Anthranilate Synthase Can Generate Sufficient Phosphoribosyl Amine for Thiamine Synthesis in *Salmonella enterica*

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In bacteria, the biosynthetic pathway for the hydroxymethyl pyrimidine moiety of thiamine shares metabolic intermediates with purine biosynthesis. The two pathways branch after the compound aminimidazole ribotide. Past work has shown that the first common metabolite, phosphoribosyl amine (PRA), can be generated in the absence of the first enzyme in purine biosynthesis, PurF. PurF-independent PRA synthesis is dependent on both strain background and growth conditions. Standard genetic approaches have not identified a gene product singly responsible for PurF-independent PRA formation. This result has led to the hypothesis that multiple enzymes contribute to PRA synthesis, possibly as the result of side products from their dedicated reaction. A mutation that was able to restore PRA synthesis in a purF gnd mutant strain was identified and found to map in the gene coding for the TrpD subunit of the anthranilate synthase (AS)-phosphoribosyl transferase (PRT) complex. Genetic analyses indicated that wild-type AS-PRT was able to generate PRA in vivo and that the P362L mutant of TrpD facilitated this synthesis. In vitro activity assays showed that the mutant AS was able to generate PRA from ammonia and phosphoribosyl pyrophosphate. This work identifies a new reaction catalyzed by AS-PRT and considers it in the context of cellular thiamine synthesis and metabolic flexibility.

Metabolic processes in a living cell are connected through the integration of biochemical pathways. This metabolic integration allows bacteria to survive under diverse conditions and is essential for their adaptation to changing environments. The structure and function of networks created by the integration and overlap of metabolic processes are largely uncharacterized. The thiamine biosynthetic pathway in *Salmonella enterica* has direct biochemical connections with other metabolic pathways, including purine and isoprenoid biosynthesis (5, 33, 40), and indirect connections with a number of other cellular processes (1, 16, 19, 25, 36). Thus, this biosynthetic pathway serves as a focal point for studies to identify and characterize metabolic interactions, redundancy, and regulation that are essential in a robust metabolism.

Thiamine consists of a 4-amino-5-hydroxymethyl-2-methyl pyrimidine (HMP) pyrophosphate moiety and a 4-methyl-5-(β-hydroxymethyl)-thiazole phosphate moiety, which are synthesized independently prior to their condensation (6) (Fig. 1). HMP is generated from an intermediate in the de novo purine biosynthetic pathway, aminimidazole ribotide, the last intermediate common to purine and thiamine biosynthesis (18, 33, 34). Mutants lacking the first step in purine synthesis, catalyzed by the PurF enzyme (phosphoribosyl pyrophosphate [PRPP] amidotransferase), lack synthesis of purines but retain HMP synthesis under a number of growth conditions and genetic backgrounds (14, 15, 36). PurF is the only purine biosynthetic enzyme that can be bypassed in thiamine synthesis, indicating that phosphoribosyl amine (PRA) is the compound generated in a PurF-independent mechanism (15, 36) (Fig. 1). Mutations in gnd (gluconate-6-phosphate dehydrogenase) or zwf (glucose-6-phosphate dehydrogenase) prevented thiamine-independent growth of a purF mutant under otherwise permissive conditions (16, 36). These results demonstrated a role for the oxidative pentose phosphate pathway in this process. The requirement for the oxidative pentose phosphate pathway in PRA formation can be overcome by exogenous ribose (16, 36) or a null mutation in yigF (17). Genetic attempts to define an activity responsible for PurF-independent PRA synthesis have been unsuccessful, leading to a model in which multiple enzymes contribute to PRA formation in vivo.

Here we report the isolation and characterization of a mutant derivative of a purF gnd strain that is capable of thiamine-independent growth. The causative mutation in this strain was in the trpD gene, encoding anthranilate synthase (AS) component II. The catalytic similarities between AS-phosphoribosyl transferase (AS-PRT) and PurF, and the results obtained in this study, support the conclusion that the AS-PRT complex can generate PRA in vivo. We suggest that the increased synthesis of PRA by the mutant complex is sufficient to allow thiamine synthesis in the absence of PurF.

MATERIALS AND METHODS

**Bacterial strains.** All strains used in this study are derivatives of *S. enterica* serovar Typhimurium strain LT2 and are listed with their genotypes in Table 1. Tn10(dTc) refers to the transposition-defective mini-Tn10 (Tn10Δ1Δ17) (44). MudJ is used to refer to the MudJ734 transposon, described previously (10).

**Culture media and chemicals.** No-carbon E medium of Vogel and Bonner (12, 43) supplemented with MgSO₄ (1 mM) and a carbon source (11 mM) was used as minimal medium. When present in the culture media, the following compounds were used at the indicated concentrations: adenine, 0.4 mM; thiamine, 0.5 μM; and tryptophan, 0.5 mM. Difco nutrient broth (8 g/liter) with NaCl (5 g/liter) and Luria-Bertani broth were used as rich media. Difco BTrk agar was added (15 g/liter) for solid medium. Antibiotics were added as needed to the following concentrations in rich and minimal media, respectively: tetracycline, 20

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and 10 µg/ml; kanamycin, 50 and 125 µg/ml; and chloramphenicol, 20 and 4 µg/ml.

Genetic methods. (i) Transduction methods. All transductional crosses were performed by using the high-frequency general transducing mutant of bacteriophage P22 (HT105/1-int-201) (39) as described previously (13). Transductants were purified by streaking on nonselective green indicator plates, and putative phage-free clones, identified by their light green color (11), were verified to be phage-free by cross-streaking with phage P22.

(ii) Isolation of the suppressor mutant. A pool of >70,000 cells containing random Tn10(Tc) insertions was generated as described elsewhere (27, 29) and mutagenized with nitrosoguanidine (12, 26). A P22 lysate grown on this pool of phage-free clones, identified by their light green color (11), was verified to be phage-free by using the high-frequency general transducing mutant of bacteriophage P22.

(iii) Isolation of the suppressor mutant. A pool of >70,000 cells containing random Tn10(Tc) insertions was generated as described elsewhere (27, 29) and mutagenized with nitrosoguanidine (12, 26). A P22 lysate grown on this pool of phage-free cells was used to transduce strain DM1231 (purF2085 gnd-174::MudJ) to thermoresistant resistance to 5-methyltryptophan (0.45 mM) to be consistent with map locations and phenotypes described for npt mutants (2, 41). The resulting trpR3612::Tn10(Tc) mutation was 80% linked to the thermore resistance of 5-methyltryptophan resistance (41).

(iv) Phenotypic analysis. Nutritional requirements were assessed both on solid and agar overlays and in liquid growth media as described below.

(a) Liquid growth. Strains to be analyzed were grown to full density in nutrient medium at 37°C. After overnight incubation, cells were pelleted and resuspended in an equal volume of saline (85 mM). A 0.2-ml aliquot of this sample was used to inoculate 5 ml of the appropriate medium (2% [vol/vol] inoculum). Culture tubes were incubated at 37°C with shaking, and growth was monitored as optical density at 650 nm on a Bausch and Lomb Spectronic 20D spectrometer. Alternatively, 2 µl of the cell suspension was used to inoculate 200 µl of the appropriate medium contained in each well of a 96-well microtiter plate. Growth at 37°C was monitored with a Spectra-Max Plus microplate spectrophotometer. The specific growth rate was determined as \[ \mu = \ln(X_f/X_0)/T, \] where \( \mu \) is optical density at 650 nm during the linear portion of the growth curve and \( T \) is time.

(b) Solid media. Nutritional requirements were measured in soft agar overlays as follows. A 0.2-ml aliquot of saline cell suspension (prepared as described above) was added to 4 ml of molten 0.7% agar and heated at 60°C on a Bausch and Lomb Spectronic 20D spectrometer. Alternatively, 2 µl of the cell suspension was used to inoculate 200 µl of the appropriate medium contained in each well of a 96-well microtiter plate. Growth at 37°C was monitored with a Spectra-Max Plus microplate spectrophotometer. The specific growth rate was determined as \[ \mu = \ln(X_f/X_0)/T, \] where \( \mu \) is optical density at 650 nm during the linear portion of the growth curve and \( T \) is time.

Molecular biology techniques. Open reading frames were amplified by PCR with cloned Pfus DNA polymerase and the appropriate primers listed in Table 2. The resulting PCR products were purified and blunt-end ligated into SmaI-cut pSU19 (3). Plasmids were transformed into Escherichia coli strain DH5α and screened for vectors containing inserts. Analysis of restriction digest patterns for

TABLE 1. Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
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<tbody>
<tr>
<td>DM1...</td>
<td>Wild type (LT2)</td>
</tr>
<tr>
<td>DM728...</td>
<td>purF2085 gnd-181</td>
</tr>
<tr>
<td>DM1231...</td>
<td>purF2085 gnd-174::MudJ</td>
</tr>
<tr>
<td>DM1936...</td>
<td>purF2085</td>
</tr>
<tr>
<td>DM6417...</td>
<td>purF2085 gnd-181 zdd-9147::Tn10(Tc) trpD3611</td>
</tr>
<tr>
<td>DM6418...</td>
<td>purF2085 gnd-181 zdd-9147::Tn10(Tc)</td>
</tr>
<tr>
<td>DM6808...</td>
<td>purF2085 gnd-181(pSU19)</td>
</tr>
<tr>
<td>DM6809...</td>
<td>purF2085 gnd-181(pIR-EDW)</td>
</tr>
<tr>
<td>DM6810...</td>
<td>purF2085 gnd-181(pIR-EDMS)</td>
</tr>
<tr>
<td>DM6811...</td>
<td>purF2085 gnd-181(pIR-DW)</td>
</tr>
<tr>
<td>DM6812...</td>
<td>purF2085 gnd-181(pIR-DMS)</td>
</tr>
<tr>
<td>DM6813...</td>
<td>purF2085 gnd-181(pIR-S40F)</td>
</tr>
<tr>
<td>DM6942...</td>
<td>purF2085 gnd-181 vigF3::MudJ(pIR-S40F)</td>
</tr>
<tr>
<td>DM7091...</td>
<td>trpR3612::Tn10(Tc)</td>
</tr>
<tr>
<td>DM7092...</td>
<td>trpR3612::Tn10(Tc)</td>
</tr>
</tbody>
</table>

* MudJ is the MudJ1734 transposon (10).
* Tn10(Tc) is the transposition-defective mini-Tn10 (Tn10ΔΔΔΔ7) (44).
the resulting plasmids was performed, and the identities and orientations of the plasmid inserts were confirmed by sequencing.

Preparation of cell extracts. Cells were grown for 16 h at 37°C with agitation in the minimal medium of Vogel and Bonner (12, 43) supplemented with tryptophan or from chorismate and NH₄Cl. The initial rate of anthranilate formation was measured by an increase in absorbance at 24°C. The clear supernatant constituted the crude extract.

Enzyme assays. AS activity was determined as described previously (20, 42, 49) by measuring the amount of anthranilate produced from chorismate and glutamine, 2 mM MgCl₂, 50 mM Tris-HCl buffer (pH 8.7), and crude cell extract in a final volume of 200 μl. One unit of activity was defined as the disappearance of 1 nmol of anthranilate in 1 min per mg of protein.

The PRT activity of AS component II was also assayed fluorometrically (20, 24) by measuring the rate of disappearance of anthranilate at room temperature. The reaction mixture contained 15 μM anthranilate, 0.3 mM PRP, 10 mM MgCl₂, 100 mM Tricine buffer (pH 7.6), and crude cell extract in a final volume of 200 μl. One unit of activity was defined as the disappearance of 1 μmol of anthranilate in 1 min per mg of protein. In all assays, between 0.5 and 0.7 μg of protein was added per reaction. To assay allosteric inhibition, tryptophan was added to a final concentration of 0.5 mM.

The PRT-forming activity was determined by using a modified assay initially described for PurF (38), where synthesis of PRA from PRP and glutamine is determined as a function of [14C]glycinamide ribonucleotide (GAR) produced in a coupled reaction catalyzed by GAR synthetase. A molecule of provided [14C]glycinamide ribonucleotide (GAR) produced in buffer. The cells were disrupted by using a French pressure cell at 104 kPa. Cell containing 1 mM EDTA and 1 mM dithiothreitol, and resuspended in the same volume of 200 μl. One unit of activity was defined as the disappearance of 1 nmol of anthranilate in 1 min per mg of protein.

RESULTS

Isolation of a mutation that allows PurF-independent PRA synthesis. In continuing efforts to identify cellular processes (other than PurF) involved in vivo PRA generation, Thi derivatives of a purF mutant strain were isolated. A pool of Tn10(Tc) insertions was mutagenized with nitrosoguanidine, and a P22 phage lysate grown on these cells was used to transduce DM1231(purF gnd) to Tc. From a screen of approximately 16,000 Tc transductants that retained the gnd-174::MudJ from the recipient, 20 grew on glucose minimal medium supplemented with adenine. Since lesions in yjgF can result in this phenotype (17), the 20 mutants were tested for the serine sensitivity associated with yjgF mutations. Eleven of the mutants were able to grow in the presence of 0.5 mM serine. Genetic reconstruction experiments determined that the causative mutation in one of these strains was linked (30%) to the selected Tn10(Tc) insertion. This strain was further characterized.

A mutation in the trpD gene allows PRA synthesis. To define the site of the lesion responsible for allowing PRA synthesis in a purF gnd background, the location of the linked Tn10(Tc) insertion [zdd-9147::Tn10(Tc)] was determined by arbitrarily primed PCR (7). Sequence analysis determined that the insertion was in the acnA-cysB intergenic region at ~28.7 min on the S. enterica chromosome. Further linkage analysis and data from three-factor cross experiments determined that the relevant mutation was located clockwise of the caba gene on the circular chromosomal map. When an insertion in the nearby trp operon was used as a selected marker in three-factor crosses, PRA formation could not be recovered, suggesting that one or more of the tryptophan biosynthetic genes were involved in this phenomenon.

When the catalytic reactions of the tryptophan biosynthetic enzymes and glutamine-PRPP amidotransferase (PurF) are compared, a similarity between AS-PRT (encoded by trpED) and PurF is obvious (Fig. 2). The causative mutation was hypothesized to be an allele of trpD or -E that resulted in a mutant AS-PRT that is able to generate PRA in vivo. To address this possibility, trpE and -D were amplified from both the wild-type (DM1) and mutant (DM6417 strains and cloned into the mid-copy-number vector pSU19 (3), generating plasmids pIR-EDW and pIR-EDMS, respectively. Sequence analysis identified a single nucleotide difference between the genes amplified from the mutant and the wild type. The insert derived from strain DM6417 carried a C-T transversion at nucleotide 1084 of trpD, causing proline 362 of the peptide to be replaced by leucine (trpD3611). Subsequent experiments confirmed the assignment of this change as the causative lesion (see below). Pro362 resides in the PRT subdomain of AS component II, which consists of residues 202 to 531 of TrpD (28, 46). A comparison of multiple ASs from a variety of organisms shows that this residue is highly conserved. The crystal structure of the dimeric anthranilate PRT of Sulfolobus solfataricus has been published (30). While Pro362 is not conserved in S. solfataricus, this amino acid is located next to a conserved residue in the proposed anthranilate binding site, R364.

Tryptophan prevents PRA formation by trpD3611. The trpD3611 mutation was selected to allow thiamine-independent

![Table 2. Plasmids](http://jb.asm.org/Downloaded/from/98.6.9.89)
growth of a purF gnd mutant on solid glucose medium. This phenotypic effect was quantified by liquid growth analyses. The specific growth rates (μ) for isogenic strains DM6418 (purF gnd) and DM6417 (purF gnd trpD3611) grown on glucose-adenine medium with the indicated supplements are shown in Table 3. These quantitative growth analyses confirmed that the trpD3611-containing strain (DM6417) grew in absence of thiamine, while the parental strain (DM6418) did not. Addition of thiamine restored growth to the parental strain and had no stimulatory effect on the growth of DM6417. A similar effect of the restoration of growth to the parental strain (DM6418) did not. Addition of thiamine containing strain (DM6417) grew in absence of thiamine, while when glutamate was provided as the sole carbon source for the purF2085 mutant strain to grow in the absence of thiamine. How-

![Chemical diagram](http://jb.asm.org/)

**FIG. 2.** TrpDE- and PurF-catalyzed reactions show biochemical similarity. Biochemical reactions catalyzed by the AS-PRT complex (TrpDE) and glutamine phosphoribosyl amidotransferase (PurF) are illustrated to emphasize similarities. Each enzyme has a glutaminase activity and transfers a phosphoribosyl group to an amidated substrate.

satisfied by providing the HMP moiety of thiamine but not by the addition of exogenous tryptophan (0.5 mM) was completely overcome by the addition of thiamine to the medium, indicating that this amino acid was simply negating the effect of the trpD3611 mutation. Nutritional analyses confirmed that the requirement for thiamine caused by the presence of tryptophan could be satisfied by providing the HMP moiety of thiamine but not by the addition of exogenous tryptophan (0.5 mM). This result was consistent with the conclusion that tryptophan had simply restored the thiamine requirement of the parental strain. Since AS is negatively regulated at the levels of both transcription and activity by tryptophan (8, 37), it would have been expected that tryptophan was having a regulatory effect.

**Catalytic activity of AS-PRT is required to generate PRA in the absence of PurF.** The trpD3611 allele could result in a form of AS-PRT that is able to generate PRA by stimulating an existing activity or generating a new one. Plasmids pIR-EDW and pIR-EDMS were used to address whether the trpD3611 allele had generated a new activity. These plasmids were introduced by electroporation into strain DM728 (purF gnd), and the resulting strains were tested for growth in the absence of thiamine. As shown in Fig. 3, both plasmids allowed the purF gnd mutant strain to grow in the absence of thiamine. However, pIR-EDMS, carrying the mutant trpD allele, resulted in an approximately twofold-higher growth rate. From this result it was concluded that the wild-type AS-PRT was able to generate PRA in vivo and that the trpD3611 mutation had stimulated this activity.

As with the strain carrying trpD3611 in the chromosome (DM6417), suppression of the thiamine requirement of purF gnd, by plasmids pIR-EDW and pIR-EDMS, was prevented by tryptophan (data not shown). Significantly, in these plasmid constructs, the native trp promoter was not present. Rather, transcription depended on the plasmid-encoded lac promoter, suggesting that tryptophan was mediating its effect via allosteric regulation of AS-PRT activity. To confirm this interpretation, a plasmid (pSTG92) that carried a feedback-resistant allele of AS-PRT (trpDE<sup>aphD</sup>) was obtained (9). From this plasmid a DNA fragment corresponding to the insert in plasmid pIR-EDW was cloned into pSU19. As expected, the resulting plasmid (pIR-S40E) restored growth to a purF gnd mutant on adenine medium, but, significantly, thiamine-inde-

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**TABLE 3.** The trpD3611 allele restores thiamine-independent growth of a purF gnd mutant

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Specific growth rate (μ) in minimal glucose medium supplemented with*:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ade</td>
</tr>
<tr>
<td>DM6417</td>
<td>purF gnd trpD3611</td>
<td>0.413</td>
</tr>
<tr>
<td>DM6418</td>
<td>purF gnd</td>
<td>NG</td>
</tr>
</tbody>
</table>

<sup>a</sup> Strains were grown in minimal glucose medium with the indicated supplements at 37°C as described in Materials and Methods. Ade, adenine; Trp, tryptophan; Thi, thiamine. The specific growth rate (μ) was determined by the equation ln(X<sub>370</sub>/X<sub>0</sub>) / T, where X is A<sub>650</sub> at time zero, and T is time (in hours).

<sup>b</sup> NG, specific growth rate was below detection (≤0.02).
that a plasmid carrying only the *trpE* gene did not restore thiamine-independent growth of the *purF gnd* mutant strain (data not shown). These results indicate that at least in multicopy, TrpD was sufficient to allow *purF* synthesis in vivo, but the *trpD3611* allele failed to stimulate that synthesis. Unexpectedly, the thiamine-independent growth allowed by pIR-DW and pIR-DMS was prevented by tryptophan, possibly reflecting inhibition of the PRT activity previously reported (20, 22, 35). Because of common problems with assessing complementation and growth phenotypes with plasmids present, data from the experiments described above were considered only as a qualitative assessment of function and regulation; continued experiments utilized the chromosomal mutation(s).

**trpR** mutations allow *PRA* synthesis in vivo. The results from the plasmid studies showed that overproduction of TrpDE, even when the mutant allele is absent, allowed PRA synthesis sufficient for thiamine synthesis in vivo. A simple extrapolation of this conclusion suggested that regulatory mutations derepressed for the *trp* operon (i.e., *trpR*) would allow thiamine-independent growth of *purF* mutants. A *trpR3612*: Tn10(Tc) mutation was transduced into strain DM1936 (*purF*), and the resulting strain was assessed for thiamine-independent growth. When assessed on solid media, the *trpR* mutation allowed thiamine-independent growth, although it was somewhat weaker than that allowed with the *trpD3611* allele (data not shown).

**Can AS catalyze *PRA* synthesis in vitro.** Crude cell extracts were generated from strains DM6418 (wild type) and DM6417 (*trpD3611*) grown under conditions previously determined to induce the *trp* operon (21, 22). Three biochemical activities previously attributed to the AS-PRT complex were assayed. These assays measured glutamine-dependent AS, ammonium-dependent AS, and PRT activities, and the results are presented in Table 4. As anticipated, the addition of tryptophan inhibited each of these reactions. Further, the data showed that the *trpD3611* allele decreased the PRT activity by ~40%, consistent with the location of the mutant residue in the enzyme. While the PRT activity was reduced, the in vivo data suggested that this decrease was not sufficient to negatively affect the tryptophan biosynthetic capacity of the cell. The other two activities of the AS-PRT complex were not altered by the mutation.

In addition to the activities described above, the proposed PRA synthetase activity in these cell extracts was assayed. These assays utilized PRPP in combination with either amm-

![Absorbance (650nm)](Image)

**FIG. 3.** TrpDE, provided in trans, allows thiamine-independent growth. Growth analyses were performed at 37°C as described in Materials and Methods. Growth of DM6808 [purF2085 gnd-181(pSU19)] (●), DM6809 [purF2085 gnd-181(pIR-EDW)] (△), DM6810 [purF2085 gnd-181(pIR-EDMS)] (▲), DM6811 [purF2085 gnd-181(pIR-DW)] (□), and DM6812 [purF2085 gnd-181(pIR-DMS)] (●) in glucose-adenine medium is shown.

**TABLE 4.** The *trpD3611* allele alters catalytic properties of the AS-PRT complex

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Activity*</th>
<th>Activity*</th>
<th>Activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PRT</td>
<td>AS(Gln)b</td>
<td>AS(NH₄)c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Without Trp</td>
<td>With Trp</td>
<td>Without Trp</td>
</tr>
<tr>
<td>DM6418</td>
<td><em>purF gnd</em></td>
<td>10.2 ± 1.3</td>
<td>3.87 ± 0.92</td>
<td>10.2 ± 1.8</td>
</tr>
<tr>
<td>DM6417</td>
<td><em>purF gnd trpD3611</em></td>
<td>6.58 ± 1.1</td>
<td>2.50 ± 0.28</td>
<td>10.5 ± 1.7</td>
</tr>
</tbody>
</table>

* Strains were grown in minimal glucose medium supplemented with acid-hydrolyzed casein, adenine, and thiamine, as indicated in Material and Methods. Except as indicated, the data represent the averages and standard deviations from three independent experiments performed in triplicate. Trp, tryptophan. For activity units, see Materials and Methods.

b AS(Gln), glutamine-dependent AS activity.

c AS(NH₄), NH₄-dependent AS activity.

d Assays were performed only once in presence of tryptophan.

ND, not detectable.
nia or glutamine. When glutamine was present, no PRA formation was detected (data not shown). However, when NH₄Cl was provided, the cell extracts of DM6417, but not those of DM6418, generated detectable PRA, as shown in Fig. 4. Significantly, the PRA-forming activity was inhibited by tryptophan, as predicted by the in vivo results. The amount of PRA detected was small, a result not unexpected given the cellular requirement for thiamine and the extremely short half-life of PRA in aqueous solution (38). However, this was the first demonstration of enzymatic generation of PRA in a strain lacking the PurF enzyme, and it served to explain the growth data presented throughout this paper.

**DISCUSSION**

This study describes the isolation of a mutation allowing thiamine synthesis to be independent of both PurF and the oxidative pentose phosphate pathway. Characterization of this mutation determined that it caused a proline-to-leucine change at residue 362 of the trpD gene product. Together, trpD and trpE encode the AS-PRT complex, which catalyzes the first two steps in the tryptophan biosynthetic pathway (Fig. 5). Since previous work demonstrated that PurF-independent thiamine synthesis is synonymous with PRA formation (15), the simplest interpretation of the thiamine-independent growth of the purF gnd trpD3611 mutant was that PRA was being generated by the mutant AS-PRT complex. This hypothesis is supported by the biochemical and genetic data presented here.

AS-PRT is a multifunctional enzyme with complex regulation and multiple intersubunit and interdomain interactions (9, 23, 24, 32). Although the different activities associated with this enzyme complex appear to be functionally independent and can be physically separated (4, 28, 46, 48), interactions between the AS-II glutamine domain, AS-II PRT domain, and AS-I have been reported (9, 20, 22, 32, 35). The primary PRA-forming enzyme in the cell, glutamine amidophosphoribosyl transferase (PurF), shares several features with AS-PRT. Both enzymes have NH₃- and glutamine-dependent activities (47, 48) that ultimately result in the condensation of a phosphoribosyl molecule and an amino group (Fig. 2). Significantly, P362

![FIG. 4. Synthesis of PRA from PRPP and NH₄Cl. PRA-forming activity in a cell extract from strain DM6417 was determined by using a coupled assay with GAR synthetase. In the coupled reaction a molecule of provided [¹⁴C]glycine is incorporated into the PRA structure. Synthesis of PRA from PRPP and NH₄Cl was determined as a function of [¹⁴C]GAR synthesis. Labeled GAR and glycine were separated on polyethyleneimine-cellulose with a methanol-pyridine-water (20:1:5) solvent system. Quantification determined that lane 3 contained two-fold more GAR than the control lane without NH₄Cl. No increase in GAR formation was seen with cell extracts from DM6418.](https://jb.asm.org/)

![FIG. 5. Schematic representation of wild-type and mutant AS-PRT complexes. AS-PRT catalyzes the first two steps in tryptophan biosynthesis, which involve amidotransferase and PRT activities of component II (TrpD). The site for allosteric inhibition by tryptophan is on TrpE, and its approximate location is indicated. Solid lines represent the defined reaction path. Dotted lines reflect the proposed reaction catalyzed by the AS complex leading to PRA synthesis. An asterisk indicates the position of the trpD3611 mutation in TrpD.](https://jb.asm.org/)
is located in the PRT subdomain of TrpD, consistent with the proposed effect of this substitution on PRA formation. Compared to the *S. solfataricus* sequence, P362 is positioned in the anthranilate binding site near the conserved R364 residue. In the recently solved crystal structure, R364 is proposed to provide a ligand to the pyrimidine ring of anthranilate (30).

The position of P362 in a critical binding site suggested that altered catalytic properties of the AS-PRT complex might be detected in vitro. Analysis of catalytic activities of AS-PRT in crude cell extract showed that the *trpD3611* mutation decreased the PRT activity of the enzyme while leaving the AS activities unchanged (Table 4). Based on growth of the mutant strains, any reduction in phosphoribosyl anthranilate caused by the *trpD3611* mutation was not sufficient to disrupt the normal role of this enzyme in tryptophan biosynthesis. Significantly, the mutant extract was able to generate detectable PRA from PRPP and ammonia. As expected, all catalytic activities detected were inhibited by the addition of tryptophan. To our knowledge, this is the first case of enzymatic PRA formation being detected in the absence of a functional PurF, and it justifies the interpretation of the in vivo growth results presented here.

Together, the biochemical results are consistent with the location of the P362L substitution within the anthranilate binding site in the PRT domain of the enzyme. One scenario for the flow of metabolites in the AS-PRT enzyme that could result in PRA formation from known substrates is schematically illustrated in Fig. 5. In the analyzed structure of AS-PRT, the binding sites for PRPP and anthranilate are too distant to undergo catalysis; therefore, domain rearrangements have been proposed to occur during substrate binding (30). We hypothesize that the P362L substitution could decrease the efficiency of anthranilate binding, affecting the proper conformational changes required for phosphoribosyl anthranilate synthesis, and favor PRA production by allowing condensation between PRPP and ammonia. Kinetic analysis of purified enzymes will be required to test this prediction.

While the *trpD3611* mutation restored full thiamine-independent growth to a *purF* gnd mutant strain, the wild-type AS-PRT enzyme or the TrpD subunit alone was able to partially satisfy the PRA requirement of thiamine synthesis if provided in multicopy. This result suggested that in the wild-type enzyme either glutamine or the NH$_3$ released on cleavage of the glutamine has the potential to react with PRPP in the AS-PRT complex to generate PRA. These results suggested that derepression of the *trp* operon might similarly generate PRA for thiamine synthesis. Although not obtained in numerous mutant screens for PRA synthesis, when *trpR* mutations were introduced into strains lacking *purF*, thiamine-independent growth was observed. This finding confirmed that under some conditions, wild-type AS-PRT is able to contribute to PRA synthesis in vivo.

While PRA formation by the wild-type AS-PRT complex was not detected in the *purF* mutant extract, it was possible that this enzyme contributed to thiamine synthesis under some conditions in vivo. The finding that tryptophan inhibited PurF-independent thiamine synthesis of a *purF* gnd *yigF* mutant strain on glucose medium (data not shown) suggested that PRA formation in this situation involved AS-PRT activity. Multiple enzymes are expected to participate in PRA synthesis, making it difficult to define the contributors in vivo. In addition, the low levels of PRA needed to satisfy the cellular thiamine requirement, combined with the instability of PRA (38), make the biochemical approach equally laborious. The work described here has taken advantage of a mutant allele to identify the AS-PRT complex as a potential contributor to the formation of PRA in vivo. In addition, a new catalytic activity for a well-studied enzyme has been demonstrated.

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