Thiamine Biosynthesis in Escherichia coli

IN VITRO RECONSTITUTION OF THE THIAZOLE SYNTHASE ACTIVITY

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The biosynthesis of thiamine in Escherichia coli requires the formation of an intermediate thiazole from tyrosine, 1-deoxy-d-xylulose-5-phosphate (Dxp), and cysteine using at least six structural proteins, ThiFSGH, IscS, and ThiH. We describe for the first time the reconstitution of thiazole synthase activity using cell-free extracts and proteins derived from adenosine-treated E. coli 83-1 cells. The addition of adenosine or adenine to growing cultures of Aerobacter aerogenes, Salmonella typhimurium, and E. coli has been shown previously to relieve the repression by thiamine of its own biosynthesis and increase the expression levels of the thiamine biosynthetic enzymes. By exploiting this effect, we show that the in vitro thiazole synthase activity of cleared lysates or desalted proteins from E. coli 83-1 cells is dependent upon the addition of purified ThiGH-His complex, tyrosine (but not cysteine or 1-deoxy-d-xylulose-5-phosphate), and an as yet unidentified intermediate present in the protein fraction from these cells. The activity is strongly stimulated by the addition of S-adenosylmethionine and NADPH.

Thiamine pyrophosphate (TPP), 1 also known as vitamin B 1, is an essential cofactor for several enzymes involved in carbohydrate metabolism. All organisms able to produce the vitamin initially assemble the corresponding monophosphate (TP) by coupling 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate (Hmp-PP) and 4-methyl-5-(β-hydroxyethyl) thiazole phosphate (Thz-P) (Fig. 1) (1). TP is then converted to the biologically active form, TPP, either by direct phosphorylation (in enteric bacteria) or by de-phosphorylation to thiamine followed by pyrophosphorylation (in aerobic bacteria and yeast) (2). The two hetrocyclic precursors of thiamine pyrophosphate, Hmp-PP and Thz-P, are biosynthesized through independent pathways. In Escherichia coli and other enteric bacteria, Hmp-PP derives from 5-aminoimidazole ribotide (AIR), a precursor shared between thiamine and de novo purine biosyntheses (3, 4). ThiC is the only enzyme known so far to be required for the rearrangement of AIR to Hmp-P (5), which is subsequently further phosphorylated to Hmp-PP by ThiD. The Thz-P is assembled from Tyr (6–8), Cys (9), and Dxp (10, 11). ThiF-SGH (1, 5), ThiH (12, 13), and IscS (14) are all involved in the thiazole formation.

Cysteine has been identified as the precursor of the thiazole sulfur atom. The sulfur atom is initially transferred to IscS, a pyridoxal phosphate-dependent cysteine desulfurase (15), in the form of a persulfide of an active site Cys residue (16). It is likely that in vivo the sulfur atom is then passed to ThiH (12) as a persulfide of residue Cys-456 (13). The sulfur atom is then transferred to the C terminus of ThiS, converting it to a thio-carboxylic acid (14, 17). This final sulfur transfer reaction requires the activation of ThiS as its acyladenylate (17). The adenylation reaction is catalyzed by ThiF, which has been observed to form a complex with ThiS (17). In addition, an unusual acyl-persulfide link between ThiS thio-carboxylic acid and Cys-184 of ThiP has been observed, and mutagenesis studies suggest it may be important for thiazole formation (18).

Recent studies with purified Bacillus subtilis proteins have resulted in the first demonstration of thiazole biosynthesis in a bacterial cell-free system (19). Although B. subtilis shares some metabolic components with E. coli, including some of the biosynthetic proteins (ThiFSG, ThiI, and IscS) and two metabolic precursors (Dxp and Cys), the biosynthetic pathways also differ significantly. Whereas B. subtilis depends upon ThiO, an oxygen-dependent flavoenzyme, to utilize glycine to provide the C2–N-3 fragment of the thiazole (1, 20), E. coli and other enteric organisms substitute ThiO with an oxygen-sensitive protein, ThiH (21–23) and use tyrosine to provide the C-2–N-3 unit.

Although genetic studies have identified ThiG and ThiH as essential for thiazole biosynthesis in E. coli, incubation of over-expressed ThiFSGH and Thi with ThiS thio-carboxylic acid, [U-14C]tyrosine, and Dxp has failed to produce any detectable amount of thiazole product (24). Under these conditions, tyrosine was cleaved to p-hydroxybenzyl alcohol, a known by-product of TPP biosynthesis (25), but sulfur transfer from 35S-ThiS thio-carboxylic acid did not occur (1).

Analysis of the ThiS sequence has identified a conserved cysteine triad motif, thought to be involved in Fe-S cluster binding and characteristic of the “radical AdoMet” family (26). Recently, hexahistidine-tagged ThiH (ThiH-His) has been isolated in a complex with ThiG and shown to contain a highly oxygen-sensitive Fe-S cluster (21), which might explain the difficulty in obtaining in vitro activity under aerobic conditions. The ThiGH complex is likely to be involved in the last steps of the thiazole biosynthesis, a cyclization reaction that could conceivably require sulfur transfer to Dxp from either the ThiS thio-carboxylic acid or the ThiFSGH conjugate, condensation with Tyr (or a derivative), and cyclization to the thiazole product.
The addition of adenosine or adenine during the growth phase of enteric bacteria, such as Aerobacter aerogenes (27), Salmonella typhimurium (28, 29), and E. coli (6, 30, 31), is known to release the repression by thiamine pyrophosphate of its own biosynthesis by decreasing the intracellular concentration of the vitamin. 5-AIR is a shared precursor of Hmp-PP and purines (32). Adenosine and adenine are negative regulators of de novo purine biosynthesis (33) and decrease the concentration of AIR, which results in a reduction in Hmp-PP biosynthesis and hence a decrease in intracellular TPP (28). Thiamine might therefore be expected to result in higher levels of enzymes suitable for thiazole biosynthesis.

As a pre-requisite to elucidating the function of the ThiGH complex and the mechanism of the thiazole cyclization in E. coli, we report here the reconstitution of thiazole biosynthetic activity in vitro using a cell-free system containing extracts from adenosine-treated E. coli 83-1 cells (6) and purified ThiGH-His under anaerobic conditions.

EXPERIMENTAL PROCEDURES

Materials—Materials were purchased from the following suppliers: aldolase, triose-phosphate isomerase (TIM) (both from rabbit muscle), and all other reagent grade chemicals from Sigma unless otherwise stated; adenosine, 4-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid, and 4-aminobenzoic acid from Across; D-glucose from BDH; HPLC grade solvents, K$_2$HPO$_4$ and KH$_2$PO$_4$, from Fisher; K$_3$[Fe(CN)$_6$] and all other reagent grade chemicals from Sigma unless otherwise stated; adenosine, 4-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid, and 4-aminobenzoic acid from Acros; Polyacrylamide/bispolyacrylamide solution (30% w/v) from Amresco. Hmp and Thz-P were prepared as described (35, 36). S-Adenosylmethionine (AdoMet) was a generous gift from Dr. H. Schroeder (BASF, Ludwigshafen, Germany). For TLC analysis, aluminum-backed TLC plates (Silica Gel 60, F-254, 250 μm) were used. A Gilson 301 HPLC system connected to a Shimadzu RF-10AXL fluorimeter and/or to a Gilson UV-visible detector was used for HPLC analysis. $^1$H NMR spectra were recorded using a Bruker AC300 (300 MHz) spectrometer.

Bacterial Strains, Plasmids, and Proteins—E. coli 83-1 cells, which are Tyr/Trp/Phe auxotrophs, were the generous gifts from Prof. R. Azerad and Prof. M. Therisod (6). BL21(DE3) cells transformed with a pET-23b-derived plasmid encoding for a hexahistidine-tagged 1-deoxy-D-xylulose-5-phosphate synthase (Dxs-His) were kindly donated by Prof. A. Boronat (37). Dxs-His was expressed from these cells and purified to ~80% purity by nickel-affinity and gel filtration chromatography. ThiG and ThiH-His were purified from E. coli BL21(DE3) pRIL1020 as described previously (21). Protein concentration was routinely estimated by the method of Bradford (38); ThiG and ThiH-His concentrations in the ThiGH-His complex were determined by SDS-PAGE analysis and gel densitometry (Syngene Gene Genius imaging system) against bovine serum albumin standards.

HPLC Purification of Dxp—Dxp was enzymatically synthesized as described by Taylor et al. (39), and the progress of the reaction was monitored by TLC analysis (40). When the reaction was judged to be complete, the proteins were removed by ultrafiltration (10-kDa molecular weight cut-off, Millipore), and Dxp was purified by semi-preparative HPLC (PerkinElmer Life Sciences Prep-10 Octyl column, 250 × 10.0 mm). Dxp was eluted with H$_2$O at 2 ml/min, and the column eluate was fractionated from $t$ = 6.0 to $t$ = 8.0 min. The TPP-containing fractions were identified by TLC analysis and discarded. The TPP-free fractions were pooled and freeze-dried. The Dxp content of the resultant white solid (which also contained residual salts) was determined by $^1$H NMR, and a stock solution of Dxp (50 mM) was prepared in 50 mM Tris-Cl, pH 8.0, 5 mM MgCl$_2$. The absence of contaminating TPP was confirmed with the thiochrome assay (41).

Cell Culture Media—The following media were used to grow or resuspend E. coli 83-1: DM1 medium, Davis and Mingioli medium (42) without glucose; DM1 medium, DM medium supplemented with glucose (0.2%, w/v), Tyr and Phe (0.2 mM each), Trp (0.1 mM), 4-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid, and 4-aminobenzoic acid (10 μM each) (6). Combinations of Tyr (0.2 mM), Hmp (14 μM), and Thz-P (14 μM) were added to the DM medium where stated. DM and DM1 media were supplemented with ampicillin (100 μg/ml) in all the experiments with E. coli 83-1 pRIL1020.

Time Course of the Adenosine Derepression—E. coli 83-1 cells derepressed in DM1 medium containing adenosine (300 μg/ml), essentially as described previously (6). The TPP content was determined in duplicate by the thiochrome assay (41) and expressed as nanograms of TPP/mg of wet cells. To be able to use the “wet weight” of the cell pellets in the calculations, the published procedure was slightly modified. Single colonies were used to inoculate DM1 medium (10 ml) and the cells grown for 8–10 h; the resultant culture was used as a 0.3% inoculum into fresh DM1 medium (300 ml) and grown overnight at 37 °C. Cells were harvested by centrifugation (Beckman JA-14, 12,000 g, 10,000 rpm for 15 min).

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**Fig. 1.** The biosynthesis of thiamine in E. coli. The positions of the known biosynthetic enzymes are shown, but salvage pathways are excluded for clarity.
Fig. 2. Derepression of thiamine biosynthesis by adenosine. A, effect of the adenosine treatment on the TPP content of E. coli 83-1. B, TPP content of washed cell suspensions of E. coli 83-1 previously incubated with (solid lines) or without (dashed lines) adenosine.

The compounds were eluted at 0.8 ml/min with linear gradients between the following time points: 0 min, 0% buffer B; t = 5 min, 0% buffer B; t = 10 min, 20% buffer B; t = 12 min, 40% buffer B; and detected by fluorescence (excitation at 360 nm and emission at 454 nm). TP and TPP, and unphosphorylated thiamine solutions of known concentration were derivatized (excitation at 360 nm and emission at 454 nm). TPP, TP, and unphosphorylated thiamine derivatives of TPP, TP, and unphosphorylated thiamine contributing to the total amount of thiamine derivatives present in samples. Unphosphorylated thiamine was not detected in any of the in vitro or in vivo enzyme assays, but the retention time was determined to exclude the possibility of unphosphorylated thiamine contributing to the total amount of thiamine derivatives present.

TTP Production by Washed Cell Suspensions—Experiments were carried out in duplicate. Cultures of E. coli 83-1 or 83-1 pRL1020 (100 ml/flask, 14 flasks) were incubated with adenosine as described above, and samples withdrawn at t = 0, 1.0, and 2.5 h to monitor the derepression phase. At the end of this phase, the cultures were quickly cooled in icy water, and the cells were harvested by centrifugation (Beckman JA-14, 12,000 rpm, 10 min at 4 °C). The cell pellets were then resuspended and washed with sterile 0.9% NaCl (two times, 10 ml), and the wet weight determined as described above. Cell pellets (27–180 mg) were stored at −80 °C until analyzed.

The thiochrome method (41) was adapted to estimate the TPP content in cell pellets. Briefly, the frozen cell pellets were thawed on ice for 10 min and then resuspended in a 1:1 mixture of H2O/7.2% HClO4 (2 ml for cell pellets of up to 80 mg of wet cells, 4 ml for heavier cell pellets); the white suspensions were sonicated on ice and the precipitated proteins removed by centrifugation. The supernatants (0.5 ml) were oxidized with a K3[Fe(CN)6] (12 mM in 3.35 M NaOH) solution (100 μl (41) and then adjusted to pH 7.0 with phosphoric acid, and an aliquot (50 μl) was analyzed by reverse phase HPLC using a Phenomenex ODS column (150 × 4.6 mm, 5-μm particle size) and eluted with buffer A (140 mM K2HPO4, 12% MeOH, 1.5% N,N-dimethylformamide, 0.3 mM tetrabutylammonium hydroxide, pH 7.0) and buffer B (70% MeOH, 30% H2O). The compounds were eluted at 0.8 ml/min with linear gradients between the following time points: t = 0 min, 0% buffer B; t = 2 min, 0% buffer B; t = 5 min, 20% buffer B; t = 10 min, 20% buffer B; t = 12 min, 40% buffer B; t = 15 min, 40% buffer B; and detected by fluorescence (excitation at 360 nm and emission at 454 nm). TPP, TP, and unphosphorylated thiamine solutions of known concentration were derivatized and analyzed by HPLC by the same method. The retention times of the thiochrome derivatives of TPP, TP, and unphosphorylated thiamine were determined to be 7.5, 9.0, and 15.9 min, respectively. TP and TPP calibration curves (0–1.3 μM) were constructed and used to quantify the thiamine derivatives present in samples. Unphosphorylated thiamine was not detected in any of the in vitro or in vivo enzyme assays, but the retention time was determined to exclude the possibility of unphosphorylated thiamine contributing to the total amount of thiamine derivatives present.

TABLE I

| Initial culture conditions | Tyr | Tyr + Hmp | +Thz-P | +Hmp |
|---------------------------|-----|-----------|--------|------|
| -Adenosine                | 5.6 ± 0.1 | 34 ± 2 | 64 ± 4 |
| Adenosine                 | 8.9 ± 0.2 | 11.4 ± 0.2 | 67 ± 1 |

The cells were initially cultured for 2.5 h in DM1 medium with the supplements indicated and then transferred to fresh DM medium supplemented as indicated for 2 h.

rpm, 10 min at 4 °C, resuspended in DM medium (10 ml), transferred to pre-weighed tubes (15 ml), and centrifuged (Beckman JA-14 with adaptors, 7000 rpm, 10 min at 4 °C). After having drained most of the medium, residual liquid was removed by gently wiping the walls of each tube with a clean tissue. Tubes were then re-weighed, and the wet weight was obtained by subtraction. The weighed cell pellets were resuspended in DM medium to yield 24 mg/ml suspensions, pooled, used as inoculum (1.3 ml) in DM1 medium (100 ml/flask, 12 flasks), and aerated in a rotary shaker. β-D-arabinose (0.2%) was added to the DM1 medium during derepression experiments with E. coli 83-1 pRL1020, where indicated. To provide duplicate data, a pair of flasks were withdrawn at t = 0 and every 30 min for 2.5 h, rapidly cooled in icy water, and the cells harvested by centrifugation. The cell pellets were then resuspended and washed with sterile 0.9% NaCl (two times, 10 ml), and the wet weight determined as described above. Cell pellets (27–180 mg) were stored at −80 °C until analyzed.

The thiochrome method (41) was adapted to estimate the TPP content in cell pellets. Briefly, the frozen cell pellets were thawed on ice for 10 min and then resuspended in a 1:1 mixture of H2O/7.2% HClO4 (2 ml for cell pellets of up to 80 mg of wet cells, 4 ml for heavier cell pellets); the white suspensions were sonicated on ice and the precipitated proteins removed by centrifugation. The supernatants (0.5 ml) were oxidized with a K3[Fe(CN)6] (12 mM in 3.35 M NaOH) solution (100 μl (41) and then adjusted to pH 7.0 with phosphoric acid, and an aliquot (50 μl) was analyzed by reverse phase HPLC using a Phenomenex ODS column (150 × 4.6 mm, 5-μm particle size) and eluted with buffer A (140 mM K2HPO4, 12% MeOH, 1.5% N,N-dimethylformamide, 0.3 mM tetrabutylammonium hydroxide, pH 7.0) and buffer B (70% MeOH, 30% H2O). The compounds were eluted at 0.8 ml/min with linear gradients between the following time points: t = 0 min, 0% buffer B; t = 2 min, 0% buffer B; t = 5 min, 20% buffer B; t = 10 min, 20% buffer B; t = 12 min, 40% buffer B; t = 15 min, 40% buffer B; and detected by fluorescence (excitation at 360 nm and emission at 454 nm). TPP, TP, and unphosphorylated thiamine solutions of known concentration were derivatized and analyzed by HPLC by the same method. The retention times of the thiochrome derivatives of TPP, TP, and unphosphorylated thiamine were determined to be 7.5, 9.0, and 15.9 min, respectively. TP and TPP calibration curves (0–1.3 μM) were constructed and used to quantify the thiamine derivatives present in samples. Unphosphorylated thiamine was not detected in any of the in vitro or in vivo enzyme assays, but the retention time was determined to exclude the possibility of unphosphorylated thiamine contributing to the total amount of thiamine derivatives present.

TABLE I

Effect of adenosine treatment on the TPP content of washed cell suspensions

The cells were initially cultured for 2.5 h in DM1 medium with the supplements indicated and then transferred to fresh DM medium supplemented as indicated for 2 h.
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Thz-P or no supplement. The 10-ml cultures were shaken at 37 °C, and the cells were harvested every 30 min for 2 h by centrifugation. The cell pellets were washed with NaCl solution, weighed, and stored at −80 °C until analyzed. The TPP content was estimated as described above.

Large Scale Growth of E. coli 83-1 pRL1020 in the Presence of Adenosine—A single colony was used to inoculate DM1 medium (10 ml), and cells were grown at 37 °C for 8–10 h. This culture (3 ml) was used to inoculate fresh DM1 medium (800 ml). After overnight incubation at 37 °C, the cells were harvested by centrifugation (Beckman JA-14, 12000 rpm, 10 min at 4 °C). The cell pellets were weighed and resuspended in DM medium to yield 100 mg/ml suspensions, and then the resultant cultures were pooled and used as inocula (5 ml) in DM1 medium (four times, 1.25 liters) supplemented by the addition of adenosine (300 mg/ml). The cells were grown at 37 °C for 2.5 h, then cooled down by swirling in icy water, and harvested. The typical yield of cell paste ranged between 5.5 and 7.5 g from 5 liters. The cell paste was stored at −80 °C until used.

Preparation of Cell-Free Extracts Under Anaerobic Conditions—Where possible cell samples were manipulated under anaerobic conditions using a Belle glove box (<2 ppm of O2). Cell-free extracts from derepressed E. coli 83-1 pRL1020 or BL21(DE3) pRL1020 were prepared by introducing weighed amounts of cell paste (0.8–1.0 g) into the glove box, and resuspending it in anaerobic reaction buffer (1.6–2.0 ml, 50 mM Tris-HCl, pH 8.0, 20 mM MgCl2, 5 mM DTT, 12.5% (w/v) glycerol). The suspensions were then withdrawn from the anaerobic box and rapidly lysed by sonication. The lysates were returned to the box and allowed to degas for 10 min and then cleared by centrifugation (Beckman JA-14 with appropriate adapters, 7000 rpm, 15 min at 4 °C) in gas-tight tubes. The protein concentration in these lysates was typically 35–45 mg/ml. The cleared lysate prepared from E. coli BL21(DE3) pRL1020 cells (grown in 2YT medium) was routinely gel-filtered through a NAP-10 column (AP Biotech), equilibrated in anaerobic action buffer to remove nearly all of the low molecular weight molecules, including TPP, and is referred to as the “ThiGH-His-enriched fraction.” Cell-free lysates and desalted protein fractions were prepared by similar methods from derepressed E. coli 83-1 pRL1020 and are termed “derepressed 83-1 lysate” and “derepressed 83-1 protein fraction,” respectively.

Measurement of the in Vitro Thiazole Synthase Activity by Enzymatic Coupling to Hmp-PP and Thiocrome Assay—Unless otherwise specified, the term “thiamine” is used to indicate any vitamin form, regardless of the extent of phosphorylation. A typical reaction mixture contained the following: 100 μl of derepressed 83-1 lysate or protein fraction, 100 μl of ThiatGH-His-enriched protein fraction or purified ThiGH-His (8.0–10.0 mg/ml, total protein concentration) in anaerobic reaction buffer, ATP (20 mM), Tyr (1.5 mM), Cys (1.5 mM), Dxp (1.0 mM), and Hmp (0.6 mM). Depending on the experiment, the total volume varied between 250 and 350 μl, as required. Tyr stock solutions (40 mM) were prepared in 100 mM HCl. Cys stock solutions (80 mM) were freshly prepared from fresh solutions of Cys stock solutions of Cys stock solutions (50 and 14 mM, respectively) were prepared in 50 mM Tris-HCl, pH 8.0, 5 mM MgCl2. The ATP stock solutions (250 mM in ThiH-His) were incubated for 2 h at 37 °C, then stopped with H2O. The precipitated proteins were removed by centrifugation and the supernatant (500 μl) assayed for TP and TPP. Due to the presence of DTT, the sensitivity of the thiazole assay had to be optimized by doubling the final K3[Fe(CN)6] concentration. For each set of conditions, the amounts of TP and TPP were estimated separately, being transferred to the AdoMet-containing reaction mixtures.

RESULTS AND DISCUSSION

Effect of the Adenosine Treatment on Thiamine Biosynthesis in Washed Cell Suspensions

E. coli 83-1 cells are among those enteric bacteria that have been shown to be sensitive to the adenosine derepression of thiamine biosynthesis (6). To optimize the potential thiamine biosynthetic activity for the planned in vitro experiments, the time course of the in vivo derepression and subsequent burst in thiamine production were investigated in detail. The experimental conditions were analogous to those adopted by Newell and Tucker (28) during their investigation of the derepression of thiamine biosynthesis in S. typhimurium LT2 (mutant T), but with modifications to the growth medium to provide the auxotrophic growth requirements for E. coli 83-1 (6), including the aromatic amino acids Phe, Trp, and Tyr. As Tyr is a known thiazole precursor, if the medium is not supplemented with tyrosine, de novo thiamine biosynthesis becomes dependent on Tyr scavenged from protein turnover in E. coli 83-1.

The addition of adenosine to the medium during the growth phase caused a drop in the initial TPP content (3–4 ng/mg wet cells), with the vitamin level remaining low for about 2 h (Fig. 2A). During this period of time, E. coli 83-1 starved of thiamine and freed from the vitamin repression could accumulate higher (derepressed) levels of biosynthetic enzymes (30). After washing, these cells were transferred to media supplemented with Tyr and Hmp and produced approximately three times more TPP than the untreated cells (negative control) (Fig. 2B). Under these conditions, the burst in the vitamin production may be ascribed to de novo synthesis of the thiazole (44), because this moiety does not accumulate in TPP-depleted cells, such as adenosine-treated cells or in mutants unable to assemble Hmp-PP (27, 29).

In a positive control experiment, using media supplemented by the addition of not only Hmp but also Thz-P, both adenosine-treated and untreated cells were able to produce 60–70 ng of TPP/mg wet cells (Table I, 3rd column). This suggests that in experiments where the medium was supplemented with Hmp and thiamine precursors, any Thz-P produced would be rapidly converted to TPP and that neither ThiD nor ThiE was limiting the TPP formation. Indeed, these results strongly suggest that, under the conditions investigated, thiazole biosynthesis was the rate-limiting step.

The observation that the pre-incubation with adenosine had lowered the intracellular TPP concentration and, as a result, may have increased the expression level of the rate-determining thiazole biosynthetic enzymes, contrasted with the activities of ThiD and ThiE, which were not equally affected. This is an unexpected result as the genes encoding these two proteins belong to the thMD and thiCEFSGH operons and therefore should be subject to coordinated repression by thiamine (30, 31, 34, 45, 46). Despite the lack of a simple explanation for these results, Newell and Tucker (29) observed a precisely analogous effect with the Sol-
monella mutant used for their studies. An alternative regulatory mechanism, operating at the translational or metabolic level, can be invoked to explain these observations, but it is clear that more than one factor is controlling the balance of the different thiamine biosynthetic enzymes.

As the assembly of Thz-P was observed to be rate-limiting for TPP production in derepressed _E. coli_ 83-1 cultures, the effect of expression plasmids encoding structural genes known to be required for Thz-P biosynthesis was investigated. For these experiments, _E. coli_ 83-1 was transformed with pRL1020, a plasmid encoding ThiFSGH-His (21). The resultant strain, _E. coli_ 83-1 pRL1020, was cultured with adenosine (to derepress thiamine biosynthesis) and β-1-arabinose (to induce the expression of ThiFSGH) for 2.5 h. However, _E. coli_ 83-1 pRL1020 produced only marginally more TPP than _E. coli_ 83-1, and comparative analysis of the proteins in the cleared cell lysates by SDS-PAGE did not show an appreciable difference in the protein expression levels (Fig. 3). This may be due to the short induction time or it may be a function of the high glucose content of the DM1 medium acting to repress expression from pRL1020 (47). As the presence of pRL1020 did not alter the response to the adenosine treatment, but conveniently conferred ampicillin resistance, _E. coli_ 83-1 pRL1020 was used in the subsequent _in vitro_ experiments.

**In Vitro Assay Strategy**

Several problems were overcome during the development of a functional thiazole synthase assay.

**Thz-P Is Difficult to Assay Directly in _E. coli_ Cell-free Systems**—Although it is known that certain mutants impaired in thiamine biosynthesis can accumulate Hmp and also excrete it into the medium (29, 32, 46, 48), Thz-P is normally present at low levels and does not accumulate. It has been proposed that this compound may exert a feedback inhibition on one or more enzymes involved in its own biosynthesis (29). In fact, the direct detection of the thiazole has been reported to be very difficult (29, 49), but this problem may be avoided by efficiently coupling any thiazole produced to Hmp-PP and then measuring the thiamine content (TP or TPP) after quantitative oxidation to the fluorescent thiochrome derivatives (41, 49–51).

**Thiamine Biosynthesis Is Repressed**—The tight regulation of thiamine biosynthesis can be overcome by the addition of adenine or adenosine to the growth medium. The adenine decreases the intracellular TPP concentration, and the cells respond by accumulating the thiamine biosynthetic enzymes (30). These derepressed cells therefore have the potential to provide a cell-free system suitable for a thiazole synthase assay. The _E. coli_ strain 83-1 was selected as it had already been employed in the _in vivo_ investigation of the thiazole biosynthesis using labeled precursors (6, 10, 44).

**_E. coli_ Accumulates Intracellular TPP When Cultured on Rich Media**—When cultured on rich media (such as 2YT or LB), _E. coli_ sequesters thiamine, resulting in very high levels of background TPP (>500 pmol/mg of protein). This makes accurate assays of _de novo_ biosynthesis difficult. Gel filtration of the cell lysates reduced the TPP background; for example, typical TPP background levels in the 83-1 protein extract and the ThiGH-His-enriched protein extract were 60–100 and 220–250 pmol/mg protein, respectively.

**Thiazole Biosynthesis Must Be the Rate-limiting Step in a Coupled Assay**—Control experiments demonstrated that derepressed 83-1 lysates were able to produce thiamine at more than 50 pmol/mg/h when supplied with Hmp and Thz-P in the presence of ATP and Mg²⁺. This was far greater than the observed rate of _de novo_ thiazole biosynthesis observed in subsequent assays and indicates that the ThiD-dependent pyro-
Thiazole Synthase Activity in E. coli Cleared Cell Lysates and Desalted Protein Extracts

De novo production of both TP and TPP was measured in the derepressed 83-1 lysate, anaerobically incubated with ATP, Hmp, and the thiazole precursors, Tyr, Cys, and Dxp. This basal activity was estimated at ~7 pmol/mg of protein/h (Fig. 4, 1st column, or Fig. 5, sample 2) and was confirmed to be higher than the activity measured in lysates derived from repressed cells (4–5 pmol/mg of protein/h, Fig. 5, sample 1), although the increase in the in vitro thiamine biosynthetic activity of the derepressed E. coli 83-1 lysate was less pronounced than in vivo. The ThiGH-His-enriched protein fraction (prepared by gel filtration of the E. coli BL21(DE3) pRL1020 cleared cell lysate) was also assayed under identical conditions and shown to be active, producing almost exclusively TPP (8–11 pmol/mg of protein/h, Fig. 4, 7th column). This was a somewhat surprising result as earlier attempts to measure in vitro thiazole synthase activity from a ThiPSGH-overexpressing extract had been unsuccessful (1, 24). However, the thiazole synthase activity described here was detected in assays maintained under anaerobic conditions, using an expression system optimized for the soluble expression of the ThiGH-His complex (21).

As in vivo studies and control experiments had demonstrated that thiazole biosynthesis was likely to be the rate-limiting step for de novo thiamine biosynthesis, the effect of mixing the ThiGH-His-enriched protein extract and the derepressed 83-1 lysate was investigated. Mixtures containing different ratios of these two extracts were assayed (Fig. 4) and showed activities higher than either individual extract (to a maximum of 16.5 pmol/mg of protein/h), which suggested that the two extracts might be providing complementary components. The increasing proportion of the ThiGH-His-enriched protein extract also increased the TPP/TP ratio when compared with the derepressed 83-1 lysate (Fig. 4), revealing that the derepressed E. coli 83-1 lysate has a lower thiamine phosphate kinase (ThiL) activity than the BL21(DE3)-derived ThiGH-His-enriched protein extract.

Comparing the Effect of the ThiGH-His-enriched Protein Fraction and Purified ThiGH-His

The increased activity obtained by mixing the ThiGH-His-enriched protein fraction and the derepressed 83-1 cell lysate suggested the possibility that the rate-enhancing components provided by the ThiGH-His-enriched protein fraction were the overexpressed proteins. Therefore, the effect of this fraction (Fig. 5, sample 3) was compared with that of purified ThiGH-His (Fig. 5, sample 4), and both showed an approximate 3-fold increase over the basal activity of the derepressed E. coli 83-1 lysate. This result may indicate that the amount of ThiGH present in the derepressed 83-1 lysate is limiting the rate of thiamine biosynthesis and provides for the first time evidence for in vitro thiazole synthase activity of the ThiGH-His complex.

Precursor Dependence of Thiazole Synthase Activity

Having established an assay for thiazole synthase activity by using purified ThiGH-His and the derepressed E. coli 83-1 lysate, the concentration dependence of the activity was determined for the known thiazole precursors Dxp, Tyr, and Cys. To remove any potential interference from endogenous amounts of these metabolites, the derepressed 83-1 cell lysate was desalted using a NAP-10 column, and the protein-containing high molecular weight fraction was isolated. Initial experiments with this derepressed 83-1 protein fraction showed that it retained at least the same level of activity of the unfractionated lysate (15–20 pmol/mg of protein/h), providing a further simplification of the system for in vitro studies.
The precursor-dependent activity was investigated by varying the concentrations of each of the known precursors, otherwise maintaining the standard assay conditions. In these experiments, the Cys concentration was increased from 0 to 2 mM and the Dxp concentration from 0 to 3.7 mM, but in both cases, the de novo thiamine production was almost completely unaffected by the presence of these two putative precursors (Fig. 6). Conversely, the thiazole synthase activity appeared to be dependent on the presence and concentration of Tyr up to 0.2 mM. These results raised the interesting question of the actual source of the C5 unit and sulfur atom that are incorporated into Thz-P during these experiments. The component that provides these atoms appears to co-elute with the proteins when the derepressed E. coli 83-1 lysate is fractionated on a NAP-10 gel filtration column. In this regard, it is interesting to note that the studies of Park et al. (19) on thiazole biosynthesis in B. subtilis have provided evidence for a C-terminal derivative of ThiS that contains the atoms originating from Dxp and Cys (Fig. 7). Such an enzyme-bound intermediate may therefore be common to the thiazole-forming pathways of tyrosine/ThiH-dependent organisms (such as E. coli) and glycine/ThiO-dependent organisms (such as B. subtilis).

**ThiGH-His Dosage and Time Course, Implication for the Thiazole Biosynthesis**

The effect of varying the concentration of ThiGH-His in the assay was investigated (Fig. 8). The amount of thiamine produced was approximately proportional to the ThiGH-His added up to a concentration of 2 mg/ml, after which additional ThiGH-His gave no increase and in fact decreased the overall yield of thiamine.

Earlier studies had shown the marked tendency of the ThiGH-His complex to precipitate (21). Careful observations of the assays revealed that a protein precipitate formed in all the reaction mixtures containing ThiGH-His but was more pronounced when ThiGH-His concentrations exceeded 1 mg/ml. This aggregation may, at least in part, account for the decreased activity in assays containing a higher concentration of ThiGH-His. Despite this clearly observable protein precipitation, a time course experiment demonstrated that thiamine biosynthesis occurred in an approximately linear manner (rate = 8 pmol/mg of protein/hour) up to 2 h and continued at a reduced rate (rate = 2 pmol/mg of protein/h, Fig. 9) up to at least 6 h.

**Effect of AdoMet and Reducing Agents**

ThiH shares the Fe-S cluster-binding motif, Cys-XXX-Cys-XX-Cys, with members of the radical AdoMet family (26), whose activity is dependent upon the presence of AdoMet. The effects of adding AdoMet and potential reducing agents, dithionite and NADPH, on the formation of thiamine were investigated (Figs. 10 and 11). Whereas AdoMet alone gave a relatively modest increase in activity, the addition of AdoMet with a reducing agent more than doubled the observed thiazole synthase activity. Iron and sulfide have been used to reconstitute the Fe-S clusters of several “radical AdoMet” enzymes (53–55) and were added to these thiazole synthase assays in an effort to maximize activity.
The deoxyadenosyl radical generates a tyrosinyl radical which
undergoes homolytic cleavage of the \( \text{C}_4-\text{C}_9 \) bond to release the quinone
methylene. Hydration of 4 leads to \( p \)-hydroxybenzyl alcohol 5, a known thiamine biosynthetic by-product. The glycinyl radical of adenosin-treated samples and an enzyme-coupled assay, the thiazole synthase activity is stimulated by the addition of AdoMet and a reducing agent, with NADPH proving more effective than dithionite.

These results are consistent with our working model in which the thiazole is formed from tyrosine and an as yet uncharacterized intermediate by the ThiGH complex in a AdoMet/NADPH-dependent reaction. Experiments to isolate and characterize the putative intermediate and to define the role of AdoMet in the thiazole synthesis reaction are under way.

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Conclusions
For the first time, using lysates or partially purified protein samples and an enzyme-coupled assay, the thiazole synthase activity of E. coli has been measured. By adding purified ThiGH-His to these assays, we have demonstrated that, under the assay conditions, this complex is involved in the rate-limiting step. The reaction is completely dependent on the addition of tyrosine, a known Thz-P precursor. A further component that co-purified with the proteins derived from a lysate of adenosine-treated E. coli 83-1 was also required and is presumed to provide the C<sub>5</sub> unit and sulfur for which Dxp and cysteine are known to be the original metabolic precursors. The thiazole synthase activity is stimulated by the addition of AdoMet and a reducing agent, with NADPH proving more effective than dithionite.

These results are consistent with our working model in which the thiazole is formed from tyrosine and an as yet uncharacterized intermediate by the ThiGH complex in a AdoMet/NADPH-dependent reaction. Experiments to isolate and characterize the putative intermediate and to define the role of AdoMet in the thiazole synthesis reaction are under way.
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