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Glutamine Phosphoribosylpyrophosphate Amidotransferase-independent Phosphoribosyl Amine Synthesis from Ribose 5-Phosphate and Glutamine or Asparagine*

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Phosphoribosylamine (PRA) is the first intermediate in the common pathway to purines and thiamine and is generated in bacteria by glutamine phosphoribosylpyrophosphate (PRPP) amidotransferase (EC 2.4.2.14) from PRPP and glutamine. Genetic data have indicated that multiple, non-PRPP amidotransferase mechanisms exist to generate PRA sufficient for thiamine but not purine synthesis. Here we describe the purification and identification of an activity (present in both *Escherichia coli* and *Salmonella enterica*) that synthesizes PRA from ribose 5-phosphate and glutamine/asparagine. A purification resulting in greater than a 625-fold increase in specific activity identified 8 candidate proteins. Of the candidates, overexpression of AphA (EC 3.1.3.2), a periplasmic class B nonspecific acid phosphatase, significantly increased activity in partially purified extracts. Native purification of AphA to >95% homogeneity determined that the periplasmic L-asparaginase II, AnsB (EC 3.5.1.1), co-purified with AphA and was also necessary for PRA formation. The potential physiological relevance of AphA and AnsB in contributing to thiamine biosynthesis *in vivo* is discussed.

In bacteria, phosphoribosylamine (PRA)[4] is the first intermediate in the biosynthetic pathways for purines and the pyrimidine moiety of thiamine (1–4) (Fig. 1). Glutamine-PRPP amidotransferase (E.C. 2.4.2.14), the product of the *purF* gene in *Salmonella enterica* (and *Escherichia coli*), catalyzes the synthesis of PRA from PRPP and glutamine. Strains lacking PurF have the expected nutritional requirement for purines; however, under some growth conditions *S. enterica* strains lacking PurF are able to grow in the absence of thiamine (5, 6). Genetic and biochemical studies have demonstrated this growth reflects the existence of a PurF-independent mechanism(s) to generate PRA that is utilized by subsequent purine and thiamine enzymes to generate the hydroxymethyl pyrimidine moiety of thiamine (7, 8). However, PRA generated in the absence of PurF is not sufficient to satisfy the cellular requirement for purines.

An inability to obtain mutants devoid of PRA formation suggests multiple activities that can generate this metabolite. PRA could be generated by a side reaction(s) of enzyme(s) involved in other metabolic pathways, redistribution of metabolic flux, or a combination of the two. Results implicating the anthranilate synthase-phosphoribosyltransferase complex (TrpDE, EC 4.1.3.27, EC 2.4.2.18), required in tryptophan biosynthesis in the synthesis of PRA (9, 10), are consistent with this scenario. Genetic studies have shown that a functional oxidative pentose phosphate pathway is required for at least one route of PRA biosynthesis independent of PurF and distinct from the Trp enzymes (6, 9, 11). Nutritional studies were consistent with a role of the oxidative pentose phosphate pathway in contributing ribose 5-phosphate (R5P) for PRA synthesis (Fig. 1) (6, 11, 12).

The study described here was initiated to test the hypothesis that a PRA-forming activity utilizing R5P as a substrate is present in bacterial cells. Here we describe the identification and purification of a PRA-forming activity present in *E. coli* and *S. enterica* which converts R5P and asparagine or glutamine to PRA. Purification of this activity from crude extract determined that it required two proteins encoded by *aphA* and *ansB*.

EXPERIMENTAL PROCEDURES

Medium and Chemicals—Culture media supplies were obtained from Difco. Glutamine, asparagine, glycine, ribose 5-phosphate, PRPP, magnesium acetate, ammonium chloride, ATP, EDTA, β-mercaptoethanol, phenylmethanesulfonyl fluoride, protamine sulfate, DNase I (EC 3.1.21.1), and lysozyme (EC 3.2.1.17) were obtained from Sigma-Aldrich. KCl, sucrose, $MgCl_2$, Tris base, methanol, and pyridine were from Fisher. K_2HPO_4, KH_2PO_4, and ammonium sulfate were obtained from Mallinckrodt Baker (Phillipsburg, NJ). [1-^{14}C]Glycine was from PerkinElmer Life Sciences. DEAE-Sepharose Fast Flow and all chromatography resins were obtained from Amersham Biosciences. Cellulose-polyethyleneimine plates were from Selecto Scientific (Suwanee, GA). Ultrafiltration YM membranes were

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[4] The abbreviations used are: PRA, phosphoribosylamine; PRPP, phosphoribosylpyrophosphate; R5P, ribose 5-phosphate; PurF, glutamine PRPP amidotransferase; GAR, 5′-phosphoribosylglycinamide; PurD, GAR synthetase; AphA, class B non-specific acid phosphatase/phosphotransferase; AnsB, L-asparaginase II; CV, column volume.

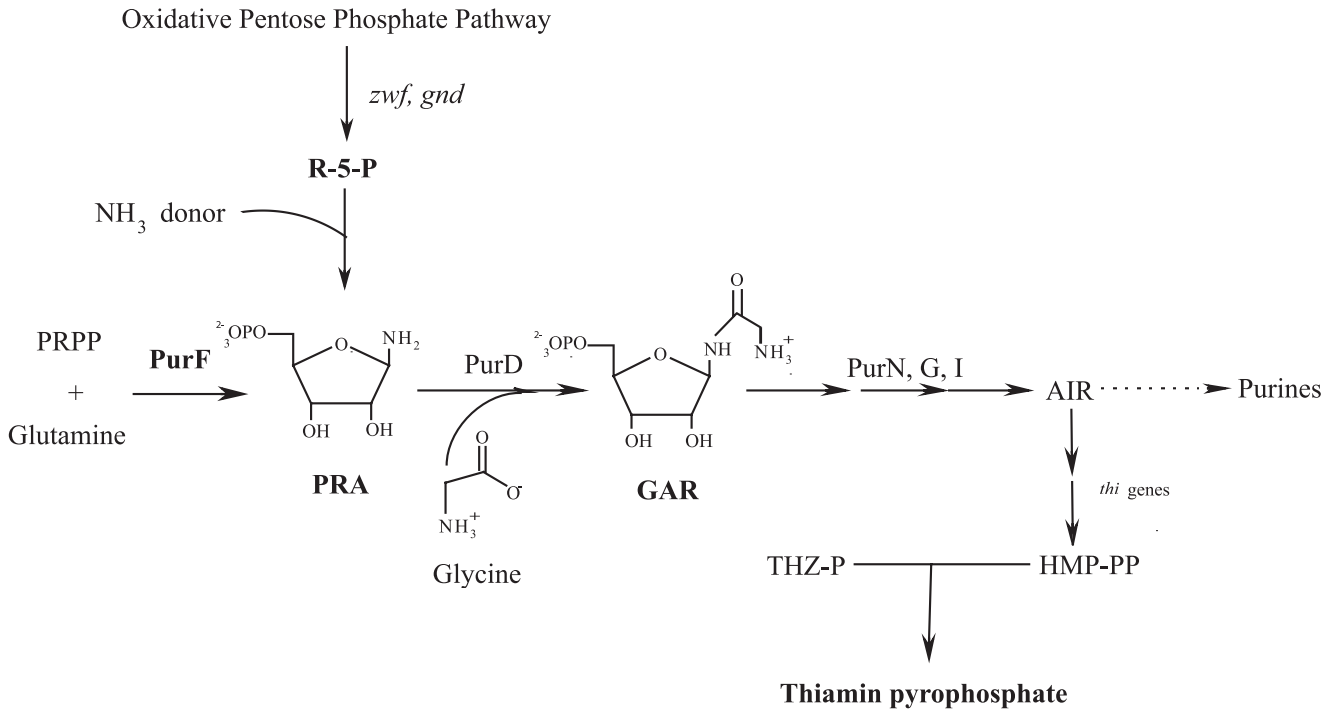

FIGURE 1. **Pathway schematics; biosynthetic pathway for purine and thiamine synthesis.** Some purine gene products are indicated above the reactions they catalyze. The proposed substrates for the alternative PRA synthesis are R5P and an unknown amino donor. *AIR*, aminoimidazole ribotide; *HMP-PP*, 4-amino-5-hydroxymethylpyrimidine pyrophosphate; *THZ-P*, 4-methyl-5-(β-hydroxyethyl)thiazole phosphate.

from Amicon (Beverly, MA). All restriction enzymes used were from Promega (Madison, WI). AnsB (EC 3.5.1.1) from *E. coli* (catalog number A4887) was obtained from Sigma-Aldrich.

Strains, Cell Growth, Extract Preparation—Strain DM1293 (*E. coli*, *purF*::Tn*10*) was grown in Luria-Bertani broth in a 16-liter batch fermentor. Strain DM1936 (*S. enterica, purF2085*) was grown in nutrient broth in a 16-liter batch fermentor. The medium was inoculated with 1 liter of an overnight culture and incubated at 37 °C with aeration for 12 h. Cells (~88 g) were harvested by centrifugation at 4 °C, washed twice with buffer A (0.05 M potassium phosphate buffer, pH 7.5, containing 5 mM β-mercaptoethanol and 1 mM phenylmethanesulfonyl fluoride), and resuspended in 200 ml of the same buffer. The cells were disrupted using a French pressure cell at 10^4 kPa. Clarified cell-free extract was obtained by centrifugation at 23,700 × *g* for 45 min at 4 °C. When grown on a smaller scale, media were inoculated with a nutrient broth overnight culture and incubated at 37 °C with agitation until full cell density was achieved. Cell-free extracts were then generated as described above.

Assay for PRA Formation—A PRA-forming activity assay described for glutamine-PRPP amidotransferase (4, 13) was modified to optimize sensitivity. PRA synthesis was assayed in 50 mM potassium phosphate buffer, pH 8.0, in the presence of 6 mM magnesium acetate, 2.5 mM ATP, 2 mM [^{14}C]glycine (26 nCi), a pool of amino acids containing equal concentrations (6 mM) of glutamine, asparagine, and 2 μg of glycinamide ribonucleotide (GAR) synthetase. Reactions were started by the addition of R5P (13 mM final concentration) and incubated at 37 °C for 4 h. Radiolabeled GAR and glycine were separated by thin-layer chromatography (TLC) on polyethyleneimine-cellulose using a methanol:pyridine:water system (20:1:5). The position of radioactive spots was detected using a Cyclone Storage Phosphor System (Packard Instrument Co.), and their identity was confirmed with standards.

Protein Purification

GAR Synthetase (PurD)—GAR synthetase was purified to 95% homogeneity from *E. coli* strain DM1295 (*purF*::Tn*10*/pJS187). Plasmid pJS187 provides for the overexpression of GAR synthetase (14). PurD protein was purified as described using ammonium sulfate fractionation, ion exchange chromatography (DEAE resin), and size exclusion chromatography (14). The activity of the purified protein was assayed by monitoring [^{14}C]GAR production from [^{14}C]glycine and chemically synthesized PRA (4, 13, 15). Alternatively, PurD was purified by affinity chromatography using the IMPACT CN Kit (New England Biolabs, Ipswich, MA). A C-terminal intein tag was created by amplifying the open reading frame of *purD* from *S. enterica* LT2 chromosomal DNA by PCR using Platinum Pfx DNA polymerase (Invitrogen). This construct was cloned into the NdeI and SmaI sites of the plasmid pTYB2. Overexpression and purification of the protein were performed as recommended by the manufacturer and resulted in a native protein containing a single additional glycine at the N-terminal end. Preparations of PurD purified by either method were used interchangeably with no affect on the ability to detect PRA-forming activity.

PRA-forming Activity—Nucleic acids were precipitated by the addition of protamine sulfate to the clarified cell-free extract at a final concentration of 0.1% with constant stirring over 10 min. The solution was then stirred for an 30 additional min and centrifuged at 23,700 × *g* for 20 min. A two-step salt precipitation was performed. The clarified cell-free extract was

brought to 35% saturation by the addition of solid ammonium sulfate with constant stirring for 30 min. The solution was then stirred for 30 additional min and centrifuged at 23,700 × *g* for 30 min. The same procedure was used in a second precipitation step performed at 70% saturation. The supernatant at 70% saturation was discarded, and the precipitate was resuspended in buffer A (0.05 M potassium phosphate buffer, pH 7.5, containing 5 mM β-mercaptoethanol, and 1 mM phenylmethanesulfonyl fluoride) and dialyzed against the same buffer. The dialyzed fraction was applied at a flow rate of 2 ml/min to a DEAE ion exchange column (200-ml column volume) equilibrated with buffer A. The column was washed with two column volumes (CV) of buffer A, and all PRA-forming activity was found to be in the flow-through and wash fractions. These fractions were pooled and concentrated using a 30-kDa cut-off Amicon ultrafiltration membrane. The concentrated fraction was applied at a flow rate of 0.5 ml/min to a HiLoad Superdex 75 size exclusion column (24-ml CV) equilibrated with buffer A containing 100 mM KCl; 1-ml fractions were collected and assayed for PRA-forming activity. The active fractions were applied at a flow rate of 2 ml/min to a Mono Q (strong ion exchange) column (8 ml of CV) equilibrated with buffer A. After a 2-CV wash with buffer A, a linear gradient from 0 to 1 M KCl was used to elute the proteins bound to the column; 1-ml fractions were collected throughout the wash and elution. After dialyzing against buffer A, the fractions were tested for PRA-forming activity and stored at 4 °C.

An alternative step was used interchangeably with the DEAE column chromatographic step. In this alternative 100 cubic centimeters of unpacked DEAE resin equilibrated with buffer A was mixed with the dialyzed fractions, and the suspension was placed on ice for 10 min. Trial experiments determined that this batch elution protocol resulted in purification of the PRA-forming activity with the same efficiency as collecting the flow-through from standard column chromatography. The supernatant was removed by filtration. The resin was washed with an additional 200 ml of buffer A, which was then pooled with the 200-ml supernatant and concentrated using a using a 30-kDa cut-off Amicon ultrafiltration membrane and applied to a Superdex 75 size exclusion column (see above).

AphA—Strain DM10089 (*purF2085 ansB1*::Kan pSU-*aphA*) was grown in nutrient broth with chloramphenicol (20 μg/ml) in a 16-liter batch fermentor, and the cells were collected by centrifugation. Proteins from the periplasmic space were isolated using a modified spheroplasting technique (16). The resulting protein preparation was subjected to the purification scheme described for the PRA-forming activity. SDS-PAGE protein bands were visualized by staining with a Silver Stain Plus kit from Bio-Rad, and the protein prep was determined to be >95% pure after passage through a Superdex 75 size exclusion column (data not shown). The protein prep was then stored at 4 °C for two months or frozen and stored at −80 °C for future use.

Protein Quantification and Manipulation

Protein was quantified by the method of Bradford (17) or by using a BCA protein assay kit (Pierce). Ultrapure bovine serum albumin was used to generate a standard curve. After SDS-polyacrylamide gel electrophoresis (SDS-PAGE), protein bands were visualized by staining with either (*a*) 40:55:5:0.05 ethanol:water:acetic acid:Coomassie G-250 and distained in 40:55:5 ethanol:water:acetic acid or (*b*) silver using a Silver Stain Plus kit from Bio-Rad.

TABLE 1
Primers used in this study

Plasmid[a]	Primers used to amplify insert
pIR-*mdh*	5′-GCCTGTGTCACGCCTCGCAAATAA-3′
	5′-GCGACCTGCATGTGCCTGTTGG-3′
pIR-*mdoG*	5′-CACGTATTCTCAGATTTTTCACCT-3′
	5′-CGCGAGTAAGTCCGATGCT-3′
pIR-*ydcG*	5′-CTGAAGAATGCTGCGTGAGG-3′
	5′-GAGCAGCACTTAATAAACCCAGAG-3′
pSU-*aphA*	5′-GTCGACCCGTTACTGGCGTTATGGTC-3′
	5′-GGATCCGTGCAGCAAGTCTGGAAAAG-3′
pSU-*cpdB*	5′-CTGCAGGGAACGATATCGGGTTTCAC-3′
	5′-GGATCCCGGGAACGTTTATCAGATGG-3′
pSU-*ansB*	5′-CTGCAGTAAACAATGGCGCAGATCG-3′
	5′-GGATCCGTGCGAGAGGTCTTCCAAAG-3′
pSU-*yghA*	5′-GAGAGTCGACGAATACGGGCGAAGCATAAG-3′
	5′-CAGAGGATCCAAGTGGCGCCTTGCTTAAC-3′
pSU-*talA*	5′-GAGACTGCAGGCCTGTCTGCTATGCTTTTTG-3′
	5′-GAGAGGATCCCATTGGCAAGGTCTTTACGG-3′

[a] For all plasmids, pSU19 (Cm[r]) was used.

Analysis of Candidate Genes

Candidate genes were amplified from *S. enterica* LT2 chromosomal DNA by PCR using Platinum *Pfx* DNA polymerase (Invitrogen) with the primers shown in Table 1. The resulting PCR products were purified and cloned into the multicloning site of pSU19 (18). Plasmids were electroporated into *E. coli* strain DH5α and screened for vectors containing inserts. Constructs containing the cloned genes under the control of the *lac* promoter were confirmed by sequence analysis and introduced into *S. enterica* strain DM1936.

Candidate proteins were partially purified from cell-free extracts of strains overexpressing them. Cells (~3 g) were resuspended in buffer A (6 ml) with 2 mg/ml lysozyme and 1 mg/ml DNase before disruption with a French pressure cell at 10^4 kilopascals. Clarified cell-free extract was obtained by centrifugation at 39,000 × *g* for 30 min at 4 °C. The clarified cell-free extract was at 65 °C for 5 min, centrifuged at 39,000 × *g* for 45 min at 4 °C, and run by gravity feed over two DEAE columns (6-ml CV) equilibrated with buffer A running in tandem at 4 °C. A 6-ml wash was combined with the flow-through fraction and concentrated with a 30-kDa cut-off Amicon ultrafiltration membrane.

RESULTS

Optimization of in Vitro Assay for PRA Formation—Past protocols for PRA-formation have utilized a coupled reaction with glycinamide ribotide synthetase (PurD) in which the substrate ([^{14}C]glycine) is separated from the product ([^{14}C]GAR) by ion exchange chromatography based on the charge conferred by the phosphate group in GAR (4). Use of this assay in crude cell-free extracts was hampered by nonspecific phosphatases that partially converted GAR to GARs and, thus, decreased the sensitivity of the assay. Because the activity of interest was expected to be weak, a TLC system using a mobile phase of methanol/pyridine/water (20:1:5) on polyethyleneimine-cellu-

lose plates was used to separate unincorporated [^{14}C]glycine from the [^{14}C]GAR/[^{14}C]GARs.

Initial screens for PRA-forming activity were preformed in *S. enterica* and *E. coli* cell-free extracts lacking glutamine-PRPP amidotransferase. Either PRPP or R5P was provided as the carbon backbone donor and a pool of amino acids containing glutamine, asparagine, alanine, and valine (each at 6 mM) as the potential source of the amino group. Subsequent experiments determined that glutamine and asparagine were equally proficient as amino donors, and no other amino acid allowed PRA formation. Control experiments determined PRA was formed in the absence of extract (data not shown) if R5P was provided to the Tris-HCl buffer used in previous work (pH 8.0, 100 mM) (4). Because PRA can be chemically synthesized from R5P and ammonia, this nonenzymatic formation was attributed to small amounts of ammonia in the buffer (15, 19). Additional buffers (HEPES-NaOH, Clark and Lubs KH_2PO_4-NaOH, pH. 8, 50 mM) were discarded for a similar reason or because the (PurD) activity was negatively affected. Potassium phosphate buffer, pH 8.0, was found to produce negligible background PRA formation and was used in subsequent experiments.

Initial Characterization of a PRA-forming Activity in Cell-free Extracts Lacking PurF—Dialyzed cell-free extracts from *S. enterica* and *E. coli* strains lacking glutamine-PRPP amidotransferase failed to support synthesis of PRA from either R5P or PRPP, glutamine, and asparagine. It was subsequently found that synthesis of PRA from R5P, glutamine, and asparagine was detectable after the cell-free extract had interacted with an anion exchange resin (DEAE, pH 7.5) and/or was subjected to 65 °C for 5 min. Detection of this activity is shown in Fig. 2. Data in Fig. 2*A* show that in the presence of fractionated extract, formation of GAR was detectable when R5P (*lane 2*), but not PRPP (data not shown), was provided with amino acids. The PRA-forming activity was decreased when heated at 98 °C for 5 min (*lane 3*) and retained after dialysis (*lane 4*). Furthermore, the activity was unaffected by treatment with either RNase or DNase (data not shown). Accumulation of GAR was proportional to the time of assay incubation up to 4 h, as shown by data from a representative experiment in Fig. 2*B*. Data in Fig. 2*C* show that GAR synthesis was proportional to protein up to 15 μg, after which a plateau was reached. The data in Figs. 2, *B* and *C*, are from a representative experiment, and GAR concentration was determined from a linear curve of radiolabeled glycine generated under visualization conditions.

Inability to detect PRA-forming activity in crude cell-free extracts before fractionation suggested the presence of an inhibitory compound. Subsequent experiments determined the target of inhibition was the PurD reaction, and it was not further characterized.

Purification of PRA-forming Activity—PRA-forming activity was followed during fractionation of an *E. coli* strain lacking glutamine-PRPP amidotransferase (DM1293 (*E. coli purF77*::Tn*10*)). A purification protocol was implemented and is summarized in Table 2. A typical purification used 250 ml of cell-free extract (8 mg/ml) fractionated by precipitation with ammonium sulfate as described under "Experimental Procedures." The proteins precipitated at 70% saturation were resuspended in buffer A and dialyzed against the same buffer. Ion exchange chromatography was performed on the dialyzed fractions (200 ml). PRA-forming activity was detectable at this stage in the flow-through fraction, and this activity was set to be 100% for subsequent yield determination.

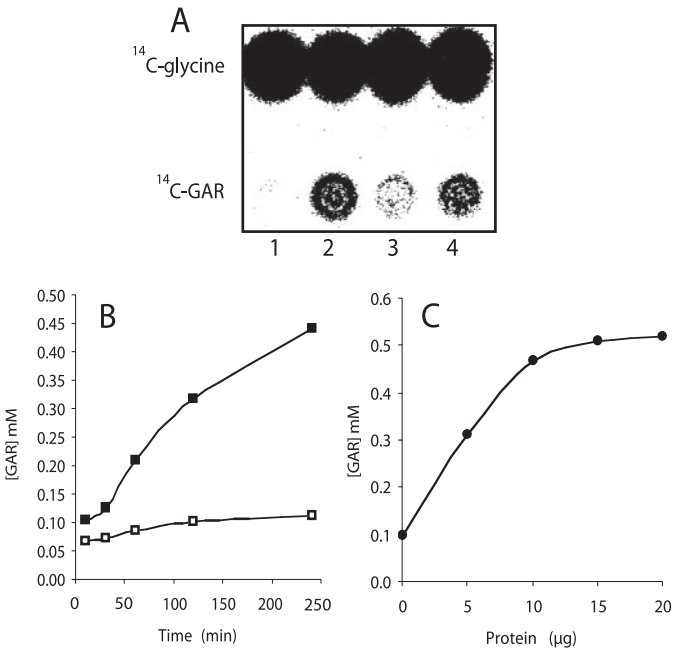

FIGURE 2. **PRA-forming activity in a partially purified extract from an *E. coli purF* mutant.** *A*, synthesis of PRA as a function of [^{14}C]GAR production. Reactions were performed as described under "Experimental Procedures" adding an amino acid pool (glutamine and asparagine) and R5P as substrates in the absence (*lane 1*) and presence of a fresh (*lane 2*), boiled (*lane 3*), or dialyzed (*lane 4*) partially purified cell extract. After incubation at 37 °C for 4 h, radiolabeled [^{14}C]GAR and [^{14}C]glycine were separated on polyethyleneimine-cellulose using a methanol/pyridine/water (20:1:5) solvent system. *B*, GAR synthesis *versus* time. GAR synthesis from R5P and an amino acid pool in the presence (■) and absence (□) of the partially purified extract (10 mg total protein). *C*, synthesis of GAR in the presence of increasing amounts of protein after 4 h of incubation at 37 °C.

TABLE 2

Purification of PRA-forming activity

PRA synthesis from R5P, glutamine, and asparagine was determined to be a function of radiolabeled [^{14}C]GAR production.

Purification step	Specific activity	Purification	Yield
	Units[b]*/mg of protein*		%
Cell-free extract	ND[a]		
$(NH_4)_2SO_4$ precipitation	ND		
DEAE ion exchange chromatography (flow-through)	506	1	
Superdex 75 size exclusion chromatography	10,848	22	68
Mono Q ion exchange chromatography	316,492	625	89

[a] ND, none detected.
[b] Arbitrary units detected by the Cyclone Storage Phosphor System.

After interaction with the DEAE resin, the PRA-forming activity was further purified and concentrated by passage through a 30-kDa cut-off Amicon ultrafiltration membrane followed by size exclusion chromatography and ion exchange chromatography as described under "Experimental Procedures." Data in Table 2 indicates a 625-fold final purification was achieved.

Proteins in the active fractions from the purification were visualized after separation on SDS-polyacrylamide gel. The

TABLE 3
Identification of potential PRA-forming enzymes by mass spectrometry analysis

Gene	Strain[a]	Gene product[b]	Size	Function
			kDa	
mdoG	DM9380	Putative periplasmic glycoprotein	57	Periplasmic glucans biosynthesis, addition of branches to linear glucan backbone
ydcG	DM9831	Putative periplasmic glycoprotein	61	Periplasmic glucans biosynthesis
mdh	DM9379	Malate dehydrogenase	32	Interconversion of L-malate and oxaloacetate
talA	DM9652	Transaldolase A	35	Reversible 3-carbon ketol unit transfer from sedoheptulose 7-phosphate (P) to glyceraldehyde 3-P to form erythrose 4-P and fructose 6-P
yghA	DM9615	Hypothetical oxidoreductase NAD(P) binding	31	Belongs to the short-chain dehydrogenases/reductases (SDR) family
cpdB	DM9520	2′,3′-Cyclic nucleotide 2′-phosphodiesterase	70	Salvage of nucleosides and nucleotides (interconversion)
ansB	DM9584	Periplasmic L-asparaginase II	35	Hydrolysis of asparagine to aspartic acid and ammonia
aphA	DM9518	Periplasmic class B phosphatase/phosphotransferase	25	Non-specific acid phosphatase/phosphotranserase

[a] *purF2085* strain with gene cloned into pSU19.
[b] At least two different peptides matching the identified protein were detected by mass spectrometry.

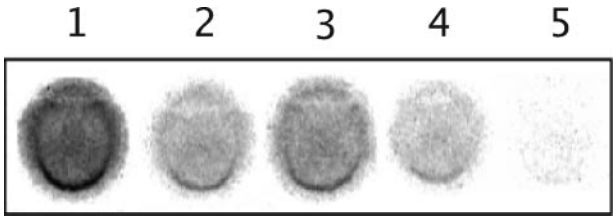

FIGURE 3. **PRA-forming activity in partially purified protein extracts.** PRA formation as a function of radiolabeled [^{14}C]GAR from R5P, asparagine, and glutamine using 10 μg of DM9518 (*purF* pSU-*aphA*) (*lane 1*), 10 μg of DM9584 (*purF* pSU-*ansB*) (*lane 2*), 10 μg of pure AnsB from Sigma (*lane 3*), 10 μg of DM1936 (*purF*) (*lane 4*), and no protein control (*lane 5*). After incubation at 37 °C for 4 h, radiolabeled [^{14}C]GAR and [^{14}C]glycine were separated by TLC, and the [^{14}C]GAR spots are shown.

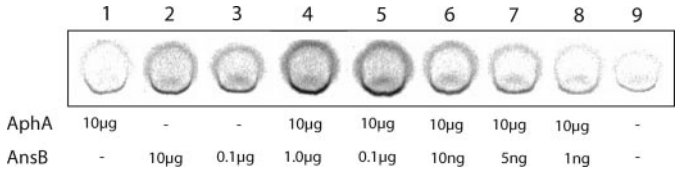

FIGURE 4. **Formation of PRA from AphA and AnsB.** PRA formation using purified protein as a function of radiolabeled [^{14}C]GAR from R5P, asparagine, and glutamine using 10 μg of AphA (*lane 1*), 10 μg of AnsB (*lane 2*), 0.1 μg of AnsB (*lane 3*), 10 μg AphA plus 1.0 μg of AnsB (*lane 4*), 10 μg of AphA plus 0.1 μg of AnsB (*lane 5*), 10 μg of AphA plus 10 ng AnsB (*lane 6*), 10 μg of AphA plus 5.0 ng AnsB (*lane 7*), 10 μg of AphA plus 1.0 ng of AnsB (*lane 8*), and no protein control (*lane 9*). After incubation at 37 °C for 4 h, radiolabeled [^{14}C]GAR and [^{14}C]glycine were separated by TLC, and the [^{14}C]GAR spots are shown.

fraction from the Mono Q column contained multiple protein bands (data not shown). Final active fractions from three independent purifications and an inactive fraction from the final column were sent to Midwest Bio Services (Overland Park, KS) for in-gel extraction and mass spectrometry analysis. Table 3 summarizes the proteins identified in all active fractions and not present in the inactive fraction.

Partially Purified AphA Has PRA-forming Activity—Genes encoding each of the eight proteins described in Table 3 were amplified from *S. enterica* and cloned into a pSU19 multicopy plasmid. The resulting constructs were introduced into the *S. enterica* strain DM1936 (*purF2085*). Cell free extracts from the resulting strains were subjected to partial purification and assayed for PRA-forming activity. Of the eight strains, two had more PRA synthesis than the parental strain (Fig. 3). The strain overexpressing AphA (*lane 1*) and the strain overexpressing AnsB (*lane 2*) were found to have increased PRA formation relative to the control strain DM1936 (*lane 4*). Purified AnsB was purchased (Sigma) and tested for its ability to make PRA. Although AnsB allowed PRA formation (*lane 3*), two points were noted. First, 10 μg of AnsB allowed less PRA formation than crude extract of the AphA-overproducing strain. Second, as an asparaginase, AnsB produces NH_3 and, thus, PRA formation is expected to be at least partially due to the nonenzymatic combination of ammonia and R5P (15).

AphA and AnsB Are Required for PRA-forming Activity—AphA was purified from strain DM9518 (*purF2085* pSU-*aphA*) to >95% homogenicity and tested for PRA-forming activity. Ten micrograms of this protein preparation generated more PRA than the partially purified extract from DM9518 (data not shown), although there was a noticeable contaminating protein band of approximately the size of the AnsB protein (~35 kDa). To address the possible contribution of AnsB to the detected PRA-forming activity, a strain lacking AnsB and PurF activities was generated. Plasmid pSU-*aphA* was moved into this strain to create DM10089 (*purF2085 ansB1*::Kan pSU-*aphA*). AphA was then purified after overexpression in the *ansB* mutant background and found to have no PRA-forming activity. Additional protein preparations confirmed that AphA is not sufficient to catalyze PRA formation.

Data in Fig. 4 showed that although neither 10 μg of AphA (*lane 1*) or AnsB (*lane 2*) allowed significant PRA formation, a mixture of the two proteins did. In the presence of less than molar amounts of AnsB, AphA was able to generate significant amounts of PRA. From these data it was concluded that the PRA-forming activity purified from crude extracts was due to a complex involving the periplasmic proteins AphA and AnsB.

Physiological Role of AphA and AnsB in Thiamin Biosynthesis—Results described here showed that AphA and AnsB have the ability to produce PRA *in vitro*. Strain DM10259 (*purF2085 ansB1*::Kan *aphA15*::Cm) was constructed and analyzed for growth under various conditions known to allow PurF-independent PRA formation. In all growth conditions tested, the strain lacking *ansB* and *aphA* grew as well as the parental *purF* mutant, indicating this PRA-forming activity was not essential for growth. When *aphA* or *ansB* was placed in multicopy, neither allowed a *purF* mutant strain to grow in the absence of thiamine under non-permissive conditions (data not shown).

DISCUSSION

This study describes the identification and purification of a cellular activity from both *E. coli* and *S. enterica* that synthe-

sizes PRA. Data herein showed that AphA, a periplasmic class B nonspecific acid phosphatase/phosphotransferase (EC 3.1.3.2), in combination with submolar ratios of the periplasmic L-asparaginase II, AnsB (EC 3.5.1.1), can produce PRA from R5P and either asparagine or glutamine *in vitro*. Titration experiments determined that (at pH 7.5) optimal PRA-formation occurred when AphA and AnsB were provided at a 13:1 molar ratio. We suggest that AphA binds R5P and catalyzes the formation of PRA when presented with the ammonia released by hydrolysis of asparagine or glutamine by AnsB.

Based on the enzymes involved, a scenario in which either one could produce PRA is feasible. Although AphA has been characterized primarily for its ability to dephosphorylate mononucleotides and various other phosphomonoesters, the enzyme has been shown to catalyze dephosphorylation of R5P. This finding indicates that the enzyme can bind R5P, although it is acted on with a relatively low catalytic efficiency (K_{cat}/K_m = 5.0×10^3 M^{-1} s^{-1}, pH 6) (20). At physiological pH the protein exhibits suboptimal activity, potentially decreasing this catalytic efficiency further, raising the possibility that R5P could bind in the active site without dephosphorylation occurring. In this scenario, AphA could serve to concentrate R5P, allowing PRA formation to occur when ammonia is present. Because AnsB hydrolyzes both asparagine and glutamine optimally at pH 7.5 (21), a simple scenario suggests that AnsB produces ammonia that allows nonenzymatic formation of PRA in the presence of R5P (15).

Although more complex scenarios for the generation of PRA by the individual enzymes could be imagined, several observations suggested AphA and AnsB together were responsible for the PRA production detected herein. First, AphA and AnsB co-purified through several chromatographic steps, consistent with at least a loose association of the two proteins. The fact that both enzymes are located in the periplasm is consistent with this interpretation. Second, the amount of PRA formed with the addition of both proteins was noticeably more than that generated by AphA even with excess ammonia. We suggest a model in which PRA is formed in the active site of AphA. By this scenario R5P would occasionally bind the AphA enzyme, blocking the active site due to the poor catalytic activity of the enzyme with this substrate. This association would remain until the glutaminase/asparaginase releases ammonia in close proximity, perhaps allowing the interaction of R5P and ammonia, resulting in PRA formation. The inability of PRPP to allow PRA formation could be due to the binding specificity of AphA. Alternatively, the inability of PRPP to participate in the reaction could support the nonenzymatic mechanism proposed. If PRA is easily released from the enzyme, this could benefit the organism by restoring the availability of the active site for mononucleotide dephosphorylation. Testing this model will require additional kinetic analyses *in vitro*.

Although AphA/AnsB could synthesize PRA *in vitro*, a condition where these proteins were essential for thiamine biosynthesis *in vivo* was not identified. Localization studies showed that AphA is present in the cytoplasm in addition to its predominant location in the periplasm (22); thus, is it rational to suggest some PRA is generated by the mechanism described herein. Previous work in our laboratory has emphasized the multiple inputs to PRA formation (9, 10, 23). In all cases separate from that described here, these inputs have been identified using sensitive *in vivo* genetic analyses. The work described here emphasizes that the cell has additional capacity to generate metabolites that may not be apparent by only addressing activities essential for growth. Furthermore, there is not a good way of determining that the appropriate condition to detect essentiality has been tested.

The robustness of cellular metabolism predicts that multiple inputs to many (if not all) metabolites will exist. Such overlap can be considered to occur by evolutionary pressure or simply be the natural result of biochemical reactions that involve compounds of similar structure. Regardless of the evolutionary questions, from the perspective of understanding the metabolic network in the cell it is important to define not only reactions that are easily recognized as essential for growth but the potential for robustness provided by minor reactions. Based on the work herein, AphA is component of the cell that can be probed with *in vitro* biochemical approaches to generate a better understanding of metabolic robustness with respect to PRA formation.

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Glutamine Phosphoribosylpyrophosphate Amidotransferase-independent Phosphoribosyl Amine Synthesis from Ribose 5-Phosphate and Glutamine or Asparagine

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