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Mutations in the tryptophan operon allow PurF-independent thiamine synthesis by altering flux *in vivo*.

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ABSTRACT

Phosphoribosyl amine (PRA) is an intermediate in purine biosynthesis and also required for thiamine biosynthesis in *Salmonella enterica*. PRA is normally synthesized by phosphoribosyl pyrophosphate (PRPP) amidotransferase, a high-turnover enzyme of the purine biosynthetic pathway encoded by *purF*. However, PurF-independent PRA synthesis has been observed in strains with different genetic backgrounds and growing under diverse conditions. Genetic analysis has shown that the anthranilate synthase-phosphoribosyltransferase (AS-PRT) enzyme complex, involved in the synthesis of tryptophan, can play a role in the synthesis of phosphoribosyl amine (PRA). This work describes the *in vitro* synthesis of PRA in the presence of the purified components of AS-PRT complex. Results from *in vitro* assays and *in vivo* studies indicate the cellular accumulation of phosphoribosyl anthranilate can result in non-enzymatic PRA formation sufficient for thiamine synthesis. These studies have uncovered a mechanism used by cells to redistribute metabolites to ensure thiamine synthesis, and may define a general paradigm of metabolic robustness.

INTRODUCTION

Phosphoribosyl amine (PRA) participates in cellular metabolism as an intermediate required for both purine and thiamine production (11, 21, 22), and is synthesized by the first enzyme in the purine biosynthetic pathway (PurF) (19, 21). In *Salmonella enterica,* the activity of PurF can be bypassed in thiamine (but not purine) synthesis, indicating the existence of alternative pathways generating at least small amounts of PRA (10, 24). We have hypothesized that PRA synthesis in the absence of PurF is the result of promiscuous enzymes involved in different metabolic processes. This hypothesis was supported by the demonstration that in at least two situations the

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anthranilate synthase- phosphoribosyltransferase (AS-PRT) complex is required for PRA formation *in vivo* (4, 27). The AS-PRT complex is a multi-functional enzyme (chorismate pyruvate-lyase, EC 4.1.3.27 and *N*-(5'-phosphoribosyl)-anthranilate pyrophosphate phosphoribosyltransferase, EC 2.4.2.18) that catalyzes the first and second steps of tryptophan biosynthesis (2, 34) (Fig. 1A). The products of the *trpE* and *trpD* genes, the first two in the tryptophan operon, compose this allosteric heterotetrameric ($TrpE_2$-$TrpD_2$) enzyme, which has been extensively studied (2, 34). The AS-PRT complex catalyzes both the formation of anthranilate from chorismate and L-glutamine (2, 14) and the formation of *N*-(5'-phosphoribosyl)-anthranilate from anthranilate and 5-phosphoribose-1-pyrophosphate (PRPP) (Fig. 1A) (2, 13). Phosphoribosyl transfer requires the carboxy-terminal domain of the TrpD subunit (12) and the ability of the TrpD subunit to perform this reaction is equivalent to that of the complex (TrpE2-TrpD2) (2, 13). Each of the enzymatic activities is negatively feedback-regulated by tryptophan at a well-defined allosteric binding site (20, 34, 35).

The step in the biosynthetic pathway for tryptophan following the action of AS-PRT is catalyzed by the product of *trpC*, N-(5'-phosphoribosyl)anthranilate isomerase, EC 5.3.1.24 (PR-AnI); indole-3-glycerol-phosphate synthase, EC 4.1.1.48 (IGPS). TrpC is a monomeric bifunctional enzyme (18). The two catalytic activities of this enzyme are located on different domains of the protein and act sequentially in the biosynthetic pathway (Fig. 1B). While the N-terminal domain contains the IGPS activity, the C-terminal domain catalyzes the isomerization of PR-anthranilate to 1-(*o*-carboxyphenylamino)-1-deoxyribulose 5-phosphate (CdRP) (15, 33), which is the first of the two reactions metabolically. Both domains consist of a parallel β-barrel core of eight strands that form a pocket where the active site is located, and eight surrounding α-helices (26).

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This study was initiated to better understand the contribution of AS-PRT to PRA formation relevant *in vivo*. Characterization of PRA formation *in vitro* with purified proteins led to the identification of PRA formation *in vivo* that was dependent on both the TrpDE complex and the status of flux through the tryptophan biosynthetic pathway. Thus, this study describes a mechanism by which the activity of the AS-PRT enzyme can contribute to PRA production in the cell in a flux dependent manner.

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MATERIAL AND METHODS

Culture media and chemicals. Culture media was obtained from Difco (Franklin Lake, NJ). Glutamine, glycine, ribose-5-phosphate (R5P), 5-phosphoribose-1-pyrophosphate (PRPP), ammonium chloride and ammonium sulfate, were obtained from Sigma (St. Louis, MO). Tris base, methanol and pyridine were from Fisher Scientific (Pittsburgh, PA). ATP was from Fisher Biotech (Pittsburg, PA). K_2HPO4 and KH_2PO_4 were obtained from Mallinckrodt LabGuard (Phillipsburg, NJ). [1-^{14}C]- glycine was from New England Nuclear (Boston, MA). Cellulose PEI plates were from Selecto Scientific (Suwanee, GA). No-carbon E medium of Vogel and Bonner (8, 31) supplemented with $MgSO_4$ (1 mM) and glucose (11 mM) was used as minimal medium. When present in the culture medium, the following compounds were used at indicated concentrations: adenine, 0.4 mM; thiamine, 0.5 μM; and tryptophan, 0.5 mM. Difco nutrient broth (8 g/l) with NaCl (5 g/l) or Luria-Bertani broth was used as rich medium. Difco BiTek agar was added (15 g/l) for solid medium. Tetracycline was added as needed to 20 μg/ml final concentration in rich media.

Abbreviations. Abbreviations used extensively throughout the manuscript are: GAR, glycinamide ribonucleotide; PRA, phosphoribosyl amine; R5P, ribose-5-phosphate; PR-Ant, phosphoribosyl anthranilate; TrpD, anthranilate synthase component II; TrpC, N-(5'-phosphoribosyl)anthranilate isomerase, indole-3-glycerol-phosphate synthase; PurD, glycinamide ribonucleotide synthetase.

Bacterial strains. All strains used in this study are derivatives of *Salmonella enterica* serovar Typhimurium strain LT2 and are listed with their genotypes in Table 1. Tn10d(Tc) refers to the transposition-defective mini-Tn10 (Tn10Δ16Δ17) (32).

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Genetic methods. *Transduction methods*. Transductional crosses were performed using the high-frequency general transducing mutant of bacteriophage P22 (HT105/1, int-201) (28) as has been described previously (9). Transductants were purified by colony isolation on nonselective green indicator plates (6), and verified to be phage-free by cross-streaking with phage P22.

Strain constructions. *Isolation of trp mutants*. A P22 lysate grown on wild-type strain DM1 previously treated with diethylsulfate (DES) was used to transduce strain DM6806 (*purF2085 gnd-181 trp*::Tn10*d*(Tc)) to Trp^+ on minimal medium with adenine and thiamine. Trp^+ transductants were screened for their ability to grow on minimal adenine medium. All putative Trp^+ Thi^+ mutants were reconstructed by transduction into strain DM8916 (*purF2085 gnd-181 ΔtrpEDCBA*), prior to further characterization. An isogenic strain, carrying a wild-type *trp* operon, was generated by transducing DM8916 to Trp^+ with a lysate grown on a wild-type donor strain (DM1).

Phenotypic analysis. *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 50-µl sample of this suspension was used to inoculate 5 ml of the appropriate medium. 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. 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 micro titer plate. Growth at 37°C was monitored using a microplate spectrophotometer Spectra-Max Plus. The specific growth rate was determined as $\mu = \ln(X/Xo)/T$, where $X = OD_{650}$ during the linear portion of the growth curve and T = time.

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Solid media. Nutritional requirements were measured by growing the strains on rich agar medium and replica plating to minimal agar medium containing the appropriate nutrients. Growth was assessed after 24 h incubation at 37°C. Cross-feeding was measured by spotting relevant samples (cells or supernatants) on a soft agar overlay seeded with a strain containing *trpE8* (old designation *trpA8* (3), kindly provided by J. Roth).

Plasmid constructs. Genomic DNA of strain DM6418 (*purF2085 gnd-181 zdd-9147*::Tn*10*d(Tc)) was purified from bacterial cells using an Easy-DNA kit (Invitrogen Life Technologies; Carlsbad, CA). Cloned Pfu DNA polymerase from *Pyroccoccus furiosus* (Stratagene; La Jolla, Calif.) was used in PCR reactions. The PCR fragment corresponding to the *trpD* (1610 bp) gene was gel-purified, digested with the appropriate restriction enzymes and cloned into the expression vector pET-28a (Novagen, Darmstadt, Germany). The ligation mix was transformed into *Escherichia coli* strain DH5α, and DNA sequencing (University of Wisconsin Biotechnology Center-Nucleic Acid and Protein Facility) was used to verify the plasmid constructs.

Purification of TrpD enzyme. An expression vector containing the *trpD* gene under control of T_7 promoter (pET-trpD) was freshly transformed into *Escherichia coli* BL21. Overnight cultures were used to inoculate Luria-Bertani (LB) broth supplemented with 50 µg/ml kanamycin and the cultures were grown to an optical density at 650nm of 0.6 at 37°C. The cells were cooled to 30°C and then induced with 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were grown for an additional 5 h at 30°C, harvested and resuspended in Binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9). All subsequent purification steps were performed at 4°C. Cells were disrupted using a French pressure cell at 10^4 kPa, followed by brief sonication using a Sonic Dismembrator 550 (Fisher Scientific). Clarified cell-free extract was

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obtained by centrifugation at 23,700 x *g* for 45 min at 4°C. The supernatant was filtered and loaded onto a 10-ml Ni-HisBind resin column equilibrated with Binding buffer. Unbound proteins were washed from the resin with 20 mM Tris-HCl pH 7.9, 6 mM imidazole, 0.5 M NaCl. A linear gradient from 0.02 to 1 M imidazole was used to elute the recombinant protein. TrpD eluted at ~ 0.3 M imidazole. Fractions containing the eluted enzyme were assayed for activity, dialyzed overnight against PED buffer (50 mM potassium phosphate pH 7.5, 0.1 mM EDTA, 0.2 mM DTT) containing 20% glycerol and stored at –80°C. Proteins were purified to >95% purity, as judged by SDS-PAGE with the yield from a typical purification of 100 mg of purified protein per 4 liters of culture.

Enzyme assays. *Phosphoribosyltransferase activity* was assayed fluorometrically (12, 13) by measuring the rate of disappearance of anthranilate at 25°C. The reaction mixture contained 15 μM anthranilate, 0.3 mM 5-phosphoribose-1-pyrophosphate (PRPP), 10 mM $MgCl_2$, 100 mM tricine buffer (pH 7.6), and 0.625 μg of purified enzyme (TrpD) in a final volume of 200 μl. Anthranilate was detected by fluorescence at 325nm excitation and 400nm emission wavelengths.

Phosphoribosylamine-forming activity was determined using a modified assay initially described for PurF (17, 29). Synthesis of PRA from PRPP and ammonium was determined as a function of [1-^{14}C]- glycinamide ribonucleotide (GAR) produced in a coupled reaction catalyzed by GAR synthetase (PurD) enzyme (Fig. 2B). A molecule of [1-^{14}C]-glycine is condensed with PRA to yield GAR. The reactions were performed in 50 mM potassium phosphate buffer pH 8.0 in the presence of 10 mM PRPP, 10 mM $MgCl_2$, 2.5 mM ATP, 25 mM ^{14}C-glycine (26 nCi), 10 mM NH_4Cl, 2 μg of GAR synthetase. PurD was overexpressed and purified as previously described (27). In a standard assay, reactions were started by the addition of 5-10 μg of purified TrpD and

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incubated at 37°C during 1 h. Various changes to this protocol are noted in the text as relevant for each experiment. Labeled GAR and glycine were separated by thin layer chromatography on polyethyleneimine (PEI)-cellulose using a methanol:pyridine:water system (20:1:5) determined experimentally. The position of radioactive spots was detected using a Cyclone Storage Phosphor System (Packard Instrument Company), and their identity was confirmed with known standards.

Protein quantification and manipulation. Protein concentration was calculated at 25°C using the equation $A=\Sigma BC$, where A equal A_{280}-Ls, Ls equal A_{330} x 1.929, Σ is the molar extinction coefficient of the pure protein and C is protein concentration in moles/l. SDS-polyacrylamide gel electrophoresis (PAGE*)* was performed by the method of Laemmli (16) and protein bands were visualized after staining with 40:55:5:0.05 ethanol:water:acetic acid:Coomassie G-250 and destained in 40:55:5 ethanol:water:acetic acid.

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RESULTS

TrpD- mediated PRA generation in vitro requires anthranilate. Previous genetic results suggested that purified TrpD would mediate PRA formation from PRPP and NH_3. TrpD containing an N-terminal His-Tag fusion was purified to >95% purity and assayed for inherent phosphoribosyl transferase activity. A typical preparation converted 1.9 μmoles of anthranilate to phosphoribosyl anthranilate per min when both anthranilate and PRPP were provided in the assay mix. Potential formation of PRA was assessed utilizing PurD in a coupled assay, when PRPP, NH_3 and TrpD were provided in the assay mixture. Under the conditions tested, PRA formation was detected only when anthranilate was also added to the reaction (Fig. 2). When provided in place of anthranilate, methylanthranilate (a competitive inhibitor) failed to allow PRA formation (data not shown). This result suggested that the synthesis of PR-anthranilate was required for production of PRA detected in the presence of TrpD.

PRA formation in the presence of anthranilate is non-enzymatic. Synthesis of PRA by TrpD was monitored as a function of anthranilate (0 - 150 μM) by the level of radio-labeled GAR ([1-^{14}C]-glycinamide ribonucleotide) detected. Data in Fig. 3 showed that GAR produced was proportional to the concentration of anthranilate added up to 75 μM, where there was a plateau of product formation. The final concentration of GAR, as estimated from a linear curve generated with precursor glycine, was approximately twice the amount of anthranilate added. This result suggested that anthranilate (or a derivative) was allowing PRA synthesis but was not participating as a substrate in the reaction.

The above result led to the hypothesis that PRA was synthesized non-enzymatically from a product of the TrpD reaction. Under the conditions used to assay phosphoribosyl transferase activity, TrpD quantitatively converted PRPP and anthranilate to PR-anthranilate after about five

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min (as measured by a loss in fluorescence at 400nm). Over the next 30 min, fluorescence increased, indicating a reappearance of anthranilate and corresponding to the breakdown of PR-anthranilate (Fig. 4).

The products of PR-anthranilate breakdown are anthranilate and R5P (30), suggesting that PRA might be generated by a non-enzymatic reaction between accumulating R5P and the ammonia present in the reaction mix. Non-enzymatic formation of PRA has been well characterized (23), and its potential contribution was determined by performing the coupled assay in two steps. PR-anthranilate was formed by TrpD from anthranilate and PRPP. Conversion of anthranilate to PR-anthranilate was judged to be complete when fluorescence in the mixture decreased to that of the blank. Proteins in the assay mix were then removed by ultra filtration with a 3 kDa cut off membrane, and the resulting eluant was provided as a source of R5P to a reaction mixture containing NH_3 and the components for ^{14}C-GAR synthesis. Data from this experiment, shown in Table 2, indicated that when PurD was the only enzyme in the assay mixture the eluant allowed GAR formation. These data allowed the conclusion that non-enzymatic PRA formation was occurring between R5P and NH_3. Taken together the above results suggested that anthranilate was being recycled by TrpD to synthesize more PR-anthranilate, which resulted in the continuous formation of R5P allowing non-enzymatic formation of PRA as depicted in Fig. 5.

Metabolite accumulation can lead to PRA formation in vivo. A physiological role for non-enzymatic formation of PRA has been discounted in the past. During steady state metabolic conditions this conclusion is validated by the thiamine requirement of a *purF* mutant strain. PR-anthranilate is the substrate for a bi-functional enzyme encoded by *trpC*. Strains lacking TrpC activity have been shown to accumulate anthranilate in the medium (30), which could suggest an

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accumulation of R5P endogenously. A *purF gnd trpC::*Tn*10* mutant strain was generated to determine if the process described *in vitro* could be forced to occur *in vivo* by manipulating metabolic flux. Two regulatory mutations were incorporated into the strain to ensure that metabolic flux reached the genetically blocked step in the presence of the required tryptophan. An insertion in *trpR* relieved repression (4, 25) and an allosterically insensitive variant of TrpE (*trpE3613*) prevented feedback inhibition (4, 5). The strain lacking *trpC* and defective in regulation by tryptophan showed significant growth in the absence of thiamine as shown in Fig. 6. This result was consistent with the hypothesis that the limiting metabolite for non-enzymatic PRA formation *in vivo* is R5P.

Disruption of flux through the tryptophan biosynthetic pathway can increase PRA formation in vivo. The experiment above employed a contrived strain that was lacking a major biosynthetic enzyme and as such the value of this result in conclusions about a physiological role for non-enzymatically generated PRA was limited. Mutations that restored PurF-independent PRA synthesis, mapped to the tryptophan operon, and allowed retention of the ability to grow in the absence of tryptophan were sought using localized mutagenesis. A phage lysate grown on a DES mutagenized strain (DM1) was used to transduce strain DM6806 (*purF2085 gnd-181 trpC*::Tn*10*d(Tc)) to Trp^+ on medium containing adenine and thiamine. Trp^+ transductants were replica-printed to minimal medium supplemented with adenine, and growth was scored. Seven Trp^+ Thi^+ mutants were isolated. Genetic reconstruction determined the causative mutation in 6 of the 7 strains was 100% linked to the tryptophan operon, and these mutations were further characterized.

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Mutations in trpC allow PRA synthesis. The region between the *trpH* and *trpC* genes was PCR-amplified and sequenced from each of the mutants. (The *trpH* gene is upstream and divergently transcribed with respect to the *trp* operon.) Six of the mutations allowing PRA synthesis were in *trpC* and one in *trpE*. Due to their prevalence, the *trpC* alleles were analyzed further. The causative lesion in each mutant strain is described in Table 3. All 6 mutations mapped to the phosphoribosylanthranilate isomerase (PR-AnI) domain of the bifunctional enzyme. The isomerase reaction is the first of the two sequential reactions catalyzed by TrpC. Two of the mutant enzymes ($TrpC^{A405T}$ and $TrpC^{G435D}$) contained changes in conserved residues of the PR-AnI active site, as determined by amino acid sequence alignments of the PR-AnI of ten different microorganisms (26). One mutation introduced an amber codon resulting in a TrpC enzyme with a predicted truncation of the PR-AnI domain ($TrpC^{G339ter}$). The amino acid change in $TrpC^{G255D}$ affected a residue that serves as a bridge between the PR-AnI and the indole-3-glycerol-phosphate synthase (IGPS) domains in *E. coli* and *S. enterica* TrpC (26). The location of these mutations in the three-dimensional structure the protein is illustrated in Fig. 7.

*Nutritional analyses of trpC mutants***.** Specific growth rates (μ) for the isolated mutant strains (DM7863-DM7870) and a control wild-type are shown in Table 4. None of the *trpC* mutant strains displayed a significant requirement for tryptophan, indicating at least a low level of enzymatic activity remained in the mutant proteins. Though unexpected for strain DM7869, low level read-through of nonsense codons could generate sufficient full length message for *in vivo* function (1). Each of the mutant strains grew in the absence of thiamine, while the strain containing a wild-type tryptophan operon (DM6418) did not. Significantly, addition of tryptophan (0.5mM) eliminated the growth of all mutant strains. The inhibition by tryptophan

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was reversed by the addition of thiamine and partially alleviated by the introduction of a feedback resistant allele of *trpE* (data not shown), as expected if flux regulation is involved. Together these results indicated that growth of these strains in the absence of thiamine was a result of PRA production by a tryptophan-regulated mechanism.

trpC mutants excrete anthranilate. The phenotypic similarities between the strains carrying *trpC* point mutations and a strain lacking *trpC*, in addition to their location in the coding sequence, suggested these alleles had compromised the isomerase activity. Given the prototrophic nature of the relevant strains, the indirect aspect of available assays, and potential complexities of the metabolic network involved, the predicted accumulation of anthranilate was tested by bioassay. A strain containing *trpE8*, which creates an auxotropic requirement for anthranilate or tryptophan, was used as an indicator strain. The tryptophan requirement of this strain is satisfied by 15 μM of anthranilate (data not shown). A variety of relevant strains were grown in the appropriate medium and the presence of exogenous anthranilate was monitored by stabbing cells on a soft-agar overlay seeded with the *trpE8* mutant strain. Results of a typical experiment are presented in Fig. 8. The figure shows that the parental strain (DM9784) fails to excrete a metabolite that allows growth of the *trpE* mutant. In contrast, the *trpC* mutant strains excrete a compound that supplies the growth requirement of the *trpE* strain and is thus assumed to be anthranilate.

DISCUSSION

All organisms maintain a complex set of biochemical interactions that make up the metabolic network. A key component of the metabolic network is the embedded robustness that

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allows an organism to compensate for defects in one metabolic branch by altering another. The studies described here were initiated to test the prediction that the AS-PRT (TrpDE) enzyme complex could generate phosphoribosyl amine (PRA) (27). However, data from the *in vitro* studies unexpectedly led to the conclusion that PRA needed for thiamine synthesis could be generated non-enzymatically if carbon flux through the tryptophan biosynthetic pathway was altered.

The *in vitro* system described herein serendipitously allowed the generation (and detection) of PRA that was formed non-enzymatically from ribose-5-P and NH_3. Manipulation of the assay system allowed the conclusion that the TrpD enzyme mediated the conversion of PRPP to R5P through a PR-anthranilate intermediate (Fig. 6). Thus in this isolated system, the critical product of the TrpD reaction was R5P, generated by breakdown of the unstable PR-anthranilate. The R5P then condensed with NH_3 present to generate PRA. It is important to note that these results did not address the possibility that TrpD can enzymatically generate PRA under some condition(s).

The instability of PR-Anthranilate has been previously described (7). However, a role for the breakdown products in metabolism had not been considered since the subsequent enzyme in the pathway (TrpC) would be expected to remove this metabolite before breakdown. The potential contribution of non-enzymatic formation of PRA to thiamine synthesis *in vivo* was explored genetically. Interruption of the *trpC* gene allowed thiamine independent growth in the absence of PurF. However, while producing a positive result, this was a harsh test of this hypothesis since it restored one pathway (thiamine) at the expense of another (tryptophan). The isolation of mutations in *trpC* that did not compromise the ability of the cell to grow in the absence of tryptophan, but allowed growth without thiamine (in cells lacking *purF*) confirmed a

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physiological potential for non-enzymatic PRA formation. Cross-feeding studies detected the expected excretion of anthranilate, making all data consistent with accumulation of PR-anthranilate being indirectly responsible for thiamine synthesis in these strains. These data allow the conclusion that R5P is the limiting metabolite for non-enzymatic formation of PRA *in vivo*, at least in the medium used. This conclusion explains why no mutations have been isolated that prevent PurF-independent thiamine synthesis when cells are grown on ribose. Use of ribose as a sole carbon source would be expected to elevate R5P levels.

Five of the *trpC* mutations restoring PRA synthesis affected residues in the PR-AnI domain and one mutation affected the single amino acid that serves as a bridge between the two domains. The diversity of mutations that resulted in a similar phenotype by distinctly altering the TrpC enzyme, yet failing to compromise tryptophan independent growth was unexpected. The presence of one mutation that generated a termination codon was noteworthy since activity sufficient for tryptophan biosynthesis was maintained. It seems likely that low levels of read-through of the UAG codon produced enough functional TrpC to allow synthesis of tryptophan. It was telling that each of the randomly generated mutations that survived the selection mapped to the isomerase domain of the protein. This activity is the first of the two sequential reactions the enzyme catalyzes in tryptophan biosynthesis and significantly is the one that acts on PR-anthranilate. This result emphasized the need for the specific accumulation of PR-anthranilate.

In a global context, this work illustrates several points. First, the potential for non-enzymatic synthesis of key metabolites is demonstrated. Strains were generated in which thiamine synthesis depended on the non-enzymatic formation of an intermediate. No significant growth defect resulted in these strains, indicating that all essential metabolic processes were functioning at a capacity that allowed growth on minimal medium. A second point of note was

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the ease with which subtle changes in enzyme kinetics appeared to alter the metabolic network. This work suggests that even in highly conserved proteins, key residues involved in catalysis are not the only critical feature to be maintained. Finally, this work demonstrates that changes in a protein can alter metabolic flux in a way that has a global impact on metabolism, yet can not be predicted, or in fact measured if only one output is monitored. As such, protein variants should be considered to be optimized for function in the native metabolic network. If this view is correct, it has implications for evaluating essential residues and protein evolution in the context of a single activity, or pathway. Rather, analysis of the relevant network, and its conservation in different organisms may provide a better understanding of the forces at work to constrain and define metabolism and allow for the critical inherent robustness.

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Table 1. Bacterial strains and plasmids.

Strain	Genotype
DM1	WT
DM6806	*purF2085 gnd181 trp*::Tn*10*d(Tc)
DM6418	*purF2085 gnd-181 zdd-9147*::Tn*10*d(Tc)
DM8916	*purF2085 gnd181* Δ*trpEDCBA::Cm*
DM7863	*purF2085 gnd181 trpC3620*
DM7864	*purF2085 gnd181 trpC3625*
DM7865	*purF2085 gnd181 trpC3621*
DM7867	*purF2085 gnd181 trpC3622*
DM7868	*purF2085 gnd181 trpC3623*
DM7869	*purF2085 gnd181 trpC3624*
DM7870	*purF2085 gnd181 trpC3626*
DM9719	*purF2085 gnd181 trpE3613* Δ*trpR::Cm trpC::*Tn*10*d(Tc)
DM9784	*purF2085 gnd181*
DM9891	*purF2085 gnd181 zdd1947::*Tn*10*d(Tc) *trpC3620*
TrpE8	*trpE8*

Plasmids	Source	Insert
pET-28a (kan^r)	Novagen	None
pET-trpD (kan^r)	This work	*trpD*

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Table 2. Non-enzymatic formation of PRA.

Step #1	Reaction #				
Additions	1	2	3	4	5
Anthranilate	+	+	+	-	+
PRPP	+	+	-	+	+
ATP	-	-	-	-	+
TrpD	-	+	+	+	+
Step#2	Eluant from reaction #				
	1	2	3	4	5
^{14}GAR formed[a]	74	374	59	65	290

Assay conditions were as described in the text with 5 initial reactions containing the indicated additions. Following incubation, protein was removed to generate the eluant of the corresponding number. The eluants were added to the reaction mixture containing NH_3, ATP, PurD and 14C-glycine, and GAR formed was monitored by phosphorimage analyses.

[a]numbers represent phosphorimager units representing the amount of GAR formed.

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Table 3. Mutations and amino acid changes that allow PurF-independent PRA synthesis.

Strain	*trp* allele	Causative mutation	Amino acid change	3-dimensional location [a]
DM7863	*trpC3620*	G-A	A405T	Conserved active site residue in PR-AnI domain[b]
DM7865	*trpC3621*	G-A	G255D	Hinge region between PR-AnI and IGPS
DM7867	*trpC3622*	G-A	A358T	Non conserved residue in PR-AnI domain
DM7868	*trpC3623*	G-A	G435D	Fully conserved active site residue in PR-AnI domain
DM7869	*trpC3624*	C-T	G339Ter (UAG)	Truncates half of the PR-AnI domain
DM7870	*trpC3626*	Insertion at bp 798	6 a acid insertion	Beginning of the first α-helix of PR-AnI domain

[a] 2.8-A resolution crystal structure of TrpC from *Escherichia coli* as solved by Priestle *et al*. (26).

[b]Conserved residues were determined by the amino acid sequence alignment of TrpC from several organisms (26).

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Table 4. Mutations in the tryptophan operon restore thiamine-independent growth.

Strain	*trp* allele	Specific growth rate (μ) in minimal glucose medium supplemented with[a]: Ade	Ade Trp	Ade Thi	Ade Trp Thi
DM6418	WT	0.087	0.072	0.459	0.439
DM7863	*trpC3620*	0.365	0.030	0.393	0.415
DM7865	*trpC3621*	0.290	0.074	0.323	0.410
DM7867	*trpC3622*	0.262	0.047	0.378	0.400
DM7868	*trpC3623*	0.324	0.080	0.390	0.409
DM7869	*trpC3624*	0.315	0.086	0.379	0.381
DM7870	*trpC3626*	0.295	0.032	0.319	0.396

[a]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 $\ln(X/X_0)/T$, where X is A_{650}, X_0 is A_{650} at time zero, and T is time in hours.

WT, wild-type.

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FIGURE LEGENDS

Fig 1. Reactions catalyzed by the TrpE, TrpD and TrpC enzymes in the tryptophan biosynthetic pathway. A) The heterotetrameric complex ($TrpE_2D_2$) catalyzes glutamine-dependent anthranilate synthesis. The second activity, PR-anthranilate synthesis, is performed by the phosphoribosyl transferase subunit (TrpD). Gln, glutamine; Glu, glutamate; Pyr, pyruvate; PRPP, 5-phosphoribosyl 1-pyrophosphate. **B)** Reactions catalyzed by the bi-functional enzyme TrpC. The carboxy-terminal domain catalyzes the isomerization of N-(5'-phosphoribosyl) anthranilate to CdRP. The amino-terminal domain catalyzes the decarboxylation of CdRP to indole-3-glycerol-phosphate. PR-Ant, N-(5'-phosphoribosyl) anthranilate; CdRP, 1-(o-carboxyphenylamino)-1'-deoxyribulose-5'-phosphate; InGP, indole-3-glycerol-phosphate

FIG. 2. ^{14}C-GAR formation in the presence of TrpD enzyme requires anthranilate. (A) Reactions were performed in 50 mM potassium phosphate buffer pH 8.0 in the presence of 150 μM anthranilate (when added), 10 mM PRPP, 10 mM NH_3, 6 mM $Mg(Ac)_2$, 2.5 mM ATP, 25 mM ^{14}C-glycine (26 nCi) and 2 μg of GAR synthetase. Reactions were started by the addition of 5 μg of TrpD enzyme and incubated at 37ºC for 1 h. Labeled GAR and glycine were separated on PEI-cellulose using a methanol/pyridine/water (20:1:5) solvent system. (+) addition; (-) no addition and visualized using a Cyclone Storage Phosphor System. (B) A schematic of the coupled assay used to detect PRA formation is shown.

FIG. 3. GAR synthesis vs. anthranilate concentration. Production of GAR by wild-type TrpD was determined in the presence of increasing concentrations of anthranilate. The assay was performed as described in Materials and Methods in the presence of 10 mM PRPP and 10 mM

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NH_3. Reactions were started by the addition of 5 μg of the TrpD enzyme and incubated at 37°C for 1 h. The data represents the average of duplicate experiments and detected ^{14}C-GAR was quantified in PhosphorImager (PI) units.

FIG 4 . Degradation of N-(5'-phosphorybosyl)-anthranilate (PR-Ant). The phosphorybosyl transferase activity of TrpD was assayed as described in Materials and Methods in the presence of 1mM PRPP, 1mM anthranilate and 0.3 μg of TrpD enzyme. Disappearance of anthranilate was followed fluorometrically (excitation wavelenght: 325nm; emission: 400nm) in the presence (O), or absence (□) of TrpD.

FIG. 5. Non-enzymatic PRA synthesis from NH_3 and R5P in the presence of TrpD. R5P released from the degradation of PR-anthranilate formed by TrpD reacts non-enzymatically with NH_3 to produce PRA. PRPP, phosphoribosyl pyrophosphate; Ant., anthranilate; PR-Ant., phosphoribosyl-anthranilate; R5P, ribose 5-phosphate; PRA, phosphoribosyl amine

FIG. 6. Metabolite accumulation allows PRA formation *in vivo*. Strains were grown in minimal medium supplemented with adenine and tryptophan. DM9784 *(purF2085 gnd181)* (■), DM9719 (*purF2085 gnd181 ΔtrpR3614 trpE3613 trpC::Tn10*) (●). Addition of thiamine restores growth of DM9784 (□). Data shown are an average of 2 independent cultures with error bars indicated.

FIG. 7. 3-dimensional localization of mutations restoring PRA synthesis. Structure of PR-AnI-IGPS (TrpC) (26). Residue changes are indicated in different colors. The region presumed

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to be predominately truncated in $TrpC_{G339Ter}$ is shown in yellow starting with residue G339 in pink. The location of the active sites of the PR-AnI and IGPS is indicated by I and S, respectively. Structural depictions were generated using PyMol software version 0.99 (http://pymol.sourceforge.net/)

FIG. 8. Anthranilate excretion by *trpC* mutants. Feeding experiments were performed as described in Materials and Methods. The indicator strain *trpE8* was seeded as an overlay on minimal medium plates supplemented with adenine and thiamine. Indicated mutant strains were stabbed into the agar lawn from overnight cultures. Mutant strains used were: DM7870 (*purF2085 gnd181 trpC3626*), 7863 (*purF2085 gnd181 trpC3620*), 7865 (*purF2085 gnd181 trpC3621*), 7869 (*purF2085 gnd181 trpC3624*) and 7868 (*purF2085 gnd181 trpC3623*). Strain DM9784 (*purF2085 gnd181*) is the parental control and the only growth seen is of the strain itself. Plates were incubated at 37°C for 24 hours.

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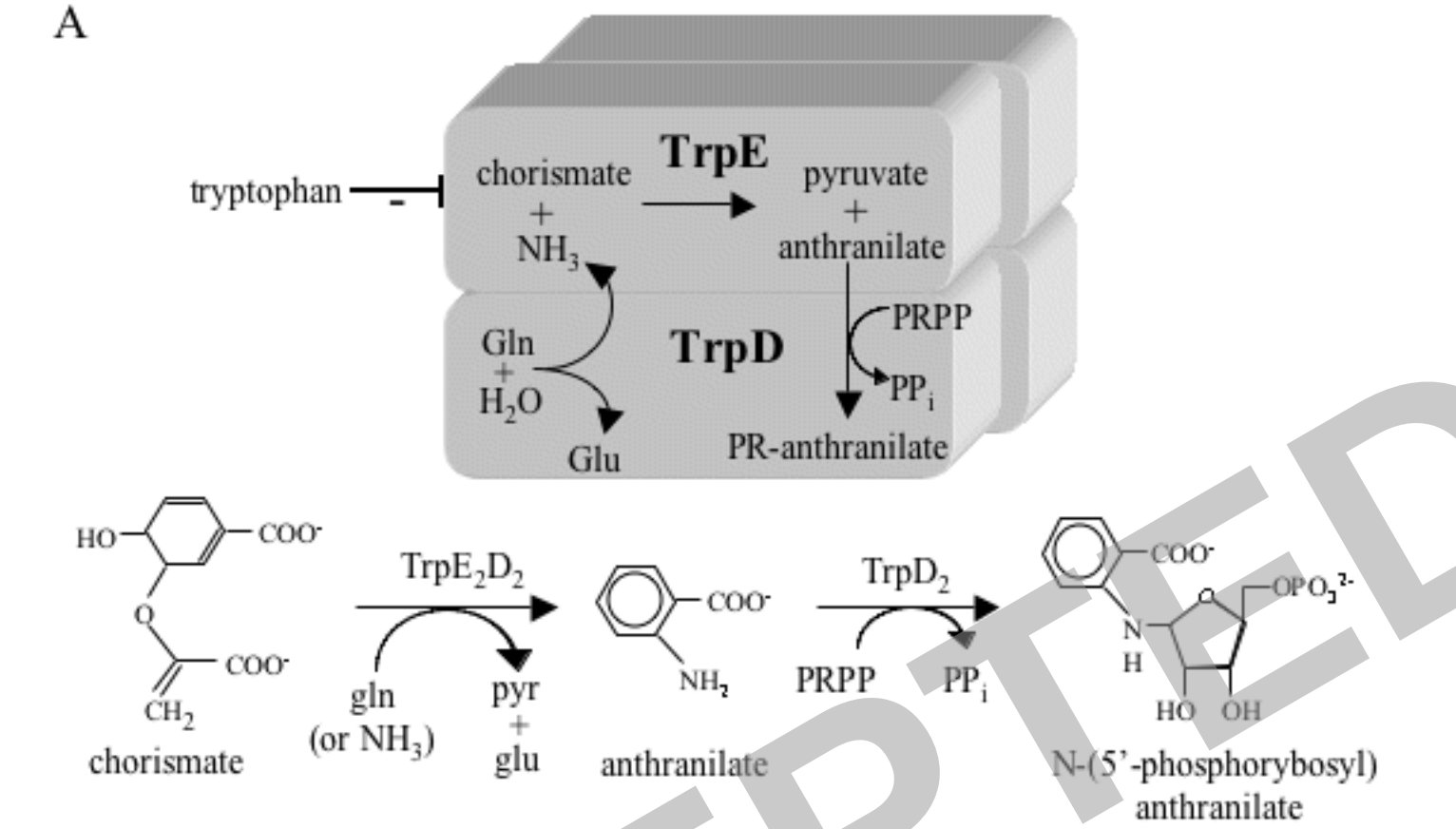

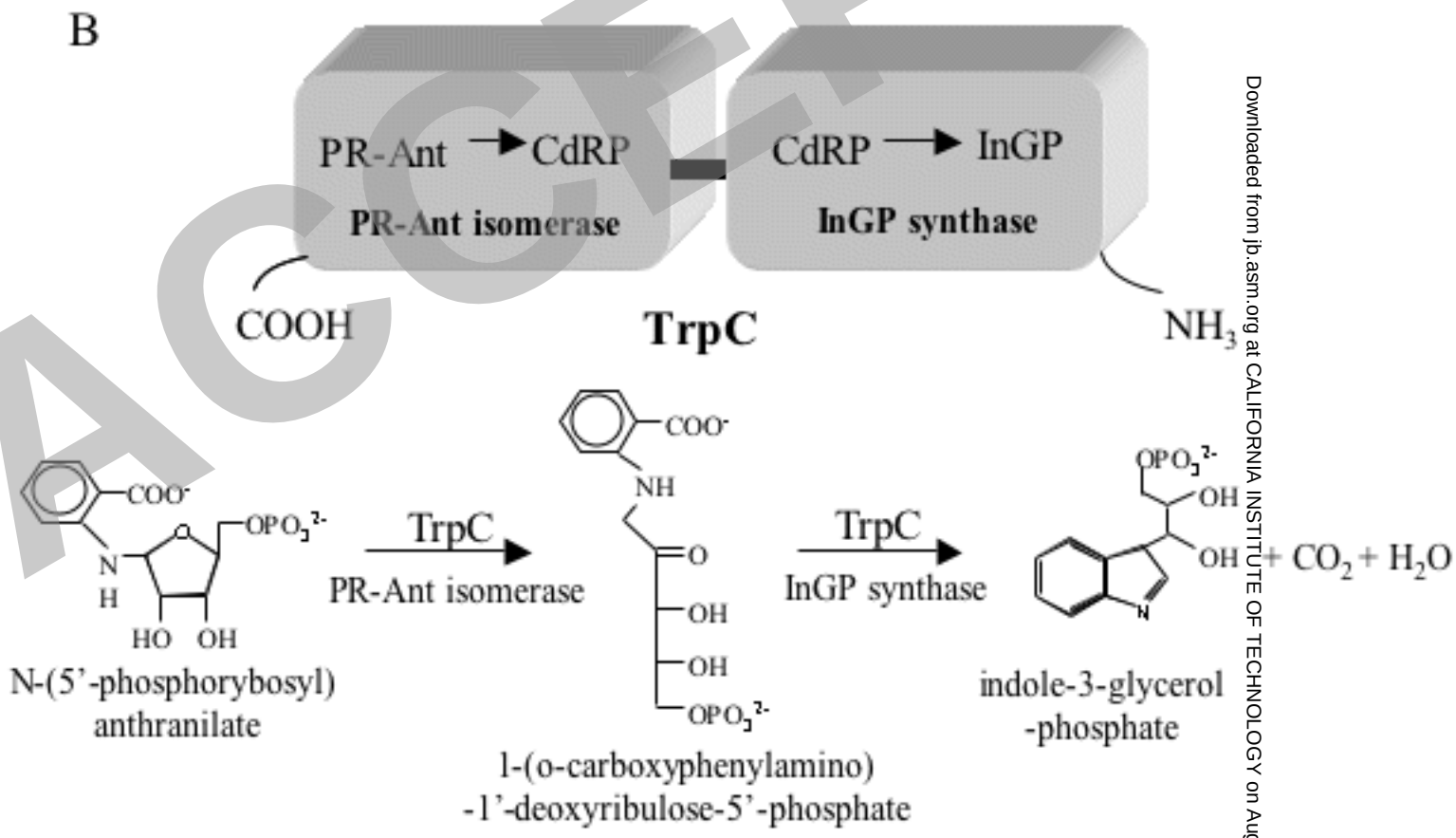

FIG. 1

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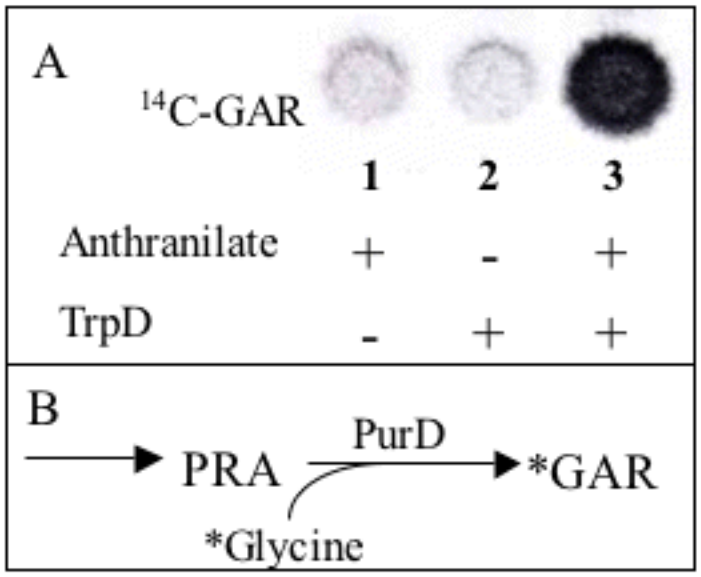

FIG.2

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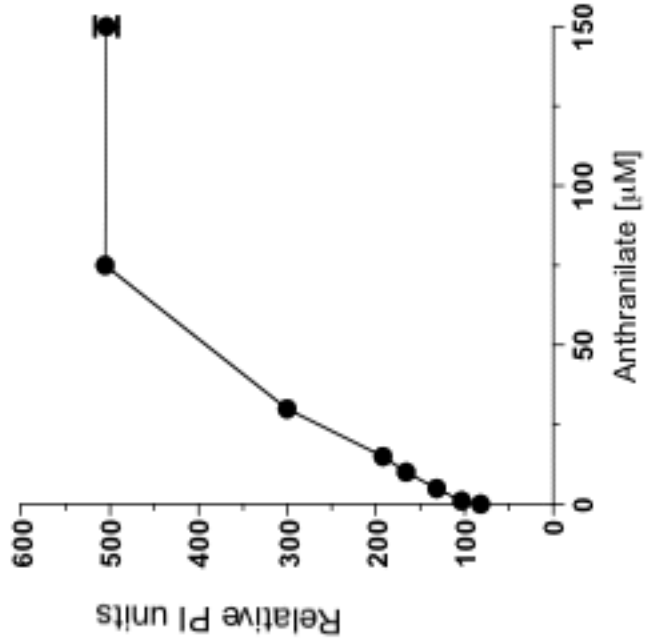

FIG. 3

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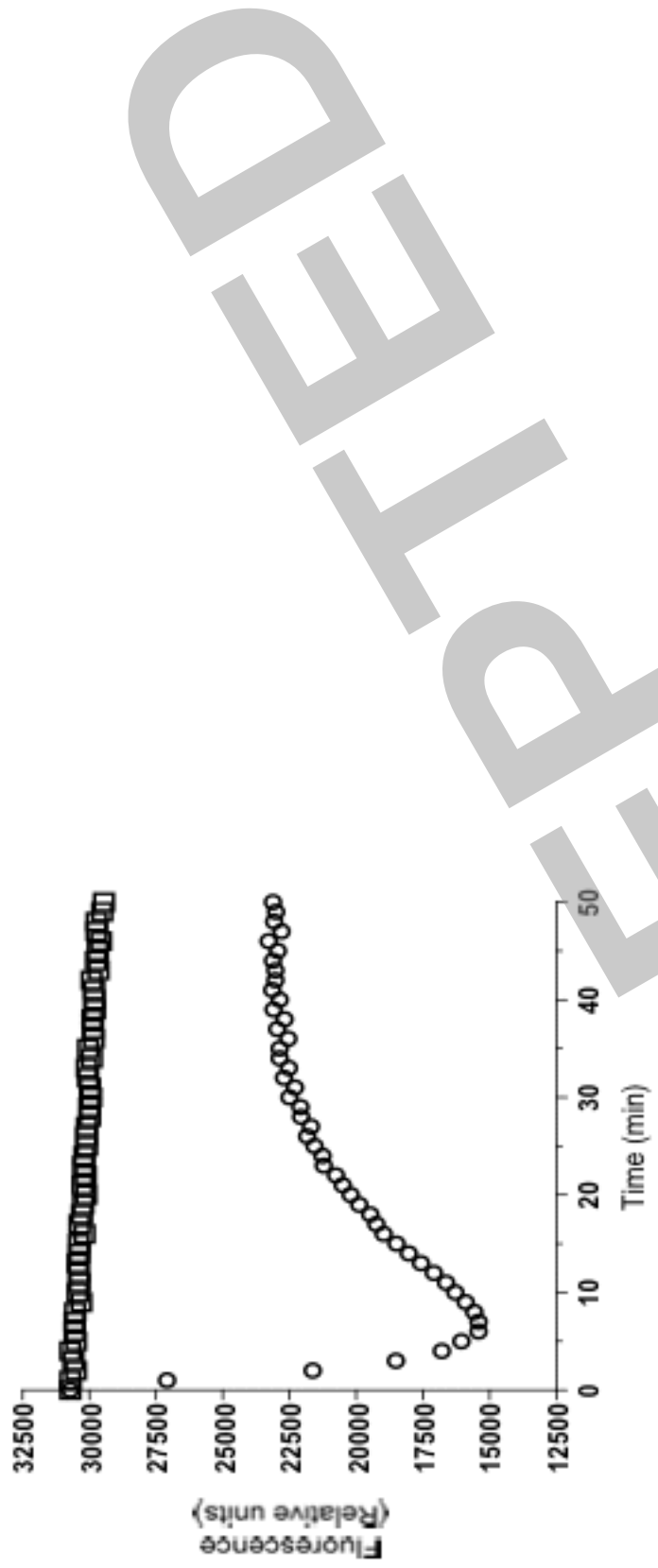

FIG. 4

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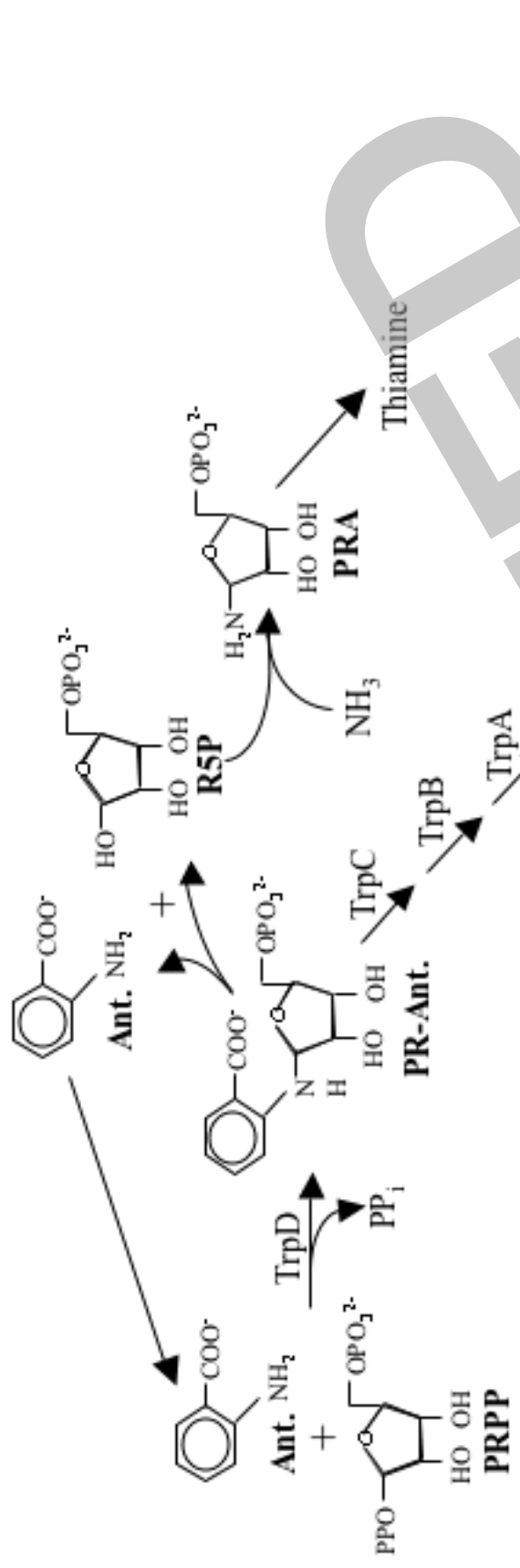

FIG. 5

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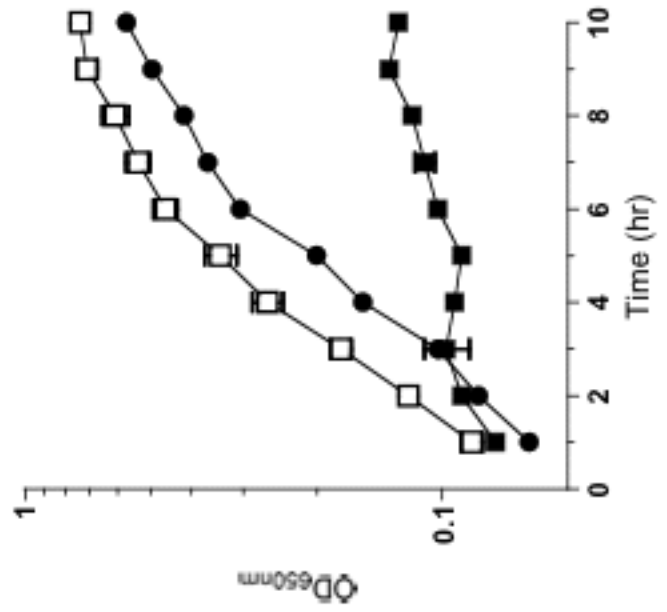

FIG. 6

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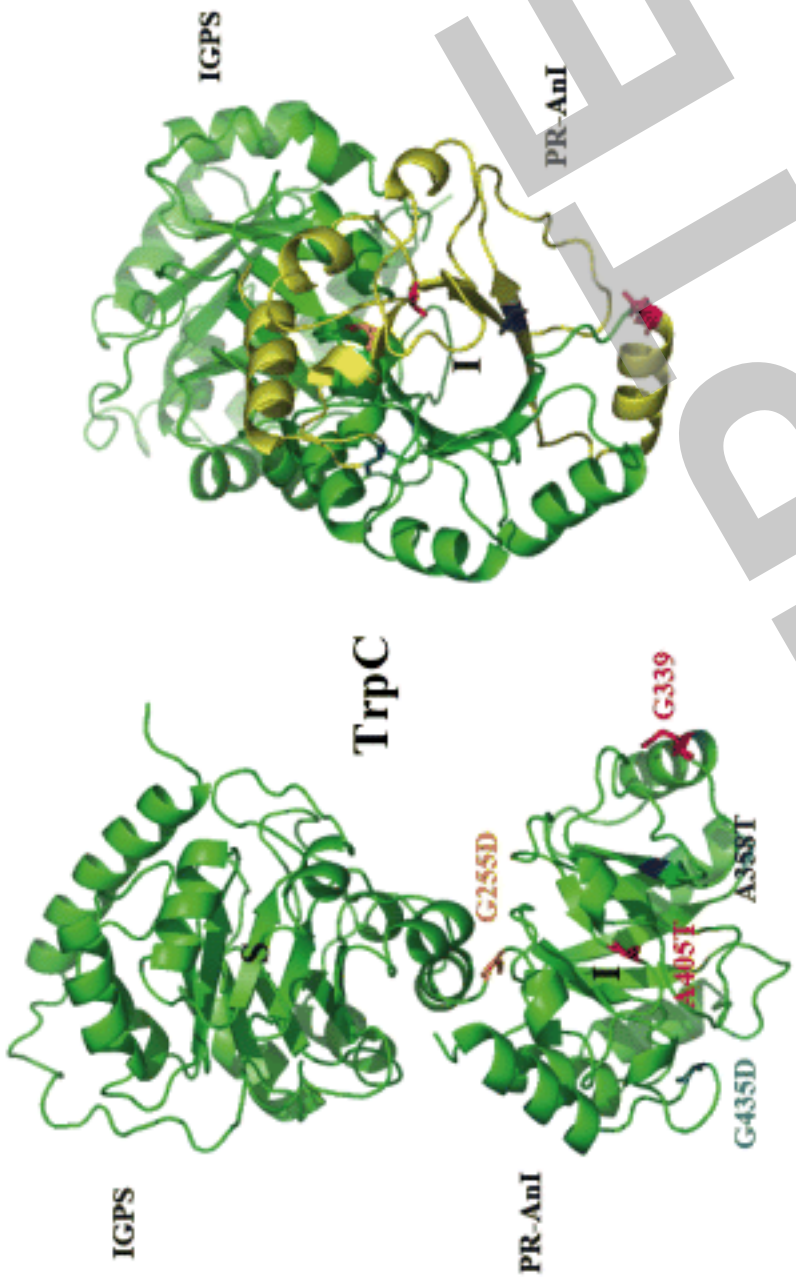

FIG. 7

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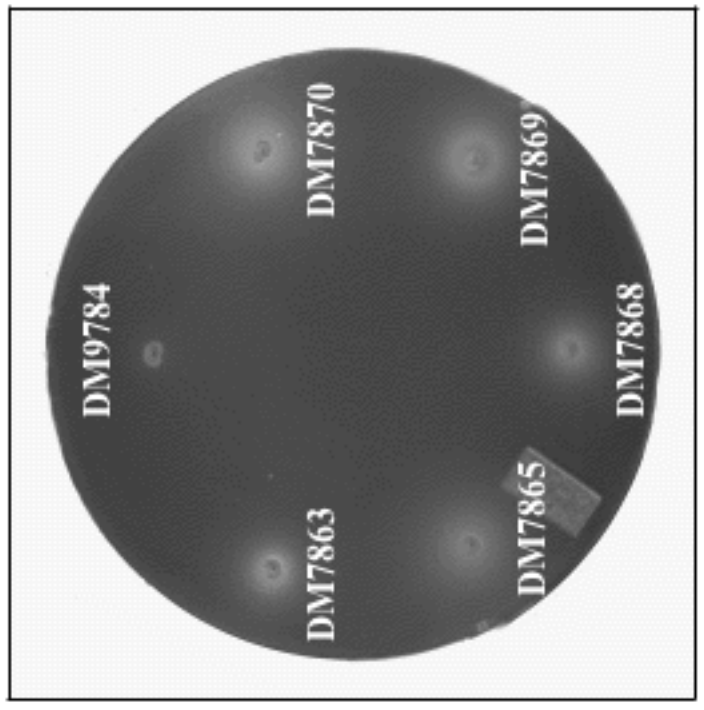

FIG. 8.

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