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Anthranilate phosphoribosyl transferase (TrpD) generates phosphoribosylamine for thiamine synthesis from enamines and phosphoribosyl pyrophosphate

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Abstract

Anthranilate phosphoribosyl transferase (TrpD) has been well characterized for its role in the tryptophan biosynthetic pathway. Here, we characterized a new reaction catalyzed by TrpD that resulted in the formation of the purine/thiamine intermediate metabolite phosphoribosylamine (PRA). The data showed that 4- and 5-carbon enamines served as substrates for TrpD and the reaction product was predicted to be a phosphoribosyl-enamine adduct. Isotopic labeling data indicated that the TrpD reaction product was hydrolyzed to PRA. Variants of TrpD that were proficient for tryptophan synthesis were unable to support PRA formation *in vivo* in *Salmonella enterica*. These protein variants had substitutions at residues that contributed to binding substrates anthranilate or phosphoribosyl pyrophosphate (PRPP). Taken together the data herein identified a new reaction catalyzed by a well-characterized biosynthetic enzyme and both illustrated the robustness of the metabolic network and identified a role for an enamine that accumulates in the absence of reactive intermediate deaminase RidA.

Bifunctional anthranilate synthase-phosphoribosyl transferase (TrpD; EC 4.1.3.27, 2.4.2.18) has been rigorously characterized from model organisms such as *Salmonella enterica* and *Escherichia coli*. TrpD forms a heterotetramer *in vivo* with TrpE (anthranilate synthase component I), and the complex is responsible for the first step in tryptophan synthesis: the transfer of an amino group from glutamine to chorismate and the formation of anthranilate (1). The TrpD component then catalyzes the second step in tryptophan synthesis, the transfer of a phosphoribosyl group to anthranilate to generate phosphoribosyl anthranilate (PR-anthranilate), without participation by TrpE (Figure 1a). In the absence of TrpE, TrpD exists as both a monomer and a homodimer (2) and can catalyze the phosphoribosyl transfer but does not retain the glutamine amidotransferase activity (3).

Despite a solid understanding of the *in vitro* biochemistry of TrpD, contributions of this enzyme to the metabolic network continue to be uncovered by *in vivo* studies. In *S. enterica*, TrpD has been implicated in two different mechanisms of generating the thiamine precursor phosphoribosylamine (PRA). PRA formation *in vivo* is determined as the ability to support growth in the absence of exogenous thiamine when the first step in the purine/thiamine biosynthetic pathway (PurF) is not present (4). In the first mechanism, if the PR-anthranilate product of TrpD was not consumed by TrpC, it accumulated and broke down to ribose 5'-phosphate (R5P) and anthranilate (5, 6). With excess ammonia present in the medium, PRA was formed non-enzymatically from R5P and free ammonia in a well characterized reaction (7).

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In a second mechanism, TrpD-, IlvA-dependent PRA synthesis occurred in strains lacking the protein RidA (formerly YjgF) and was independent of excess ammonia in the growth medium (8–10). The latter mechanism was reconstituted *in vitro* with the pyridoxal 5'-phosphate (PLP)-dependent enzyme threonine dehydratase (IlvA; EC 4.3.1.19), threonine, phosphoribosyl pyrophosphate (PRPP) and TrpD (11). In the *in vitro* system, an uncharacterized metabolite generated by IlvA served as the nitrogen donor for PRA formation. The enzyme RidA reduced PRA synthesis by this mechanism, suggesting that RidA and TrpD were competing for the same metabolite substrate (11). RidA has been shown recently to increase the rate of hydrolysis of enamine/imine intermediates generated by the PLP-dependent mechanism of IlvA, facilitating formation of the ketoacid products (12). The demonstration that the enamine derived from threonine was a substrate for RidA suggested this metabolite could also be the substrate used by TrpD to generate PRA.

This study was initiated to define the substrate in TrpD-dependent PRA formation and gain insight into the mechanism of this reaction. The data showed that TrpD used 2-aminocrotonate, the enamine derived from threonine, and PRPP to generate PRA. Further, results suggested that this mechanism proceeded through a phosphoribosyl-enamine intermediate that was then hydrolyzed to PRA (Figure 1b). This is the first characterized role for the 2-aminocrotonate that accumulates *in vivo* in the absence of RidA. Thus, this work identified a new substrate for the well-characterized enzyme TrpD and highlighted a role for an unrelated short-lived metabolite to satisfy a biosynthetic need *in vivo*.

RESULTS AND DISCUSSION

TrpD-dependent PRA formation involves an unstable metabolite

Threonine dehydratase (IlvA) generated a metabolite from threonine that served as the nitrogen donor in TrpD-dependent PRA formation (12). The enamine/imine intermediates derived from threonine have short half-lives estimated to be 3 minutes or less under relevant *in vitro* conditions (13). Thus, to see if TrpD could be using one of the short-lived intermediates generated by IlvA as a substrate, PRA assays were performed with TrpD added at various times after the reaction had begun. PRA was monitored by a coupled assay that detected the stable product ¹⁴C-glycinamide ribonucleotide (¹⁴C-GAR), generated from the condensation of ¹⁴C-glycine and PRA by GAR synthetase (PurD) (11, 14, 15). Assays containing PRPP and IlvA were initiated with threonine, and TrpD was added at subsequent times. As the length of time before TrpD addition increased, the amount of product GAR (reflecting PRA) that was made decreased (Figure 2a), indicating that the substrate of TrpD was not stable. The IlvA-catalyzed disappearance of threonine mirrored the decrease in substrate available for GAR formation (Figure 2b). As was previously reported, only ~1% of the threonine was converted into GAR (11). Taken together these data supported the hypothesis that the unstable enamine/imine product of IlvA catalysis was a substrate for TrpD in the formation of PRA.

PRA formation proceeds via a phosphoribosyl-enamine intermediate

The data above, and results from previous *in vivo* and *in vitro* experiments (9, 11) were consistent with a mechanism in which TrpD combined PRPP with the enamine/imine metabolite generated by IlvA from threonine. (Control experiments showed that TrpD did not generate PRA from PRPP and ammonia (11).) In the proposed mechanism, a C-N bond would be formed that is akin to the one in the product of the phosphoribosyl transfer catalyzed by TrpD in tryptophan biosynthesis (Figure 1a). Two different mechanisms could be imagined for the conversion of the resulting phosphoribosyl-enamine adduct to PRA. In one scenario, the adduct would directly break down into PRA, either non-enzymatically or facilitated by TrpD (Figure 1b, pathway I). In an alternative scenario, the phosphoribosyl-

enamine adduct would break down into R5P and 2-aminocrotonate, which would tautomerize to 2-iminobutyrate and spontaneously hydrolyze to 2-ketobutyrate and ammonia. R5P and ammonia could then combine non-enzymatically to generate PRA (Figure 1b, pathway **II**). Both scenarios were consistent with the finding that in a *ridA* mutant, PRA formation did not require excess ammonia in the medium (10).

The two mechanistic scenarios were distinguished by labeling experiments. Equimolar ^{15}N -threonine and ^{14}N -ammonium chloride were added to a PRA formation reaction. The composition of the resulting GAR was determined by mass spectral analysis at several time points during the reaction. At early times (10, 20, and 30 minutes), the GAR consisted of 2 species in approximately a 1:2 ratio (^{14}N -GAR: ^{15}N -GAR) (Figure 3a). However, after 4 hours (the full reaction incubation), the 2 species existed in a 1:1 ratio (Figure 3b). The result at the four hour timepoint could be explained by if there was non-enzymatic combination of 2-ketobutyrate and ammonia to form the imine/enamine intermediate, which then re-entered the reaction cycle to form GAR. To address this possibility, reactions containing 2-ketobutyrate and excess ammonia were performed. In these reactions, 200 μM GAR was generated after 4 hours, despite the lack of threonine. It is possible that this mechanism of GAR formation accounted for the lesser amount of ^{14}N -GAR seen at earlier timepoints as well.

It was also possible that the labeling of GAR was impacted by exchange of the amino group on either PRA or the enamine intermediate with solvent ammonia. Two experiments ruled out these possibilities. First, the labeling experiment described above was repeated with two- and ten-fold more PurD activity present. Increased levels of PurD would decrease the PRA available for possible exchange with solvent ammonia. The mass ratios of GAR were unchanged by the concentration of PurD, ruling out significant exchange of the PRA nitrogen with solvent ammonia (data not shown). This result was consistent with the characterized properties of PRA and PurD: the half-life of PRA is 35 sec (at 37 °C, pH 7.5; higher with increasing pH (16)), and PurD has a low K_m (10 μM for PRA (7)) and high turnover (7 sec^{-1} (16)), suggesting that PurD would act on PRA as fast as it was generated. An additional labeling experiment was set up with threonine and excess ^{15}N -ammonium chloride. The enamine/imine intermediates of the IlvA reaction were reduced with sodium borohydride at various times during the reaction to generate stable product 2-aminobutyrate. The mass composition of the resulting 2-aminobutyrate was determined, and the vast majority of the species were ^{14}N -labeled (Figure 3c), indicating that there was not significant nitrogen exchange between the intermediates and solvent ^{15}N -ammonia.

Taken together, the labeling data suggested that a majority of the GAR was derived directly from threonine, especially in light of the higher fraction of ^{15}N -labeled GAR compared to ^{14}N -labeled GAR found at early timepoints. However, it is formally possible that the presence of both ^{14}N -GAR and ^{15}N -GAR at these early timepoints is due to a contribution of both mechanisms (pathways **I** and **II**) occurring at the same time. The only model that can confidently be ruled out by these data is that all of the PRA is generated via R5P and ammonia intermediates (pathway **II** exclusively). All of the previously identified mechanisms of PurF-independent PRA generation depended on R5P and ammonia combining non-enzymatically to generate PRA. However, each of those mechanisms relied on increasing the levels of R5P in the cell while utilizing excess ammonia from the growth medium. The mechanism depicted here in pathway **II** relies on the generation of R5P and ammonia in equimolar amounts. It is unlikely that the levels of these intermediates *in vivo* would result in sufficient non-enzymatic production of PRA to support growth.

Substrate specificity of TrpD

The activity characterized above identified a new substrate for TrpD, and raised the question of whether other enamine metabolites would support PRA formation by this mechanism. L-threonine, D-threonine, D/L-3-hydroxynorvaline, L-serine and D-serine were tested for supporting PRA formation in the presence of an appropriate dehydratase. D-serine dehydratase (DsdA; EC 4.3.1.18) was used to dehydrate D-amino acids. All of the 3-hydroxy amino acids resulted in a detectable amount of PRA (Table 1; Figure 4). The IlvA isozyme, TdcB, also generated PRA from L-threonine (data not shown). When serine was used as a substrate, less PRA was generated than when the substrates had 4 or 5 carbons. This result could be because the serine-derived enamine, 2-aminoacrylate, has a significantly shorter half-life than the threonine-derived 2-aminocrotonate (1.5 sec versus approximately 3 min, respectively at pH 8) (13, 17). Alternatively, the difference in product formation could reflect specificity of an active site. Performing the PRA formation assay at a higher pH to increase the stability of the intermediate (18, 19), did not increase the product formation from serine (data not shown). This result suggested that TrpD preferred the 4- and 5-carbon enamines. Further, the threonine analog 2-aminobutyrate did not support PRA formation, emphasizing the role of the reactive enamine in catalysis. In the absence of TrpD, a background level of GAR was produced in the reactions with IlvA, PRPP and threonine (Table 1, line 6). PRA in this reaction was formed non-enzymatically from R5P, a contaminant of PRPP stocks, and ammonia released from threonine deamination as has been reported previously (11). These data suggested the mechanism of PRA formation facilitated by TrpD is specific for 4- or 5- carbon enamines, which was consistent with the *in vivo* data that found threonine was the only supplement that increased the TrpD-dependent PRA formation (9).

Variants of TrpD unable to support PRA synthesis retain tryptophan biosynthesis

Since the mechanism of PRA formation proposed here was analogous to the characterized phosphoribosyl transferase reaction of TrpD with anthranilate and PRPP, it was reasonable to suggest that the enamine substrate used the same binding site. Crystal structures of several TrpD homologs have been solved with substrates bound (20, 21). The structure of the *Sulfolobus solfataricus* TrpD showed two molecules of anthranilate in the active site. The amino group of one anthranilate molecule was stabilized by hydrogen bonding with a backbone glycine, and an asparagine residue analogous to Asn309 in the *S. enterica* protein bound the carboxylic acid. The second anthranilate also interacted with Asn309 and hydrogen bonded with a residue analogous to the Arg364 position in *S. enterica* TrpD (21). Two additional residues (His307 and Gly308 in the *S. enterica* protein) were proposed to interact with the anthranilate substrate based on a structure of the *Mycobacterium tuberculosis* protein (20). In both the *S. solfataricus* and *M. tuberculosis* structures, residues analogous to Gly278, Lys306 and Asp282 in *S. enterica* were involved in binding the PRPP substrate (20, 21).

To address the hypothesis that TrpD could bind enamine substrates in the same place(s) as anthranilate, *in vivo* analyses were performed with TrpD variants. Informed by the residues implicated in the crystal structure studies (20, 21), site directed mutagenesis was used to generate *S. enterica trpD* alleles encoding TrpD^{T279A}, TrpD^{D282A}, TrpD^{K306A}, TrpD^{H307A}, TrpD^{G308L}, TrpD^{N309A}, and TrpD^{R364A} variants. Function of the variants in thiamine and tryptophan biosynthesis was assessed *in vivo* using a *purF gnd ridA trpD* strain (DM9813) that has an auxotrophic requirement for purines, thiamine, and tryptophan. The mutant *trpD* alleles were individually expressed at low levels *in trans*, and the ability of each variant to restore thiamine and/or tryptophan synthesis sufficient to allow growth was assessed. Expression of the wild-type *trpD* allowed growth of the *purF gnd ridA trpD* strain on minimal adenine medium, which confirmed that a functional TrpD protein was required for

both thiamine and tryptophan biosynthesis in this strain background. None of the TrpD variants generated sufficient PRA to allow growth in the absence of thiamine (Figure 5a and data not shown). These data indicated that the variant proteins were unable to support enough PRA formation to satisfy the cellular thiamine requirement. In contrast, each of the TrpD variant proteins, except TrpD^{K306A} and TrpD^{R364A}, synthesized sufficient tryptophan to allow growth (Figure 5b). Growth of strains carrying TrpD^{H307A} and TrpD^{N309A} in the absence of tryptophan was not significantly different than the strain carrying the wild-type TrpD. The remaining three variants (TrpD^{T279A}, TrpD^{D282A}, and TrpD^{G308L}) supported slower growth than the wild-type protein, suggesting they were partially defective in the anthranilate phosphoribosyl transferase activity. All strains were able to grow when both tryptophan and thiamine were present in the minimal adenine medium.

The growth results in the absence of tryptophan obtained here were consistent with the limited *in vitro* analysis of TrpD variants performed previously with the *S. solfataricus* TrpD (21). In the previous study, variants with substitutions at the residues analogous to *S. enterica* Arg364, His307, and Lys306 had increased K_m values for anthranilate (21), with the largest defect observed in the variant analogous to TrpD^{R364A}. Consistently, the TrpD^{R364A} variant described here was unable to synthesize enough tryptophan or PRA to support growth. No catalytic residues were identified in the *S. solfataricus* study. Rather, the authors suggested that the role of TrpD was to orient the substrates, facilitating the attack by anthranilate and the concomitant release of pyrophosphate from PRPP (21). If the enamine substrates described here bound in the same pocket in TrpD as anthranilate, then hydrogen-bonding interactions between the enamine and the enzyme could stabilize the short-lived substrate long enough for it to attack PRPP (Figure 1b). In this scenario, it was surprising that TrpD variants that supported tryptophan synthesis were unable to allow PRA synthesis sufficient for growth. It is possible that the enamines required a specific conformation of the active site while anthranilate could bind more flexibly. A more likely explanation is that the K_m of TrpD for enamine substrates is high in the context of the physiological level of these metabolites. In this case substitution of a residue involved in substrate binding could raise the K_m for the enamine above a concentration that would accumulate *in vivo*, even in the absence of the enamine deaminase RidA. It is also possible that TrpD performs two roles in PRA formation: generating the phosphoribosyl-enamine adduct and then the subsequent hydrolysis to form PRA. In this scenario, the enzyme could be more sensitive to mutations that affect either activity.

PRA forming activity is not a characteristic of other phosphoribosyl transferases

S. enterica has at least 11 other phosphoribosyl transferase enzymes in addition to TrpD, some of which combine PRPP with different nitrogenous substrates. Two of these enzymes were tested for ability to facilitate PRA formation *in vitro*. Neither adenine phosphoribosyl transferase (Apt; EC 2.4.2.7) nor ATP phosphoribosyl transferase (HisG; EC 2.4.2.17) could substitute for TrpD in the PRA forming reaction (data not shown). This result suggested the ability to facilitate PRA formation from enamine intermediates is specific to TrpD and not a general feature of PRPP-binding enzymes with similar catalytic mechanisms. This result was consistent with the genetic data that identified TrpD as essential for the PurF-independent mechanism of PRA formation that occurs in a *ridA* mutant (9, 11).

Conclusions

In the absence of the first step in purine biosynthesis (PurF) and the enamine/imine deaminase RidA, thiamine synthesis in *S. enterica* depends on anthranilate phosphoribosyl transferase (9, 12). A previous study demonstrated that RidA eliminated enamine intermediates generated by PLP enzymes (12). In the absence of RidA the reactive enamines are expected to react with other cellular targets, resulting in the diverse metabolic

phenotypes of a *ridA* (*yjgF*) mutant in *S. enterica* (8, 9, 22, 23). This is the first characterized role for the 2-aminocrotonate that accumulates in the absence of RidA. This study showed that TrpD could utilize this reactive intermediate to generate a metabolite critical for thiamine synthesis. TrpD likely facilitated the formation of a phosphoribosyl-enamine adduct and possibly the subsequent conversion of this adduct to PRA, though the second step could also be envisioned to be non-enzymatic. Though the precise mechanism of TrpD-dependent PRA formation requires further study, it is clear from the labeling data presented here that R5P and ammonia intermediates are not always required to generate PRA in the absence of PurF. Thus, this study identified both a new function for the biosynthetic enzyme TrpD and the mechanism of direct PRA formation that occurred in the absence of PurF.

METHODS

Chemicals

L-1-¹⁴C-glycine was purchased from DuPont and both L-¹⁵N-threonine and ¹⁵N-ammonium chloride were purchased from Cambridge Isotope Laboratories. All other chemicals were purchased from Sigma-Aldrich Chemical Co.

Protein Purification

PurD, IlvA, and TrpD proteins were purified using His₆ tags as previously described (11, 24). DsdA was purified from an N-terminal His₆-tagged construct in the ASKA (A Complete Set of *E. coli* K-12 ORF Archive) collection (JW2363) (25). 10-mL overnight cultures were grown in Luria-Bertani broth with chloramphenicol (20 mg L⁻¹) and used to inoculate 3 liters of superbroth (32 g tryptone, 20 g yeast extract, 5 g sodium chloride, and 1 mL 5 N sodium hydroxide L⁻¹) with chloramphenicol (20 mg L⁻¹). When an optical density (650 nm) of 0.3 was reached, isopropyl-β-D-thiogalactoside (0.1 mM) was added and growth continued for 8 hours at 37 °C. Cells were then pelleted, resuspended in 50 mM Tris-HCl pH 8.0 with 100 mM NaCl and 11% (v/v) glycerol, and disrupted with a French pressure cell. Extract was clarified by centrifugation (48,000 *g* for 45 min) and applied to nickel-nitrilotriacetic superflow resin. The relevant protein was purified according to manufacturer's protocol (Qiagen) using column chromatography. The purified protein was concentrated, dialyzed three times against 50 mM Tris-HCl pH 8.0 with 100 mM NaCl and 11% (v/v) glycerol, and stored at -80 °C.

Phosphoribosylamine Formation Assay

Synthesis of PRA was determined as a function of ¹⁴C-GAR formation with a coupled assay described previously (11, 14, 15). Briefly, standard assays contained 100 mM Tris-HCl, 10 mM magnesium acetate, 2.7 mM adenosine triphosphate, 27 μM PLP, 1 mM PRPP, 5 mU (1 μM) GAR synthetase (PurD; EC 6.3.4.13), 1 μM IlvA, 2 μM TrpD, and 2 mM (26 nCi) ¹⁴C-glycine. Reactions were initiated by the addition of substrates at indicated concentrations, and mixtures incubated for 4 h at 37 °C. ¹⁴C-glycine and ¹⁴C-GAR were separated by thin-layer chromatography in a 1:1 *n*-propyl alcohol:water system and detected by phosphorimage.

For GAR labeling experiments, the PRA formation reaction contained unlabeled glycine, 50 mM ¹⁵N-threonine, and 50 mM ammonium chloride. The reaction was incubated for times ranging from 10 minutes to 4 hours at 37 °C before proteins were removed by running the reaction through a 10,000 molecular weight cut-off centrifugal filter unit (Millipore). Samples were submitted for liquid chromatography negative time-of-flight (-TOF) mass spectral analysis, carried out at the University of Wisconsin Biotechnology Center. For 2-aminobutyrate labeling experiments, reactions contained IlvA, 50 mM threonine, and 50

mM ^{15}N -ammonium chloride. Duplicate reactions were stopped by addition of sodium borohydride (50 mM final concentration) at times ranging from 30 seconds to 30 minutes after initiating reactions with threonine. This treatment resulted in the reduction of the aminocrotonate/iminobutyrate intermediates to 2-aminobutyrate. Samples were submitted for liquid chromatography positive (+) TOF mass spectral analysis, carried out at the University of Wisconsin Biotechnology Center.

Bacterial strains and growth analyses

All strains used for growth analysis were derivatives of *S. enterica* serovar Typhimurium strain LT2 and contained the *purF2085 gnd181 ridA3::MudJ trpD16* mutations (DM9813). The *purF2085 gnd181 ridA3::MudJ* strain (DM7436) has been described (9). The *trpD16* allele was obtained from the Salmonella Genetic Stock Center in strain SA2437 and was moved by transduction. Plasmids used in this study are listed in Table 2. The *trpD* gene was cloned in to pET14b at a SacI site (added as a linker between the NdeI and XhoI sites) and a BamHI site, producing an N-terminal His₆-tagged construct. Site-directed mutations were introduced using the QuikChange II site-directed mutagenesis kit according to manufacturer's protocol (Agilent Technologies). Mutations were confirmed by sequence analysis performed at the University of Wisconsin Biotechnology Center.

Growth analyses were performed in liquid no-carbon E media (26) with MgSO₄ (1 mM), glucose (11 mM), and adenine (0.4 mM, to satisfy the purine auxotrophy). When indicated, tryptophan (0.1 mM) and/or thiamine (100 nM) were added to growth media. Strains were grown overnight in rich medium with ampicillin (150 mg L⁻¹) at 37 °C, subcultured 1:40 into the relevant minimal media in a 96-well microtiter plate and grown at 37 °C with shaking for 18 hours in a BioTek ELx808 Absorbance Microplate Reader. Growth was monitored by optical density at 650 nm.

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Figure 1. Reaction mechanisms of TrpD-dependent PRA formation

a) Characterized reactions of TrpD: TrpD combines anthranilate and PRPP to generate PR-anthranilate. b) Two possible pathways for PRA formation from enamine 2-aminocrotonate: TrpD facilitates the attack by the nitrogen of 2-aminocrotonate on the 1' C of PRPP, displacing pyrophosphate. A PR-enamine adduct intermediate is formed, which could have one of two fates. In pathway **I**, the intermediate breaks down directly into PRA and 2-KB (a reaction either mediated by TrpD or non-enzymatic). In pathway **II**, the intermediate breaks down non-enzymatically into R5P, 2-KB, and ammonia. R5P and ammonia combine non-enzymatically to form PRA. Abbreviations: PRPP, phosphoribosyl pyrophosphate; PR-anthranilate, *N*-phosphoribosyl anthranilate; PR-enamine, phosphoribosyl-aminocrotonate adduct; 2-KB, 2-ketobutyrate; R5P, ribose-5'-phosphate; PRA, phosphoribosyl amine.

Figure 2. The alternative substrate of TrpD is unstable

PRA forming reactions were performed as described in Methods. a) The stability of the substrate used by TrpD to generate PRA was assessed by monitoring the ability of TrpD to generate PRA when the protein was added at various times after initiating reactions with threonine. Reactions were incubated for 4 hours post-addition of TrpD, and the amount of resulting GAR was quantified. Error bars represent ranges of two replicates. b) ^{14}C -threonine was quantified from a standard PRA forming reaction mixture over time. ^{14}C -threonine and product ^{14}C -2-ketobutyrate were separated by thin-layer chromatography and visualized and quantified by phosphorimage. The amount of ^{14}C -threonine is shown. Error bars represent ranges of two replicates.

Figure 3. GAR formed in PRA assay is mostly ^{15}N -labeled

a, b) Shown are mass spectral data from $-$ TOF analysis of the GAR reaction products described in the text that contained ^{15}N -threonine and ^{14}N -ammonium. Expected m/z of unlabeled GAR in this mode is 285.0482, and ^{15}N -GAR should be +1 amu. a) Reaction incubated 30 minutes (reactions from 10–60 minutes were not significantly different). b) Reaction incubated 4 hours. c) Shown are mass spectral data from $+$ TOF analysis of 2-aminobutyrate generated by reducing unlabeled enamine/imine intermediates with borohydride, in the presence of excess ^{15}N -ammonium chloride. Shown is 30-minute reaction; data from shorter reactions were not significantly different. Expected m/z of unlabeled 2-aminobutyrate in this mode is 104.0706, and ^{15}N -labeled 2-aminobutyrate is +1 amu.

Figure 4. TrpD uses multiple substrates

Multiple enamine substrates and a non-enamine analog were tested for PRA formation by TrpD. a) substrates TrpD used well: **1** [2-aminocrotonate (derived from threonine)] and **2** [2-amino-2-pentenoate (derived from 3-hydroxynorvaline)]. b) substrate TrpD used to a lesser degree: **3** [2-aminoacrylate (derived from serine)]. c) substrate TrpD did not use: **4** [2-aminobutyrate].

Figure 5. TrpD variants fail to support PRA synthesis *in vivo*
purF gnd ridA trpD strains containing plasmids encoding different TrpD variants (Table 1) were grown in minimal adenine media with various supplements. a) A derivative of a *purF gnd ridA trpD* strain (DM9813) containing a plasmid encoding TrpD^{H307A} was grown in minimal adenine media (no add.), with supplements of tryptophan (+ trp), thiamine (+ thi), or both tryptophan and thiamine (+ trp, thi). b) Derivatives of a *purF gnd ridA trpD* strain encoding TrpD variants were grown in minimal adenine medium supplemented with thiamine (lacking tryptophan). An empty vector or plasmids containing wild-type TrpD, TrpD^{T279A}, TrpD^{D282A}, TrpD^{H307A}, TrpD^{G308L}, and TrpD^{N309A} were present in the strain. Growth of strains expressing TrpD^{K306A} and TrpD^{R364A} (not shown) were indistinguishable from the empty vector control. Growth curves were performed in triplicate, and mean values are shown. Errors were insignificant and have been removed for simplicity. Active site schematics displaying these residues in relationship to the TrpD substrates can be found in Lee *et al.* (20) and Marino *et al.* (21).

Table 1

TrpD uses 4- or 5-carbon enamines in PRA formation.

Substrate ^a , Enzyme	GAR formed (μM)
L-threonine, IlvA	438 ± 26
D-threonine, DsdA ^b	262 ± 14
D/L-3-hydroxynorvaline, IlvA	250 ± 11
L-serine, IlvA	67 ± 3
D-serine, DsdA	59 ± 3
L-threonine, IlvA; No TrpD	37 ± 1
2-aminobutyrate ^c	9 ± 1

PRA formation assays were performed with the indicated dehydratase enzyme and substrate as described in Methods. Product is represented as μM GAR formed at the end of 4 hours, and data represent average and ranges of 2 replicates.

^aSubstrates were present in the following concentrations: L-threonine, 50 mM; D-threonine, 50 mM; DL-3-hydroxynorvaline, 100 mM; L-serine, 80 mM; D-serine, 80 mM; and 2-aminobutyrate, 50 mM.

^bDsdA was used to dehydrate D-amino acids, but was not used with D/L-3-hydroxynorvaline as preliminary studies indicated that the enzyme used the substrate very poorly.

^cAssays with 2-aminobutyrate did not have a dehydratase enzyme present.

Table 2

Plasmid list

Plasmid	Vector and protein encoded
pDM1353	pET14b-TrpD (WT)
pDM1354	pET14b-TrpD ^{R364A}
pDM1355	pET14b-TrpD ^{T279A}
pDM1356	pET14b-TrpD ^{D282A}
pDM1357	pET14b-TrpD ^{K306A}
pDM1358	pET14b-TrpD ^{H307A}
pDM1359	pET14b-TrpD ^{N309A}
pDM1360	pET14b-TrpD ^{G308L}

All plasmids were generated using pET14b (Novagen) as a vector, encoded an ampicillin resistance marker, and used the T7 promoter for expression. The *S. enterica* strains did not have T7 polymerase, therefore expression from these constructs was low, when judged by SDS-PAGE.