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Thiamin Biosynthesis - still yielding fascinating biological chemistry

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Abstract

This paper will describe the biosynthesis of the thiamin thiazole in *Bacillus subtilis* and *Saccharomyces cerevisiae*. The two pathways are quite different: in *B. subtilis*, the thiazole is formed by an oxidative condensation of glycine, deoxy-D-xylulose- 5-phosphate and a protein thiocarboxylate, while in *S. cerevisiae* the thiazole is assembled from glycine, NAD and Cys205 of the thiazole synthase.

Keywords

Thiamin biosynthesis; thiazole; suicide enzyme; THI4; ThiG

The major thiamin biosynthetic pathway in bacteria is outlined in Figure 1.(1, 2) In this pathway, glycine **3** undergoes an oxidative condensation with deoxy-D-xylulose- 5-phosphate **5** and ThiS-thiocarboxylate **6**, to give the thiazole tautomer 7, which then aromatizes to form carboxythiazole **8**.(3, 4) The thiamin pyrimidine **15** is formed by a remarkable rearrangement of AIR **14**, an intermediate on the purine pathway.(5) Coupling the thiazole and the pyrimidine, with concomitant decarboxylation, yields thiamin phosphate **2**.(6, 7) A final phosphorylation gives thiamin pyrophosphate **1**, the biochemically active form of the cofactor.(8)

Our understanding of this biosynthetic pathway is now at an advanced stage. All the biosynthetic genes have been identified and cloned, all of the enzymes have been overexpressed, reconstituted and structurally characterized and mechanisms for all of the biosynthetic reactions, except for the pyrimidine synthase (ThiC) are reasonably clear (1, 5). The entire biosynthetic pathway has been fully reconstituted using pure enzymes. In this lecture, I will describe the biosynthesis of the thiamin thiazole in *B. subtilis* and compare this pathway with the very different thiazole biosynthesis recently elucidated in *S. cerevisiae*.

Thiamin thiazole biosynthesis in B. subtilis

Each of the steps involved in the assembly of the thiamin thiazole in bacteria will be described in the following sections.

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Glycine Oxidation

The ThiO gene product encodes a flavin-dependent glycine oxidase that catalyzes the oxidation of glycine **3** to the glycine imine **4** (9). In the absence of the other thiazole biosynthetic enzymes, the glycine imine is hydrolyzed to glyoxal.

The structure of this enzyme, with N-acetyl glycine bound at the active site, has been determined (PDB code = 1NG3). This structure and studies with substrate analogs are consistent with a hydride transfer mechanism for glycine oxidation (9).

In anaerobes, the glycine imine is formed from tyrosine in a reaction catalyzed by ThiH, a radical SAM enzyme (10–12).

ThiS-thiocarboxylate Formation

The chemistry involved in the formation of ThiS-thiocarboxylate is outlined in Figure 1. Activation of ThiS-COOH **9**, at its carboxy terminus, by adenylation, gives **10** which then acylates the IscS persulfide to give **13**. Reduction of **13**, by DTT in the reconstitution reaction mixture, gives the ThiS thiocarboxylate **6** (13, 14). The biochemical reduction of **13** is not yet understood. In some bacteria, an additional protein (ThiI) mediates the sulfur transfer to **10**.(15) ThiS-thiocarboxylate **6** can also be efficiently synthesized by treating intein-activated ThiS-COOH with ammonium sulfide (16).

The structures of the ThiF/ThiS complex and the ThiF/ATP complex have been determined (17, 18) (PDB codes = 1ZUD and 1ZFN). The IscS protein probably does not form a specific complex with ThiS/ThiF because all four IscS paralogs in *B. subtilis* are competent persulfide donors. (14, 19)

Protein thiocarboxylates as sulfide carriers in other biosynthetic pathways

Protein thiocarboxylates have now been found to play a role as sulfide carriers in several other biosynthetic pathways and sequence analysis suggests that this strategy may be quite general (Figure 2).

In molybdopterin biosynthesis, MoaE catalyzes the transfer of sulfide from MoaD-thiocarboxylate to give **19** (Figure 2A)(20, 21). A protein thiocarboxylate dependent cysteine biosynthetic pathway has been found in *M. tuberculosis* (Figure 2B). In this pathway, CysM thiocarboxylate reacts with phosphoserine **21** in a PLP-mediated reaction to form thioester **22**. This then undergoes an N/S acyl shift to give **23** followed by release of cysteine in a hydrolysis reaction catalyzed by the Mec protease (22–26). A closely related pathway for the biosynthesis of homocysteine was discovered in *Wolinella succinogenes* (Figure 2C). In this pathway HcyS-thiocarboxylate **25** adds to O-acetyl homoserine **26** to give thioester **27**. An N/S acyl shift to give **28** followed by HcyD-catalyzed amide hydrolysis generates homocysteine **30** (27, 28). A fourth example is found in the biosynthesis of the siderophore thioquinolobactin **34** (Figure 2D). In this pathway, QbsE thiocarboxylate forms a mixed thioanhydride **33** with quinolobactin **31**. Hydrolysis of **33** generates the siderophore **34**.(29, 30) A reagent for the sensitive detection of protein thiocarboxylates in a proteome, that uses a click reaction between the protein thiocarboxylate and a fluorophore-tagged sulfonyl azide, has been described. (31)

Formation of the thiazole tautomer 7

The bacterial thiazole synthase catalyzes the condensation of DXP (**5**), ThiS-COSH **6** and the glycine imine **4** to form the thiazole tautomer **7** (Figure 1)(3). A mechanistic proposal for this reaction is outlined in Figure 3. In this mechanism, DXP **5** forms an imine with Lysine 96 of the thiazole synthase. Tautomerization to **38** followed by thiocarboxylate addition

gives **39**. An O/S acyl shift followed by loss of water generates thioketone **41**. Tautomerization of **41**, followed by loss of ThiS-COOH generates **43**. Addition of the glycine imine **4** followed by transimination gives the thiazole tautomer **7**.

In support of this mechanism, enzyme-catalyzed exchange of the DXP carbonyl oxygen has been observed and the DXP/K96 imine has been trapped by borohydride reduction and characterized by MS analysis. Intermediate **37** is supported by the observation of enzyme-catalyzed exchange of the C3 proton of DXP. The unanticipated O/S acyl shift to give **40** is supported by the observation of oxygen incorporation from DXP and not the buffer into the nascent ThiS-COOH. Thioenol **42** has also been trapped and characterized by MS analysis and the final product **7** has been fully characterized by spectroscopic analysis (3, 14).

The structure of the ThiG/ThiS complex, with phosphate bound at the active site, has been determined (PDB code = 1TYG). In this structure the phosphate and Lys96 define the DXP binding site, which suggests that Glu98 and Asp182 are also likely to play a role in the catalysis of thiazole formation (32).

Thiazole tautomerase

The thiazole tautomer **7** is surprisingly stable and the aromatization reaction to produce the thiazole **8** requires enzymatic catalysis. In *B. subtilis*, the TenI protein has recently been identified as the thiazole tautomerase.

The structure of the enzyme product **8** complex has been determined (PDB code = 3QH2). A model of the enzyme substrate complex generated from this structure suggests that His122 mediates the deprotonation at C2 and that the substrate phosphate group functions as the proton donor for the exocyclic double bond protonation (4). TenI shows high sequence similarity to thiamin phosphate synthase and the two enzymes are frequently incorrectly assigned in genome annotation.

Thiamin thiazole biosynthesis in *S. cerevisiae*

The thiamin biosynthetic pathway in *S. cerevisiae* is outlined in Figure 4.(33) The biosynthesis of the thiazole and the pyrimidine heterocycles (**5** &**10**) occurs by very different chemistry from that used for the bacterial biosynthesis. Labeling studies have demonstrated that the thiazole is formed from an unidentified C5 carbohydrate, glycine **3** and cysteine **11**(34–36) and that the pyrimidine **10** is formed from histidine **48** and PLP **49** (37–39). Thiamin biosynthesis in yeast requires fewer enzymes than the bacterial pathway. The biosynthesis of the thiazole requires only one protein (THI4p) in contrast to the bacterial pathway, which requires six (ThiOFSG, IscS and TenI).

All attempts to reconstitute the THI4p-catalyzed reaction, using a variety of C5 carbohydrates, initially failed. However, a breakthrough was achieved by the detection of three metabolites (**56**, **63** and **64** in Figure 5) released from the protein by heat denaturation (40, 41).

The identification of product **64** demonstrated that complete thiazole biosynthesis could be achieved using THI4p expressed in *E. coli*. In addition, this structure demonstrated that the thiazole was adenylated, suggesting that NAD **45**, and not a simple pentose, might be the donor of the C5 carbohydrate. Initial attempts to detect Thi4p-catalyzed modification of NAD failed. However, after the structure of THI4p was determined (PDB code = 3FPZ)(42) it was possible to prepare an active site mutant (C204A) that was free of the tightly bound metabolites **56**, **63** and **64** (42). This form of the enzyme catalyzed the conversion of NAD

45 and glycine **3** to **56** via intermediates **51** and **52** and confirmed NAD at the C5 carbohydrate donor. (43)

The discovery that metabolite-free THI4p could be isolated when the *E. coli* overexpression strain was grown at low iron concentrations provided a source of native enzyme with an unoccupied active site. Treatment of NAD and glycine with this form of the enzyme generated intermediate **56**. Addition of Fe(III) to this reaction mixture resulted in the transfer of sulfide from Cys205 of THI4p to generate **63** and **64**. MS analysis of the protein in this reaction mixture confirmed Cys205 as the sulfide donor.(44) These observations led to the mechanistic proposal outlined in Figure 5.

In this proposal hydrolysis of the N-glycosyl bond of NAD **45** gives **51**. Ring opening, tautomerization and imine formation give **53**. Tautomerization, loss of water and a second tautomerization generates compound **56**, the most labile of the three intermediates released in the heat denaturation experiment. Tautomerization to **57** followed by sulfide transfer from Cys205 of the THI4 protein gives **60**. Cyclization and two dehydrations gives the thiazole tautomer **63**, the second of the heat released metabolites. A final tautomerization completes the thiazole formation. Our mechanism suggests that the THI4 protein may be a single turnover enzyme. This was confirmed by demonstrating a 1:1 ratio of THI4p to thiamin produced.

In conclusion, we have explored here the mechanistic biochemistry of thiamin thiazole biosynthesis in *B. subtilis* as a representative prokaryote and in *S. cerevisiae* as a representative eukaryote. The biosynthetic routes are quite different between the two systems and the reasons for these differences are not yet known. The mechanism of thiazole biosynthesis in bacteria is at an advanced stage, while our understanding of the mechanism of thiazole biosynthesis in yeast is still growing with many unanswered questions remaining. We have not yet identified most of the residues involved in catalyzing the conversion of **45** to **64**. We also do not yet understand the role of iron in the sulfur transfer or the physiological role of inactive THI4p.

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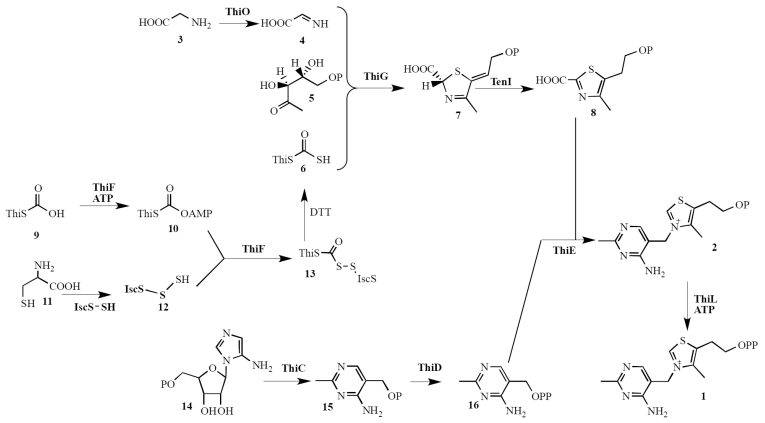

Figure 1.
The bacterial thiamin biosynthetic pathway.

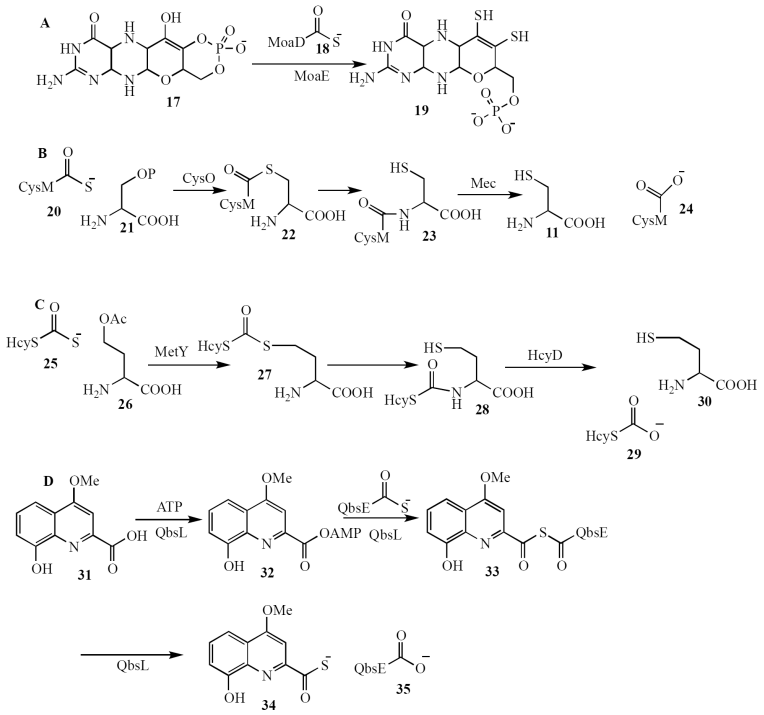

Figure 2.

Four additional examples of protein-thiocarboxylate-dependent biosynthetic pathways. A) Molybdopterin biosynthesis in bacteria, B) Cysteine biosynthesis in *Mycobacterium tuberculosis*, C) Homocysteine biosynthesis in *Wolinella succinogenes*, D) Thioquinolobactin biosynthesis in *Pseudomonas fluorescens*.

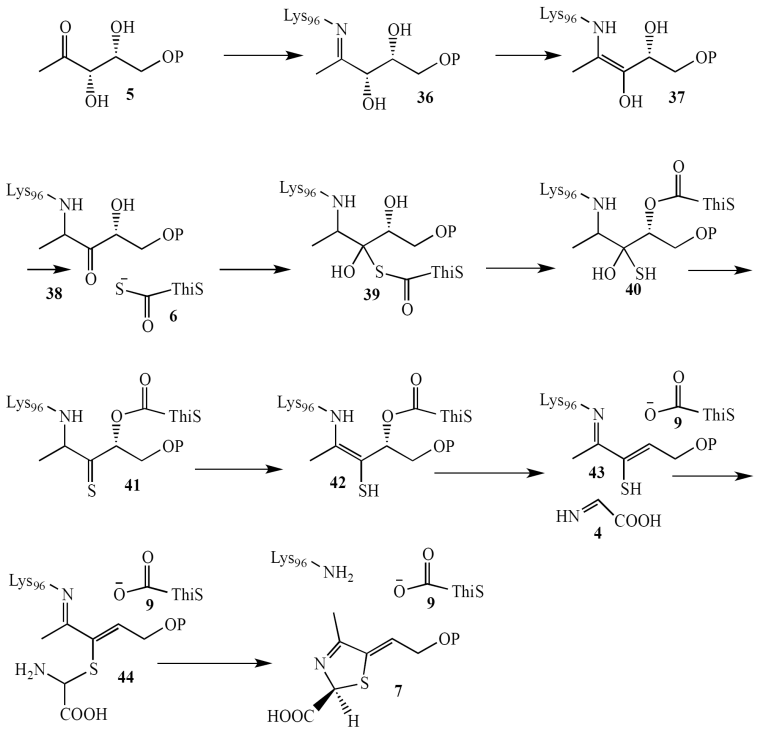

Figure 3.
Mechanistic proposal for the formation of the thiazole tautomer **7**.

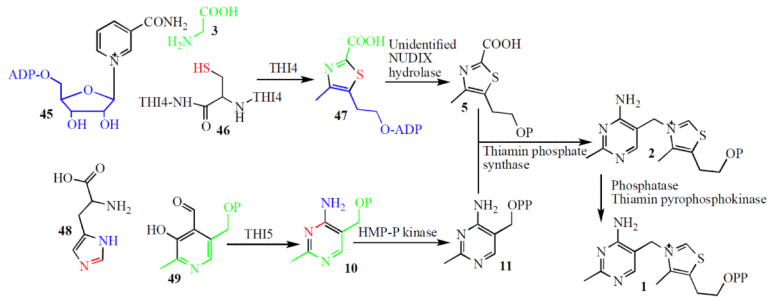

Figure 4.
Thiamin pyrophosphate biosynthesis in *S. cerevisiae*.

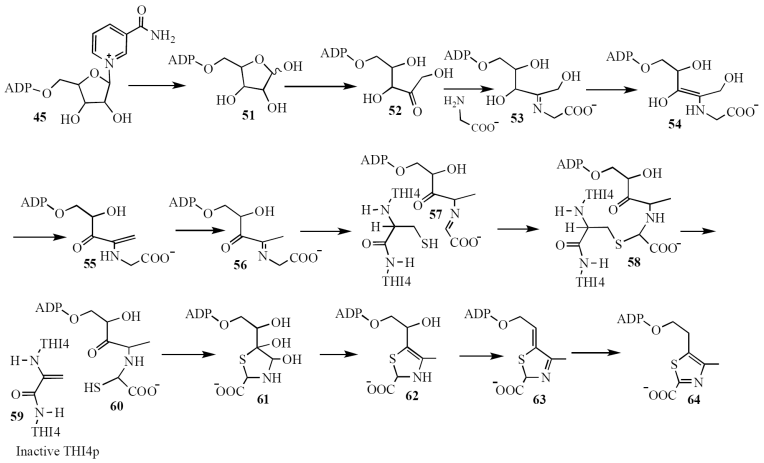

Figure 5.
Mechanistic proposal for the THI4 mediated formation of ADP-thiazole **64**.