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MINI-REVIEW

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Thiamin biosynthesis in prokaryotes

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Abstract Twelve genes involved in thiamin biosynthesis in prokaryotes have been identified and overexpressed. Of these, six are required for the thiazole biosynthesis (*thiFSGH*, *thiI*, and *dxs*), one is involved in the pyrimidine biosynthesis (*thiC*), one is required for the linking of the thiazole and the pyrimidine (*thiE*), and four are kinase genes (*thiD*, *thiM*, *thiL*, and *pdxK*). The specific reactions catalyzed by ThiEF, Dxs, ThiDM, ThiL, and PdxK have been reconstituted in vitro and ThiS thiocarboxylate has been identified as the sulfur source. The X-ray structures of thiamin phosphate synthase and 5-hydroxyethyl-4-methylthiazole kinase have been completed. The genes coding for the thiamin transport system (*thiBPQ*) have also been identified. Remaining problems include the cloning and characterization of *thiK* (thiamin kinase) and the gene(s) involved in the regulation of thiamin biosynthesis. The specific reactions catalyzed by ThiC (pyrimidine formation), and ThiGH and ThiI (thiazole formation) have not yet been identified.

Key words Thiamin · Biosynthesis · Prokaryotes · Transport · Kinase · Mechanism · Structure

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Introduction

Thiamin is an essential component of the human diet, with a required daily allowance of 1.4 mg. Thiamin is added to many commercial foods since, unlike microorganisms, humans cannot synthesize this vitamin. Annual production, by chemical synthesis, is on the order of 3,300 tons (Burdick 1998). Thiamin-dependent enzymes play a particularly important role in carbohydrate metabolism and include transketolase, α-ketoacid decarboxylase, α-ketoacid dehydrogenase, and acetolactate synthase. Our understanding of this class of enzymes is sophisticated. In all cases studied, the mechanistic role of thiamin is to stabilize the acyl carbanion (Schowen 1998). In contrast, our understanding of the mechanistic enzymology of thiamin biosynthesis is still at a relatively early stage (Begley 1996; Estramareix and David 1996; Spenser and White 1997).

The thiamin biosynthesis pathway in prokaryotes is outlined in Fig. 1, and the status of the gene/enzyme characterization is summarized in Table 1. The numbers in bold in the text refer to the numbered compounds in Figs. 1–3. Thiamin biosynthesis involves the separate formation of the pyrimidine (**12**) and the thiazole (**8**). These are then coupled to form thiamin phosphate (**14**). Largely due to available molecular genetic techniques, studies on thiamin biosynthesis are most advanced in *Escherichia coli*, *Salmonella typhimurium*, and *Bacillus subtilis*. Based on genome sequence comparisons, it is likely that the pathway in these three microorganisms is a paradigm for thiamin biosynthesis in other prokaryotes.

Studies on thiamin biosynthesis are significant for at least three reasons. First, since this vitamin is a required component of the human diet, it is important to understand how it is biosynthesized. Second, the biosynthetic pathway involves an unusually large amount of unprecedented chemistry. The mechanism of the complex oxidative condensation reaction to give the thiazole (**8**) and the remarkable rearrangement involved in the conversion of AIR (5-aminoimidazole ribotide, **10**) to the pyrimidine (**11**) have not yet been elucidated. Finally, biosynthetic studies will facilitate the construction of overexpression

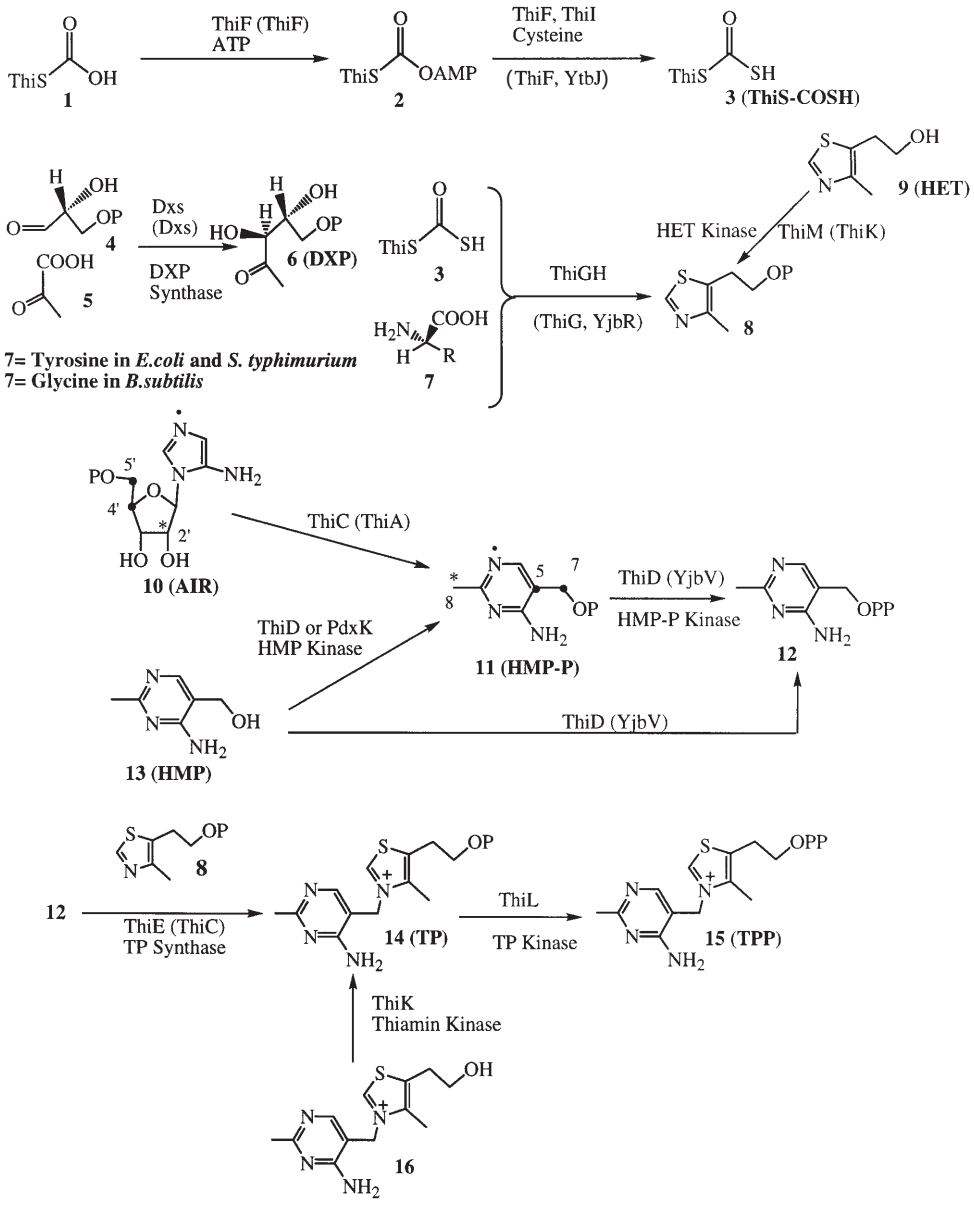

Fig. 1 Thiamin pyrophosphate (**15**) biosynthesis in prokaryotes (*Escherichia coli, Salmonella typhimurium*, and *Bacillus subtilis*). The *B. subtilis* gene names are given *in parenthesis*. YjbS is the *B. subtilis* homolog of ThiS. The marked atoms of **11** indicate the origin of these atoms in **10**

strains that may be of use for the commercial production of thiamin or its components by fermentation.

Gene organization

As can be seen in Table 1, the genes involved in thiamin biosynthesis, salvage, and transport are scattered throughout the chromosome. Thus far, genes that have been identified in *E. coli* and in *S. typhimurium* comprise three operons and four single gene loci. The sequence of the *thiK* gene has not been identified; thus, its physical structure is unknown. Each of the *thi* operons is transcriptionally regulated by thiamin pyrophosphate (**15**), whereas the single gene loci are not (Webb et al. 1996; Petersen and Downs 1997; Webb and Downs 1997; Webb et al. 1997, 1998). It is not surprising that *dxs* and *thiI* are not regulated because both gene products are involved in additional biosynthetic pathways (Hill et al. 1996; Arigoni et al. 1997; Sprenger et al. 1997; Eisenreich et al. 1998; Mueller et al. 1998). The lack of transcriptional regulation of *thiL* may indicate that the primary point of regulation in thiamin pyrophosphate synthesis is prior to the formation of thiamin phosphate (**14**). Alignment of sequences upstream of several genes regulated by thiamin has defined the consensus for a proposed "thi box" (Miranda-Rios et al. 1997; E. A. Webb and D. Downs, unpublished work). However, standard genetic screens have thus far failed to identify the protein predicted to be involved in the regulation of thiamin biosynthesis. In *B. subtilis*, two thiamin biosynthesis operons and a single gene locus have been characterized (Zhang and Begley1997; Zhang et al. 1997; K. Eichler, S. Taylor, C. Vockler, Y. Zhang, V. Delague, T. P. Begley, and A. P. G. M. van Loon, unpublished work).

Table 1 Characterization of genes involved in thiamin biosynthesis, salvage and transport in prokaryotes

Gene	Map position	Purified?	Reaction reconstituted	Source and reference
thiC (*thiA*)	90.4[a] 90.0[b] 81.6[c]	Yes	No	[a] Vander Horn et al. (1993), Costello (1996), Kelleher et al. (1998); [b] Webb et al. (1996); [c] Zhang and Begley (1997)
thiE (*thiC*)	90.4[a] 90.0[b] 335.7[c]	Yes	Yes	[a] Vander Horn et al. (1993), Backstrom et al. (1995), Kelleher et al. (1998); [b] Webb et al. (1996); [c] Zhang et al. (1997)
thiF (*thiF*)	90.3[a] 90.0[b] 105[c]	Yes (With ThiS)	Yes	[a] Vander Horn et al. (1993), Taylor et al. (1998a), Kelleher et al. (1998); [c] K. Eichler, S. Taylor, C. Vockler, Y. Zhang, V. Delague, T.P. Begley, and A.P.G.N. van Loon (unpublished work)
thiS (*yjbS*)	90.3[a] 90.0[b] 105[c]	Yes	Yes	[a] Vander Horn et al. (1993), Taylor et al. (1998a), Kelleher et al. (1998); [c] K. Eichler, S. Taylor, C. Vockler, Y. Zhang, V. Delague, T.P. Begley, and A.P.G.N. van Loon (unpublished work)
thiG (*thiG*)	90.3[a] 90.0[b] 105[c]	Yes (With ThiS)	No	[a] Vanderhorn et al. (1993), Kelleher et al. (1998); [b] Webb et al. (1996); [c] K. Eichler, S. Taylor, C. Vockler, Y. Zhang, V. Delague, T.P. Begley, and A.P.G.N. van Loon (unpublished work)
thiH (*yjbR*)	90.3[a] 90.0[b] 105[c]	Yes	No	[a] Vanderhorn et al. (1993), Kelleher et al. (1998a); [b] Webb et al. (1996); [c] K. Eichler, S. Taylor, C. Vockler, Y. Zhang, V. Delague, T.P. Begley, and A.P.G.N. van Loon (unpublished work)
thiI (*ytbJ*)	9.5[a] 9.65[b] 258.5[c]	Yes	No	[a] C. Kinsland and T.P. Begley (unpublished work) [b] Webb et al. (1997)
thiM (*thiK*)	47.1[a] 46.6[b] 335.8[c]	Yes	Yes	[b] Petersen and Downs (1997) [c] Zhang et al. (1997)
thiD (*yjbV*)	47.0[a] 46.6[b] 333[c]	Yes	Yes	[a] Kinsland and Begley (1998, unpublished work) [b] Reddick et al. (1998), Petersen and Downs (1997)
thiL	9.4[a] 10[b]	No	Yes	[b] Webb and Downs (1997)
thiK	25[a]	No	Yes	[a] Fujio et al. (1990)
dxs	9.5[a]	Yes	Yes	[a] Sprenger et al. (1997), Lois et al. (1998)
thiBPQ	1.5[a] 1.5[b]	No	Yes	[b] Webb et al. (1998)

[a] *E.coli*
[b] *Salmonella typhimurium*
[c] *Bacillus subtilis*; the *B. subtilis* genes are given in parentheses

Phenotypic analysis directed early efforts to determine the function of the various gene products. Loss of function mutations in *thiI*, *thiF*, *thiG*, or *thiH* results in a thiazole auxotrophy, mutations in *thiC* result in a nutritional requirement for the pyrimidine, and mutations in *thiD* result in a requirement for thiamin. Surprisingly, although null mutants of *thiE* require thiamin, an *E. coli thiE* mutant that requires only thiazole has been identified (Backstrom et al. 1995). It is likely that this strain contains a mutant ThiE protein with an increased K_m for thiazole. Mutations in *thiL* result in a requirement for thiamin pyrophosphate, and mutations in *dxs* have not yet been constructed in *E. coli*. In contrast, lesions in *thiM*, *thiK*, or *thiBPQ* are prototrophic, consistent with the role of these gene products in thiamin salvage and/or transport.

Formation of 5-hydroxyethyl-4-methylthiazole phosphate (8)

Labeling studies in *E. coli* and *S. typhimurium* have demonstrated that the thiazole (**8**) is formed from tyrosine (**7**; Estramareix and Therisod 1972; Bellion et al. 1976; White and Rudolph 1978), deoxy-D-xylulose (**6**, without the phosphate; David et al. 1982; Himmeldirk et al. 1996), and cysteine (De Moll and Shive 1985; Tazuya et al. 1987a). In *B. subtilis*, the thiazole is formed from glycine rather than tyrosine (Tazuya et al. 1987b). The de novo biosynthesis of this small molecule is complex and involves at least six gene products (ThiFSGH, ThiI, and Dxs), with an additional gene product (ThiM) involved in salvage of the thiazole from the medium.

DXP synthase (Dxs) catalyzes the condensation of glyceraldehyde 3-phosphate (**4**) and pyruvate (**5**) to give 1-deoxy-D-xylulose-5-phosphate (**6**, DXP). This enzyme

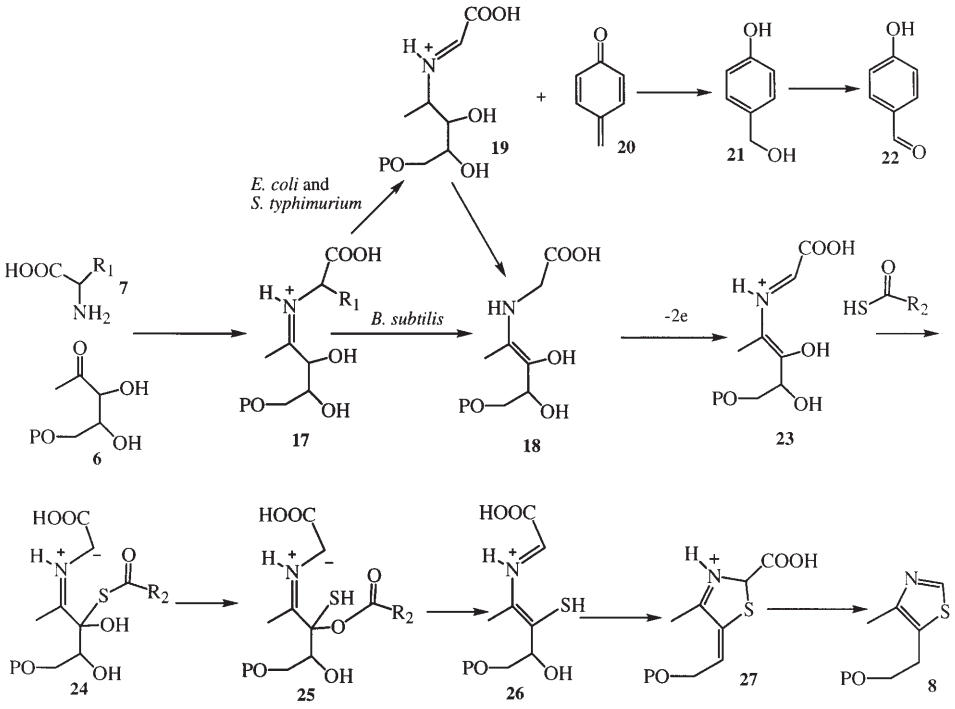

Fig. 2 Mechanistic proposal for the formation of the thiazole (**8**). For *Escherichia coli* and *Salmonella typhimurium*, **7** = tyrosine and R_2 = ThiS; for *Bacillus subtilis*, **7** = glycine and R_2 = YjbS

utilizes thiamin pyrophosphate as a cofactor (Sprenger et al. 1997; Lois et al. 1998). DXP or DX is also required for the biosynthesis of pyridoxal (Himmeldirk et al. 1996) and for the biosynthesis of terpenes by the nonmevalonate pathway (Arigoni et al. 1997; Eisenreich et al. 1998; Kuzuyama et al. 1998).

The ThiF/ThiS complex, when isolated from an overexpression strain, is post-translationally modified by conversion of the carboxy terminus of ThiS to a thiocarboxylate (Taylor et al. 1998a). In contrast, ThiF/ThiS isolated from a *thiI* mutant lacks this post-translational modification (Taylor et al. 1998 a). This suggests that ThiS thiocarboxylate (**3**) is the sulfur source for the thiazole formation and that ThiI catalyzes one of the reactions involved in the transfer of the sulfur of cysteine to ThiS. This key intermediate has been overexpressed using the intein cleavage system (Kinsland et al. 1998). The complexity of ThiS–COSH as a sulfur source may reflect the need to keep small reactive intermediates bound to a protein complex.

Thiocarboxylate formation has been reconstituted in a cell-free system using ^{35}S-cysteine. ThiF catalyzes the adenylation of ThiS to give **2**. The sulfur transfer from ^{35}S-cysteine to **2** is also catalyzed by ThiF and by an additional as yet uncharacterized enzyme in the *E. coli* crude extract. This protein is now the limiting factor in achieving efficient sulfur transfer (Taylor 1998; Taylor et al. 1998 a; J. Xi, S. Taylor, and T. Begley, unpublished work). In contrast to the in vivo production of ThiS–COSH, ThiI is not required for the in vitro reaction. The proposed dual function of ThiF is analogous to the function of the ubiquitin-activating enzyme E1. This enzyme also catalyzes an adenylation/thioester formation sequence on ubiquitin and shows high sequence similarity to ThiF (Hershko and Ciechanover 1992; Rajagopalan 1996).

In vitro reconstitution of the thiazole biosynthesis has not yet been achieved. When DOX-P (**6**; Taylor et al. 1998b), U^{14}C-tyrosine (**7**), and ThiS–COSH (**3**) were incubated in the presence of *E. coli* cell-free extract, overproduced ThiFSGH, and ThiI, thiazole formation was not detected using a TLC/autoradiography assay. However, tyrosine cleavage to give 4-hydroxybenzyl alcohol (**21** in Fig. 2) and 4-hydroxybenzaldehyde (**22**) was observed. Since 4-hydroxybenzyl alcohol (**21**) has been previously identified as a byproduct of thiamin biosynthesis in *E. coli* (White 1979), it is likely that the aldehyde is formed in an oxidation reaction that is not related to the thiazole biosynthesis. To further localize the blocked reaction, the reconstitution experiment was repeated using ThiFS–CO^{35}SH. Analysis of this reaction mixture by SDS-PAGE/autoradiography demonstrated that none of the components of the reaction mixture catalyzed the release of ^{35}S from ThiS–CO^{35}SH (J. Xi, S. Taylor, and T. Begley, unpublished work).

A mechanistic proposal for the formation of the thiazole is outlined in Fig. 2. This proposal suggests that **21** is formed by loss of quinone methide from **17** and that sulfur transfer from ThiS–COSH to **18** does not occur because **18** is in the wrong oxidation state. Thiazole formation from **3**, **6**, and **7** is a two-electron oxidation; none of the purified components in the reaction mixture (ThiFSGH, ThiI) contain a redox cofactor, and addition of NAD(P)$^+$ to the reaction mixture did not result in thiazole formation. It is possible that ThiH is the missing oxidase. This protein contains an iron sulfur cluster sequence motif, but the overproduced protein does not contain any cofactor. The thiazole biosynthesis cluster in *B. subtilis* does not contain a ThiH homolog. Instead, YjbR shows significant sequence similarity to sarcosine oxidase, to other D-amino

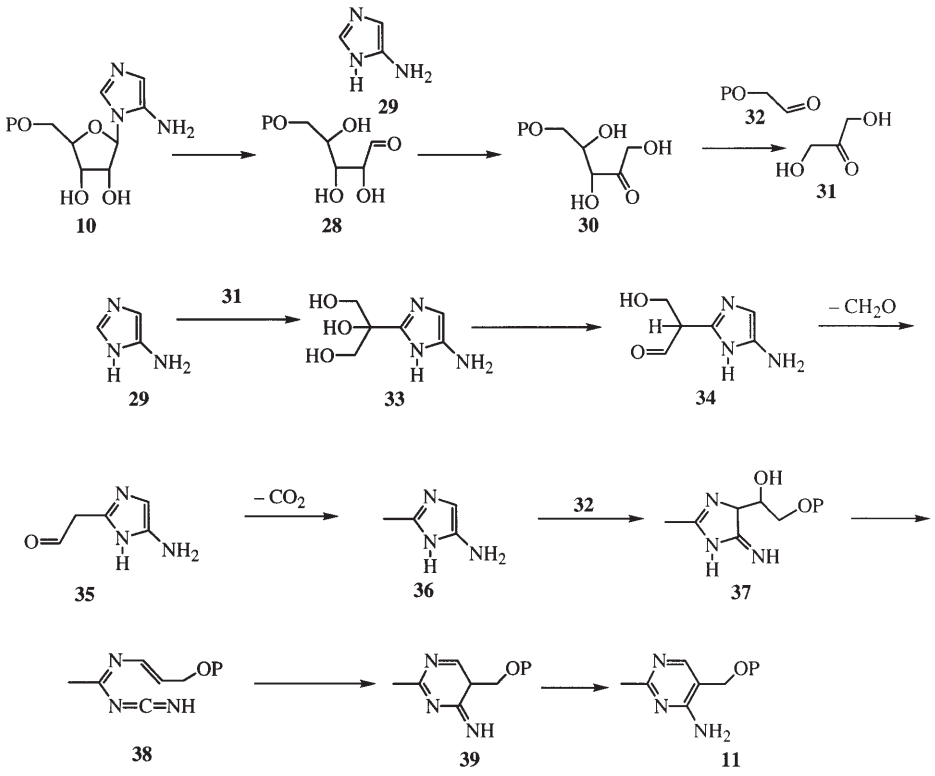

Fig. 3 Mechanistic proposal for the formation of the pyrimidine (**11**)

acid oxidases, and to the recently identified ThiO from *Rhizobium etli* (Miranda-Rios et al. 1997). This suggests that YjbR may catalyze the oxidation of **18** to **23**, which could then react with the thiocarboxylate to give **24**. Thioester hydrolysis followed by ring closure and decarboxylation would give the thiazole (**8**).

In addition to the de novo biosynthesis of the thiazole phosphate (**8**), thiazole alcohol (**9**) can be incorporated from the medium into the thiamin pool by the *thiM* gene product, which catalyzes its phosphorylation (Fig. 1). The ThiM homolog from *B. subtilis* (ThiK) has been overproduced and characterized, and the crystal structure has been solved (Zhang et al. 1997; N. Campobasso, T. Begley, and S.E. Ealick, unpublished work).

Formation of 4-amino-5-hydroxymethyl-2-methyl pyrimidine pyrophosphate (12)

The pyrimidine phosphate (**11**) is formed from 5-aminoimidazole ribotide (**10**, AIR), an intermediate in de novo purine biosynthesis. This reaction involves a complex rearrangement in which C5, C7, and C8 of the pyrimidine **11** are derived from the 4′ (or 5′), 5′ (or 4′), and 2′ ribose carbons of **10** (Estramareix and Therisod 1984; Estramareix and David 1990; Himmeldrik et al. 1998). ThiC (ThiA) is the only required gene product that has been identified for the pyrimidine biosynthesis in *E. coli*, *S. typhimurium*, and *B. subtilis* (Costello 1996; Zhang and Begley 1997). However, overproduced ThiC (ThiA) did not catalyze any detectable reaction involving AIR (**10**) or the close biosynthetic relatives of AIR on the purine biosynthetic pathway (Costello 1996). The inability to detect a biochemical activity for ThiC (ThiA) suggests that an additional enzyme is required to convert AIR (**10**) to the substrate for ThiC (ThiA). A mechanistic proposal for the conversion of AIR to the pyrimidine that is consistent with the labeling studies is outlined in Fig. 3.

ThiD catalyzes the phosphorylation of **11** and is essential for the synthesis of thiamin (Fig. 1; Petersen and Downs 1997). The recent demonstration that this enzyme also catalyzes the phosphorylation of **13** makes it likely that *thiD* is the affected gene in mutants that lack this second activity; therefore, *thiD* could be the gene previously described as *thiJ* (Mizote et al. 1996; Reddick et al. 1998). This latter kinase reaction is likely to be involved in the salvage of **13** from the culture medium and is therefore nonessential for thiamin biosynthesis. The product of the *pdxK* gene, pyridoxine kinase, is also able to catalyze the phosphorylation of compound **13** (HMP), but the physiological significance of this redundancy is not clear (Reddick et al. 1998).

The biosynthesis of the pyrimidine has been the focus of extensive genetic analysis in *S. typhimurium*. This work has determined that in the absence of the first enzyme in purine biosynthesis, phosphoribosyl pyrophosphate amidotransferase (PurF), the pyrimidine can be synthesized under some growth conditions using a novel, as yet undefined mechanism for formation of phosphoribosyl amine (Downs and Roth 1991; Enos-Berlage and Downs 1999). Analysis of thiamin synthesis in strains lacking the PurF enzyme have clearly demonstrated that a

variety of cellular functions impinge on synthesis of the pyrimidine under these conditions (Enos-Berlage and Downs 1996; Petersen et al. 1996; Beck et al. 1997; Petersen and Downs 1996; Beck and Downs 1998). In most cases, these effects are specific to conditions where the synthesis of AIR is independent of PurF.

Formation of thiamin pyrophosphate (15)

Thiamin phosphate synthase catalyzes the coupling of the pyrimidine pyrophosphate (**12**) and the thiazole phosphate (**8**) to give thiamin phosphate (**14**) (Fig. 1; Backstrom et al. 1995). Mechanistic and structural studies have been carried out on the *B. subtilis* enzyme because the overproduced *E. coli* enzyme forms inclusion bodies.

The crystal structure of thiamin phosphate synthase, complexed with the reaction products, shows that the pyrophosphate and the thiazole moiety are on opposite sides of the pyrimidine and that the pyrophosphate is stabilized as a leaving group by coordination to a magnesium ion and by extensive electrostatic and hydrogen bonding interactions with the enzyme (H. J. Chiu, J. J. Reddick, T. P. Begley, and S. E. Ealick, unpublished work). These interactions are similar to the enzyme pyrophosphate interactions found in epi-aristolochene synthase (Starks et al. 1997) and farnesyl pyrophosphate synthase (Kellog and Poulter 1997), two enzymes that generate carbocations from pyrophosphate esters. This suggests that thiamin phosphate formation may occur by an SN1 mechanism. This is supported by the demonstration that the enzyme is very sensitive to perturbations that are designed to destabilize the putative pyrimidine cation/pyrophosphate anion pair. Replacement of the methyl group of the pyrimidine pyrophosphate with a trifluoromethyl group greatly reduces the catalytic activity (> 10^3-fold reduction; Nicewonger 1997). Replacement of Ser-130, which forms a hydrogen bond to the oxygen linking the pyrophosphate and the pyrimidine, also greatly reduced the catalytic activity (> 10^4-fold reduction for the S130A mutant).

A final phosphorylation, catalyzed by thiamin phosphate kinase (ThiL), gives thiamin pyrophosphate (**15**), the biologically active form of the vitamin (Webb and Downs 1997). A point mutation in *thiL* that fails to cause a nutritional requirement but reduces repression of transcription by thiamin has been characterized. This mutant is thought to be defective in the phosphorylation of thiamin phosphate, as evidenced by the ninefold increase in thiamin phosphate levels in the mutant strain (Webb et al. 1996). Mutants defective in thiamin kinase have also been identified, and the affected gene has been designated *thiK*, although its sequence has not been determined. This enzyme is likely to be involved in the salvage of thiamin (**16**) from the growth medium because in prokaryotes the biosynthetic product (**14**) is already phosphorylated.

Transport of thiamin and its phosphoesters

A locus encoding an ABC transporter required for transport of thiamin pyrophosphate has been identified in *S. typhimurium* (Webb et al. 1998). Uptake assays determined directly that the *thiBPQ* genes were required for uptake of both thiamin and thiamin pyrophosphate. Additional genetic experiments were consistent with this system also transporting thiamin phosphate.

Conclusions and perspective

Twelve genes involved in thiamin biosynthesis in prokaryotes have been identified and overexpressed. Of these, six are required for the thiazole biosynthesis (*thiFSGH*, *I*, and *dxs*), one is involved in the pyrimidine biosynthesis (*thiC*), one is required for the linking of the thiazole and the pyrimidine (*thiE*), and four are kinases (*thiD*, *thiM*, *thiL*, and *pdxK*). The specific reactions catalyzed by ThiEF, Dxs, ThiD, ThiM, ThiL, and PdxK have been reconstituted in vitro and ThiS–COSH has been identified as the sulfur source for the thiazole formation. The X-ray structures of thiamin phosphate synthase and 5-hydroxyethyl 4-methylthiazole kinase have been completed. The genes coding for the thiamin transport system (*thiBPQ*) have been identified. The genes coding for ThiK and the protein(s) involved in the regulation of thiamin biosynthesis have not yet been identified.

The reactions involved in the assembly of the thiazole and the pyrimidine moieties of thiamin involve unprecedented chemistry and have not yet been reconstituted in vitro. While it is relatively easy to identify the phenotype of mutations in ThiC, ThiGH, and ThiI, it is considerably more difficult to identify the specific reactions catalyzed. This large knowledge gap between sequence information and function for enzymes involved in biosynthetic pathways involving unprecedented reactions is a major challenge facing the field of functional genomics. For *E. coli*, the best-studied microorganism, only 63% of the reading frames have identified functions, and many of these functions correspond to phenotypes rather than to specific reactions. The rapid progress made in the biosynthesis of thiamin over the past five years illustrates that the narrowing of this gap is a fertile research area at the interface of chemistry and biology.

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