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Cofactor biosynthesis – still yielding fascinating new biological chemistry

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INTRODUCTION

Cofactor biosynthetic pathways use a larger amount of novel organic chemistry than any of the other pathways in primary metabolism and mechanistic studies on cofactor biosynthetic enzymes continue to be a rich area of investigation. In this perspective, we will describe recent advances in the biosynthesis of thiamin, molybdopterin, pyridoxal phosphate, nicotinamide adenine dinucleotide, heme and vitamin B₁₂, with a focus on reactions that pose novel mechanistic problems.

Thiamin biosynthesis in bacteria

The bacterial thiamin biosynthetic pathway involves the separate biosynthesis of thiazole 1 and pyrimidine 2. These are then coupled to give thiamin phosphate 3. The mechanism of the coupling enzyme is now well understood: the intrinsic rate of carbocation formation has been determined and the pyrimidine carbocation intermediate has been structurally characterized. [1]

The current mechanistic proposal for thiazole formation is shown in Figure 2. DXP 5 forms an imine with Lys96 of the thiazole synthase (ThiG) to give 6. Tautomerization followed by thiocarboxylate addition to the C3 ketone of 7 gives 9, which then undergoes an S/O acyl shift followed by water loss to give thioketone 11. Tautomerization followed by elimination of the sulfur carrier protein (ThiS, 15) gives 13. Addition of the glycine imine 14, formed in a separate reaction, followed by a transamination generates the thiazole tautomer 17. A separate enzyme (TenI) is required to catalyze the aromatization of 17 to 1. This proposal is supported by product characterization, substrate analog studies, the trapping of the imine 6, the demonstration of enzyme catalyzed H/D exchange at C3 of DXP, the demonstration that a carboxy terminal oxygen of ThiS 15 is derived from DXP 5, the trapping of 13 and the structures of the ThiSG and ThiFS complexes.[2–8]

In most bacteria, glycine oxidation to give 14 is catalyzed by an oxygen-requiring flavoenzyme (ThiO).[9] In *Escherichia coli* and some other proteobacteria, in which thiamin biosynthesis can occur under anaerobic conditions, the glycine imine is formed from tyrosine in a reaction catalyzed by ThiH - a radical SAM enzyme. A mechanistic proposal is outlined in Figure 3. Hydrogen atom abstraction from tyrosine 18 gives the phenoxy radical 20, which can then

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undergo fragmentation to 22 and 23. A second electron transfer gives the glycine imine 14. [10]

The biosynthesis of the pyrimidine moiety of thiamin occurs by a remarkable rearrangement of aminoimidazole ribotide catalyzed by the ThiC gene product. The results of a comprehensive labeling study are shown in Figure 4.[11] This reaction has now been reconstituted in a defined biochemical system. The pyrimidine synthase activity was found to be dependent on a functional iron-sulfur cluster and on S-adenosyl methionine (SAM). Production of 5-deoxyadenosine from SAM during pyrimidine formation suggests that this enzyme belongs to the radical SAM superfamily of enzymes. However, the mechanism of the rearrangement reaction has not yet been elucidated.

Thiamin biosynthesis in *Saccharomyces cerevisiae*

While the later steps in thiamin biosynthesis in *Saccharomyces cerevisiae* are similar to those in bacteria, the biosynthesis of the two heterocycles is different.

Considerable progress has been made with the enzymology of thiazole formation. While the reaction catalyzed by the thiazole synthase (THI4) has not yet been fully reconstituted, the enzyme copurified with three tightly bound metabolites (35, 40 and 41) and the structure of the enzyme complexed to 41 has been determined.[12–15] The identity of these metabolites suggested that NAD is the precursor to the thiazole. This was confirmed by the identification of three partial reactions catalyzed by the C204A mutant of THI4 (28 to 29, 29 to 30 and 29 to 35 in Figure 5).[12] Combining this information suggests the mechanism of thiazole formation outlined in Figure 5.

In this mechanism, cleavage of the N-glycosyl bond of NAD 28 followed by ring opening and tautomerization gives 30. Imine formation with glycine 31, tautomerization and water loss gives 34. Two tautomerizations followed by sulfide addition gives 37 which then cyclizes to 38. Loss of two molecules of water to give 40 followed by thiazole aromatization completes the reaction. The sulfur source depicted in the conversion of 36 to 37 has not yet been unambiguously identified.

The thiamin pyrimidine in *S. cerevisiae* is derived from histidine 42 and PLP 43 in a reaction catalyzed by the THI5 gene product (Figure 6).[16] The enzymology of this remarkable process is not yet understood.

Thiamin salvage

A new pathway for the salvage of the thiamin pyrimidine from thiazole-degraded thiamin has recently been discovered (Figure 7). In this pathway, thiazolium degraded forms of thiamin (e.g. 45) bind to the periplasmic component of an ABC transporter (ThiY) and are then transported into the cell. Deformylation to 46 followed by hydrolysis gives 47, which can then be incorporated into the thiamin biosynthetic pathway. This pathway is widely distributed in bacteria, archaea and eukaryotes.[17]

Molybdopterin biosynthesis

The enzyme catalyzing the formation of Precursor Z (49) from GTP (48) is a radical SAM enzyme (Figure 8). The structure of this enzyme has been determined but the reaction mechanism is still unknown.[18]

Pyridoxal phosphate (PLP) Biosynthesis

Just at the point when we thought PLP biosynthesis was a solved problem,[19] an entirely new pathway emerged from studies on singlet oxygen resistance in *Cercospora nicotianae*. [20]

This new pathway has now been reconstituted using ribose-5-phosphate glutamine and glyceraldehyde-3-phosphate as substrates and the new PLP synthase has been structurally and mechanistically characterized.[21–24] A mechanistic proposal is outlined in Figure 9.

In this mechanism, ribose-5-phosphate 50 undergoes ring opening and forms an imine 52 with an active site lysine. Tautomerization followed by ammonia addition gives 54. Loss of water followed by a tautomerization gives 56. Elimination of lysine from C1 followed by its addition to C5 gives 58. Loss of phosphate from 58 gives 59. Addition of glyceraldehyde-3-phosphate 60 gives imine 61. A double tautomerization to 63 followed by an electrocyclic ring closure to 64 and dehydration gives 65. Imine hydrolysis completes the reaction.[25,26]

Nicotinamide adenine dinucleotide (NAD) biosynthesis

The formation of quinolinic acid 68, the precursor to the pyridine ring of NAD 28, is a longstanding unsolved problem in biosynthesis. Two routes have been identified: one involving the condensation of aspartic acid imine 66 with dihydroxyacetone phosphate 67, the other involving the oxidation of hydroxyanthranilate 69.

The condensation of the imine of aspartic acid 66 with dihydroxyacetone phosphate 67 to form quinolinic acid 68 has only recently been reconstituted using purified enzyme. This enzyme contains an iron sulfur cluster and a structure of the apoenzyme has been solved.[27,28] No mechanistic studies have yet been reported.

The mechanism for the oxidation of hydroxyanthranilate 69 to 2-amino-3-carboxymuconic acid semialdehyde 70 is now relatively well understood and proceeds by an extradiol dioxygenase type mechanism.[29] Compound 70 then undergoes a nonenzymatic tautomerization/isomerization/electrocyclization sequence followed by a dehydration to form quinolinic acid as outlined in Figure 11.[30]

Porphyrin biosynthesis

The characterization of the oxygen-independent coproporphyrinogen III oxidase (HemN) is an exciting recent advance in porphyrin biosynthesis. This enzyme catalyzes the reaction shown in Figure 12 and is a radical SAM enzyme. The structure of this enzyme has been solved and a substrate derived radical characterized by ESR spectroscopy.[31,32] The reaction is likely to proceed by hydrogen atom abstraction to give 76 followed by decarboxylation and a second electron transfer to give 78. Repetition of this sequence would give 75.

Vitamin B₁₂

The dimethylbenzimidazole ligand 87 of vitamin B₁₂ is formed from flavin mononucleotide (FMN). The enzyme catalyzing this remarkable transformation has now been identified and an early intermediate in which molecular oxygen is poised for attack on the flavin has been structurally characterized.[33] A mechanistic proposal, consistent with the structure, previous labeling studies and model chemistry is outlined in Figure 13.[34] In this mechanism, reduced FMN 79 reacts with molecular oxygen to form the hydroperoxide 80. A Baeyer-Villiger like rearrangement to 81 followed by four hydrolysis reactions gives 82. A 2-electron oxidation of 82 to bisimine 83, a retroaldol fragmentation to 84, and a cyclization would give 85. A final 2-electron oxidation followed by a tautomerization would complete the formation of the DMB ligand.

Conclusions

While our knowledge of cofactor biosynthetic pathways is now at an advanced stage, the mechanistic chemistry of several of the reactions involved remains to be elucidated. These reactions include the formation of the pyrimidine moiety of thiamin in both prokaryotes and

eukaryotes, the formation of precursor Z on the molybdopterin pathway, the formation of quinolinic acid in bacteria and the formation of the Vitamin B12 DMB ligand. It is also likely that new pathways to some of the cofactors remain to be discovered in the wealth of information now available from genome sequences and environmental-DNA libraries. In addition to the discovery of new biosynthetic chemistry our increasing understanding of cofactor biosynthetic chemistry will facilitate the production of vitamins by fermentation and the exploration of cofactor biosynthetic enzymes as antibiotic targets.

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Figure 1.

Thiamin phosphate 3 is biosynthesized by coupling the thiazole 1 with the pyrimidine 2.

Figure 2.

Mechanistic proposal for the formation of the thiamin thiazole 1 in bacteria. ThiS represents the sulfide carrier protein.

Figure 3.
Mechanistic proposal for the ThiH-catalyzed formation of glycine imine (14) under anaerobic conditions.

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Figure 5.
Mechanistic proposal for the formation of the thiamin thiazole (41) in *S. cerevisiae*.

Figure 6.

The pyrimidine moiety of thiamin in *S. cerevisiae* is formed by a remarkable reaction sequence in which the histidine atoms labeled in blue and the PLP atoms labeled in red are incorporated into the pyrimidine 44.

Figure 7.
A new thiamin salvage pathway for the recycling of the pyrimidine of thiazole-degraded thiamin.

Figure 8. MoaA and MoaC catalyze the formation of Precursor Z (49) on the molybdopterin biosynthetic pathway.

Figure 9.

A mechanistic proposal for the formation of PLP (43). Pdx1 represents the pyridoxal phosphate synthase.

Figure 10.

Shown is the major bacterial pathway for the formation of the quinolinic acid (68) precursor to NAD 28.

Figure 11.

Shown is the mechanistic proposal for the formation of quinolinic acid 68 from hydroxyanthranilate (69).

Figure 12.

Mechanistic proposal for the oxidative decarboxylation of coproporphyrinogen III (74) to protoporphyrinogen IX (75).

Figure 13.
Mechanistic proposal for the oxidation of FMN 79 to dimethylbenzimidazole 87.