Characterization of thiL, Encoding Thiamin-monophosphate Kinase, in Salmonella typhimurium*

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Thiamin pyrophosphate is an essential cofactor that is synthesized de novo by Salmonella typhimurium. In bacteria, the end product of the de novo biosynthetic pathway is thiamin monophosphate, which is then phosphorylated by thiamin-monophosphate kinase (EC 2.7.4.16) to form thiamin pyrophosphate. We have isolated and characterized the thiL gene of S. typhimurium and showed that thiL is a 978-base pair open reading frame encoding a 35-kDa protein with thiamin-monophosphate kinase activity. thiL was located in the 10-centisome region of the S. typhimurium chromosome. We demonstrated that altered thiamin-monophosphate kinase activity resulted in decreased repression of transcription of thiamin pyrophosphate-regulated thiamin biosynthetic genes. In contrast to other thi loci, thiL is not transcriptionally regulated by thiamin pyrophosphate. This result is consistent with a dual role for ThiL in de novo biosynthesis and in salvage of exogenous thiamin.

Thiamin pyrophosphate (TPP) is an essential cofactor for a number of well characterized enzymes in the cell (e.g. pyruvate dehydrogenase (EC 1.2.4.1), α-ketoglutarate dehydrogenase (EC 1.2.4.2), and acetolactate synthase (EC 4.1.3.18). Despite the important role it plays in metabolism, the TPP biosynthetic pathway and its regulation are not well understood in any organism.

Thiamin consists of a 4-amino-5-hydroxymethylpyrimidine (HMP) moiety, and a 4-methyl-5-(α-hydroxyethyl)thiazole (THZ) moiety (see Fig. 1). Phosphorylated derivatives of these two moieties (HMP3P and THZ3P) are condensed to form thiamin monophosphate (TMP), the product of de novo thiamin biosynthesis in both bacteria and yeast. This condensation is catalyzed by thiamin-phosphate pyrophosphorylase (EC 2.5.1.3), the product of the thiE gene in Escherichia coli (1) and of the thi6 gene in Saccharomyces cerevisiae (2). After the formation of TMP, the strategies to generate TPP differ between enterics and yeast. In yeast, TMP is first dephosphorylated to generate thiamin, and TPP is then formed by the addition of a thiamin pyrophosphate group from ATP in a step catalyzed by thiamin pyrophosphokinase (EC 2.7.6.2), encoded by the thi80 gene in S. cerevisiae (3). In contrast, in both E. coli and Salmonella typhimurium, TMP is directly phosphorylated to generate TPP by action of thiamin-monophosphate kinase (EC 2.7.4.16) (4). Thus, unlike yeast, E. coli and S. typhimurium require a salvage enzyme (thiamin kinase (EC 2.7.1.99), encoded by thiK (4)) to incorporate exogenous thiamin into the TPP pools (Fig. 1) (for review, see Ref. 5).

Previous work demonstrated that cell-free extracts of wild-type E. coli contained thiamin-monophosphate kinase activity, and mutants lacking this activity were subsequently isolated. These mutants required TPP for growth and defined the thiL locus (6). Hfr mapping experiments located thiL at centisome (Cs) 10 on the E. coli chromosome (4), and it was proposed that this locus encoded thiamin-monophosphate kinase.

During work on the regulation of thiamin biosynthesis in S. typhimurium, we isolated a point mutation (thiR927) that eliminated the ability of exogenous thiamin or TMP to repress transcription of the thi-operon located at 90 Cs (7). This mutation did not, however, affect the ability of TPP to repress transcription of this operon (7). Since both thiR927 and thiL mapped to the 10 Cs region, our results were consistent with thiR927 being an allele of thiL that resulted in altered enzyme activity. We suggested that thiR mutants had lower TPP pools resulting in constitutive expression of genes normally repressed by TPP. To test this model, characterization of thiL was initiated in S. typhimurium.

We report here the identification of the thiL gene at Cs 9.5 on the S. typhimurium chromosome. We show herein that the thiL gene encodes a 35-kDa protein with thiamin-monophosphate kinase activity. Further, we demonstrated that the thiR927 mutation is an allele of thiL resulting in a glycine to aspartate change at position 132 of the predicted amino acid sequence of the protein, and thus we re-designated this allele thiL927. This work supports the hypothesis that TPP is the regulatory molecule for thiamin synthesis in S. typhimurium and predicts the existence of a sensor/regulatory protein.

EXPERIMENTAL PROCEDURES

Bacterial Strains

All strains used in this study are derivatives of S. typhimurium LT2, unless noted, and are listed in Table I. MudJ is used throughout the paper to refer to the MudI1734 transposon, which has been described (8), and Tn10d(Tc) refers to the transposition defective mini-Tn10(Tn10d16317) (9).

Culture Media and Biochemicals

No-carbon source E medium supplemented with 1 mM MgSO4 and 11 mM glucose was used as minimal media (10, 11). Difco nutrient broth (8 g/liter) with NaCl (5 g/liter) added was used as rich medium. Luria broth was used for experiments involving plasmid manipulation. Difco BiTek agar (15 g/liter) was added for solid medium. Thiamin and its phosphoesters were added, where indicated, to a final concentration of 100 nM. Antibiotics were added as needed to the following concentra-
Thiamin Synthesis in S. typhimurium

The biosynthetic pathway for TPP in S. typhimurium is schematically represented. Enzymatic steps generating HMP and THZ are presently unknown, but labeling studies have shown the indicated metabolites to be involved in the pathway. THZ-P and HMP-P are condensed by ThiE (thiamin-phosphate pyrophosphorylase) to form TMP, which is then phosphorylated by ThiL (thiamin-monophosphate kinase) to form the coenzymic form of thiamin, TPP.

Genetic Methods

Transduction Methods—All transductional crosses were performed by using the high frequency transducing bacteriophage P22 mutant HT 1051/1 int-201 (12) as described (13). Transductants were purified and identified as phage-free by cross-streaking on green plates (14).

Mutant Isolation—Strains auxotrophic for thiamin pyrophosphate (TPP) were isolated by insertional mutagenesis with one of two defective transposons; Thn10(Tc) or MudJ elements. The complete insert from pThiL1.8 was ligated into T7 overexpression vector (Qiagen, Madison, WI). The complete plasmid was purchased from Fisher Biotech (Chicago, IL).

Molecular Biological Techniques

Recombinant DNA Techniques—Plasmid DNA was isolated by either alkaline lysis or a QIAprep Spin Plasmid kit (Qiagen, Chatworth, CA), and was electroporated into different backgrounds using the E. coli Pulser (Bio-Rad). Standard methods were used for DNA restriction and ligation. Restriction endonucleases and ligase were purchased from New England Biolabs, Inc. (Beverly, MA) in a Thermylone Temp-Thermocycler. Reaction conditions were as follows: 95 °C denaturation for 1 min, 55 °C annealing for 1 min, and 72 °C extension for 1 min. Primers used were: thiL1-4 (5'-GACAAAGATGTTGATCCACCTATAC-3'), which hybridizes to the 66-bp inverted repeat Tn10 sequence; MudL (5'-ATCCGGAATACTCAGATAATCC-3'), which hybridizes to the left end of the MudJ insertion; MudR (5'-GAAACGCCCCTGCTTTTTGTGCG-3'), which hybridizes to the right side of the MudJ, and ThiL-2–40 (5'-GACGACTGCACGATT-3') and ThiL-2–48 (5'-GTGATCGTGTCACGACAAAATCG-3'), which annealed downstream of the thiL, respectively, and whose relative locations are indicated in Fig. 2.

β-Galactosidase Assays

β-Galactosidase assays were performed using the Miller method (24) as described previously (25).

Overexpression and Visualization of ThiL

The complete insert from pThiL1.8 was ligated into T7 overexpression vectors pT7-5 and pT7-6 (26) using an EcoRI-PstI double digest (sites shown in Fig. 2). The resulting plasmids, pThiL-5 and pThiL-6, were electroporated into E. coli strain BL21(DE3), generating strains DM2571 and DM2572, respectively. These strains contain the T7 RNA polymerase in a λ lysogen, under the control of an IPTG-inducible promoter. The induction protocol was as follows. Strains were grown shaking at 37 °C to 60 Klett units, IPTG was added to 400 μM, and incubation was continued at 37 °C for 3.5 h. After this time, cells were pelleted, resuspended in 2 ml of 50 mM Tris-HCl (pH 7.5), and sonicated for 40 s (2 × 20) at 50% duty cycle using a model 550 sonic dismembrator (Fisher, Itasca, IL). Membrane and soluble fractions were separated by centrifugation at 40,000 × g for 1 h. Proteins from both supernatant and pellet fraction were separated by 12% SDS-PAGE and visualized by Coomassie Blue staining.

Thiamin-monophosphate Kinase Assay

Thiamin-monophosphate kinase was assayed by a modification of a previously described protocol (6). The assay mixture contained 1 mM TMP, 5 mM ATP, 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, and 0.35 mM KCl. The reaction was started by the addition of ~0.16 mg of protein to a final volume of 200 μl in 1.5-ml Eppendorf tubes. The reaction mixture was incubated for 30 min at 37 °C. To precipitate the protein after the incubation, 10 μl of 1.6 M HCl was added, and the reaction mixture was analyzed using the Genetics Computer Group (GCG) (Madison, WI) DNA sequence analysis program, BLAST (22), and SeqEd (Applied Biosystems Inc., Foster City, CA).

The thiL297 mutation was sequenced as follows. ThiL was amplified from the chromosome of a strain containing the thiL297 mutation (strain DM946) using primers ThiL-48–2 and ThiL-40–2 under conditions described below for PCR amplification. This amplification produced a 1.2-kb fragment containing thiL, which could be visualized on a 2% agarose gel. This fragment was further purified using QIAquick Gel Extraction Kit (Qiagen) and was entirely sequenced on both strands at least 3 times.

Mapping Insertions by PCR—Insertions were mapped by modifying a PCR mapping protocol, described elsewhere (23). Amplification between two primers was performed using Vent (exo-) polymerase (New England Biolabs, Inc., Beverly, MA) in a Thermolyne Temp-Thermocycler. Reaction conditions were as follows: 95 °C denaturation for 1 min, 55 °C annealing for 1 min, and 72 °C extension for 1 min. Primers used were: ThiL-48a (5'-GACAAAGATGTTGATCCACCTATAC-3'), which hybridizes to the 66-bp inverted repeat Tn10 sequence; MudL (5'-ATCCCGGAATACTCAGATAATCC-3'), which hybridizes to the left end of the MudJ insertion; MudR (5'-GAAACGCCCCTGCTTTTTGTGCG-3'), which hybridizes to the right side of the MudJ, and ThiL-2–40 (5'-GACGACTGCACGATT-3') and ThiL-2–48 (5'-GTGATCGTGTCACGACAAAATCG-3'), which annealed downstream of the thiL, respectively, and whose relative locations are indicated in Fig. 2.

Size of amplified fragments was determined by using agarose gel electrophoresis with standard size markers.

β-Galactosidase Assays

β-Galactosidase assays were performed using the Miller method (24) as described previously (25).
was incubated at 100 °C for 15 min. Addition of 10 μl of 1 M KOH neutralized the sample, and centrifugation removed the precipitated proteins. Thiamin and its phosphoesters in the supernatant were derivatized to thiochromes by mixing 150 μl of the assay supernatant with 250 μl of 2.65% KFe(CN)6 in 0.01 N NaOH.  

Quantitation of TMP and TPP

TMP and TPP concentrations in strains DM3286 (zaj-8048: Tn10d(Tc)) and DM3287 (zaj-8048::Tn10d(Tc)thiL927) were determined via a modification of CNBr thiochrome derivatization, (27). Briefly the protocol is as follows. Cells were grown in minimal medium to 100 Klett units and pelleted, resuspended in 1 ml of 0.1 M HCl, and divided into two aliquots. One aliquot was used to determine dry weight, and the other was adjusted to pH 2.0 with 1 M HCl and boiled for 20 min. The sample was then centrifuged at 30,000 × g for 20 min to remove cellular debris. The supernatant was used for quantitation via derivatization to TMP and TPP by Waters Millenium software and quantitated utilizing a standard curve generated with Waters Millenium detector set at 375 nm (excitation) and 432 nm (emission). The Elution of thiochromes was monitored with a Waters 990 spectrofluorometer.

RESULTS

Isolation of thiL Mutations—Six independent insertions (2 Tn10d(Tc) and 4 MudJ), which caused a TPP auxotrophy, were identified by screening 10,000 independent insertion mutants. Transductional analysis found that all six insertions were −50% co-transducible with P22 with an insertion in thiL, a locus known to be located at 9.6 Cs (28).

Cloning of thiL—To confirm that the above insertions were in a single gene that was thiL, a complementing clone was isolated using MudP/Q technology (see “Experimental Procedures”). Plasmid pThiL, contained a 7.2-kb fragment and complemented DM1683 (thiL933::Tn10d(Tc)). This plasmid was sequentially subcloned to more precisely localize thiL (Fig. 2). Digestion of pThiL with SacI released a 3-kb band that is contained in pThiL3.0. A Sau3A partial digest and self ligation of pThiL3.0 generated pThiL1.8. Arrows show the relative positions of primers Thil-48–2, Thil-213 and Thil-72, for spans indicated by horizontal bars (Fig. 2).

Identification of thiL ORF—The insert of pThiL1.8 was sequenced completely, and a 1173-bp contiguous sequence be-
All of the insertions were in the 978-bp ORF. The identification resulted in a product separated by approximately 1 kb of unsequenced DNA.

Recent analysis of the internal sequence of pThiL-1.8 identified two putative ORFs initiating at nucleotide 1 or 388 and molecular mass of 35 kDa for ThiL.

ThiL contains thiamin-monophosphate kinase activity. The thiamin-monophosphate kinase assay was performed as described under "Experimental Procedures." TPP formed in the reaction after 30 min was derivatized to a fluorescent thiochrome with KFe(CN)₆, separated from substrate by HPLC, and quantitated by comparison to a known TPP standard. Shown are HPLC tracings from reaction mixes performed with extracts of strains DM2571 (lane A) and DM2572 (lane B).

Overexpression of ThiL—To confirm our assignment of the 978-bp ORF to the thiL gene, we cloned the entire insert from E. coli strains DM2571 and DM2572, which contain the T7-RNA polymerase and plasmids pThiL-5 and pThiL-6, respectively. Following the induction, proteins in the crude extract were then subjected to T7-specific expression. The overexpressed protein correlated well with the predicted molecular mass of 35 kDa for ThiL.
ThiL Has Thiamin-monophosphate Kinase Activity—To show that thiL encoded thiamin-monophosphate kinase, activity assays were performed on crude extracts of DM2571 and DM2572 after induction of T7 polymerase. The assays were performed by providing TMP and ATP as substrates and measuring TPP accumulation after 30 min of incubation (see “Experimental Procedures”). Crude extract from strain DM2572 had a specific activity of 9.01 ± 0.90 nmol TPP formed/mg of protein/min, whereas strain DM2571 had 1.93 ± 0.80 nmol TPP formed/mg of protein/min, when data from three independently performed experiments were analyzed. Representative HPLC tracings detecting the products of this assay are shown in Fig. 5. A small peak with the retention time of TPP is visible in the control tracing in Fig. 5. Control experiments determined that this peak reflected a contaminant in the TMP purchased from Sigma.

thiR927 Is an Allele of thiL—Results presented above confirmed that thiL encoded thiamin-monophosphate kinase, and we sought to determine if the point mutation previously designated thiR927 was an allele of thiL, as we had proposed. The thiL gene from DM946 (thiL927) was amplified from the chromosome and sequenced. A comparison of the resulting sequence to wild-type thiL using SeqEd identified thiL927 as a missense mutation in thiL at residue 132, causing a glycine (GGT) to an aspartate (GAT) change in the predicted amino acid sequence. The affected codon is underlined in Fig. 3. The A to G transition was consistent with the use of hydroxylamine, a mutagen, to generate this mutation (7).

TMP and TPP Quantitation in thiL927—A simple explanation for the regulatory phenotype caused by thiL927 would be that it resulted in an enzyme unable to maintain optimal TPP pools. To test this prediction, an isogenic pair of strains, DM3286 (zaj-8048::Tn10d(Tc)) (A) and DM3287 (zaj-8048::Tn10d(Tc) thiL927) (B) monitoring fluorescence of the generated thiochromes.

FIG. 6. thiL927 increases the TMP/TPP ratio. TMP and TPP concentrations were quantitated by modifying a reported procedure (27) (J. Enos-Berlage, unpublished results) as described under “Experimental Procedures.” Shown are HPLC tracings of the derivatized extracts from strains DM3286 (zaj-8048::Tn10d(Tc)) (A) and DM3287 (zaj-8048::Tn10d(Tc) thiL927) (B) monitoring fluorescence of the generated thiochromes.

1.77 ± 0.36 and 0.242 ± 0.31 for DM3287 and DM3286, respectively, and are shown in Fig. 6 as representative HPLC tracings.

Transcriptional Regulation—The transcription of thiL was investigated since some genes involved in thiamin biosynthesis are transcriptionally regulated in S. typhimurium (7). Regulation of thiL was tested in a strain containing insertion thiL953::Mud that had been shown by PCR amplification to be in the correct orientation for transcription from the thiL promoter. To account for the possibility that ThiL may be involved in its own expression, transcriptional analysis was performed in a strain that also contained pThiL1.8. Strain DM3154 (thiL953::MudJ/pThiL1.8) was grown in minimal medium in the presence and absence of exogenous thiamin, TMP, or TPP (100 μM), and β-galactosidase activity was measured. In all media, a basal level of ~50 Miller units was obtained, indicating that thiL was in a class of thiamin biosynthetic genes not transcriptionally regulated by TPP.

DISCUSSION

Results presented herein showed that thiamin-monophosphate kinase in S. typhimurium is encoded by thiL, a 978-bp ORF that produces a 35-kDa protein. The chromosomal location of the thiL gene in S. typhimurium was determined genetically and physically to be 9.5 Cs.

This work also showed that altered ThiL activity can cause a regulatory phenotype. The increased TMP/TPP ratio found in the thiL927 containing strain was consistent with the mutant enzyme being unable to increase TPP pools to repressing levels, even when supplied with exogenous thiamin. Such a scenario explains the lack of thi gene repression found in strains carrying this lesion when grown in excess thiamin. The similar TPP pools maintained by the mutant and wild-type strains after growth on minimal medium is consistent both with the lack of thi derepression in the mutant strain carrying allele thiL927 and the lack of an obvious growth defect. Based on the measured pool sizes and phenotypic considerations, the thiL927 mutation appears to result in a protein with either an altered equilibrium or subject to increased allosteric inhibition by TPP.

Interestingly, thiL was not transcriptionally regulated by

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TPP, making it the second of four characterized *thi* loci that appear to be constitutively transcribed. Identification of previously isolated mutations that cause regulatory effects as alleles of *thiL* predicts that there is a regulatory protein involved in the transcription of thiamin genes. Such a protein remains to be identified.

At this time, thiamin biosynthesis is not well understood in any organism. Although in both yeast and enteric bacteria TMP is the product of *de novo* synthesis, the conversion of TMP to TPP differs in these organisms. Unlike *E. coli* and *S. typhimurium*, the yeast fail to directly phosphorylate TMP to form TPP, but rather dephosphorylate it to form thiamin prior to adding a pyrophosphate group to generate TPP. This reaction in *S. cerevisiae* is catalyzed by thiamin pyrophosphokinase and is encoded by the TPP repressible gene *thi80* (3). In contrast, we have shown that in *S. typhimurium* the terminal step in TPP synthesis, encoded by *thiL*, is not transcriptionally regulated by thiamin or its phosphoesters. These data emphasize two important differences between the terminal reactions in TPP biosynthesis in *S. typhimurium* and *S. cerevisiae*. Not only do the two organisms utilize different substrates (TMP and thiamin, respectively), they regulate the step in distinct ways. The physiological relevance of these differences remains to be clarified with additional work on thiamin biosynthesis in both organisms.

REFERENCES

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