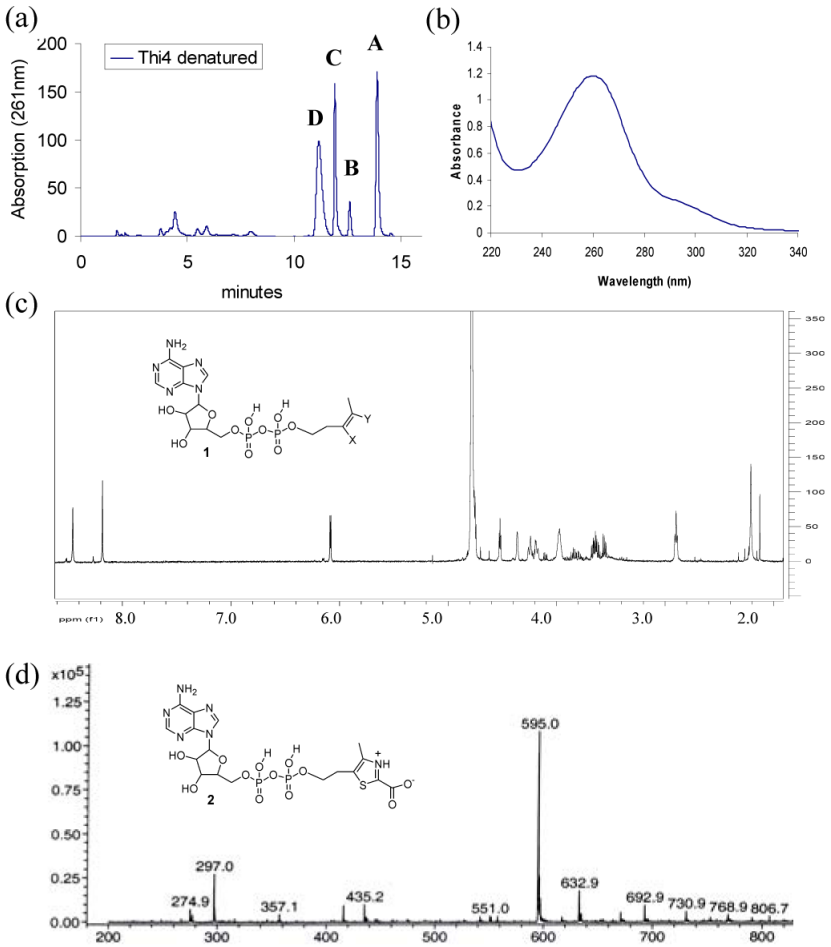

Figure 1.

Identification of Thi4 bound small molecules by HPLC (a). Analysis of the HPLC purified peak A : by UV-Vis spectroscopy (b), by 1H NMR spectroscopy (c) and by negative mode ESI-MS (d).

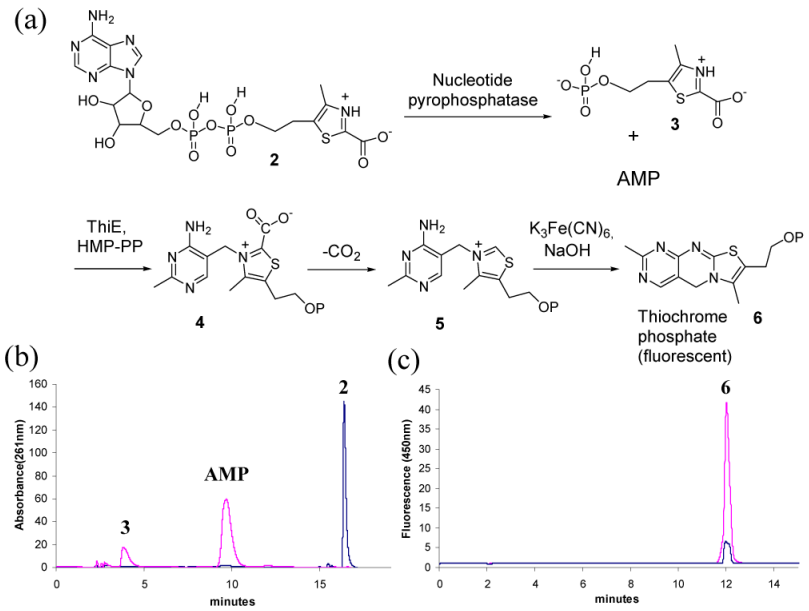

Figure 2.

Confirmation of the structure **3** by its enzymatic conversion to thiamin phosphate **5**: (a) Scheme showing the reactions performed. (b) HPLC analysis of the cleavage reaction of **2** to **3** and AMP: The blue trace shows the control reaction where no pyrophosphatase has been added to **2;** The purple trace shows the cleavage reaction of **2** by the pyrophosphatase. (c) HPLC detection of the thiochrome phosphate **6** produced from **2**: blue trace shows the control reaction with no HMP-PP added; Purple trace shows the actual coupling reaction.

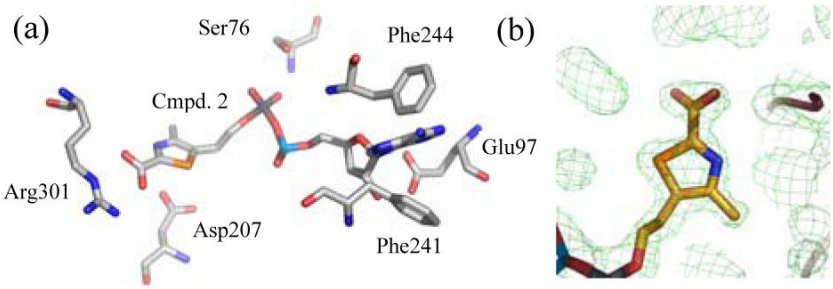

Figure 3.

Active site structure of Thi4 (PDB code: 2GJC): (a) The structure of Compound 2 and its interactions with the enzyme. (b) Electron density confirming the thiazole carboxylic acid moiety of 2. The density is a composite omit map contoured at the 1σ level.

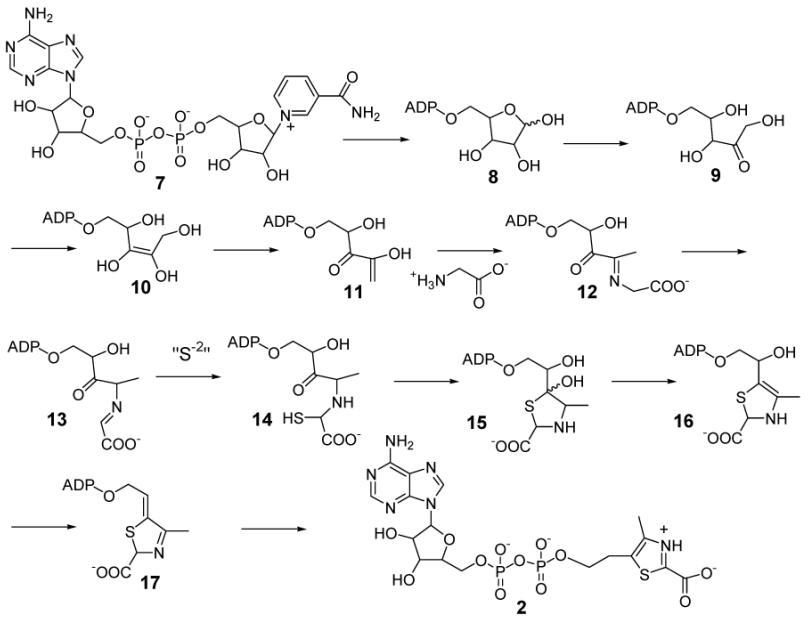

Figure 4.
A proposed mechanism for thiazole biosynthesis in eukaryotes.