Thiamine Biosynthesis

DOI: 10.1002/anie.200702554

Thiamine Biosynthesis in *Escherichia coli*: Identification of the Intermediate and By-Product Derived from Tyrosine**

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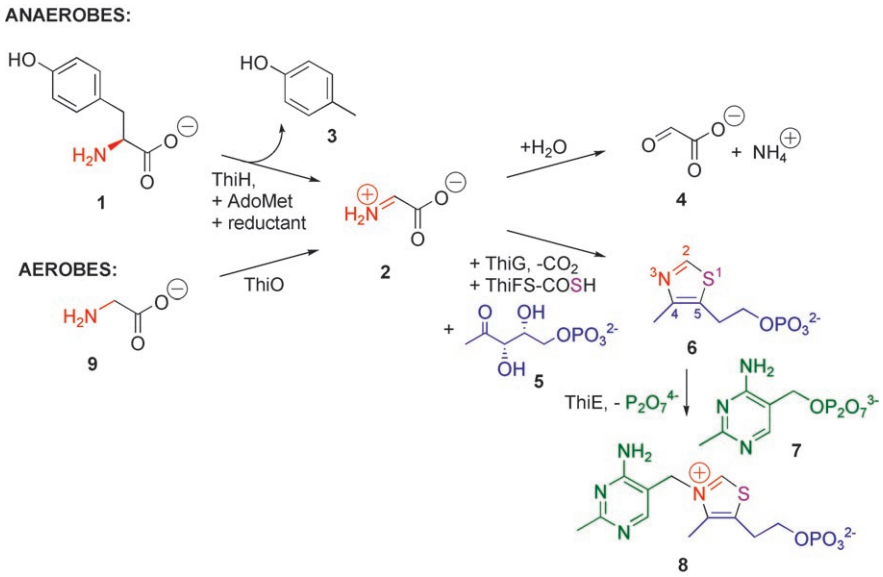
Thiamine is an essential cofactor in many metabolic pathways and is required by all living organisms. The biosynthesis of thiamine phosphate is convergent, linking two independently biosynthesized heterocyclic precursors (Scheme 1, **6** and **7**) in the final step.[1–4] The biosynthesis of the thiazole moiety of thiamine has been well characterized in the aerobe *Bacillus subtilis*,[4,5] which obtains the C2–N3 fragment of the thiazole by oxidation of glycine **9** to dehydroglycine **2** using ThiO[6] (Scheme 1). This hydrolytically unstable intermediate is incorporated into the thiazole **6** in a multistep reaction requiring the thiazole synthase (ThiG),[7] the sulfur donor ThiFS thiocarboxylate,[8,9] and 1-deoxyxylulose 5-phosphate (Dxp, **5**). Dehydroglycine **2** has been proposed as the first common intermediate for bacterial thiazole biosynthesis,[10] but microorganisms growing under anaerobic conditions probably cannot oxidize glycine. Instead anaerobes, such as *Escherichia coli,* utilize an alternative pathway forming dehydroglycine from tyrosine **1** in a ThiH-dependent reaction.[11,12] This biosynthetic step therefore requires the cleavage of the C_α–C_β bond and release of the aromatic side chain. ThiH shows sequence similarity to the "radical *S*-adenosylmethionine (AdoMet)" family of proteins,[13] including conserved ligands to an essential [4Fe-4S] cluster[14] and has been shown to form a complex with ThiG.[15] Studies on the reconstitution of the thiazole-forming reaction[10,16] with purified *E. coli* proteins have shown that ThiGH requires AdoMet and a reductant for activity, but (like other proteins in the "radical Adomet" family[17,18]) it is not catalytically active in vitro. In this paper, we elucidate the reaction products formed in vitro, demonstrating that the aromatic by-product derived from the side chain of tyrosine is *p*-cresol and the remaining fragment of tyrosine yields glyoxylate, the expected product of in situ hydrolysis of dehydroglycine. Finally, these experiments are shown to be relevant to in vivo metabolism by the detection of *p*-cresol in *E. coli* that are expressing ThiH.



Scheme 1. The biosynthesis of thiamine phosphate **8**.

To follow the fate of tyrosine in the reaction, ^{14}C-labeled tyrosine was incubated with the heterodimeric ThiGH complex, AdoMet and the natural electron-donor system consisting of flavodoxin (FldA), flavodoxin reductase (Fpr), and NADPH. After 2 h, the reaction was analyzed by thin-layer chromatography (TLC) and monitored by autoradiography (Figure 1). By using two TLC systems, two radiolabeled products with very different polarities were observed. The highly polar component co-eluted with glyoxylate (**4** in Scheme 1 and Figure 1 A) and comparison of the less polar product with several aromatic standards showed that it co-eluted with *p*-cresol (**3** in Scheme 1 and Figure 1 B). This experiment also demonstrated the absolute requirement for AdoMet as the negative control showed no activity.

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[**] This work was supported by grants from the Biotechnology and Biological Sciences Research Council (M.K. and M.R.C.), the Engineering and Physical Sciences Research Council (F.T.M.), and the Royal Society (University Research Fellowship to P.L.R.). We thank J. Street, N. Wells, G. J. Langley, and J. Herniman for help with spectroscopy and spectrometry.

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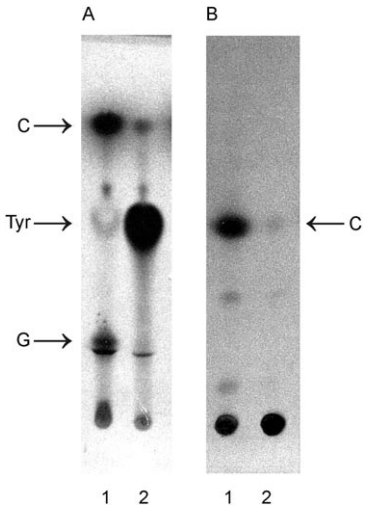


Figure 1. Autoradiograms of a ThiH assay. Two TLC systems (plates A and B) were used to resolve the products. The complete assay (lane 1 on each plate) contained L-[*U*-^{14}C]tyrosine, reconstituted ThiGH, AdoMet, and a reductant. The negative control (lane 2) lacks AdoMet. Spots corresponding to *p*-cresol, tyrosine, and glyoxylate standards are indicated by C, Tyr, and G, respectively.

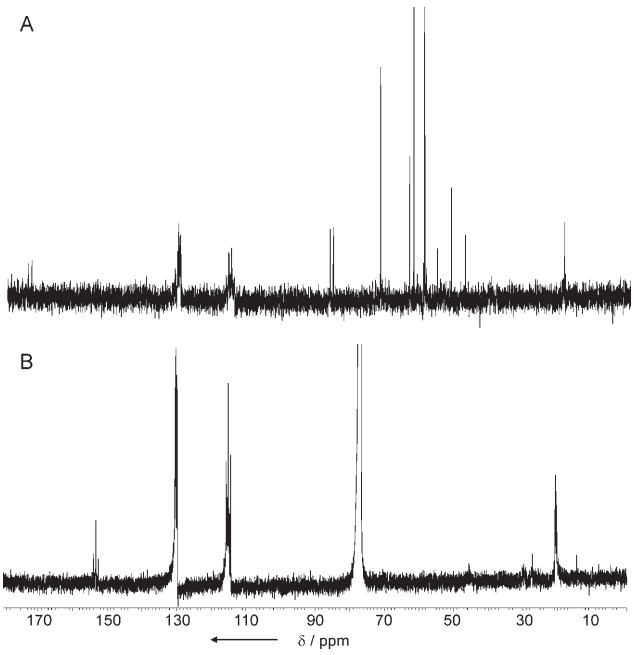
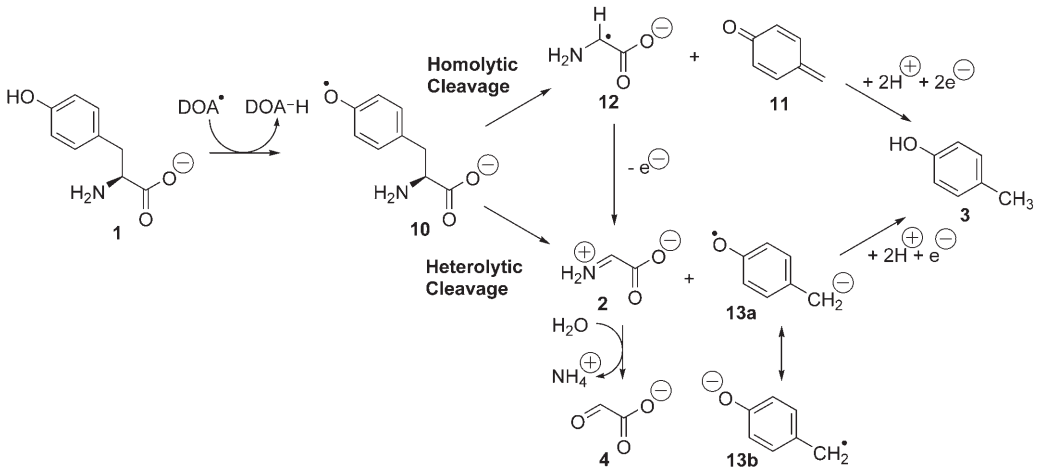


Figure 2. ^{13}C NMR spectra of ThiGH reactions. The reaction mixtures contained reconstituted ThiGH, L-[*U*-^{13}C]tyrosine, AdoMet, and a reductant, and were incubated at 37 °C for 2 h. Panel A shows the ^{13}C NMR spectrum of the supernatant obtained after the proteins were removed by acid precipitation and centrifugation. Glyoxylate gives signals at 173 and 86 ppm under these conditions, observed as doublets in here because of the ^{13}C labeling. Panel B shows the ^{13}C NMR spectrum of an organic extract prepared from the supernatant and is consistent with ^{13}C-labeled *p*-cresol [^{13}C NMR (100 MHz; $CDCl_3$) $\delta = 153.2$ (t, $J = 66$ Hz, COH), 130.1 (m, 2 CH, CCH_3), 115.0 (m, 2 CH), 20.4 ppm (m, CH_3)].

The formation of these two products was confirmed by ^{13}C NMR spectra of reactions containing [*U*-^{13}C]tyrosine. After precipitation of the protein components, the spectrum of the aqueous supernatant showed signals typical of glyoxylate (Figure 2 A). The spectrum of an organic extract prepared from this supernatant confirmed the formation of *p*-cresol (Figure 2 B). This sample was also analyzed by GCMS, which showed the retention time (13.38 min) and expected mass of *p*-cresol derived from [^{13}C]tyrosine. Taken together, the results demonstrate unequivocally that *p*-cresol and glyoxylate were produced from tyrosine during in vitro reactions.

The role of ThiH in the reaction can be explained by the mechanism proposed in Scheme 2. The reductive cleavage of AdoMet yields the highly reactive 5′-deoxyadenosyl radical, which can abstract the phenolic hydrogen atom from tyrosine. The resultant radical **10** may react by at least two pathways. Homolytic cleavage of the C_α–C_β bond yields the quinone methide **11** and the radical **12**. The alternative heterolytic pathway leads directly to **2** and the stabilized radical anion **13a**↔**13b**. To form *p*-cresol and dehydroglycine by either of these pathways requires further electron-transfer steps, which may be facilitated by the proximity of the intermediates to the [4Fe-4S] cluster in the active site of ThiH. DFT and ab initio calculations suggest that the homolytic mechanism is ther-



Scheme 2. Proposed mechanism for ThiH. The 5′-deoxyadenosyl radical (DOA•) is derived from the reductive cleavage of AdoMet.

modynamically more favorable, with increased polarity improving the relative stability of the heterolytic pathway, but not enough for this to be identified as the preferred pathway. Judicious placement of specific residues may therefore result in enough stabilization to tip this balance, and further experimental data will be required to differentiate between these pathways. The subsequent fate of the dehydroglycine **2** depends on the reaction conditions: if the components required for thiazole formation are present (including ThiG, the sulfur donor ThiFS thiocarboxylate, and Dxp), then it will be incorporated into the thiazole phosphate **6**.[10] If any of these components are absent, the dehydroglycine is likely released from the ThiGH complex into the aqueous medium, where it is rapidly hydrolyzed to glyoxylate and ammonia.

ThiH is not unique in mediating this type of C–C bond cleavage of a phenolic compound. For example, *Clostridium difficile* produces *p*-cresol by the decarboxylation of *p*-hydroxyphenylacetate using a glycyl radical enzyme *p*-hydroxyphenylacetate decarboxylase (HPAD).[19–21] It seems probable that ThiH and HPAD use different carbon-centered radicals to initiate mechanistically related cleavage reactions.

To investigate the kinetics and stoichiometry of product formation, the *p*-cresol and glyoxylate formed in a ThiH reaction were quantified using HPLC over a two-hour period (Figure 3). The reaction produced *p*-cresol and glyoxylate in an approximate 1:1 ratio over the time course and approached completion after 1 h (Table 1). In a subsequent experiment, the amount of 5′-deoxyadenosine formed after 2 h was measured as 1.3 mol equivalents relative to *p*-cresol, suggesting some uncoupled turnover of AdoMet had occurred.

To demonstrate the formation of *p*-cresol in vivo, we used a mutant *E. coli* K strain that lacks a genomic copy of *thiH*.[22] Two derivative strains of this mutant were prepared, one transformed with a ThiH expression plasmid[15] and the other with a control plasmid containing no thiamine biosynthetic genes. These strains were cultured in defined medium for 24 h, and an organic extract was prepared from the cleared medium. Comparison of the extracts by GCMS analysis (Figure 4) revealed a new peak at 13.38 min, corresponding to *p*-cresol, in the sample derived from the ThiH-expressing cells. In contrast *p*-cresol was not detectable in the negative control. Using cultures of an *E. coli* B strain and [3,3-$^2H_2$]tyrosine, White[23] reported the isolation of 4-hydroxybenzyl alcohol as a by-product from thiamine biosynthesis, a result that seemingly contradicts our observations. However,

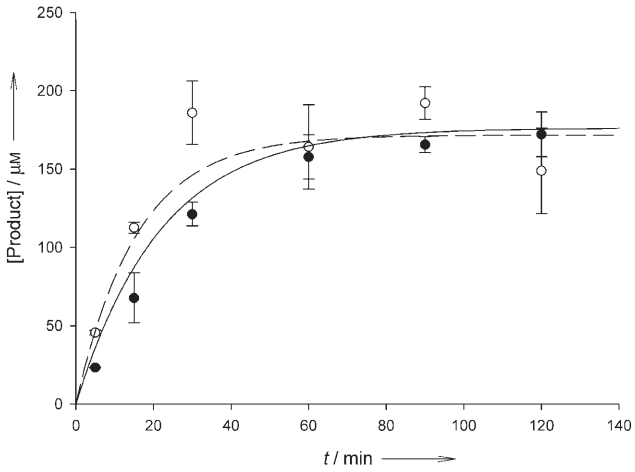


Figure 3. Time course of product formation in a ThiH assay. The graph shows the increase in the concentration of *p*-cresol (○, dashed line) and glyoxylate (●, solid line) with time. The assays, which contained reconstituted ThiGH complex, tyrosine, AdoMet, and a reductant, were incubated for the indicated time at 37 °C.

Table 1: Kinetic parameters for product formation. Data from Figure 3 were fitted to first-order processes to determine the calculated final concentration of products and the apparent first-order rate constant. Results are shown with standard errors.

Product	Rate constant [10^{-2} min^{-1}]	Final conc. [μM]
p-cresol	4.6 ± 0.7	176.2 ± 7.8
glyoxylate	6.3 ± 1.1	171.6 ± 7.9

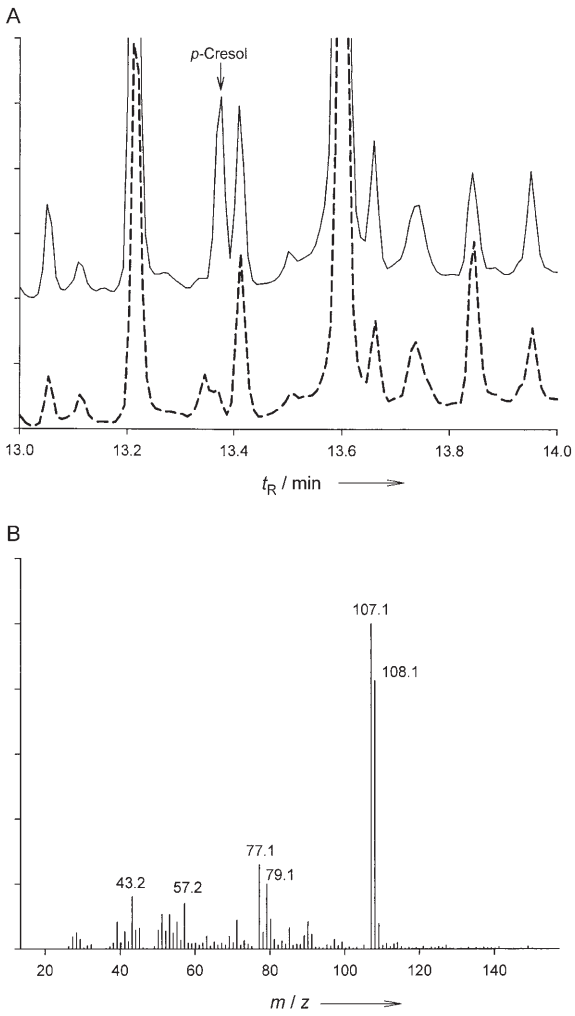


Figure 4. Evidence for the ThiH-dependent formation of *p*-cresol in vivo. A) Section of the GCMS chromatogram of an extract of medium from *E. coli* cultures that express ThiH (solid line, displaced upwards for clarity) and the negative control sample (no ThiH, dashed line). The *p*-cresol peak at 13.38 min is marked. B) The mass spectrum corresponding to the new peak at 13.38 min showing the expected M^+ signal for *p*-cresol (*m*/*z* 108.1).

E. coli B strains (but not K strains) express an oxidase that can accept *p*-cresol as a substrate.[24] It is therefore probable that tyrosine cleavage in *E. coli* produces *p*-cresol, but those strains with a suitable oxidase convert *p*-cresol to *p*-hydroxybenzyl alcohol.

In conclusion, we have characterized the products of tyrosine cleavage in the presence of the "radical Adomet" protein ThiH, AdoMet, and a reductant. The side chain of tyrosine is converted to *p*-cresol during this reaction. The remaining fragment of tyrosine likely forms the intermediate dehydroglycine, which can be combined with Dxp and a sulfur donor during thiazole biosynthesis in vivo. During in vitro experiments that lacked these other thiazole precursors, glyoxylate was observed to accumulate from the hydrolysis of the dehydroglycine.

Received: June 12, 2007
Published online: October 30, 2007

Keywords: biosynthesis · enzyme mechanisms · radicals · thiamine

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