Communications to the Editor

Biosynthesis of Thiamin I: The Function of the *thiE* Gene Product

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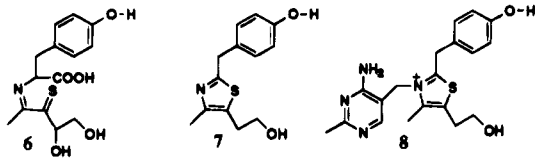
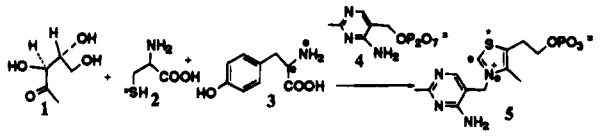
The proposed thiamin phosphate (**5**, vitamin B_1) biosynthetic pathway in *Escherichia coli*[1] and in the closely related *Salmonella typhimurium*[2] is outlined in Scheme 1. The thiazole moiety is formed from 1-deoxy-D-*threo*-2-pentulose (**1**),[3] cysteine (**2**),[4] and tyrosine (**3**).[5] The pyrimidine **4** is formed from 5-aminoimidazole ribonucleotide[6] and is coupled with the thiazole to give thiamin phosphate (**5**). A final phosphorylation gives the biologically active form of the cofactor. None of the enzymes involved in this important biosynthetic pathway have been mechanistically characterized because of their very low levels in wild-type *E. coli*. As a first step toward such mechanistic studies, a cluster of five genes from *E. coli* (*thiCEFGH*), involved in the biosynthesis of thiamin, has recently been cloned and characterized.[7] The *thiC* gene product is required for the synthesis of the pyrimidine. The *thiFGH* gene products are required for the synthesis of the thiazole. Here we report the function of the *thiE* gene product.

During the biosynthesis of thiamin, the *p*-hydroxybenzyl group of tyrosine is cleaved.[8] Three possible points of cleavage can be considered: before thiazole assembly (e.g., from **6** or from a related thiazole precursor), after thiazole assembly but before coupling to the pyrimidine (e.g., from **7**), and after coupling to the pyrimidine (e.g., from **8**). The latter possibility

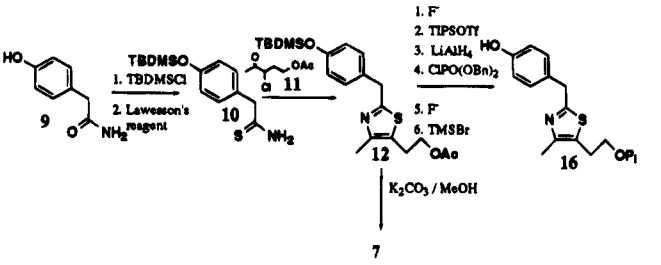


was particularly attractive, as it involved the formation of the well-characterized, stabilized thiamin ylide as the leaving group.[9] Because **6** and related intermediates are likely to be chemically unstable in aqueous solution and therefore unsuitable for phenotypic testing, our strategy for determining the point of cleavage of the *p*-hydroxybenzyl group involved testing **7**, **8**, and **16** as thiamin precursors.

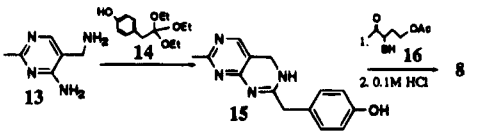
Scheme 1



Scheme 2



Scheme 3



The synthetic routes to **7**,[10] **8**,[11] and **16**[12] are summarized in Schemes 2 and 3.

When **7** was tested for its ability to overcome the thiazole requirement of *E. coli* mutated in the *thiE*, *thiF*, *thiG*, and *thiH* genes, we found that the *thiF* $^-$, *thiG* $^-$, and *thiH* $^-$ strains all grew on this compound, while the *thiE* $^-$ mutants required the unphosphorylated form of the thiazole **18** (or thiamin) for growth. This suggested that the *thiE* gene product was required for the cleavage of the *p*-hydroxybenzyl group from **7**. However, cleavage activity was not detected when enzymatic assays were carried out using **7** or **16** as substrates. The possibility that *thiE* catalyzed the cleavage of the *p*-hydroxybenzyl group from **8** was considered next. Again, cleavage activity was not detected in enzymatic assays. However, we observed that *E. coli* mutated in the *thiE*, *thiF*, *thiG*, and *thiH* genes grew on **8**. This suggested that, while **8** appeared to be a stable compound in aqueous buffer at pH = 7, a low level of cleavage, sufficient to sustain growth of the thiamin mutants, was occurring during the feeding experiment. This also suggested that the *thiE* gene product might catalyze the coupling of the pyrimidine **4** and the thiazole **16** to give **17**, which might then undergo nonenzymatic conversion to **5** (Scheme 4, path a).

The purified enzyme, isolated from a high-level overexpression strain,[13] had a specific activity of 1nmol/mg/min for the coupling of **16** and **4**.[14] This low specific activity, coupled with the observed stability of **8**, raised serious doubts that the coupling of **4** and **16** was the physiological reaction. We therefore tested **18** as a substrate and found that the specific

(1) For a useful review, see: (a) Brown, G. M.; Williamson, J. M. In *Escherichia coli* and *Salmonella typhimurium*; Neidhardt, F. C., Ed.; American Society for Microbiology: Washington, DC, 1987. (b) Young, D. *Nat. Prod. Rep.*, **1986**, 395.

(2) The thiamin biosynthetic pathway appears to be identical in both microorganisms.

(3) David, S.; Estramareix, B.; Fischer, J.-C.; Therisod, M. *J. Chem. Soc., Perkin Trans.* 1, **1982**, 2131.

(4) (a) Tazuya, K.; Yamada, K.; Nakamura, K.; Kumaoka, H. *Biochim. Biophys. Acta* **1987**, *924*, 210. (b) DeMoll, E.; Shive, W. *Biochem. Biophys. Res. Commun.* **1985**, *132*, 217.

(5) Estramareix, B.; Therisod, M. *Biochim. Biophys. Acta* **1972**, *273*, 275.

(6) Estramareix, B.; David, S. *Biochim. Biophys. Acta* **1990**, *1035*, 154.

(7) Vander Horn, P.; Backstrom, A.; Stewart, V.; Begley, T. P. *J. Bacteriol.*, **1993**, *175*, 982.

(8) White, R. *Biochim. Biophys. Acta* **1979**, *583*, 55.

(9) Haake, P. In *Enzyme Mechanisms*; Page, M. I., Williams, A., Eds.; Royal Society of Chemistry: London, 1987; Chapter 19.

(10) (a) Benner, S. *Tetrahedron Lett.* **1981**, *22*, 1851. (b) Schellenberger, A.; Heinroth, I.; Hubner, G. *Hoppe-Seyler's Z. Physiol Chem.* **1967**, *348*, 506. Compound **11** was a gift from Hoffmann-La Roche.

(11) Contant, P.; Forzy, L.; Hengartner, U.; Moine, G. *Helv. Chim. Acta* **1990**, *73*, 1300.

(12) (a) Kenner, G.; Todd, A.; Weymouth, F. *J. Chem Soc.* **1952**, 3675. (b) McKenna, C.; Schmidhauser, J. *J. Chem. Soc., Chem. Commun.* **1979**, 739. The TBDMS ether was not stable under the phosphorylation conditions.

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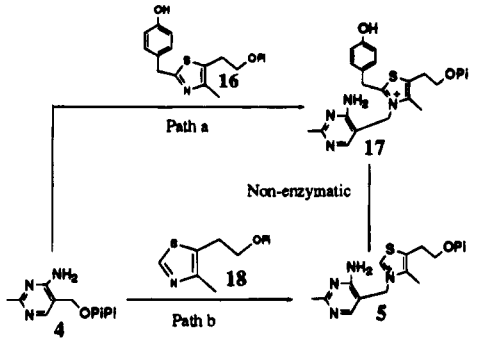
Scheme 4



activity increased 600-fold, suggesting that the coupling of **18** and **4** (Scheme 4, path b) is the reaction catalyzed by the *thiE* gene product *in vivo*.[15] The *p*-hydroxybenzyl group is therefore removed prior to the formation of the thiazole moiety (e.g., from **6** or from a related thiazole precursor).[16] The K_m for the pyrimidine pyrophosphate **4** and for the thiazole **18** are 1 and 2 μM, respectively, and k_{cat} is 14 min^{-1}.[17] The enzyme is monomeric with a molecular weight of 23 kDa. The high levels of overexpression and the small size of this enzyme make it an attractive system for further mechanistic studies.

Acknowledgment. We thank Anne Rammelsberg for developing an improved synthesis of **4**, Drs. Dolf van Loon, Hans-Peter Hohmann, and Gerard Moine of Hoffmann-La Roche for providing the synthetic intermediates **11** and 2-methyl-4-amino-5-chloromethylpyrimidine, and Colleen Costello and Dr. John Helmann for helpful discussions. This work was supported by a grant from the National Institutes of Health (DK44083).

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(13) The *thiE* gene was overexpressed in *E. coli* BL21 (DE3) by insertion into the pET-17b expression vector. The enzyme can be readily purified by ammonium sulfate fractionation, followed by gel filtration (Sephadex G 50) and anion (DEAE) exchange chromatography. Backstrom, A.; Chiu, H.-J.; Begley, T. P. Unpublished results.

(14) Thiamin was detected using the thiochrome assay. Leder, I. *J. Biol. Chem.* **1961**, *236*, 3066. This assay involves oxidation of thiamin under basic conditions to the highly fluorescent thiachrome and requires the C2 position of the thiazolium to be unsubstituted. As thiamin **5** and *p*-hydroxybenzylthiamin **8** can be detected with equal sensitivity in this assay, nonenzymatic cleavage of the p-hydroxybenzyl group occurs readily under the assay conditions.

(15) The coupling enzyme (2-methyl-4-amino-5-(hydroxymethyl)pyrimidine diphosphate:4-methyl-5-(2-phosphoethyl)thiazole 2-methyl-4-aminopyrimidine-5-methenyl transferase, EC 2.5.1.3) has been previously purified (5000-fold) from yeast. The identification of the point of cleavage of the *p*-hydroxybenzyl group is not an issue, in yeast as the N1 and C2 atoms are derived from glycine. Leder, I. *J. Biol. Chem.* **1961**, *236*, 3066. A similar coupling enzyme activity has been detected at low levels in *E. coli* and has been partially purified. Kawama, Y.; Kayasaki, T. *Arch. Biochem. Biophys.* **1973**, *158*, 242. The cloning of the yeast coupling enzyme gene was reported while this paper was in press. Nosaka, K.; Nishimura, H.; Kawasaki, Y.; Tsujihara, T.; Iwashima, A. *J. Biol. Chem.* **1994**, 30510. Zurlinden, A.; Schweingruber, E. *J. Bacteriol.* **1994**, 6631.

(16) Pyrimidine transferase mutants were previously assumed to require thiamin for growth since they are unable to couple the thiazole and the pyrimidine. This assumption is clearly not valid for the *thiE*$^-$ strain since this mutant grows on thiazole. A possible explanation is that the *thiE* mutation results in an increase in the K_m for **18**. This may have been compensated for in the feeding experiment, which used a relatively high concentration of thiazole. Vander Horn, P.; Backstrom, A.; Stewart, V.; Begley, T. P. *J. Bacteriol.*, **1993**, *175*, 982.

(17) The syntheses of the thiazole **18** and the pyrimidine **4** have been previously described. (a) Leder, I. *Methods Enzymol.* **1970**, *18A*, 166. (b) Brown, G. *Methods Enzymol.* **1970**, *18A*, 162.